THE ROLE OF THE LYMPHATIC SYSTEM IN LIPID AND ENERGY METABOLISM, AND IMMUNE HOMEOSTASIS DURING OBESITY AND DIABETES

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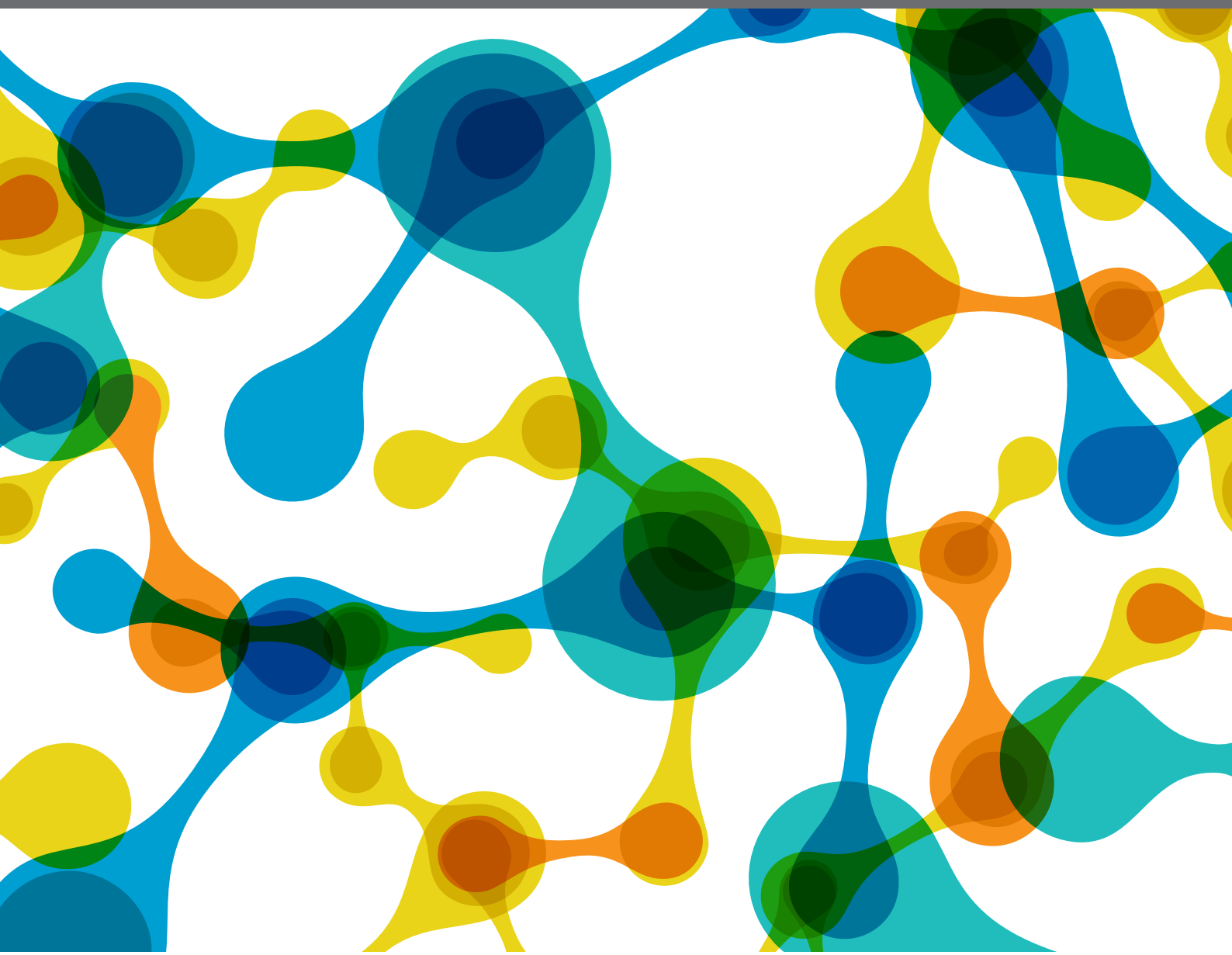

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THE ROLE OF THE LYMPHATIC SYSTEM IN LIPID AND ENERGY METABOLISM, AND IMMUNE HOMEOSTASIS DURING OBESITY AND DIABETES

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Table of Contents

04 ***Editorial: The Role of the Lymphatic System in Lipid and Energy Metabolism, and Immune Homeostasis During Obesity and Diabetes***
Vincenza Cifarelli, Hong Chen and Joshua P. Scallan

07 ***The Lymphatic System in Obesity, Insulin Resistance, and Cardiovascular Diseases***
Xinguo Jiang, Wen Tian, Mark R. Nicolls and Stanley G. Rockson

17 ***Why Do Men Accumulate Abdominal Visceral Fat?***
Andromeda M. Nauli and Sahar Matin

27 ***Emerging Roles for Lymphatics in Chronic Liver Disease***
Matthew A. Burchill, Alyssa R. Goldberg and Beth A. Jirón Tamburini

35 ***The Role of Interstitial Matrix and the Lymphatic System in Gastrointestinal Lipid and Lipoprotein Metabolism***
Anna Zhou, Jie Qu, Min Liu and Patrick Tso

45 ***Lymphatic Vasculature in Energy Homeostasis and Obesity***
Yen-Chun Ho and R. Sathish Srinivasan

55 ***Emerging Role of Lymphatics in the Regulation of Intestinal Lipid Mobilization***
Changting Xiao, Priska Stahel, Avital Nahmias and Gary F. Lewis

63 ***Lymphatic Programing and Specialization in Hybrid Vessels***
John B. Pawlak and Kathleen M. Caron

75 ***The Unresolved Pathophysiology of Lymphedema***
Syaza Hazwany Azhar, Hwee Ying Lim, Bien-Keem Tan and Veronique Angeli

86 ***Characterizing Lymphangiogenesis and Concurrent Inflammation in Adipose Tissue in Response to VEGF-D***
Adri Chakraborty, Caroline K. Scogin, Kinza Rizwan, Thomas S. Morley and Joseph M. Rutkowski

102 ***The Role of Lymphatic Vascular Function in Metabolic Disorders***
Pieter R. Norden and Tsutomu Kume

118 ***Regulation of Lymphatic Function in Obesity***
Raghu P. Kataru, Hyeong Ju Park, Jung Eun Baik, Claire Li, Jinyeon Shin and Babak J. Mehrara

131 ***Extracellular Vesicles as Potential Prognostic Markers of Lymphatic Dysfunction***
Andreea Milasan, Maya Farhat and Catherine Martel

146 ***Lymphatic Endothelial Cell Junctions: Molecular Regulation in Physiology and Diseases***
Feng Zhang, Georgia Zarkada, Sanjun Yi and Anne Eichmann

163 ***Systems Biology Will Direct Vascular-Targeted Therapy for Obesity***
Yingye Fang, Tomasz Kaszuba and P. I. Imoukhuede

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EDITORIAL
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Editorial: The Role of the Lymphatic System in Lipid and Energy Metabolism, and Immune Homeostasis During Obesity and Diabetes

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Keywords: lymphatic system, lacteal, obesity, Prox1, chylomicron, hypercholesterolemia, endothelial plasticity, chronic liver disease

Editorial on the Research Topic

The Role of the Lymphatic System in Lipid and Energy Metabolism, and Immune Homeostasis During Obesity and Diabetes

The Research Topic *"The Role of the Lymphatic System in Lipid and Energy Metabolism, and Immune Homeostasis During Obesity and Diabetes"* summarizes the rapid development of the field of lymphatic vessel biology over the last two decades emphasizing its relevance to metabolism and metabolic dysregulated diseases. The Research Topic, available in *Frontiers in Physiology—Lipid and Fatty Acid Research*, comprises 11 Reviews of the current literature, one Original Research Article, one Perspective, and one Hypothesis and Theory by experts in the field.

The involvement of the lymphatic system in the transport and distribution of lipids has been known for centuries (Suy et al., 2016), despite receiving relatively modest attention due to the wrong belief of passive unregulated drainage of the lymph. Although much uncharted scientific territory remains to be explored in this still nascent field, the regulation of gut lymphatics is experiencing a surge in interest as the associated metabolic outcomes are now starting to be elucidated, and appreciated as potential targets to manage obesity and its complications (Oliver et al., 2020). The Research Topic includes Reviews that summarize regulation of lipid trafficking and absorption, and impact on metabolism by the intestinal lymphatic capillaries (lacteals) and collecting vessels. Xiao et al. discuss the emerging evidence that oral glucose and intestinal hormone glucagon-like peptide-2 (GLP-2) mobilize retained enteral lipid into the lymphatic system by two different mechanisms; (i) via glucose, by mobilizing cytoplasmic lipid droplets and (ii) via GLP-2, by targeting post-enterocyte secretory mechanisms. A second contribution by Zhou et al., discusses the importance of composition and properties (i.e., degree of hydration) of the matrix in the lamina propria in regulating chylomicron diffusion and their entry in the lacteals. Zhou et al. provide insights into the activation of mucosal mast cells by fat absorption and the associated metabolic consequences highlighting the link between chylomicron transport, mucosal mast cell activation, leaky gut, and the microbiome. In the *Hypothesis and Theory Article*, Nauli and Matin discuss a potential mechanism related to higher accumulation of abdominal visceral fat in males as compared to pre-menopausal women. The authors propose that the accumulation of abdominal visceral fat in men, a strong

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independent predictor of mortality, is due to the higher dietary fat uptake by abdominal visceral fat. During the post-prandial state, the larger chylomicrons observed in males congest the lamina propria and the low-pressure lymphatics, predisposing the chylomicron triglycerides to hydrolysis by lipoprotein lipase. The liberated fatty acids are then stored by the nearby abdominal visceral adipocytes, leading to the accumulation of abdominal visceral fat. These mechanisms perhaps explain the reason of bigger and higher production of chylomicrons in males.

The link between the lymphatic system, metabolic dysfunction, inflammation, and the metabolic syndrome is not completely understood and likely to involve multiple mechanisms and different organs. A growing body of evidence suggest that dysfunctional lymphatic vasculature might promote obesity by impairing adipogenesis and promoting inflammation. This is somehow not surprising given the adipogenic properties of lymph (Harvey et al., 2005) and the role of inflammation in adipose tissue expansion (Crewe et al., 2017). These concepts are explored in the review by Kataru et al., which provides an overview of how metabolic disease and inflammation interact to alter lymphatic function in the context of obesity, hypercholesterolemia, and diabetes. Particular attention is given to the role of inflammatory cytokines and immune cells with respect to various aspects of lymphatic function, including lymph transport, junctional integrity, permeability, and contractile function. A study by Chakraborty et al. demonstrates that overexpression of the inflammatory growth factor, VEGF-D, in adipose tissue induces the growth of new lymphatic vessels by lymphvasculogenesis and investigates how inflammatory cytokines and immune cell populations change over time. Ho and Srinivasan and Norden and Kume discuss the bi-directional relationship between obesity and the lymphatic vasculature, reviewing mechanisms and signaling pathways of dysfunctional lymphatic vasculature in adipose tissue expansion and obesity-associated inflammation. The reciprocal correlation between obesity and lymphatic dysfunction is further discussed by Ho and Srinivasan which summarizes clinical correlations and differences between obesity, lymphedema, and lipedema, given that these three diseases all present excessive adiposity, in particular in the lower limbs, and dysfunctional lymphatic vessels. Pharmacological interventions as well as behavioral and lifestyle changes that aim to improve lymphatic function are also reviewed (Norden and Kume). Mouse models of obesity with defective lymphatic function are summarized in Norden and Kume.

The Research Topic reviews the contribution of lymphatic biology in metabolically dysregulated diseases, including type 2 diabetes, atherosclerosis, myocardial infarction, and chronic liver disease. Jiang et al. review how insulin resistance, diabetes, atherosclerosis, and myocardial infarction interact with the lymphatic vasculature. Lymphatic vessels are also located in the adventitial and periadventitial region of arterial walls and exert an important function in mobilizing efficiently cholesterol out of the vessel wall. The role of the lymphatic system in the onset and pathogenesis of atherosclerosis is summarized in Milasan et al., which also discuss the contribution and the diversity of lymph extracellular vesicles (exosomes, microvesicles, and apoptotic bodies) within the lymphatic function and progression of atherosclerosis. Another critical metabolic organ where lymphatic vessels are currently understudied is the liver. Burchill et al. review the inherent difficulties of using lymphatic markers to identify lymphatic vessels in the liver, where hepatic cells express many of the same proteins. The focus of the review, however, is summarizing newly identified roles for lymphatic endothelium in non-alcoholic steatohepatitis and hepatitis C infection.

Lymphatic endothelial cell (LEC) junctions are crucial for the maintenance of vessel integrity and proper lymphatic vascular function. Zhang et al. provide a comprehensive review of the molecular regulation of LEC junctions during development, and diseases namely obesity and inflammation-associated conditions. The authors provide a comprehensive summary of lymphatic vascular phenotypes following genetic deletion of junctional proteins.

Vascular diversity and endothelial plasticity are reviewed by Pawlak and Caron. The authors review five highly specialized hybrid vessel beds that adopt partial lymphatic programing for their specialized vascular functions namely (i) the high endothelial venules of secondary lymphoid organs, (ii) the liver sinusoid, (iii) the Schlemm's canal of the eye, (iv) the renal ascending vasa recta, and (v) the remodeled placental spiral artery. The authors summarize the morphology and endothelial expression pattern of these vessels and interrogate their specialized functions within the broader blood and lymphatic vascular systems (Pawlak and Caron). Finally, Azhar et al. discuss the current knowledge on the pathophysiology of lymphedema, a chronic and progressive disease arising from impaired lymphatic drainage, which causes accumulation of interstitial fluid and results in tissue swelling (Rockson, 2001). Authors review lymphedema diagnosis, assessment and available treatments and further discuss the cellular and molecular mechanisms involved in the expansion and remodeling of adipose tissue, as well as fibrosis and collagen accumulation occurring during lymphedema, listing animal models of lymphedema available for pre-clinical studies.

In conclusion, our understanding of the regulation of the lymphatic system in contributing to tissue remodeling, inflammation, and metabolic outcome during disease has greatly improved in the last two decades. However, more translational studies are needed to pave the way for novel therapeutic approaches to treat metabolically dysregulated diseases, including type 2 diabetes, atherosclerosis, myocardial infarction, and chronic liver disease, as well as lymphedema and lipedema.

AUTHOR CONTRIBUTIONS

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REVIEW
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The Lymphatic System in Obesity, Insulin Resistance, and Cardiovascular Diseases

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Obesity, insulin resistance, dyslipidemia, and hypertension are fundamental clinical manifestations of the metabolic syndrome. Studies over the last few decades have implicated chronic inflammation and microvascular remodeling in the development of obesity and insulin resistance. Newer observations, however, suggest that dysregulation of the lymphatic system underlies the development of the metabolic syndrome. This review summarizes recent advances in the field, discussing how lymphatic abnormality promotes obesity and insulin resistance, and, conversely, how the metabolic syndrome impairs lymphatic function. We also discuss lymphatic biology in metabolically dysregulated diseases, including type 2 diabetes, atherosclerosis, and myocardial infarction.

Keywords: lymphatic, LEC, insulin resistance, type 2 diabetes, atherosclerosis, myocardial infarction

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INTRODUCTION

Obesity, characterized by increased storage of fatty acids in expanded adipose tissues, is becoming a major health problem in modern society, as humans increasingly embrace a relatively sedentary lifestyle. The chronic obese status predisposes individuals to the development of the metabolic syndrome and increases the incidence of type 2 diabetes (T2D) and cardiovascular diseases (Kahn and Flier, 2000; O'Neill and O'Driscoll, 2015; Catrysse and van Loo, 2017). Dysregulated lipid metabolism and low-grade chronic inflammation are among the notable pathologies in obese adipose tissue (Ruotolo and Howard, 2002; Konner and Bruning, 2011). More recently, there is increasing evidence that dysfunction of the lymphatic vasculature is involved in the pathogenesis of obesity and obesity-associated dyslipidemia and low-grade chronic inflammation (Harvey et al., 2005; Aspelund et al., 2016), presumably because the lymphatic system is important for immune homeostasis and lipid transport (Jiang et al., 2018). This review provides an overview of the interplay between the function of lymphatic system and presence of obesity and insulin resistance. We also discuss how the lymphatic system may be harnessed to treat T2D and cardiovascular diseases associated with obesity and insulin resistance.

THE LYMPHATIC SYSTEM

Lymphatic Structure

The lymphatic system is comprised of lymphatic vessels and the secondary lymphoid organs that include lymph nodes, spleen, tonsils, and Peyer's patches (Ruddle and Akirav, 2009; Choi et al., 2012). The lymphatic vasculature is a unidirectional circulatory network that begins as blunt-ended capillaries composed of a single layer of lymphatic endothelial cells (LECs) that tether directly to

the interstitial tissue through anchoring filaments, with discontinuous basement membrane coverage (Jiang et al., 2018). Capillary LECs are interconnected by unique button-like structures that are formed by discontinuous layers of junctional proteins such as VE-cadherin, claudin, Zonula occludens-1 (ZO-1), connexin, and occludin (Baluk et al., 2007). The presence of these buttons serves to create overlapping LEC flaps that serve as *de facto* primary valves. In response to interstitial fluid pressure fluctuation, these flaps can open and close to regulate fluid reabsorption as well as the uptake of macromolecules and immune cells (Yao et al., 2012; Jiang et al., 2018). The lymphatic capillaries converge into precollectors and these in turn coalesce into the collecting lymphatics, in which LECs are joined by zipper-like, continuous, seamless junctions and invested with basement membrane along with smooth muscle cell coverage. Intraluminal lymphatic valves divide the collecting lymphatics into contractile segments designated as lymphangions, thus providing a structural basis for the unidirectional lymph flow (Aspelund et al., 2016; **Figure 1**). The collecting lymphatics travel through chains of lymph nodes, which allows the delivery of free antigens and antigen-loaded dendritic cells (DCs) from interstitial tissue for immune priming; the central lymphatic vasculature eventually joins the subclavian veins via the thoracic duct(s) conveying the lymph node-filtered interstitial fluid back to the blood circulatory system (Thomas et al., 2016; Jiang et al., 2018).

Early Lymphatic Development and Lymphangiogenesis

Lymphatic vasculature originates from the cardinal vein and subsequently develops independently from the blood circulatory system (Wigle and Oliver, 1999). The homeobox gene *Prox-1* appears to be the master control gene for lymphatic differentiation and development (Choi et al., 2012). In E9.5 mouse embryos, Prox-1 expression begins in a subset of endothelial cells (ECs) of the cardinal vein and specifies them as LECs. Prox-1 then upregulates lymphatic markers such as LYVE-1, VEGFR3, and Chemokine (C–C motif) ligand (CCL)21, and concurrently downregulates blood vascular signature genes (Wigle et al., 2002). This molecular differentiation enables LEC budding from the cardinal vein to form the rudimentary lymphatic vessels, known as the jugular lymph sac, at E11.5 (Choi et al., 2012). Conditional Prox-1 downregulation reprograms LECs into blood endothelial cells (BECs) in both developing and adult mice as well as in cell culture (Johnson et al., 2008), supporting the notion that Prox-1 is an indispensable transcriptional factor for the maintenance of LEC identity. Following the initial specification and budding, the lymph sac then expands through lymphangiogenesis, a process of new lymphatic vessel sprouting from preexisting structures (Aspelund et al., 2016). VEGF-C/D-activated VEGFR3 signaling is the most central pathway for lymphangiogenesis (Zheng et al., 2014). VEGF-C deficiency leads to lymphatic insufficiency and lymphedema, a defect that can be rescued by VEGF-D during development (Karkkainen et al., 2004); VEGFR3 activation is also critical for pathophysiological lymphangiogenesis following lymphatic injury (Szuba et al., 2002). VEGFR3 missense mutations in the tyrosine kinase domains underlie the etiology of 70% cases of primary congenital lymphedema, known as Milroy disease (Karkkainen et al., 2000; Connell et al., 2009), indicating that the VEGFR3 signaling is important for the normal development of human lymphatic vasculature. The axon guidance protein neuropilin (NRP)-2 enhances VEGFR3 signaling by acting as its co-receptor (Yuan et al., 2002; Xu et al., 2010). By facilitating VEGF-C maturation, the collagen- and calcium-binding EGF domains 1 (CCBE1) protein also promotes lymphangiogenesis (Bos et al., 2011; Jha et al., 2017). Thus, both NRP2 and CCBE1 are factors involved in lymphangiogenesis by regulating the VEGF-C/VEGFR3 signaling.

Following the initial sprouting, lymphatic capillaries mature through activating the Notch signaling pathway (Zheng et al., 2011), resembling the well-known tip–stalk cell paradigm in angiogenesis (Potente et al., 2011). Maturation of collecting lymphatic vessels requires concerted smooth muscle cell recruitment and patterning as well as valve development. Although angiopoietin (ANG)2 antagonizes TIE2 receptor activation during blood angiogenesis, it appears to stimulate TIE2 signaling in LECs and to promote postnatal lymphatic remodeling (Gale et al., 2007); ANG2-deficient mice do not develop normal hierarchical lymphatic vascular system (Dellinger et al., 2008). The forkhead box protein FOXC2 modulates lymphatic capillary development by controlling SMC recruitment and basement membrane formation (Petrova et al., 2004). In coordination with Prox-1, FOXC2 senses lymph flow and induces the expression of gap junction protein connexin 37 (Cx37) and activates calcineurin/nuclear factor of activated T-cells (NFAT) signaling, which regulates lymphatic valve formation (Sabine et al., 2012). While FOXC2 controls ANG2 expression in angiogenesis (Xue et al., 2008), it induces TIE2 in LECs (Thomson et al., 2014), suggesting that FOXC2 may promote lymphatic maturation by regulating the ANG2/TIE2 signaling.

Other signaling pathways, including fibroblast growth factor (FGF)2/FGF receptor (FGFR)1, sphingosine-1-phosphate (S1P)/S1P receptor (S1PR)1, and bone morphogenetic protein (BMP)9/activin receptor-like kinase 1 (ALK1), also regulate lymphangiogenesis (Zheng et al., 2014). Additionally, pro-inflammatory cytokines influence lymphangiogenesis and affect immune activation and resolution. In general, type 1 cytokines including IL-1, IL12, IL-18, and TNF-α promote, and type 2 and anti-inflammatory cytokines such as IL-4, IL-5, IL13, and IL-10 suppress lymphangiogenesis (Sainz-Jaspeado and Claesson-Welsh, 2018). The pro-inflammatory lipid molecule leukotriene B4 (LTB_4) promotes lymphatic regeneration at low concentration but suppresses lymphangiogenesis at high pathological concentrations (Tian et al., 2017). In summary, lymphangiogenesis is regulated by growth factors and cytokines, as well as by interstitial fluid flow (**Figure 1**).

THE INTERPLAY BETWEEN LYMPHATIC VASCULATURE AND OBESITY

Interaction of genetic, epigenetic, environmental, and psychological factors regulates the production of physiological

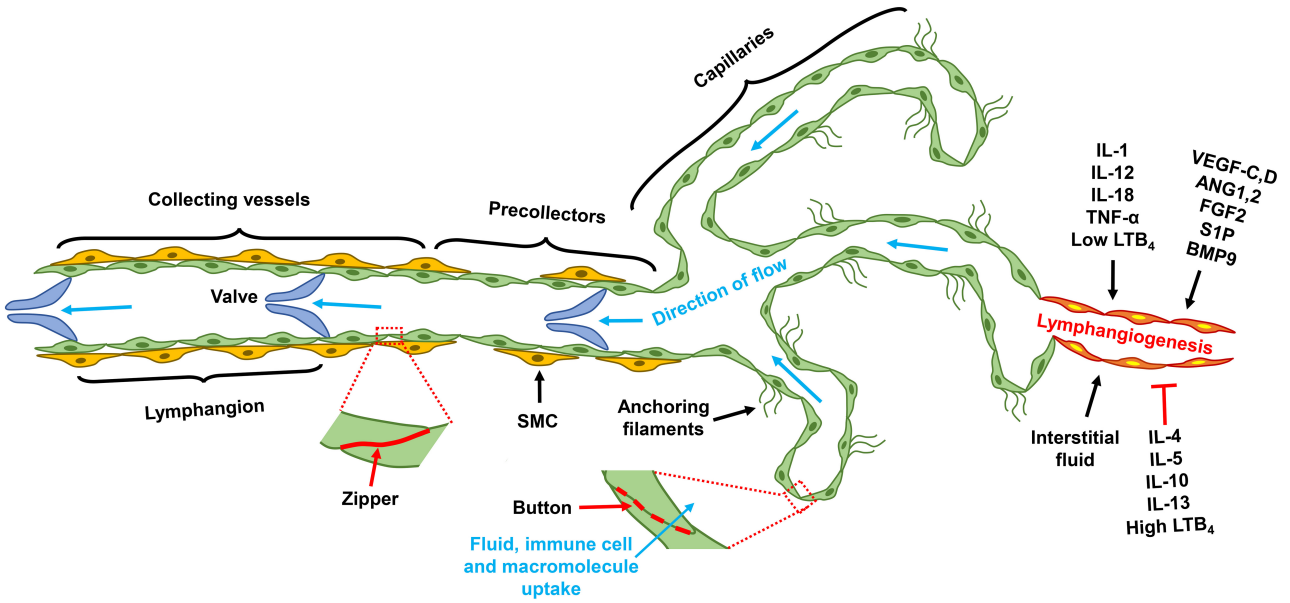

FIGURE 1 | Lymphatic vascular tree and lymphangiogenesis. Lymphatic capillaries are comprised of single layer LECs that tether directly to the extra cellular matrix. Junctional proteins form button structure that interconnects capillary LECs; and those buttons allow the formation of overlapping LEC flap, through which interstitial fluid and macromolecule enter the blind-ends of the capillaries. Capillaries converge into pre-collectors, which in turn coalesces into collecting lymphatics. SMCs loosely cover the precollector, but invest collecting lymphatics more completely. Junctional proteins of the collecting lymphatics form a continuous structure, known as zipper. Collecting lymphatics are comprised of lymphangions that are demarcated by intravascular valves, the segment between two valves is designated as one lymphangion. Factor regulates lymphangiogenesis include pro-lymphangiogenic growth factors, pro-inflammatory cytokines, and interstitial fluid. Type 2 cytokines, and high concentrations of LTB_4, are molecules that suppress lymphangiogenesis. LEC, lymphatic endothelial cell; VEGF, vascular endothelial growth factor; ANG, angiopoietin; FGF, fibroblast growth factor; S1P, sphingosine 1 phosphate; BMP, bone morphogenetic protein; IL, interleukin; LTB_4, leukotriene B_4; TNF, tumor necrosis factor; SMC, smooth muscle cell.

mediators that control the balance of energy intake and expenditure (Gonzalez-Muniesa et al., 2017). When energy intake is in surplus, about 70–80% of the excessive intake is stored as fat, and the remainder is converted into glycogen or protein or lost as heat; long-term positive energy balance ultimately leads to obesity (Oussaada et al., 2019). Genetic mediation of obesity can be monogenic or polygenic. Monogenic mutation is relatively rare and primarily affects the genes involved in the leptin–melanocortin pathway, the central regulator of food intake and energy balance (Oussaada et al., 2019); as examples, the leptin receptor (LEPR) (Farooqi et al., 2007), pro-opiomelanocortin (POMC) (Krude et al., 2003), melanocortin 4 receptor (MC4R) (Krude et al., 2003), and MC3R (Lee et al., 2002) have all been linked to early onset of obesity in humans. Polygenic causation, however, is more common and accounts for >90% cases of childhood-onset obesity (Kleinendorst et al., 2018). Notably, common variants in certain loci within the fat mass and obesity-associated gene (FTO) have been linked to higher BMI in human populations (Claussnitzer et al., 2015; Oussaada et al., 2019). DNA methylation studies have identified epigenetic modification of several genes that are associated with obesity, as detailed elsewhere (Rohde et al., 2019). Whether obesity promotes epigenetic change and further deteriorates the imbalance between energy intake and expenditure to exacerbate obesity is an open question.

The lymphatic vasculature regulates both dietary lipid absorption and peripheral cholesterol removal. The intestinal lacteals are lymphatic vessels comprised of both capillary and collecting lymphatic elements. The lacteals absorb dietary lipids packaged as chylomicrons (Iqbal and Hussain, 2009; Randolph and Miller, 2014). Intravital imaging indicates that lacteals possess a spontaneous contractile feature; they actively absorb and transport enterocyte-processed lipids to the systemic circulation in concert with contractile forces produced by adjacent smooth muscle cells controlled by the autonomic nervous system (Choe et al., 2015). It was recently revealed that lacteal function controls dietary lipid absorption and, consequently, body weight, supporting the concept that the lacteals are the gatekeepers of lipid intake from the environment (McDonald, 2018; Zhang et al., 2018; Cifarelli and Eichmann, 2019).

In peripheral tissues, the lymphatic vasculature is generally considered to be the only route for the return of lipoprotein to the blood circulation (Cooke et al., 2004); removal of interstitial cholesterol by the lymphatic route is known as reverse cholesterol transport (RCT) (Huang et al., 2015). Although both lacteals and peripheral lymphatics may selectively uptake cargoes based on their size (Randolph and Miller, 2014), they may preferentially absorb lipoproteins depending on specific receptor expression. As an example, LEC expression of the scavenger receptor class B type I (SR-B1) is required for RCT in skin lymphatic capillaries

but not for intestinal cholesterol absorption (Bura et al., 2013; Lim et al., 2013). Impaired RCT has been observed in ob/ob mice with induced obesity (Duong et al., 2018), suggesting that defective RCT may be a prerequisite for the development of obesity.

Several lines of evidence support the notion that lymphatic functionality impacts the pathogenesis of obesity. In patients with lymphatic injury-induced (secondary) lymphedema, fat hypertrophy in the lymphedematous tissues is prominent, accompanying tissue swelling and fibrosis (Jiang et al., 2018); the Chy mutant mouse, with its defective lymphatic development, also display abnormal lipid accumulation adjacent to affected hypoplastic lymphatic vessels (Karkkainen et al., 2001). *Prox-1* haploinsufficiency causes lymphatic dysfunction and leads to adult-onset of obesity (Harvey et al., 2005), in those mice, lymphatic restoration rescues them from the development of obesity (Escobedo et al., 2016). Accumulated interstitial fluid, including retrograde lymph leakage from the dysfunctional lymphatics, may promote adipocyte differentiation and enhance local fat deposition (Harvey et al., 2005; Escobedo and Oliver, 2017). Increasing lymphatic density in adipose tissue by overexpressing VEGF-D reduces local immune cell accumulation and improves systemic metabolic responsiveness in high-fat diet (HFD)-induced obese mice (Chakraborty et al., 2019), although it cannot be excluded that VEGF-D may also exert effects on blood vascular cells because it also binds to VEGFR2 (Achen et al., 1998). Increased expression of Apelin, an endogenous peptide identified as a ligand of the orphan G protein-coupled receptor APJ, was shown to inhibit HFD-induced obesity by promoting both lymphatic and blood vascular integrity (Sawane et al., 2013). Collectively, these studies suggest that lymphatic dysfunction promotes obesity, and that improving lymphatic function inhibits the development of obesity and alleviates obesity-caused metabolic syndrome. Studies have also illustrated that obesity promotes lymphatic abnormalities, such as decreased initial lymphatic density, heightened lymphatic leakiness, impaired collecting lymphatic pumping, and diminished macromolecule transport; but those phenotypic and functional changes are reversible in response to dietary modification and weight control (Garcia Nores et al., 2016; Nitti et al., 2016). Decreased collecting lymphatic pumping may result from the perilymphatic accumulation of iNOS-expressing macrophages that can affect lymphatic contractility and damage LECs by conversion of NO to a powerful oxidant, peroxynitrite (Nitti et al., 2016; Torrisi et al., 2016). Consistent with experimental animal data, a clinical study showed that obesity is a risk factor for the development of lymphedema in post-surgical breast cancer patients (Helyer et al., 2010). Also, severely obese individuals often develop acquired lymphedema of the extremities (Greene et al., 2012). *In vitro* cell culture study showed that leptin compromises LEC proliferation and tube formation by enhancing STAT3 phosphorylation, although leptin also induces IL-6, which, on the other hand, promotes lymphatic tube formation. In aggregate, the net effect of high concentrations of leptin on lymphangiogenesis appears to be suppressive (Sato et al., 2016). These findings suggest that high concentrations of leptin produced by adipose tissue maybe responsible for suppressing lymphatic vasculature in obese individuals. In summary, lymphatic dysfunction sensitizes individuals to develop obesity, and obesity worsens lymphatic function.

LYMPHATIC ENDOTHELIAL CELL INSULIN RESISTANCE

Insulin signaling regulates glucose, lipid, and energy homeostasis, predominantly through its action on adipose tissues, liver, and skeletal muscles (Boucher et al., 2014). Insulin exerts its known function by binding to the insulin receptor (INSR) expressed on target cells; ligand engagement leads to INSR autophosphorylation, followed by recruitment of various phosphotyrosine-binding scaffold proteins, which in turn activates downstream effectors (Taniguchi et al., 2006). The most crucial INSR substrates for metabolic regulation are insulin receptor substrate (IRS)1 and IRS2 proteins. They exert downstream effects by activating the PI3K/AKT signaling pathway (Petersen and Shulman, 2018). Insulin signaling in skeletal muscle promotes glucose uptake and net glycogen synthesis; IRS1 appears to be the primary scaffold protein for this process (Bouzakri et al., 2006; Thirone et al., 2006). In the liver, insulin promotes the synthesis of major classes of metabolic macromolecules, including glycogen, lipids, and proteins, and, concurrently, it reduces hepatic glucose production by controlling the PI3K/AKT/GSK3α/β or mTORC1 or FOXO1 signaling cascades (Cherrington et al., 1998; Petersen and Shulman, 2018). In white adipose tissue (WAT), insulin signaling suppresses lipolysis; but its role in glucose uptake is relatively minor, accounting for about 5–10% of whole body glucose uptake (Virtanen et al., 2002; Ng et al., 2012). Attenuation or reversal of cAMP/PKA-mediated lipolysis induced by adrenergic signaling is the best understood mechanism for insulin suppression of lipolysis (Jaworski et al., 2007). Insulin resistance arises under the condition of chronic energy surplus (Samuel and Shulman, 2016). Continuous overnutrition with insulin resistance impairs insulin secretion by pancreatic β-cells, which eventually leads to overt T2D (DeFronzo et al., 2015). At the molecular level, both decreased INSR expression and impaired intracellular signaling transduction contribute to typical obesity-induced insulin resistance (Petersen and Shulman, 2018).

While WAT, liver, and skeletal muscles have been generally recognized as major insulin target tissues, ECs, including both BECs and LECs, are also sensitive to insulin. It was suggested that blood vascular ECs might act as "first-responders" to overnutrition (Barrett and Liu, 2013). Impaired insulin signaling in BECs diminishes AKT-dependent NO production and simultaneously increases Endothelin 1 (ET-1) activity, which leads to endothelial dysfunction (Mather et al., 2002; Federici et al., 2004; Okon et al., 2005; Shemyakin et al., 2006; Muniyappa and Sowers, 2013). Unhealthy microvasculature hampers insulin delivery to muscle and adipose tissue and affects glucose disposal and lipid homeostasis (Barrett and Liu, 2013). LEC insulin signaling has only recently been explored. One study showed that LECs derived from human dermal tissue (HDLECs) express much higher levels of INSR than that of adipose tissue microvascular ECs; an insulin level as low as 2.5 nM can induce

AKT phosphorylation in HDLEC (Jaldin-Fincati et al., 2018), although circulating insulin concentrations in healthy individuals may still well-below the 2.5 nM range, which are around 100 pmol/L (Ford et al., 2006), whether similar responses can be induced at physiological conditions are unknown. Insulin-induced downstream signaling appears to be required for normal lymphatic vascular structure and function (Lee et al., 2018). Diminished LEC insulin signaling decreases eNOS phosphorylation and NO production, reduces mitochondria oxygen consumption, which alters LEC metabolism, and causes increased expression of proinflammatory molecules (Lee et al., 2018); these results suggest that physiological insulin signaling is essential for normal functioning of LECs. Supporting the role of insulin signaling in lymphatic function, blockade of IRS1 suppresses lymphangiogenesis (Hos et al., 2011). Collectively, these studies indicate that insulin signaling likely plays important roles in regulating both LEC metabolism and lymphangiogenesis (**Figure 2**); LEC insulin resistance diminishes lymphatic function, and exacerbates obesity and metabolic abnormality.

LYMPHATIC VASCULAR PATHOPHYSIOLOGY IN T2D

Type 2 diabetes is characterized by dysregulation of carbohydrate, lipid, and protein metabolism caused by impaired insulin signaling resulting from reduced insulin secretion, insulin resistance, or the combination of both (DeFronzo et al., 2015). The lymphatic pathology likely co-evolves with the pathogenesis of diabetes. In an alloxan-induced rat diabetes model, the lymph flow through the thoracic duct in diabetic rats is significantly higher than in that of healthy controls, possibly because increased interstitial glucose levels increase tissue colloid pressure, which then enhances interstitial fluid absorption and lymph production. By contrast, lymph node uptake of ^{99m}Tc−dextran 500 is impaired in diabetic rats, a result that might explain the observation that patients with diabetes often have decreased function for immune priming (Moriguchi et al., 2005). Interestingly, insulin treatment normalizes both lymph flow and lymph node dextran retention, but glucose normalization through diet-control only corrects lymph flow (Moriguchi et al., 2005), suggesting that fluid transport and immune regulatory function of the lymphatics are regulated by discrete mechanisms. It is worth noting that the alloxan model simulates type 1 diabetes (Ighodaro et al., 2017), it nevertheless will provide insights about how hyperglycemia may impact the lymphatic system. In a clinical study, skin specimens of diabetic patients displayed increased lymphatic density, and transcriptional analysis of isolated dermal LECs indicated that cells from diabetic patients exhibit signatures of inflammation, adhesion, migration, growth, and lymphangiogenesis (Haemmerle et al., 2013). Increased lymphatic density found in human patient samples seemingly correlates with the phenomenon observed in diabetic rats that have enhanced lymphatic return to the systemic circulation. However, it is possible that the lymphatic function may decompensate after prolonged interstitial fluid overload caused by hyperglycemia (Kanapathy et al., 2015). Increased lymphatic collecting vessel permeability caused by diminished NO availability appears to be a contributing factor for lymphatic dysfunction (Scallan et al., 2015).

One common complication of diabetes is diabetic retinopathy, which is characterized by a pathology that involves vascular, glial, and neuronal components and causes significant visual loss (Duh et al., 2017). A recent study investigating the lymphatics in diabetic retinopathy detected the expression of LEC markers, such as VEGFR3 and Prox-1, in excised human specimens (Loukovaara et al., 2015), providing the first clinical evidence that abnormal lymphatic growth, in addition to pathological microvascular remodeling, occurs in diabetic retinopathy. A follow-up study demonstrated that soluble pro-growth factors in vitreous fluid promoted lymphatic sprouting of patient-derived tissues with diabetic retinopathy (Gucciardo et al., 2018a), further supporting the notion that the microenvironment of the eyes of diabetic retinopathy contains sufficient cue for abnormal lymphatic expansion. Pathological lymphangiogenesis observed in the eyes of diabetic retinopathy may, therefore, be regarded as a feature for therapeutic targeting (Yang et al., 2016; Gucciardo et al., 2018b). Ongoing clinical trials targeting VEGFR- or RTK-mediated signaling may provide possibilities to suppress pathological lymphatic overgrowth, but identification of novel lymphatic specific targets is probably necessary for better therapeutic outcomes (Williams et al., 2010).

LYMPHATICS IN ATHEROSCLEROSIS AND MYOCARDIAL INFARCTION

The metabolic syndrome poses a significant risk for the development of cardiovascular diseases, such as atherosclerosis and its severe complication, myocardial infarction (MI) (Wilson et al., 2005; Mottillo et al., 2010). Following the revelation of the interplay between metabolic syndrome and lymphatic dysfunction, a multiplicity of research has shown that the lymphatic vasculature is also actively involved in the progression of atherosclerosis and development of MI (Aspelund et al., 2016). Atherosclerosis is a chronic inflammatory disease of the arterial wall with dyslipidemia as the root cause. The atherosclerotic lesion is characterized by a prominent population of lipid-filled foam cells, which are macrophages that contain an excess of plasma-derived, modified lipoproteins (Back et al., 2019). In this inflammatory pathology, the balance between pro- and anti-inflammatory molecules dictates whether a nascent atherosclerotic lesion will reverse course to normal or progress to more advanced stages. Among all the inflammatory cells that populate an atherosclerotic locus, the lipid-laden macrophage is central to disease progression. Macrophage uptake and modification of lipoprotein are physiologically important for lipid removal from intima, but uncontrolled, excessive lipid infiltration and inflammation exhausts macrophages and creates a hypoxic environment; this, in turn, promotes macrophage death and release of oxidized lipid and perpetuates the vicious cycle of foam cell formation, death, and intimal lipid accumulation (Tabas, 2010). Tipping the balance of local inflammation to an

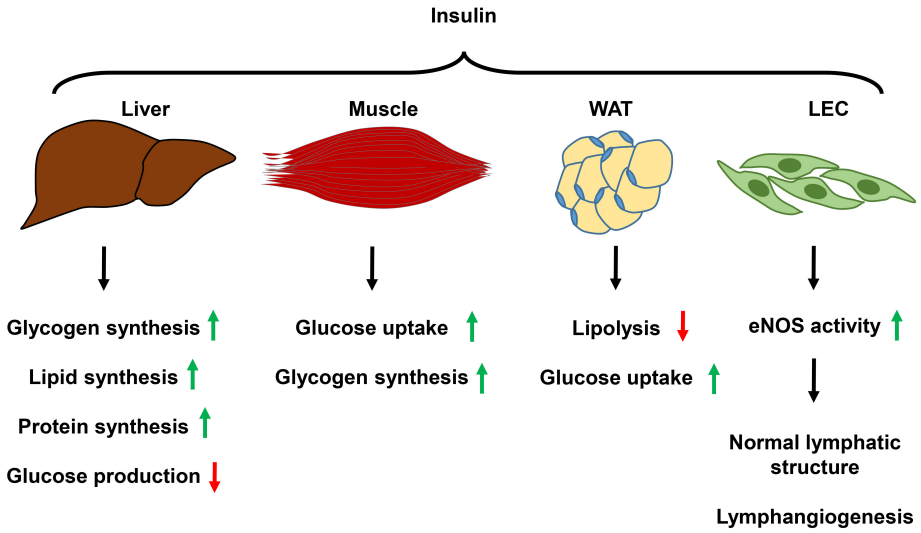

FIGURE 2 | Summary of the function of insulin signaling in metabolic organs and LECs. Insulin signaling in the liver promotes glycogen, lipid, and protein synthesis while decreases glucose production. In the skeletal muscle, insulin mediates glucose uptake and glycogen synthesis. In white adipose tissue, insulin suppresses lipolysis and promotes glucose uptake. Insulin activation of its receptor in LECs promotes eNOS activity, which keeps normal lymphatic structure, and promotes lymphangiogenesis. WAT, white adipose tissue; LEC, lymphatic endothelial cell; eNOS, endothelial nitric oxide synthase.

anti-inflammatory profile is, therefore, a therapeutic concept in treating atherosclerosis (Ruparelia et al., 2017). Recent studies have delved into lymphatic biology in the pathogenesis of atherosclerosis, presumably because the lymphatics play an essential role in immune trafficking and lipid transport (Milasan et al., 2015; Csanyi and Singla, 2019).

Lymphatic vessels are present in the adventitial and periadventitial regions of arterial walls (Sacchi et al., 1990; Martel et al., 2013; Milasan et al., 2015). Early observations that associated diminished lymphatic drainage to atherosclerosis were reported nearly three decades ago (Miller et al., 1992; Solti et al., 1994). Utilizing a mouse aorta transplantation model, the lymphatic vasculature was shown to be critical for RCT in arterial wall of the large vessel (Martel et al., 2013). Another study demonstrated that mice fed a HFD were prone to atheroma formation when the lymphatic vasculature is defective (Vuorio et al., 2014), supporting the notion that lymphatic dysfunction diminishes cholesterol removal and promotes atherosclerosis. In atherosclerosis-prone $Ldlr^{-/-}$; $ApoB100^{+/+}$ mice, lymphatic dysfunction, mainly of the collecting lymphatic vessels, occurs before the onset, and during the progression, of atherosclerosis (Milasan et al., 2016), suggesting that lymphatic vascular abnormalities likely promote atherosclerosis. In agreement with these hypotheses, the rescue of lymphatic vasculature during the early phase of atherogenesis retards disease progression, through reducing tissue inflammation and, likely also through increased lymphatic cholesterol transportation (Milasan et al., 2019). These studies in aggregate link lymphatic vascular functionality to atherosclerosis pathogenesis.

While the lymphatic vasculature is critical for immune trafficking and immune regulation, inflammatory molecules, produced by infiltrated local immune cells, also impact lymphatic vascular remodeling and function. Targeting inflammation may, therefore, not only ameliorate tissue inflammation but also improve lymphatic function. In a mouse tail surgery-induced tail lymphedema model, we have previously shown that high concentrations of LTB_4 sustain local tissue inflammation, which is characterized by infiltration of cells of both innate and adaptive immunity; blockade of LTB_4 signaling not only reduces tissue inflammation but also improves lymphatic function and alleviates lymphedema (Tian et al., 2017). In a series of *in vivo* and *in vitro* experiments, we showed that >100 nM LTB_4 induces LEC apoptosis and suppresses lymphangiogenesis, while LTB_4 in the 10 nM range enhances lymphatic regeneration; a physiological LTB_4 level appears to be essential for surgery-induced wound healing (Tian et al., 2017); these experiments indicate that inflammation and lymphatic dysfunction are closely associated. LTB_4 is an important proinflammatory molecule that promotes atherosclerosis by magnifying monocyte chemotaxis and foam cell formation (Subbarao et al., 2004), LTB_4 may also directly impact endothelial survival and angiogenesis (Tian et al., 2013) and exacerbates insulin resistance (Li et al., 2015). It is therefore possible that targeting the LTB_4 signaling pathway may simultaneously limit tissue inflammation, boost both lymphatic and blood vasculature, increase insulin sensitivity, and normalize cellular metabolism. Several Phase I atherosclerosis clinical trials targeting the LTB_4 pathway are underway (Bhatt et al., 2017).

Myocardial infarction can be triggered by rupture or erosion of vulnerable atherosclerotic plaque, which results in blood clot formation by the exposure of thrombogenic core and matrix components of plaque (Anderson and Morrow, 2017). MI is followed by robust inflammatory responses that can be categorized into the initial pro-inflammatory responses that function to remove necrotic debris, and reparative anti-inflammatory responses poised to repair the damaged tissue (Ong et al., 2018). Targeting the inflammatory response

in different phases of MI appears to be logical, but several such interventions have failed to improve patient outcomes (Ong et al., 2018), indicating additional mechanisms are likely at play for post-MI recovery. A recent study revealed significant lymphangiogenic responses following MI. Promotion of lymphangiogenesis by exogenous administration of VEGFC results in a transient improvement of post-MI myocardial function (Klotz et al., 2015). Improved outcome by VEGFC-induced lymphangiogenesis depends on lymphatic-mediated immune cell clearance through a pathway involving LEC expressed LYVE-1 (Vieira et al., 2018). Consistent with a protective role of the lymphatic vasculature in promoting post-MI recovery, downregulation of the LEC marker VEGFR3 alters cardiac lymphatic structure, increases lymphatic leakage, and raises MI-induced mortality (Vuorio et al., 2018). These recent studies suggest that the lymphatic vasculature might be a viable therapeutic target for post-MI cardiac repair (Vuorio et al., 2017).

CONCLUDING REMARKS

Type 2 diabetes and cardiovascular diseases associated with obesity are the leading cause of death in the developed world (Benjamin et al., 2019). There is an unmet need to improve the medical care for these patients. Our understanding of mechanisms underlying these diseases has grown substantially but remains incomplete. Promoting lymphatic function has the apparent capacity to reduce pathology in preclinical obesity and cardiovascular disease models. More in-depth study of lymphatic biology is therefore urgently needed. Discoveries that derive from these investigations will likely provide novel therapeutic targets and improve disease survival.

AUTHOR CONTRIBUTIONS

XJ contributed to the writing and editing of the manuscript, and creation of figures. WT and MN contributed to the concepts and editing of the manuscript. SR contributed to the concepts, editing, and final formatting of the manuscript.

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Why Do Men Accumulate Abdominal Visceral Fat?

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Men have a higher tendency to accumulate abdominal visceral fat compared to pre-menopausal women. The accumulation of abdominal visceral fat in men, which is a strong independent predictor of mortality, is mainly due to the higher dietary fat uptake by their abdominal visceral fat. Since dietary fat is absorbed by the enterocytes and transported to the circulation in the forms of chylomicrons and very low density lipoproteins (VLDLs), it is crucial to understand how these lipoproteins are different between men and women. The chylomicrons in men are generally bigger in size and more in quantity than those in women. During the postprandial state, these chylomicrons congest the lamina propria and the low-pressure lymphatics. In this paper, we propose that this congestion predisposes the chylomicron triglycerides to hydrolysis by lipoprotein lipase (LPL). The liberated fatty acids are then stored by the nearby abdominal visceral adipocytes, leading to the accumulation of abdominal visceral fat. These mechanisms perhaps explain why men, through their bigger and higher production of chylomicrons, are more likely to accumulate abdominal visceral fat than pre-menopausal women. This accumulation eventually leads to belly enlargement, which confers men their apple-shaped body.

Keywords: visceral, adipocyte, gender, lipoprotein, chylomicron, intestine, absorption, belly

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BODY FAT

The sex differences in body composition have been well established (Karastergiou et al., 2012; Palmer and Clegg, 2015). Women have a higher body fat percentage than men, and men have a higher muscle mass percentage than women. As the BMI increases in both sexes, the body fat percentage remains higher in women than men (Schorr et al., 2018). Importantly, the differences between the two sexes are not only in the percentage of total body fat but also in its distribution to the different parts of the body (Grauer et al., 1984).

Types of Body Fat

Body fat can be classified into brown, beige, and white fat (Harms and Seale, 2013). In terms of relative abundance in mitochondria, brown fat is the most abundant and white fat is the least. Unlike brown and beige fat, white fat is not capable of thermogenesis. Note that body fat in adult humans consists of mostly white fat.

Abbreviations: Angptl4, angiopoietin-like 4; BMI, body mass index; GPIHBP1, glycosylphosphatidylinositol-anchored high density lipoprotein-binding protein 1; HSPGs, heparin sulfate proteoglycans; LPL, lipoprotein lipase; VLDL, very low density lipoprotein.

Based on its location in the body, white fat can be further subcategorized into subcutaneous, visceral, and ectopic fat. Ectopic fat, which is the least in quantity, is located within the internal organs. Intrahepatocellular fat, intrapancreatic fat, intramyocellular fat, and intracardiomyocellular fat are all considered as ectopic fat. The fat that surrounds the internal organs is generally considered as visceral fat. The epicardial fat and the abdominal visceral fat surround the myocardia and gastrointestinal organs, respectively, and are both considered as visceral fat (Bertaso et al., 2013; Frank et al., 2018). Subcutaneous fat, which is more abundant in women (Karastergiou et al., 2012), is located throughout the layer deep to the skin (hypodermis).

As visceral fat in the abdomen accumulates, the belly becomes visibly bigger—a phenomenon that is commonly known as belly fat development. Of note, belly fat consists of not only abdominal visceral fat but also abdominal subcutaneous fat. Although waist circumference correlates strongly with total belly fat, it does not correlate as strongly with abdominal visceral fat (Grundy et al., 2013). Furthermore, the correlation of waist circumference with abdominal visceral fat is weaker in women than in men. Therefore, the inference of the amount of abdominal visceral fat from waist circumference should be made cautiously, especially in women.

Abdominal Visceral Fat

This paper focuses specifically on the visceral fat in the abdomen. In order to understand abdominal visceral fat, a closer look at the anatomy of mesenteries and retroperitoneum is warranted (see **Figure 1**). Mesenteries connect the gastrointestinal organs that are located within the abdominal cavity to the wall of the abdominal cavity. Most of the connections are made to the posterior rather than the anterior wall of the abdominal cavity. As such, organs that reside within the posterior wall of the abdominal cavity do not have any mesenteries. These organs are commonly known as retroperitoneal organs. The two retroperitoneal organs depicted in **Figure 1** are pancreas and duodenum. The fat that surrounds these retroperitoneal organs is known as retroperitoneal fat. Note that other retroperitoneal organs, such as kidneys, ascending colon, and descending colon, are not shown in **Figure 1**.

In addition to adhering the gastrointestinal organs to their abdominal wall, mesenteries protect numerous nerves, blood vessels, and lymphatic vessels of the gastrointestinal system. Importantly, mesenteries are also capable of storing a significant amount of fat. The greater omentum, lesser omentum, mesentery proper, and mesocolon are examples of mesenteries. As indicated in **Figure 1**, the fat that is located within these mesenteries is known as the greater omental fat, lesser omental fat, mesenteric fat, and mesocolonic fat, respectively. The fat in these mesenteries is collectively referred to as intraperitoneal fat.

Both intraperitoneal fat and retroperitoneal fat constitute abdominal visceral fat, which explains why many investigators include retroperitoneal fat when measuring abdominal visceral fat (Hung et al., 2014). There are several reasons to consider the retroperitoneal fat as part of the abdominal visceral fat. First, retroperitoneal fat surrounds retroperitoneal organs. Thus, it should be categorized as visceral instead of ectopic or subcutaneous fat. Second, the lymph fluid of the gastrointestinal tract drains through the smaller lymphatic vessels within the mesenteries before entering the larger lymphatics. The larger lymphatics, such as cisterna chyli, are retroperitoneal. Consequently, the adipocytes that are present in the mesenteries and retroperitoneum receive the same supply of lipid-rich chyle. This chyle will eventually be drained into the systemic blood circulation before supplying the subcutaneous fat. Hence, from the nutrient supply perspective, retroperitoneal fat is more similar to intraperitoneal fat than subcutaneous fat. For further discussions on the anatomy of the gastrointestinal circulation, please refer to our previously published paper (Nauli and Nauli, 2013). Third, unlike subcutaneous fat, both retroperitoneal and intraperitoneal fat increase the risk of metabolic syndrome (Hung et al., 2014). Therefore, it is conceivable that abdominal visceral fat should include both intraperitoneal and retroperitoneal fat.

The fact that abdominal visceral fat is often simply referred to as "visceral fat" can sometimes cause confusion. When the amount of "visceral fat" is measured from the abdominal region, it is arguably more appropriate to label it as abdominal visceral fat instead of "visceral fat." As mentioned above, visceral fat includes not only the abdominal visceral fat but also the fat depots that surround other non-abdominal organs, such as the epicardial fat. **Figure 2** shows how different types of body fat relate to abdominal visceral fat.

Women Are Shaped Like a Pear and Men Like an Apple

Due to their tendency of accumulating abdominal visceral fat (Grauer et al., 1984), men are more likely to develop an apple-shaped body. The excessive accumulation of abdominal visceral fat is also known as android obesity. In contrast, the pear-shaped is often ascribed to pre-menopausal women because of their tendency of accumulating subcutaneous fat in the thigh (femoral) and buttock (gluteal) regions (Karastergiou et al., 2012). The obesity resulted from the predominant subcutaneous fat accumulation is also known as gynoid obesity.

A common misconception is that beer consumption can specifically lead to belly fat accumulation. This misconception gave rise to the term "beer belly." Studies have shown that beer consumption does not specifically increase the belly size but rather increases the overall body weight (Schutze et al., 2009). Therefore, it is unlikely that beer consumption specifically increases the abdominal visceral fat or is particularly responsible for android obesity.

Abdominal Visceral Fat Is Associated Strongly With Metabolic Abnormalities

Although the abdominal subcutaneous fat and the intrahepatocellular fat are associated with a higher risk of mortality in men, only the abdominal visceral fat is a strong independent predictor of mortality in men (Kuk et al., 2006). The association of abdominal visceral fat with mortality is not unique to men as abdominal visceral fat is also a strong predictor of mortality in obese women (Koster et al., 2015). Consequently,

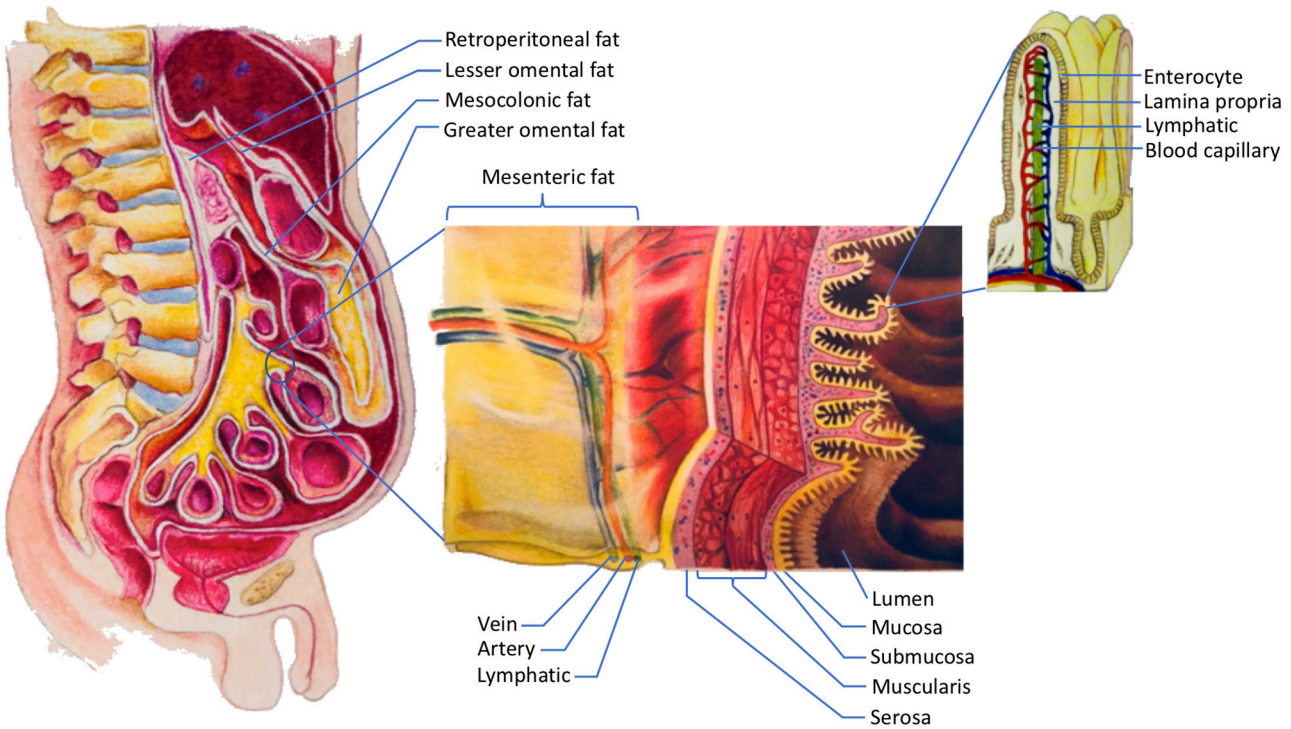

FIGURE 1 | The storage of dietary triglycerides by abdominal visceral fat. Dietary triglycerides are digested and taken up by the enterocytes that line the intestinal lumen. The enterocytes secrete the dietary triglycerides in the form of VLDLs and chylomicrons to the lamina propria. Within the lamina propria, there are numerous blood capillaries (red/blue) and lymphatic capillaries (green). Due to their smaller size, some of the VLDLs can readily enter the lumen of the blood capillaries. In contrast, chylomicrons that are produced more by males are more likely to be retained in the lamina propria as they attempt to gain access to the lumen of the lymphatics. The higher retention of chylomicrons in the lamina propria predisposes their triglycerides to LPL hydrolysis. The liberated fatty acids, which are the products of LPL hydrolysis, can then be delivered to the abdominal visceral adipocytes that are located within the retroperitoneum and mesenteries. The fat that lies within the retroperitoneum is called retroperitoneal fat, and the fat in the mesenteries is known as intraperitoneal fat. The intraperitoneal fat depots shown here are mesocolonic, lesser omental, greater omental, and mesenteric fat. Note that the liberated fatty acids supply the abdominal visceral adipocytes prior to the subcutaneous adipocytes. VLDL = very low density lipoprotein; LPL = lipoprotein lipase.

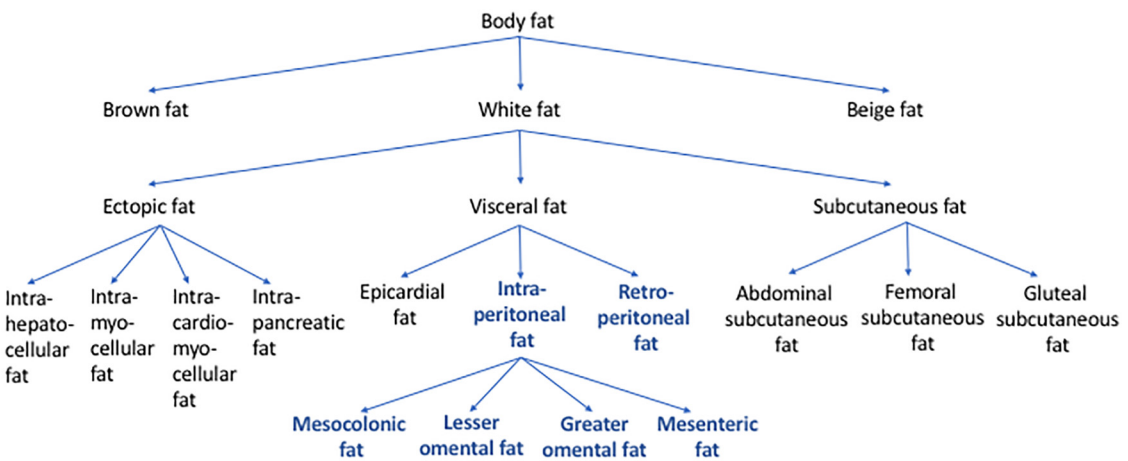

FIGURE 2 | Types of body fat in relation to abdominal visceral fat. Body fat can be categorized into brown, beige, and white fat. Based on its location, white fat can be further categorized into ectopic, subcutaneous, and visceral fat. Some of the examples of ectopic fat are intrahepatocellular, intrapancreatic, intramyocellular, and intracardiomyocellular fat. Subcutaneous fat includes abdominal, femoral, and gluteal subcutaneous fat. Visceral fat includes epicardial, retroperitoneal, and intraperitoneal fat. The intraperitoneal fat can be further subcategorized into mesocolonic, lesser omental, greater omental, and mesenteric fat. Abdominal visceral fat includes both retroperitoneal and intraperitoneal fat (bold/blue).

it is important to understand the pathogenesis of abdominal visceral fat and its association with metabolic complications.

Obesity can impede the functions of microvasculature. Studies in male hamsters have revealed that android obesity is associated not only with insulin resistance but also a diminution of capillary density and an increase in macromolecular permeability (Costa et al., 2011). This microvascular dysfunction may eventually lead to the development of hypertension (Covassin et al., 2018), which is one of the criteria of metabolic syndrome. Interestingly, the male hamsters that were subjected to the high-fat diet in these studies accumulated fat almost exclusively in the abdominal visceral region with minimal fat accumulation in the subcutaneous region. This important observation suggests that the high intake of dietary fat in men promotes fat accumulation that is rather specific to the abdominal visceral depot. The increase in vascular permeability in the android obesity described above (Costa et al., 2011) also suggests that fat accumulation in the abdominal visceral depot is caused by the dysregulation of the vascular endothelial cells. In this regard, the endothelial cells that line the affected vasculatures may be a key contributor in the development of android obesity as suggested in a recent proposed two-way communication hypothesis of vascular dysfunction in obesity (Graupera and Claret, 2018).

We have previously described how android obesity may lead to insulin resistance (Nauli, 2012). As the abdominal visceral fat accumulates, macrophages infiltration increases (Xu et al., 2003). The infiltrating macrophages are known to release inflammatory cytokines. These cytokines, which include TNFα, are capable of causing the surrounding abdominal visceral adipocytes to become insulin resistant and liberate their fatty acids (Samuel and Shulman, 2016). This flux of fatty acids is detrimental to the liver and pancreas (Matsuzawa et al., 1995). Note that the flux of fatty acids to the liver also occurs after a bolus feeding of triglycerides. Bolus feeding of fat has been shown to increase unesterified fatty acid concentration in the portal vein (Kristensen et al., 2006). Therefore, it is possible that both the abdominal visceral fat and the frequent consumption of high-fat diet contribute to the pathogenesis of metabolic syndrome through the increased flux of fatty acids to the portal venous circulation.

Perhaps it is worthwhile to examine the results of the omentectomy studies. One of the earliest omentectomy studies shows that patients who received omentectomy and adjustable gastric banding had a better metabolic profile than those with the adjustable gastric banding alone (Thorne et al., 2002). The patients with omentectomy and adjustable gastric banding lost more weight than the patients with adjustable gastric banding alone, albeit not significantly. Studies by Dillard et al. (2013) similarly show that omentectomy improved the metabolic profile without causing significantly more weight loss than the control group. On the contrary, studies by Fabbrini et al. (2010) show that omentectomy did not improve the metabolic profile when the amount of weight loss was matched between the omentectomy and the control groups. Several other studies did not also show any significant metabolic improvements in the omentectomy group (Csendes et al., 2009; Sdralis et al., 2013; Andersson et al., 2017). The conflicting results reported from these omentectomy studies could be due to several factors: the limited number of the participants, the pre-existing metabolic conditions of the participants, the procedures during surgery, the time intervals selected to assess the metabolic outcome of the omentectomy, and/or the participants' dietary lifestyle.

Based on the discussion above, it can be concluded that more studies are needed to elucidate the exact roles of the abdominal visceral fat in the pathogenesis of metabolic syndrome. It is clear, however, that abdominal visceral fat is associated with many detrimental effects (Booth et al., 2014; Santosa and Jensen, 2015).

MEN'S GUT FAVORS ABDOMINAL VISCERAL FAT ACCUMULATION

The underlying mechanisms of why men are more likely than pre-menopausal women to accumulate abdominal visceral fat remain unclear. Evidence indicates that once dietary fat is absorbed by the gut, the intestinal lipoproteins produced between males and females are not identical (Vahouny et al., 1980). How these intestinal lipoproteins may contribute to the sex differences in the regional body fat distribution will be discussed below.

More Uptake of Fat by Men's Abdominal Visceral Fat Depot

Accumulation of fat is the result of a higher calorie intake relative to the energy expenditure. From the adipocyte perspective, this corresponds to more uptake of nutrients than the breakdown of fat by adipocytes. The fat catabolism of adipocytes, also known as lipolysis, is mediated partly by epinephrine. Upon binding of epinephrine to β adrenergic receptors, lipolysis is stimulated. On the contrary, the binding of epinephrine to α2A adrenergic receptors results in the inhibition of lipolysis (Richelsen, 1986). In essence, β receptors are lipolytic and α2A receptors are anti-lipolytic.

Studies have shown that estrogen reduces the lipolysis in the gluteal subcutaneous adipocytes (Gavin et al., 2013). The reduced lipolysis in the gluteal subcutaneous adipocytes in women is likely due to estrogen receptor α-mediated increase in α2A receptors. The estrogen-stimulated increase of these anti-lipolytic receptors in the subcutaneous adipocytes, but not in the abdominal visceral adipocytes, may contribute to the more pronounced lipolysis in the abdominal visceral adipocytes relative to the subcutaneous adipocytes in women (Pedersen et al., 2004).

The net fat accumulation in a particular fat depot, however, depends not only on its adipocyte lipolysis but also on the nutrient uptake of its adipocytes as well as its total number of adipocytes. Since estrogen is capable of stimulating human pre-adipocyte proliferation (Anderson et al., 2001), the reduced gluteal subcutaneous adipocyte lipolysis may not necessarily lead to an overall reduction in the lipolysis of the gluteal subcutaneous fat depot. In fact, studies comparing the lipolysis and nutrient uptake of various fat depots indicate that women have more lipolysis than men in the lower body fat depot, whereas men have more lipolysis than women in the abdominal visceral fat depot (Santosa and Jensen, 2008). The studies suggest that relative to lipolysis, fat uptake contributes more significantly to sex differences in body fat distribution. In other words, women

accumulate more fat in the subcutaneous depot primarily because that depot takes up more fat in women than men. Likewise, men accumulate more fat in the abdominal visceral depot because their fat depot takes up more fat than women.

Some factors contributing to the tendency of non-obese women to accumulate subcutaneous fat include their high LPL activities in subcutaneous fat depots (Arner et al., 1991) and their high catabolic rate of hepatic-derived lipoproteins (Matthan et al., 2008). LPL activities are critical for body fat accumulation (Serra et al., 2017) because most of the fatty acids that are taken up by the fat depots are derived from the hydrolysis of lipoprotein triglycerides (Weinstock et al., 1997). The high LPL activities in the lower part of women's body are evident in both preprandial and postprandial state (Votruba and Jensen, 2006). A recent study shows that testosterone is capable of suppressing the LPL activity and fat storage in the femoral region (Santosa et al., 2017). Furthermore, it shows a significant correlation between the LPL activity and the fat storage, implying that women's tendency to accumulate fat in their subcutaneous depots is due to their high subcutaneous fat LPL activities. Another factor that promotes subcutaneous fat accumulation in non-obese women is their high hepatic-derived lipoprotein catabolic rate, which partly explains why they have lower plasma concentrations of apolipoprotein B-100 (Watts et al., 2000) and triglycerides (Mittendorfer et al., 2016). In addition to their high catabolic rate, women are also capable of secreting triglyceride-rich VLDLs when their liver is challenged with more fat (Hodson et al., 2015). Consequently, women are more effective than men in redirecting fat storage from liver to subcutaneous fat (Palmisano et al., 2018).

It can be concluded that women accumulate more fat in the subcutaneous depot because they have higher subcutaneous fat LPL activities and higher catabolic rate of hepatic-derived lipoproteins. The factors that allow men to accumulate more fat in the abdominal visceral depot will be discussed below.

Dietary Fat Is Preferentially Stored as the Abdominal Visceral Fat by Men

The fat that is taken up by the adipocytes is primarily from lipoproteins, lipid particles with triglycerides in their core. The fact that men and women have different intestinal lipoproteins can potentially determine which body fat depot the dietary fat will be deposited to.

The organ that arguably secretes the most amount of fat is the small intestine, particularly during the postprandial state. Recall that the small intestine is surrounded by the abdominal visceral fat. Therefore, it is not surprising that the abdominal visceral fat can take up quite a significant amount of dietary fat from the intestinal lipoproteins. Studies have shown that about 21% of the ingested fat is stored in the intraperitoneal fat and about 6% of it is stored in the retroperitoneal fat by men (Marin et al., 1996). In contrast, only about 5% of the ingested fat is stored in the intraperitoneal fat by women (Votruba et al., 2007). These studies further support the notion that sex difference in regional body fat distribution is primarily determined by fat uptake rather than lipolysis.

Men Produce Bigger and More Chylomicrons Than Women

To better understand why men's abdominal visceral fat takes up more dietary fat than women's, a closer look at the process of dietary fat absorption is necessary. Dietary fat is digested and absorbed by the small intestine. The absorbed dietary fat is secreted by the enterocytes in two major forms: chylomicrons (>80 nm in diameter) and VLDLs (30–80 nm).

Unlike VLDLs that can be produced during the preprandial states, the production of chylomicrons is primarily driven by dietary fat intake (Nauli et al., 2003, 2006, 2014; Drover et al., 2005). Importantly, when the small intestine is challenged with a higher amount of fat, it will produce bigger chylomicrons (Lo et al., 2008). These bigger chylomicrons tend to accumulate in the intestinal mucosa, as reflected by the higher recovery of the intraduodenally infused lipids in the intestinal mucosa, lower recovery in the lymph, and minimal recovery in the lumen at the end of the 6-h study. Since men generally consume a higher amount of dietary fat due to their higher energy intake (Wright and Wang, 2010), they are expected to produce bigger and more chylomicrons than women.

Studies comparing the postprandial chylomicrons in the plasma, in fact, indicate that chylomicrons transport significantly more dietary fat in men than in women (Knuth and Horowitz, 2006). The elevated plasma level of postprandial chylomicrons is also more prolonged in men than in women, suggesting further that it takes more time for men to transport bigger chylomicrons to the general circulation. Since the male participants were provided with more dietary fat in the studies, it remains to be determined if the reported effects were primarily due to their higher intake of fat. However, considering that men do normally have a higher dietary fat intake than women (Wright and Wang, 2010), these studies are still highly relevant physiologically. Of note, all of the female participants in the studies were in their follicular phase.

Since the serum level of estrogen is significantly higher than progesterone in the mid-to-late follicular phase, the estrogen effect on the size of chylomicrons warrants more investigation. Studies utilizing rodents indicate that even with a comparable amount of fat entering the lumen of the digestive tract, the chylomicrons produced by males transport more dietary fat than those produced by females; and the VLDLs produced by females transport more dietary fat than those produced by male (Vahouny et al., 1980).

From the animal to human studies discussed above, we can conclude that men transport more dietary fat through chylomicrons most likely because these chylomicrons are bigger and more than those of women. These sex differences in lipoprotein secretion can be further attributed to men's higher intake of fat and possibly hormonal regulation.

Chylomicrons May Preferentially Promote the Accumulation of the Abdominal Visceral Fat

There are several similarities and differences between chylomicrons and intestinal VLDLs. Regarding their similarities,

both are apolipoprotein B-48-containing lipoproteins. They are also secreted to the capillary-rich lamina propria by the enterocytes (see **Figure 1**). However, their transport routes are quite different, which may determine the fat depot they will preferentially supply their dietary fat to.

Chylomicrons preferentially promote the accumulation of the abdominal visceral fat. High-fat meal, which triggers more chylomicron production, decreased the proportions of meal fat stored in the subcutaneous fat of both men and women (Votruba and Jensen, 2006). The unaccounted for meal fat in that study was likely stored in the abdominal visceral fat. It is important to note that the unaccounted for meal fat was found previously to be correlated with the amount of abdominal visceral fat (Romanski et al., 2000).

Chylomicrons Are Retained Longer in the Intestinal Mucosa

Since the lymphatic capillaries of the intestine allow bigger particles to enter their lumen relative to the blood capillaries, the lymphatic system is the predominant transport route for most of the chylomicrons. In addition to the transport by the lymphatics, VLDLs are capable of entering the lumen of the blood capillaries (Takahara et al., 2013). Particles as large as 30 nm in diameter have been shown to pass through the walls of the abdominal visceral blood capillaries (Simionescu et al., 1972). In fact, up to about 39% of the dietary triglycerides have been shown to be transported by the portal blood circulation (Mansbach et al., 1991; Mansbach and Dowell, 1993). Due to the fact that chylomicrons transport more dietary fat in males than in females, it is not surprising that males also have a higher lymphatic transport of dietary triglycerides than females (Vahouny et al., 1980).

Relative to the blood circulation, the lymphatic circulation is a low-pressure system that depends on the contraction of the surrounding muscles, such as the diaphragm and other abdominal muscles, to propel the lymph fluid. Consequently, the movement of the chylomicrons within the lamina propria and low-pressure lymphatics becomes a challenge during the postprandial state. This, again, is supported by the prolonged elevation of plasma chylomicrons in men (Knuth and Horowitz, 2006) as well as the higher recovery of the infused lipids in the intestinal mucosa when the intestine is challenged with more fat (Lo et al., 2008). Abdominal visceral fat accumulation has also been shown to correlate significantly with the delayed in postprandial lipid metabolism (Taira et al., 1999), supporting the notion that bigger chylomicrons that are retained longer in the intestinal mucosa promote the accumulation of the abdominal visceral fat.

Electron microscopy studies also show that during the postprandial state the lipoproteins in the lamina propria are bigger than those in the lymphatic lumen, further supporting the notion that chylomicrons do not enter the lymphatic lumen as readily as their smaller counterparts VLDLs (Takahara et al., 2013). Importantly, unlike the lumen of the blood capillaries, the lamina propria and the lymphatic lumen are visibly congested with chylomicrons.

Chylomicron Triglycerides Are Likely Susceptible to Hydrolysis in the Gut

The retention of chylomicrons in the lamina propria may predispose their triglycerides to the LPL hydrolysis. Although it has been reported that mesenteric fat expresses LPLs (Shimomura et al., 1993), it is still not known if the LPL activity in the abdominal visceral fat is different between men and women. As mentioned above, the LPL activities in the subcutaneous fat depots are generally higher in women, particularly in the lower part of the body (Arner et al., 1991; Votruba and Jensen, 2006).

LPLs can be tethered in the extracellular matrix by the HSPGs (Young et al., 2011). Therefore, it is tempting to speculate that relative to VLDLs, chylomicron triglycerides are more likely to be hydrolyzed by LPLs due to their higher retention in the lamina propria. In this regard, HSPGs may not simply serve as LPL reservoir for GPIHBP1 tethering on the capillary endothelia but may directly facilitate the LPL hydrolysis of the chylomicron triglycerides.

Alternatively, the LPLs that are present in the lymph (Huang et al., 1990; Qin et al., 2011) may also hydrolyze the chylomicron triglycerides. Angptl4, which is secreted by adipocytes and liver during fasting (Cushing et al., 2017), inhibits the LPLs that are not stabilized by GPIHBP1 (Sonnenburg et al., 2009; Lichtenstein et al., 2010). This autocrine/paracrine inhibition prevents fat accumulation by adipocytes during fasting. As the Angptl4 expression is reduced during postprandial state, the chylomicron triglycerides can then be potentially hydrolyzed by the disinhibited LPLs in the lymph.

Interestingly, the LPL expression in the mesenteric fat can be reduced by exercise (Shimomura et al., 1993). This suggests that exercise does not simply increase the energy expenditure but may also inhibit the dietary fat uptake by the abdominal visceral fat. Additionally, exercise may help reduce the retention of the chylomicrons in the intestinal mucosa (Havas et al., 1997, 2000). Contraction of the surrounding muscles during exercise should help propel the congested chylomicrons into the lymph and reduce further the susceptibility of their triglycerides to LPL hydrolysis.

Uptake of the Liberated Fatty Acids by the Abdominal Visceral Adipocytes

The liberated fatty acids that are the products of the LPL hydrolysis will then be delivered to the mesenteries either through the veins or lymphatics within the mesenteries (see **Figure 1**). Note that these veins and lymphatics are the convergences of the blood and lymphatic capillaries of the lamina propria, respectively. Since these veins and lymphatics are surrounded by the abdominal visceral adipocytes, the liberated fatty acids supply the abdominal visceral adipocytes prior to the subcutaneous adipocytes. Consequently, the LPL hydrolysis of the chylomicron triglycerides in the lamina propria should preferentially lead to the accumulation of abdominal visceral fat instead of subcutaneous fat. The transport of the liberated fatty acids by the veins within the mesenteries is supported by the fact that the unesterified fatty acid concentration is elevated in the portal vein

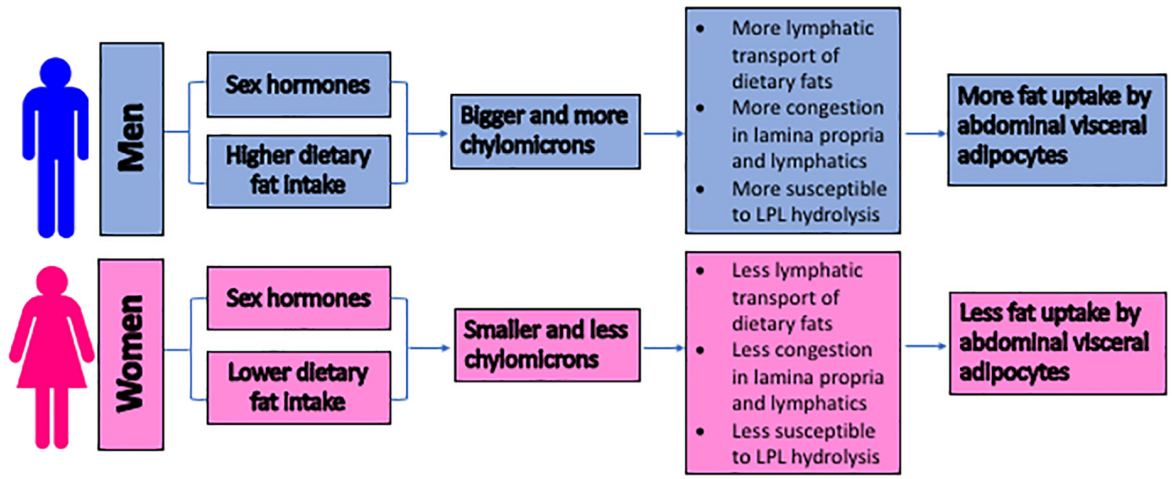

FIGURE 3 | The proposed mechanisms for the sex differences in the development of the abdominal visceral fat. The size of lipoproteins is proposed to serve as an important factor in regulating the abdominal visceral adiposity. Due to the higher intake of dietary triglycerides and the potential hormonal regulation, men produce bigger and more chylomicrons. These chylomicrons trigger congestion within the lamina propria and lymphatics, subjecting their triglycerides to LPL hydrolysis. The subsequent uptake of the hydrolysis products by the surrounding adipocytes results in the accumulation of the abdominal visceral fat. LPL = lipoprotein lipase.

after a bolus feeding of triglycerides (Kristensen et al., 2006). Of note, the hepatic portal vein receives blood from the mesenteries before draining them into the systemic blood circulation.

Leakiness of the Visceral Lymphatics Promotes the Abdominal Visceral Fat Accumulation

The idea that chylomicron retention in the extracellular matrix of the intestine can lead to the abdominal visceral fat accumulation is supported by the *Prox1* haploinsufficiency studies (Harvey et al., 2005). The studies show that *Prox1* ± mice, which have very leaky visceral lymphatics, accumulate a significant amount of abdominal visceral fat such that they develop obesity in their adulthood. The leaky intestinal lymphatics conceivably allow more chylomicrons to leave the lymphatic lumen and be retained in the extracellular matrix. Their enormous size may further prolong their retention, allowing their dietary fat to be hydrolyzed and stored by the surrounding abdominal visceral adipocytes. A recent study indicates that repairing the leaky lymphatics in *Prox1* ± mice prevents them from becoming obese, further confirming that the leaky lymphatics are responsible for their obesity phenotype (Escobedo et al., 2016). The study also suggests that the free fatty acids that are presumably liberated from the hydrolysis of chylomicron triglycerides may serve as the inducer of adipogenesis.

Lymphatic leakiness, which can be reduced by exercise (Hespe et al., 2016), is a normal part of aging (Zolla et al., 2015). The leakiness of the aging lymphatics may explain why the sex differences in the abdominal visceral fat accumulation become less pronounced with aging (Camhi et al., 2011) as well as the sex differences in lipid metabolism cannot all be attributed to sex hormones (Wang et al., 2011). It is becoming more apparent now that the roles of lymphatics in the development of android obesity cannot be disregarded.

Figure 3 summarizes our proposed mechanisms of why men are more likely to develop abdominal visceral fat than pre-menopausal women. Note that the aging and exercise effects are not depicted in the figure.

CONCLUSION

Of all of the factors contributing to the accumulation of abdominal visceral fat, lifestyle is arguably the most important. Based on our proposed mechanisms, spreading out the amount of dietary fat intake into several smaller meals should reduce the likelihood of abdominal visceral fat accumulation by reducing both the size and number of chylomicrons. Reducing the lipid load to the small intestine is also beneficial to the functions of the collecting lymphatics as high lipid load reduces their contraction frequency and amplitude (Kassis et al., 2016). By maintaining the lymphatic contraction, smaller meals may reduce the retention time of the chylomicrons in the lamina propria. This would consequently reduce the likelihood of abdominal visceral fat accumulation. In overweight minority youth studies, the higher calorie-consuming nibblers, indeed, accumulate less abdominal visceral fat than the lower calorie-consuming gobblers (House et al., 2014). The studies also take gender into account, that is, males are more likely to gobble and accumulate abdominal visceral fat than females.

Another important aspect of lifestyle is exercise. Besides increasing the energy expenditure, exercise may slow down the accumulation of abdominal visceral fat by increasing the flow of the chylomicrons within the lamina propria and lymphatics as well as reducing both the LPL expression in the mesenteric fat and the leakiness of the lymphatics.

There are other potential factors that may contribute to the development of abdominal visceral fat. But considering that it is a strong independent predictor of mortality, understanding the

mechanisms of its development is critical. Based on our proposed mechanisms, exercising and eating a diet low in fat—or at least spreading the fat intake into several smaller meals—should help in slowing down the development of abdominal visceral fat.

DATA AVAILABILITY STATEMENT

The data supporting the conclusions of this article are from previously published articles. Please refer to the reference section of this article.

AUTHOR CONTRIBUTIONS

AN wrote the manuscript. SM generated the figures.

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Emerging Roles for Lymphatics in Chronic Liver Disease

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Chronic liver disease (CLD) is a global health epidemic causing ∼2 million deaths annually worldwide. As the incidence of CLD is expected to rise over the next decade, understanding the cellular and molecular mediators of CLD is critical for developing novel therapeutics. Common characteristics of CLD include steatosis, inflammation, and cholesterol accumulation in the liver. While the lymphatic system in the liver has largely been overlooked, the liver lymphatics, as in other organs, are thought to play a critical role in maintaining normal hepatic function by assisting in the removal of protein, cholesterol, and immune infiltrate. Lymphatic growth, permeability, and/or hyperplasia in non-liver organs has been demonstrated to be caused by obesity or hypercholesterolemia in humans and animal models. While it is still unclear if changes in permeability occur in liver lymphatics, the lymphatics do expand in number and size in all disease etiologies tested. This is consistent with the lymphatic endothelial cells (LEC) upregulating proliferation specific genes, however, other transcriptional changes occur in liver LECs that are dependent on the inflammatory mediators that are specific to the disease etiology. Whether these changes induce lymphatic dysfunction or if they impact liver function has yet to be directly addressed. Here, we will review what is known about liver lymphatics in health and disease, what can be learned from recent work on the influence of obesity and hypercholesterolemia on the lymphatics in other organs, changes that occur in LECs in the liver during disease and outstanding questions in the field.

Keywords: lymphatic endothelial cell, liver lymphatics, ascites, chronic liver disease, fatty acids, cholesterol, obesity, portal hypertension

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INTRODUCTION

The lymphatic system is comprised of highly permeable capillaries found within the tissue and are required to transport lymph which contains cellular proteins, lipoproteins and lymphocytes (Dixon et al., 2009; Lim et al., 2009; Platt et al., 2013; Hansen et al., 2015; Thomas et al., 2016; Schineis et al., 2019). The capillaries drain into collecting lymphatic vessels (LV) surrounded by lymphatic muscle cells (LMC) that pump the lymph through highly specialized lymphatic valves (Chakraborty et al., 2015). How or if liver disease changes the normal functions of the lymphatics in the liver is not well understood. Here, we will outline current knowledge regarding the liver lymphatic system and lymphatic dysfunction in other organs to highlight underlying questions of how lymphatics may be affected by chronic liver disease (CLD).

LIVER LYMPHATICS IN HEALTH

The liver is the largest contributor to lymph production in the body and accounts for up to 50% of lymph entering the thoracic duct (Tanaka and Iwakiri, 2016). This lymph originates predominantly from the blood vascular system, which is characterized by a portal vein (PV) and hepatic artery (HA) in the portal triad spanning the hepatic lobule to the central vein (CV). Lymphatic structures are found within the portal triad near the PV and bile duct (**Figure 1**). These are likely lymphatic capillaries (LC) with highly permeable button like junctions and are not surrounded by smooth muscle actin (Tamburini et al., 2019). Fluid from the blood vasculature leaks into the interstitium through highly permeable liver sinusoidal endothelial cells (LSEC). These highly permeable sinusoids are thought to lead to the higher protein content of hepatic lymph as compared to lymph in other organs (Courtice et al., 1962; Witte C.L. et al., 1969; Aukland et al., 1984). Cellular byproducts from hepatocytes, hepatic stellate cells (HSCs) and Kupffer cells also flow into the interstitium.

From the interstitium of the liver, fluid flows into the space of Disse located between the hepatocytes and the blood sinusoids of the liver. The lymphatic fluid then predominantly flows through the space of Mall at the interface of the portal tract and the adjacent hepatocytes. Eighty percent or more of the lymph then flows into LC found along the portal tract (Ritchie et al., 1959) with lymph flow in the same direction as bile and prevented from re-entering the liver by lymphatic valves in downstream collecting vessels (Trutmann and Sasse, 1994). The LC then drain into LV which are surrounded by lymphatic muscle cells (LMC). These LMCs are needed to pump the lymph from the liver into the portal and the celiac lymph nodes (LNs) in mice (Barbier et al., 2012; Zheng et al., 2013; Chakraborty et al., 2015; **Figure 1**). The portal and celiac LNs in mice correlate to analogous hepatic draining LN clusters in humans (Harrell et al., 2008), from which lymph drains into the cisterna chyli.

In addition to portal lymph there is also sublobular and superficial lymph. Sublobular LV are found along the inferior vena cava (Ohtani and Ohtani, 2008). Superficial lymph drains through various LC toward the periphery of the liver before entering LV draining to regional LNs (Pupulim et al., 2015). These studies describing the normal flow and function of the lymphatics in the liver lay the groundwork for understanding how lymphatic function in the liver could be compromised during disease.

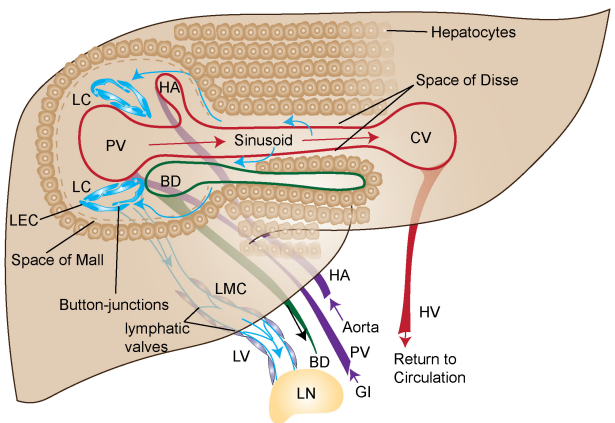

FIGURE 1 | Graphical illustration of liver blood and lymph circulation. Blood (purple arrows) flows into the portal vein (PV) from the gastrointestinal tract (GI) or into hepatic artery (HA) from the aorta. PV and HA as depicted in this figure are branches of the PV and HA. The blood is then transported through the liver sinusoids (red arrows) made up of liver sinusoidal endothelial cells (LSEC) and exits the liver through the central vein (CV) where it returns to circulation via the hepatic vein (HV). The primary source of lymph (blue arrows) travels from the sinusoids, through the Space of Disse, where it enters the Space of Mall and then enters the lymphatic capillaries (LC). The LC are made up of highly permeable button-like junctions that allow cells and protein to easily pass through. Upon entry into the lymphatic capillary the fluid and cells are then propelled through the lymphatic vessels (LV) where lymphatic muscle cells (LMC) pump the lymph toward the draining lymph node (LN). The lymphatic vessels contain valves for unidirectional flow. More than 80% of lymph is transported to portal lymphatic vessels. Superficial and sublobular lymph flow are not shown here.

LIVER LYMPHATICS IN DISEASE

During inflammation, lymphangiogenesis occurs in response to vascular endothelial growth factors (VEGF) expressed by infiltrating macrophages (Kataru et al., 2009). While the precise cell types that produce VEGFC/D to induce lymphangiogenesis in the liver are unknown, several liver resident cells including Kupffer Cells and LSECs have been shown to produce VEGFC/D (Dingle et al., 2018; McGettigan et al., 2019). During end-stage liver disease such as Hepatitis C Viral infection (HCV), Hepatitis B viral infection (HBV), Non-Alcoholic Steatohepatitis (NASH), and Alcoholic Liver Disease (ALD), hepatic lymph production and lymphatic vessel frequency increases in both human patients and animal models (Oikawa et al., 1998; Yamauchi et al., 1998; Yokomori et al., 2010; Tamburini et al., 2019). This same phenomenon of increased lymphatic vessel density, especially in the periportal area, has also been observed in idiopathic portal hypertension and in primary biliary cirrhosis (Oikawa et al., 1998; Yamauchi et al., 2002). The increase in lymphatic vessel density correlates with documented increases in VEGFC/D in the cirrhotic liver (Tugues et al., 2005). In patients with cirrhosis there is also an increase in the volume of lymph fluid draining the liver (Dumont and Mulholland, 1960, 1962; Witte et al., 1980; Granger et al., 1984). However, several studies have reported that the lymph fluid released from the liver, normally rich in protein, has a low protein content in humans (Witte M.H. et al., 1969; Witte et al., 1981) and rats (Barrowman and Granger, 1984) with cirrhosis. These findings suggest that despite the increase in frequency of lymphatics during disease, there may be a decrease in lymphatic permeability which prevents the proper removal of inflammatory mediators and cells from the liver. Despite these findings, it has yet to be determined if the lymphatics in the liver are functioning properly or if these changes in lymph content occur as a consequence of other disease-dependent mechanisms.

LIVER LYMPHATIC ENDOTHELIAL CELLS

The network of liver LV develops in the prenatal period (Paupert et al., 2011) by differentiation from blood vasculature through the expression of *Prox1* (Wigle et al., 2002). *Prox1*, the transcription factor required for lineage commitment of lymphatic endothelial cells (LEC) (Wigle and Oliver, 1999; Petrova et al., 2002), is also expressed by hepatocytes (Dudas et al., 2004, 2006; Tamburini et al., 2019). As such, the lymphatic vasculature in the liver lacks a single specific marker, however, LV can specifically be imaged using PDPN (D2-40) in humans. The D2-40 stain in humans is fairly specific for lymphatic vasculature and not the vascular endothelium nor liver epithelium (Yokomori et al., 2010; Tamburini et al., 2019). However, this is not as straight forward in mice as cholangiocytes are positive for the 8.1.1 anti-mouse PDPN clone (Finlon et al., 2019). Other common lymphatic endothelial markers include LYVE-1, but in the liver LYVE-1 is also expressed on LSECs and macrophages (Mouta Carreira et al., 2001). VEGFR3, which is an essential tyrosine kinase receptor in vascular endothelial growth, is also expressed by other cells in the liver including LSECs and cholangiocytes (Gaudio et al., 2006; Tamburini et al., 2019). Other less common lymphatic markers include CCL21, MMR1, desmoplakin, and integrin α9 (Petrova et al., 2002), all of which are also expressed by other liver resident cells and thus distinguishing liver lymphatics, especially in mouse models, has been difficult. **Table 1** outlines common markers and overlap among other liver specific cell populations in mice. Combinations of these markers can be used for the identification of LEC in the liver by flow cytometry and immunofluorescence as described in Finlon et al. (2019).

To address this, our recent studies evaluated liver LECs by single cell mRNA sequencing. However, we were unable to uncover a marker that is specific to LECs in the liver (Tamburini et al., 2019). We were able to differentiate LECs from other cells in the liver through the combined expression of *PDPN*, *LYVE1*, *PROX1*, VEGFR3 (*FLT4*), and *CCL21*. While the expression of these markers individually are not unique to the LECs in the liver, LECs are the only population of cells that express all of these markers together. Using these cell markers, we identified that the LECs within a cirrhotic liver are actively proliferating, consistent with increased lymphatic vessel density (Tamburini et al., 2019). We also discovered that LECs in disease upregulate pathways involved in the production of superoxide and reactive oxygen species consistent with the LECs responding to liver inflammation (Tamburini et al., 2019).

The transcriptional profile of LECs in the liver is dependent on disease etiology. Specifically, during chronic HCV infection the LECs upregulate genes associated with responses to increased interferon in the liver through Signal Transducer and Activator of Transcription 1 (STAT1), among others. In contrast, LECs isolated from the livers of patients with obesity associated NASH, upregulated genetic programs related to downstream cholesterol signaling and IL13 signaling, potentially through the upregulation of CD36 and p38 mitogen activated protein kinase (MAPK) (Tamburini et al., 2019). This study was the first to report the transcriptional profile of LECs isolated from healthy or cirrhotic human livers. Whether these transcriptional differences cause lymphatic dysfunction can only be inferred based on responses in other tissues.

TABLE 1 | Marker distribution that can be used to distinguish lymphatic endothelial cells (LEC) (Iwakiri, 2016; Finlon et al., 2019; Tamburini et al., 2019) from hepatocytes (Dudas et al., 2006; Goto et al., 2017), cholangiocytes (Gaudio et al., 2006; Li et al., 2013; Lua et al., 2014, 2016), liver sinusoidal endothelial cells (LSEC) (Schrage et al., 2008; Ding et al., 2010; Strauss et al., 2017), and portal endothelial cells (PEC) (Schrage et al., 2008) for flow cytometry or immunofluorescence.

	Hepatocytes	Cholangiocytes	LSEC	PEC	LEC
PDPN	−	+	−	−	+
Prox1	+	−	−	−	+
LYVE-1	−	−	+	−	+
Vegfr3/Flt4	−	+	+	+	+
CK19	−	+	−	−	−
Albumin	+	−	−	−	−
CCL21	−	−	±	−	+
CD146	−	−	+	+	−/low

OBESITY AND LYMPHATICS

In recent years obesity has been implicated as the cause of many diseases including cardiovascular disease, diabetes, and NASH. As excess stress on the body caused by obesity results in a type of chronic inflammatory state in the liver, heart and other organs it is perhaps not surprising that the lymphatic system is also affected. In an elegant report, Garcia Nores et al. (2016) demonstrated that obesity is a root cause of lymphatic dysfunction in the skin. Additionally, recent reports suggest that peri-lymphatic inflammation results in lymphatic structures that are functionally impaired causing defective interstitial fluid drainage and decreased dendritic cell (DC) migration (Weitman et al., 2013). Lymphatic dysfunction together with inflammation caused by disease can worsen inflammation and disease pathology (Cuzzone et al., 2014). In these studies, it was demonstrated that stearic acid, a long chain fatty acid commonly associated with obese tissues was highly toxic to LECs *in vitro*. This toxicity could be prevented by increasing phosphatidylinositol-triphosphate (PIP3) kinase signaling (Platt et al., 2013; Hansen et al., 2015; Thomas et al., 2016), presumably through phosphatase and tensin homolog (PTEN) activity (Garcia Nores et al., 2016). PTEN can be activated by VEGFR3 tyrosine kinase receptor activity (Garcia Nores et al., 2016). Furthermore, the peri-lymphatic inflammation seen was described to be a result of $CD4^+$ T cell and/or macrophage activity as lymphatic defects were ameliorated in $CD4^+$ T cell depleted mice fed a high fat diet (Torrisi et al., 2016); and macrophage crown like structures were observed in close proximity to defective LV (Garcia Nores et al., 2016). Intriguingly, lymphatic dysfunction, inflammation and iNOS activity can all be reversed by weight loss (Nitti et al., 2016). This is perhaps not too surprising as many of the co-morbidities associated with obesity are also resolved after weight loss (Magkos et al.,

2016). Finally, in *Prox1* heterozygous mice, a genetic model of lymphatic dysfunction (Wigle and Oliver, 1999; Harvey et al., 2005; Rutkowski et al., 2006), the LV become ruptured and "leaky" (Escobedo and Oliver, 2017). The ruptured and leaky lymphatics to lead to the accumulation of free fatty acids in the surrounding adipose tissue and increased adipogenesis (Harvey et al., 2005; Escobedo et al., 2016). These findings suggest that obesity not only drives lymphatic dysfunction, but that lymphatic dysfunction can drive obesity. Thus, in the setting of obesity associated liver disease it is possible that increased steatosis and the resulting inflammation, steatohepatitis, may occur around LC or vessels. This peri-lymphatic inflammation could cause toxicity or changes in signaling pathways in LECs that lead to decreased lymphatic integrity and increased adipogenesis in the surrounding tissues.

VEGF AND CHYLOMICRON TRANSPORT

The lymphatics within the intestinal villi, lacteals, are required for dietary fat uptake. The lacteals have highly permeable "button-like" junctions that allow for the acquisition of dietary fat, in the form of chylomicrons. As such, genetic deletion of *Neuropillin 1* together with *Vascular endothelial growth factor receptor 1* in endothelial cells, resulted in a decrease of button-like junctions and increase in zipper-like junctions in intestinal lacteals that led to the malabsorption of chylomicrons (Zhang et al., 2018). Additionally, complete loss of VEGFC expression by LECs in adult mice led to severe defects in lacteal regeneration and compromised dietary fat absorption (Nurmi et al., 2015). VEGFD (a VEGFR3 ligand) has also been shown to participate in the removal of chylomicron remnants from the blood (Tirronen et al., 2018). In a recent report, Tirronen et al. (2018) found that loss of VEGFD resulted in the downregulation of syndecan 1 (SDC1) on the sinusoidal surface of hepatocytes (Tirronen et al., 2018). SDC1 is a receptor for APO-B found in chylomicron remnants. APO-B is also important for hepatic uptake of tri-glyceride rich lipoproteins. These new findings demonstrate that VEGFD can mediate crosstalk between the LSECs and hepatocytes for chylomicron remnant removal from the blood. However, what contribution loss of VEGFD had to liver lymphangiogenesis or reverse cholesterol transport was not investigated. This work suggests an intimate crosstalk between the vascular and lymphatic endothelium with liver and intestine specific cells. This crosstalk appears to have both specific and non-specific consequences for lipid and cholesterol metabolism, lymphatic growth and differentiation, and/or transport of fatty acids and chylomicrons throughout the body.

HYPERCHOLESTEROLEMIA AND LYMPHATICS

In addition to the interplay between obesity and lymphatics, recent studies have highlighted the role of lymphatics in reverse cholesterol transport and the impact of hypercholesterolemia on lymphatic function. Reverse cholesterol transport is the process of the removal of cholesterol from peripheral tissues back to the liver where it is excreted through the bile (Brufau et al., 2011). This process requires cholesterol to be received by the lymphatic vasculature before entering into the blood stream at the thoracic duct (Huang et al., 2015). Recent reports have suggested that LV are not just passive transporters of cholesterol, but instead may be actively involved in the regulation of fatty acids and cholesterol transport from the tissue similar to how lymphatics regulate immune cell transport (Baluk et al., 2007; Pflicke and Sixt, 2009; Lim et al., 2013; Martel et al., 2013). In light of this, several studies have recently been done to understand the impact of cholesterol on lymphatic function. Many of these studies have been performed in *ApoE*−/− mice fed a high fat diet. These mice have exceptionally high levels of cholesterol and LV are more tortuous, dilated, and have areas of smooth muscle cell coverage that are irregular (Lim et al., 2009). As a result, using lymphocyntigraphy and DC migration assays, these LV were functionally impaired in their ability to transport fluid from the tissue or DCs to the LN and this could be rescued by treatment with a cholesterol reducing agent, ezetimibe (Lim et al., 2013). Hypercholesterolemia also led to increased levels of cholesterol in the tissues and LNs which could be rescued by VEGFC or VEGFD treatment (Lim et al., 2013). Further, loss of the cholesterol receptor SR-BI completely ablated cholesterol transport through the lymph (Lim et al., 2013). Finally, it was shown that hypercholesterolemia increased LN cellularity by disrupting normal lymphatic structures (Tay et al., 2019). This was due to decreased S1P secretion into the lymph, decreased *Sphk1* transcript (which catalyzes the biosynthesis of SIP) by LECs and increased *Spgl1* transcript which degrades S1P thereby decreasing lymphocyte egress from the LN (Tay et al., 2019). The effect of cholesterol on LV has yet to be fully understood, but could have a major impact on lymphatic function across systems resulting in defects in lymphocyte trafficking, cholesterol or triglyceride accumulation in the tissue, chylomicron uptake or chylomicron remnant removal. Cholesterol and oxidized cholesterol species are increased in the livers of patients with CLD, thus understanding the impact of cholesterol on lymphatics is essential for understanding lymphatic function in the liver during disease.

To gain insight into specialized roles for liver LECs during disease our recent report examined how LECs respond to cholesterol species retained in the liver during disease, such as highly oxidized low density lipoprotein (OX-LDL). While OX-LDL is a key player in the pathogenesis of atherosclerosis (Di Pietro et al., 2016; Gao and Liu, 2017), it has only recently been appreciated that OX-LDL can contribute to the progression of liver disease. OX-LDL accumulates as a result of free radicals generated by inflammation and can directly induce liver injury (Zhang et al., 2014). Further, levels of OX-LDL are elevated in patients with NASH (Ampuero et al., 2016), HCV (Nakhjavani et al., 2011), ALD (Alho et al., 2004; Schroder et al., 2006), and cholestasis (Comert et al., 2004; Karadeniz et al., 2008). This increased OX-LDL in the pathogenesis of CLD is consistent with our transcriptional data from LECs from livers of people with NASH, in that pathways involved in free radical scavenging, IL13 signaling and genes involved in cholesterol sensing were

upregulated (Tamburini et al., 2019). In our studies, OX-LDL induced the production IL13 by LECs in livers of mice but not LN LECs and in contrast to the interferon inducing toll-like receptor (TLR) agonist, polyI:C (Tamburini et al., 2019). LECs have been shown to produce, IL-1β, IL-6, IL-7, IL-8, and iNOS during inflammation and TGFβ at baseline (Card et al., 2014), however, IL-13 production by LECs had yet to be reported. These findings were interesting as recent evidence demonstrated that IL4 and IL13 can reduce *Prox1* expression (Savetsky et al., 2015; Shin et al., 2015). In addition to the expression of IL13, LECs are one of the few cell types to express both IL13Rα1 and IL13Rα2, suggesting that LECs have the ability to both produce and respond to IL13 (Heng et al., 2008). When OX-LDL was administered to human LECs *in vitro*, *Prox1* and *Vegfr3* expression were decreased (Tamburini et al., 2019), similar to what was seen when LECs were treated with recombinant IL13 (Savetsky et al., 2015; Shin et al., 2015). This loss of *Prox1* could significantly affect lymphatic differentiation as in an elegant paper by Wong et al. (2017), it was demonstrated that LECs upregulate fatty acid oxidation (FAO) in a positive feedback loop that that requires PROX1, p300 and CPT1a to promote lymphatic growth and lymphatic specific chromatin modifications. These findings are important as we begin to investigate how OX-LDL and/or IL13 could manipulate the function of LEC and also how the metabolic state of the LEC can be the consequence and/or cause of chronic disease states. This is especially intriguing in the liver as blocking IL-13 can reduce liver fibrosis-at least in a Schistosoma infection model (Chiaramonte et al., 2001). Thus, understanding how different inflammatory mediators within the liver affect lymphatic function will be important for developing novel and specific therapeutic targets for liver lymphatics.

BYPASS OF FIRST PASS METABOLISM IN THE LIVER BY TARGETING LYMPHATICS

Another recent and intriguing area of research regarding lymphatics and the liver is currently being explored within the pharmacologic community. First pass metabolism is a process in which oral drug administration results in drug metabolism in the liver. When the drug passes directly from the intestine into the hepatic PV it is rapidly cleared in the liver via drug metabolizing enzymes, transporters and secretion through the bile (Andrews et al., 2017; Argikar et al., 2017; Mariappan et al., 2017; Miron et al., 2017). This rapid clearance of drugs decreases therapeutic efficacy of the orally administered drug. To circumvent these issues, the lymphatic system is being targeted to bypass first pass metabolism by the liver. As stated above, the lacteals are required for dietary fat uptake from enterocytes in the form of chylomicrons. Through microemulsion formulations of lipids (Khoo et al., 2003), self-microemulsifying drug delivery systems (SMEDDS) (Li et al., 2017), chemical modification with fatty acyl groups/esterification (Borkar et al., 2015, 2016) and other lipid formulations (Han et al., 2015), several groups have targeted the lacteals for oral drug uptake. Targeting lymphatic uptake in the gut results in both localized and systemic drug delivery by bypassing the liver. This method for drug delivery has many potential uses, of which a particularly intriguing area of research is to improve delivery of drugs that modulate immune function. To this end, delivery of lipophilic alkyl derivatives or triglyceride derivatives of the immunosuppressive drug mycophenolic acid (MPA) caused MPA to enter into the lymphatic system, pass into the mesentery and ultimately into the mesenteric LNs to the targeted immune cells (Han et al., 2014). These studies demonstrate a novel way for specifically reaching the lymphatic and the immune system with lipophilic drugs. This is important as we negotiate new ways to manipulate lymphatic or immune function in different organ systems.

CONCLUSION AND FUTURE GOALS

As we move forward into understanding differences in LECs based on their tissue microenvironment we are likely to come across novel and specific pathways that could be targeted for disease therapeutics. Of particular interest are atherosclerosis, inflammatory bowel disease, or CLD in which LC and vessels are important for the clearance of inflammatory infiltrate, protein, cholesterol and triglycerides from the tissue. VEGF-C and VEGF-D have pleiotropic effects, but could be developed for use as recombinant proteins, agonistic antibodies or small molecules to potentiate lymphatic growth and/or function across the board to improve organ health. Similarly, the mTOR inhibitor, sirolimus, has been shown to limit downstream VEGFR3 signaling thereby aiding in diseases where lymphatic malformations occur (Baluk et al., 2017; Ozeki and Fukao, 2019; Ozeki et al., 2019a,b,c). Presumably, based on preclinical models of myocardial infarction promoting lymphangiogenesis could improve organ function (Klotz et al., 2015). However, based on recent findings it may be more relevant to focus our studies on understanding the specific contributors to lymphatic dysfunction, such as OX-LDL, and target the removal of those inflammatory mediators instead. In parallel, it will be important to understand downstream signaling pathways in LECs caused by inflammatory mediators or inflammatory cells with the end goal of maintaining lymphatic function through the prevention of signals induced by the inflammation. In the end, it is important for us to understand differences in lymphatics in each organ system, the specific stressors these cells encounter and how they can be targeted using lipophilic drug derivatives. At this time it is fairly evident that a common function of lymphatics across systems is to promote lipid and protein homeostasis to maintain organ health.

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REVIEW
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The Role of Interstitial Matrix and the Lymphatic System in Gastrointestinal Lipid and Lipoprotein Metabolism

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This review emphasizes the events that take place after the chylomicrons are secreted by the enterocytes through exocytosis. First, we will discuss the journey of how chylomicrons cross the basement membrane to enter the lamina propria. Then the chylomicrons have to travel across the lamina propria before they can enter the lacteals. To understand the factors affecting the trafficking of chylomicron particles across the lamina propria, it is important to understand the composition and properties of the lamina propria. With different degree of hydration, the pores of the lamina propria (sponge) changes. The greater the hydration, the greater the pore size and thus the easier the diffusion of the chylomicron particles across the lamina propria to enter the lacteals. The mechanism of the entry of lacteals is discussed in considerable details. We and others have demonstrated that intestinal fat absorption, but not the absorption of protein or carbohydrates, activates the intestinal mucosal mast cells to release many products including mucosal mast cell protease II in the rat. The activation of intestinal mucosal mast cells by fat absorption involves the process of chylomicron formation since the absorption of both medium and short-chain fatty acids do not activate the mast cells. Fat absorption has been associated with increased intestinal permeability. We hypothesize that there is a link between fat absorption, activation of mucosal mast cells, and the leaky gut phenomenon (increased intestinal permeability). Microbiome may also be involved in this chain of events associated with fat absorption. This review is presented in sequence under the following headings: (1) Introduction; (2) Structure and properties of the gut epithelial basement membrane; (3) Composition and physical properties of the interstitial matrix of the lamina propria; (4) The movement of chylomicrons across the interstitial matrix of the lamina propria and importance of the hydration of the interstitial matrix of the lamina propria and the movement of chylomicrons; (5) Entry of the chylomicrons into the intestinal lacteals; (6) Activation of mucosal mast cells by fat absorption and the metabolic consequences; and (7) Link between chylomicron transport, mucosal mast cell activation, leaky gut, and the microbiome.

Keywords: lymphatic circulation, chylomicron, mucosal mast cell, microbiome, lipids, gastrointestinal tract, enterocytes, intestinal absorption

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INTRODUCTION

The absorption and transport of lipids by the gastrointestinal tract involve the uptake of digested lipids by the enterocytes and the formation and secretion of chylomicrons. These different steps of intestinal fat absorption have been so ably reviewed by a number of reviews over the past few years (Wang et al., 2013; Dash et al., 2015; Levy, 2015; D'Aquila et al., 2016; Mansbach and Siddiqi, 2016; Auclair et al., 2018; Xiao et al., 2019). In most textbooks and review articles, fat absorption is described as involving the packaging of the absorbed lipids into chylomicrons, the exit of the chylomicrons from the enterocytes through exocytosis, and the subsequent entry of the chylomicrons into the lymphatics of the gastrointestinal tract. Electron microscopic studies have contributed significantly to our understanding of the formation and secretion of chylomicrons and the review by Sabesin and Frase (1977) remain one of the best articles describing this process. The role of the lymphatic system in fat absorption has been recognized for centuries. As early as the 1600s, Italian physician Gaspare Aselli demonstrated a post-prandial rise in the concentration of particulate fat in the mesenteric lymph of a dog fed shortly before its death (Yoffey and Courtice, 1970; Barrowman, 1978). A major advancement in our understanding of the transport of fat by the lymphatic system was enabled by the conscious lymph fistula rat and mouse studies (Hayashi et al., 1990; Kohan et al., 2012; Ko et al., 2019).

In fact, many events are involved from the secretion of the chylomicrons by the enterocytes to the subsequently transport of these triglyceride-rich lipoproteins by the lymphatic system. One of the goals of this review is to cover those events that occur from the secretion of chylomicrons *via* exocytosis by the enterocytes to the final entry of the chylomicrons into the lymph lacteals of the intestinal villi. After exiting from the enterocytes, the chylomicrons accumulate in the intercellular space. The basement membrane with which the enterocytes are attached to offers considerable resistance for the passage of chylomicrons from the intercellular space into the lamina propria. We will discuss how we think the chylomicrons cross the basement membrane to enter into the lamina propria. We will also discuss the properties of the lamina propria and the factors (e.g., hydration of the lamina propria) influencing the diffusion of the chylomicrons across the lamina propria and how the chylomicrons subsequently enter the lacteals located in the central core of the villus. The lacteals transporting the chylomicrons drain initially into the intestinal lymph duct, then the thoracic duct, and finally empty into the left subclavian vein.

Interest in the lymphatic system increased dramatically recently in its role in lipid metabolism and gastrointestinal function. We and others have demonstrated that in addition to chylomicrons, the lymphatics of the gastrointestinal tract also carry molecules secreted by the mucosal mast cells [mucosal mast cell protease II in the rat (Ji et al., 2011; Sato et al., 2016)] and mucosal mast cell protease I in the mouse (Miller and Pemberton, 2002) when these cells are activated during fat absorption. This finding is not surprising given the close association between the lymphatic vessels and the mucosal mast cells that has been so elegantly demonstrated by Chatterjee and Gashev (2012). Our better understanding of lymphatic transport of particles ranging in size from large chylomicrons to small mast cell activation products and incretin hormones (GLP-1 and GIP) clearly emphasizes the importance of the intestinal lymphatic system in the transport of many important molecules during health and the diseased state. With the recent surge in interest in the intestinal microbiome, we anticipate a better understanding of the molecules derived from the microbiome carried by the lymphatic system. We may also gain insight into the importance of the microbiome in the well-being of the gastrointestinal tract as well as the origin and development of conditions associated with the leaky gut and gut inflammation.

STRUCTURE AND PROPERTIES OF THE GUT EPITHELIAL BASEMENT MEMBRANE

During active lipid absorption, monoglycerides and fatty acids produced from the digestion of triglycerides are taken up by enterocytes. Here, they are re-esterified to produce triglycerides and are packaged into chylomicrons for export into the lymphatic system. For a more comprehensive discussion of these processes, readers are referred to the following excellent treatises on the subject (Levy, 1992, 2015; Abumrad and Davidson, 2012; Wang et al., 2013; Hussain, 2014; Dash et al., 2015; D'Aquila et al., 2016; Mansbach and Siddiqi, 2016; Auclair et al., 2018; Xiao et al., 2019).

Basement membranes are present in every tissue of the human body and are found either as enveloping cell coats or as sheets underlying cell layers. The gut basement membrane is a specialized structure composed of extracellular matrix components that supports and separates the epithelium from the underlying lamina propria made up of connective tissue (Paulsson, 1992; Timpl, 1996). The primary constituents of most basement membranes include collagens (predominantly collagen IV), laminins, proteoglycans, calcium-binding proteins, as well as other structural or adhesion proteins (Timpl, 1996). Basement membranes are formed by a process of self-assembly (Paulsson, 1992). Initiation of basement membrane assembly in a tissue requires the synthesis of α, β, and γ laminin subunits and the formation of laminin heterotrimers (Yurchenco et al., 1997). Secreted laminins anchor to the cell surface through several receptors (Oh et al., 1997; Edwards et al., 1998; James et al., 2000) and self-assemble into a polymeric network (Yurchenco et al., 1985). This lattice serves as scaffolding for further basement membrane assembly, including the addition of a matrix polymer of collagen IV molecules (Pöschl et al., 2004). The compact network of overlaid laminin and collagen lattices creates a dense meshwork with pores ranging in size from ~10 to 130 nm (depending on the tissue involved) that allow the basement membrane to function as a selective barrier (Yurchenco and Ruben, 1987; Yurchenco et al., 1992; Abrams et al., 2003).

During the absorption of fat, the gut basement membrane acts as a temporary barrier preventing chylomicrons, particles empirically defined as having a diameter equal to or larger than 800 Å, from entering the lamina propria (**Figure 1**). However, occasional discontinuities in the basement membrane allow the passage of the chylomicrons from the intercellular space to the lamina propria (**Figure 2**; Rubin, 1966; Tytgat et al., 1971; Sabesin and Frase, 1977). It is not uncommon to see the intercellular space between the gut epithelial cells full of chylomicrons during active fat absorption and in some images; the basement membrane appears to be broken. Thus, it has also been proposed that the basement membrane would be stretched under these conditions, causing breaks that would facilitate the transport of chylomicrons from the intercellular space to the lamina propria (Tso and Balint, 1986). However, this notion has not been tested experimentally. Kvietys et al. (1991) have demonstrated mucosal injury associated with intestinal fat absorption, but not with the absorption of the other macronutrients, which lends support to the notion that fat absorption is injurious to the integrity of the intestinal tract.

During fat absorption, the accumulation of chylomicrons in the intercellular space undoubtedly puts physical stress to the tight junctions. Furthermore, passage through breaks in the intestinal basement membrane is likely non-specific, which could allow molecules other than chylomicrons to enter the lamina propria. It is conceivable that this mode of entry may play a role in increasing intestinal permeability and the development of leaky gut. Thus, it is important for us to gain a better understanding of this process.

COMPOSITION AND PROPERTIES OF THE INTERSTITIAL MATRIX OF THE LAMINA PROPRIA

The lamina propria of the small intestine is made up of connective tissue that occupies the central cone of the intestinal villus and offers physical support for the villus structure. Many cell types are present in the lamina propria, including mast cells, plasma cells, lymphocytes, eosinophils, neuronal cells, and fibroblasts. It also contains blood and

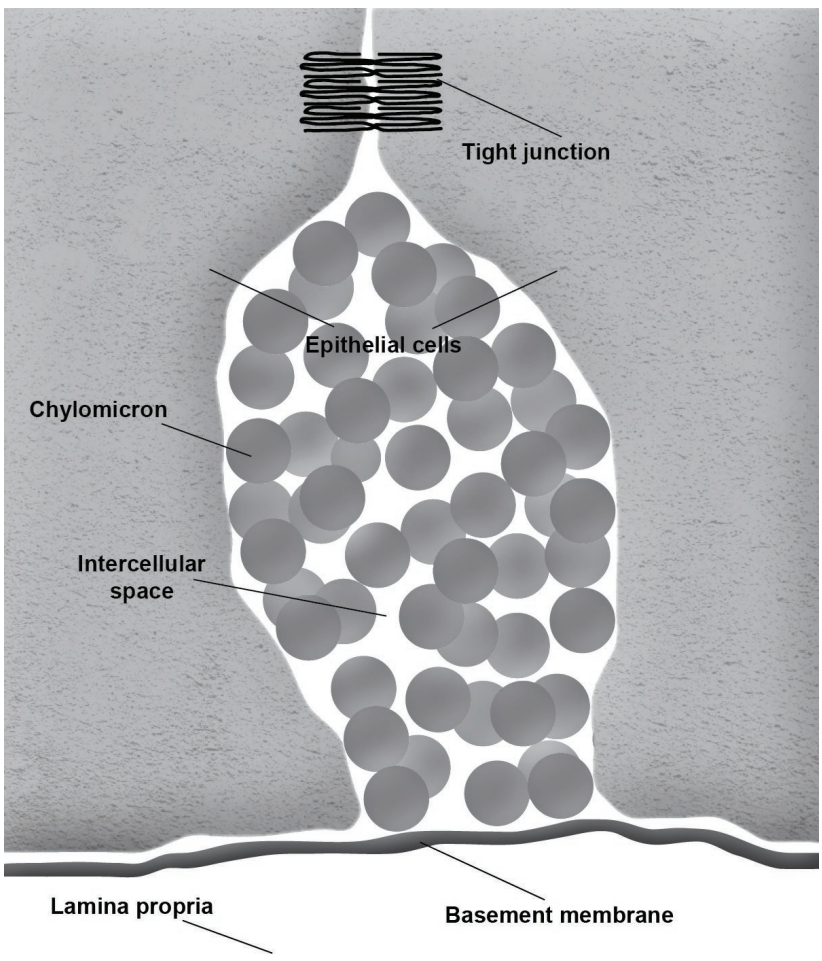

FIGURE 1 | Accumulation of chylomicrons causes distension of the intercellular space. The intact basement membrane serves as a barrier preventing the chylomicrons from entering the underlying connective tissue of the lamina propria.

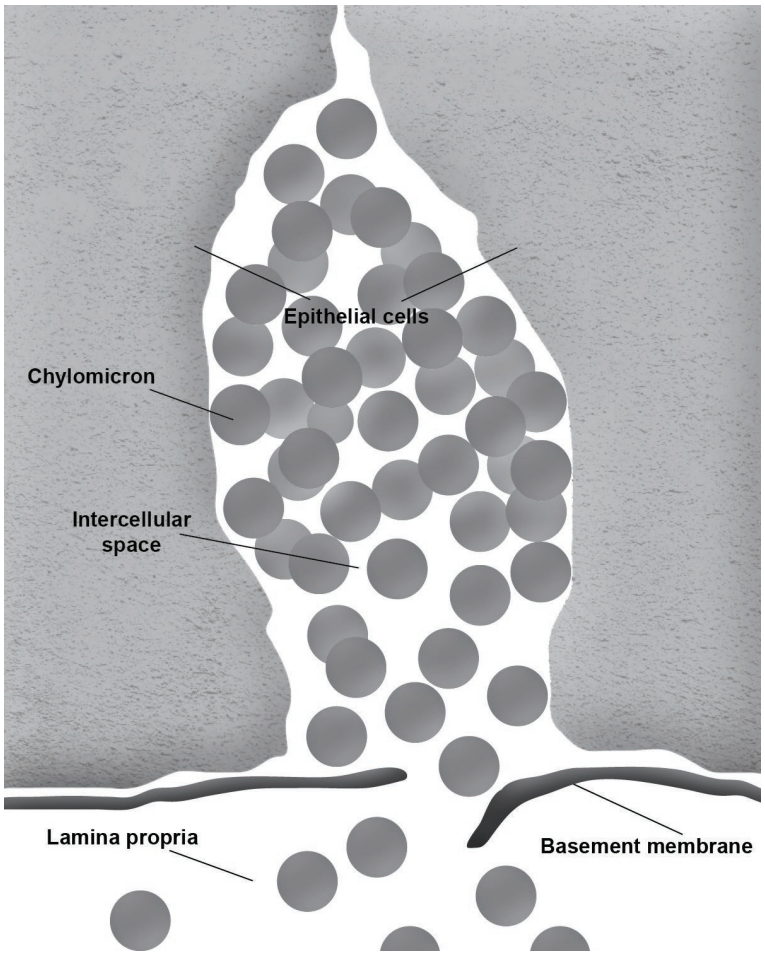

FIGURE 2 | Chylomicrons enter the lamina propria through a break in the basement membrane.

lymph vessels, unmyelinated nerve fibers, and smooth muscle cells (Tso and Balint, 1986). The interstitial matrix of the lamina propria consists of a fluid phase that is distributed within fibrillar and non-fibrillar macromolecules. The primary fibrillary components include elastin and collagens, while non-fibrillar components include glycosaminoglycans, the major one being hyaluronic acid. The glycosaminoglycans trap water, which allows them to regulate the hydration of the interstitial matrix (Kvietys and Granger, 2010). The extensive crosslinking of the collagens and glycosaminoglycans results in a gel-like matrix within the lamina propria that behaves like a mesh and excludes large molecules from the fluid phase (Kvietys and Granger, 2010).

The interstitial volume and hydration are carefully maintained by the coordinated efforts of the blood and lymph vessels as well as fluid absorption from the intestinal lumen. During the resting in between meals, the balance of hydrostatic and oncotic forces governing trans-capillary fluid exchange, as described by the Starling principle, favors net filtration from the blood to the interstitium. This is balanced by an equal rate of fluid removal by the lymphatic vessels. During fluid absorption, alterations in the Starling forces allow the capillaries and lymphatics to remove the excess fluid in the interstitium and maintain the interstitial volume.

THE MOVEMENT OF CHYLOMICRONS ACROSS THE INTERSTITIAL MATRIX OF THE LAMINA PROPRIA AND THE IMPORTANCE OF THE HYDRATION OF THE MATRIX

The properties of the lamina propria interstitial matrix play an important role in regulating chylomicron transport to the lymph lacteal centrally located within the intestinal villus. Once chylomicrons are transported across the epithelial basement membrane, they must then travel ≈50 μm through the interstitium before being taken up into the lacteal (Granger, 1981). Fluid absorption usually increases with the absorption of lipids and other nutrients, which subsequently increases the hydration of the interstitial matrix of the lamina propria. Ji et al. demonstrated that mucosal mast cells (MMC) are activated during fat absorption and the degranulation of the mast cells consequently releases

molecules such as histamine (Ji et al., 2012). Histamine increases the permeability of the intestinal capillaries, thus dramatically increasing the filtration of molecules and fluid into the interstitial space of the villus. Evidence from previous studies (Shepherd and Simmonds, 1959; Baraona and Lieber, 1975; Granger, 1981; Granger et al., 1984; Tso et al., 1985) has suggested that the hydration of the interstitial matrix facilitates the transport of chylomicrons across the lamina propria. Two possible mechanisms have been proposed for this phenomenon.

First, hydration increases the permeability of the interstitial matrix. Under resting, non-absorptive conditions, the matrix has an average pore size of 250 Å, allowing it to exclude large molecules from the fluid phase. Thus, during the non-absorptive phase, the relatively small pore size of the interstitial matrix offers significant resistance to the diffusion of chylomicrons (particles empirically defined as having a diameter equal to or larger than 800 Å) through the intercellular space. During fluid absorption, the volume of the interstitium increases, resulting in the expansion and disentanglement of the matrix components. Under these conditions, the average porosity increases to 1,000 Å (Granger, 1981; Tso et al., 1985) which likely facilitates the movement of chylomicrons across the interstitial space to the central lacteals.

It has also been proposed that chylomicron transport could be facilitated by the convective fluid movement of lymph associated with fluid absorption. During lipid absorption, the subsequent increase in fluid uptake results in an increase in interstitial fluid formation and lymph flow. Previous studies have suggested that the rate of lymph formation has a major effect on chylomicron transport (Shepherd and Simmonds, 1959; Baraona and Lieber, 1975; Tso et al., 1985). Tso et al. studied the effect of lymph flow on chylomicron appearance time (time between infusion of radioactive fatty acid into the intestinal lumen and the appearance of radioactive lipid as chylomicrons in the intestinal lacteal). They reported that in a rat model, chylomicron appearance time was inversely proportional to lymph flow rate when the flow rate was <40 µl/min. When the flow rate exceeded 40 µl/min, chylomicron appearance rate plateaued at a minimal value of 13.6 min. This likely represented the minimum time necessary for the re-esterification of absorbed fatty acids and monoglycerides, chylomicron formation, and discharge into the lacteals (Tso et al., 1985).

It is still unclear if the increase in chylomicron transport associated with fluid absorption is a result of increased permeability of the interstitial matrix or the convective fluid movement. However, in both cases, it is apparent that hydration of the interstitial matrix plays an important role in the movement of chylomicrons across the lamina propria.

ENTRY OF THE CHYLOMICRONS INTO THE LACTEALS

There is some controversy regarding the mechanism of chylomicron entry into the intestinal lacteals (**Figure 3**). The first two electron microscopic studies on the entry of fat as chylomicrons into the lacteals presented incompatible results. Palay and Karlin (1959) reported that chylomicrons passed through open intercellular junctions, while Ashworth et al. (1960) showed that they passed through "pores" in the lymphatic wall. A later study by Casley-Smith (1962) appeared to confirm the findings of Palay and Karlin. The study reported numerous electron microscopic images of open gaps between adjacent lymphatic endothelial cells containing chylomicrons, but they also observed chylomicrons entering the external endothelial membranes of the lacteal. However, Casley-Smith noted that the intercellular junctions of the intestinal lymph lacteals usually lack adhesion plates and that it is more likely that materials pass through the open junctions than through the endothelial cells. Furthermore, he suggested that Ashworth et al. may have mistaken these open junctions for "pores" (Casley-Smith, 1962). Several later studies concurred with Casley-Smith's original assumption that a paracellular route was the most likely path of chylomicrons into the lymph lacteals (**Figure 4**). However, none conclusively ruled out transcellular transport through the endothelial cells (Casley-Smith, 1964; Rubin, 1966; Tytgat et al., 1971).

These conclusions were challenged by Dobbins (Dobbins and Rollins, 1970; Dobbins, 1971). With over 500 images examined, they concluded that a majority of the intercellular junctions remained tightly closed. Furthermore, he demonstrated that chylomicron-containing vesicles occupied approximately 15% of the cytoplasm within the endothelial cells. The average size of the chylomicrons is in the range of 1,600 Å. Thus, Dobbins concluded that transcytosis must be the primary means chylomicron uptake into the lacteals. Controversy over two transport mechanisms has continued and evidence supporting both has been reported. The findings of multiple studies by Azzali supported Dobbins's conclusion (Azzali, 1982, 1990; Azzali et al., 2002). Meanwhile, Collan and Kalima (1974) demonstrated that two types of junctions were present between the endothelial cells of the lacteals: complicated and simple valve-like. They noted that the complicated joint areas served as a means of fixing adjacent endothelial cells together, while simple joint areas had an additional function as valves through which materials could be transported into the lacteal (Collan and Kalima, 1974).

A more recent study by Choe et al. (2015) has provided further insight into the mechanism of lipid drainage through the lacteals. Their study suggests that the contractility of the intestinal lacteals plays an important role in the uptake of chylomicrons into the lymphatic system. Furthermore, their findings suggest that rather than serving as passive conduits for lipids, the lacteals function as active pumps for lipid transport. Previous studies of villus motility in a dog model have described piston-like retractions and extensions. These motions were speculated to be mediated by the smooth muscles longitudinally aligned along the lacteal within in the intestinal villus (Womack et al., 1987, 1988). However, Choe and colleagues have demonstrated that the lacteals in a mouse model also undergo lateral contraction. In addition, they reported that interactions between the lacteals and surrounding smooth muscle resulted in enhanced absorptive ability and spontaneous contractibility that is regulated by the autonomic nervous system. They demonstrated that administration of acetylcholine and norepinephrine resulted in an increase and decrease in lateral contraction, respectively. These observations suggest that through

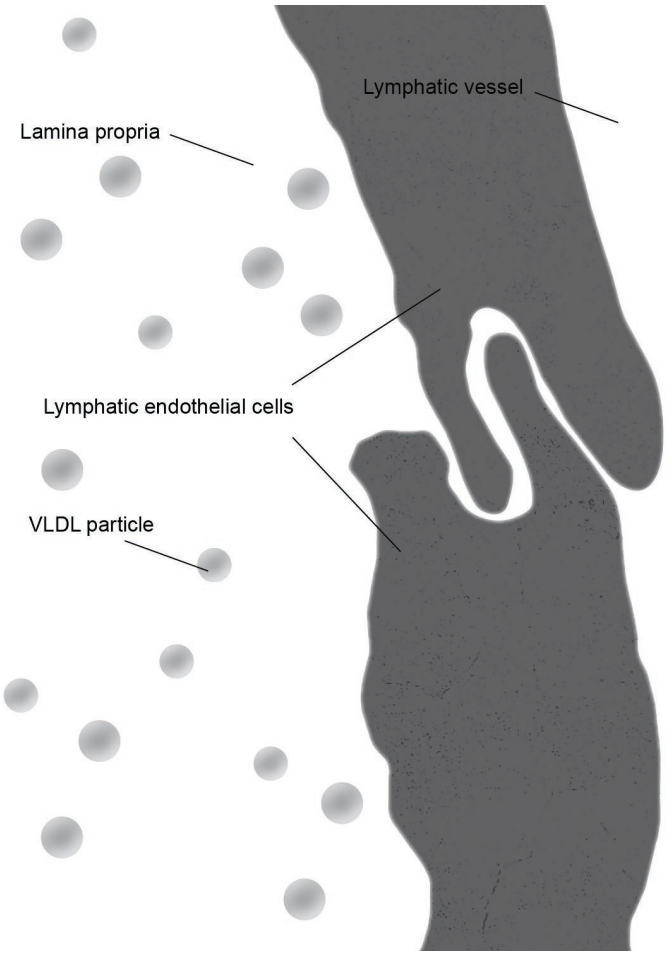

FIGURE 3 | During the fasting state, junctions between lymphatic endothelial cells form a continuous cellular lining of the central lacteal of an intestinal villus. The primary lipoprotein particles present in the lamina propria are very low density lipoprotein (VLDL) particles which are significantly smaller than chylomicrons.

autonomic regulation, the lymph lacteals serve a role in lipid transport as active pumps, which may also provide a critical mechanistic explanation for how the lacteals are able to drain such large quantities of dietary lipids. It should be emphasized that the role of the lacteals as pumps still need to be carefully studied and the same scenario may not apply to all animal species.

ACTIVATION OF MUCOSAL MAST CELLS BY FAT ABSORPTION AND THE METABOLIC CONSEQUENCES

In addition to its role in the digestion and absorption of nutrients (e.g., fat), the gut also serves an important function in host defense. The gastrointestinal tract, a tube-like structure that is covered by mucosa from the oral cavity to the anus, is continuous with the external environment. Therefore, the gastrointestinal tract is a major entry site for many bacterial and viral pathogens and has a vastly diverse microbial community (Guarner and Malagelada, 2003; Palmer et al., 2007). Despite its constant and direct exposure to large quantities of microorganisms as well as foreign and dietary antigens, the gut mucosa maintains intestinal homeostasis by utilizing the mucosal immune system (Kagnoff, 2014; Mowat and Agace, 2014). Furthermore, the mucosal surfaces in the small intestine are protected from unfavorable antigen entry into the body and pathogen invasion through non-specific and specific immunological defense mechanisms (Turner, 2009). Non-specific mechanisms include a mucus layer and antimicrobial peptides (e.g., defensins and lysozymes) covering intestinal epithelial cells that provide a first line of defense against pathogen invasion (Johansson et al., 2008; Tokuhara et al., 2019). As an example of a specific mechanism, in response to luminal stimuli, intestinal epithelial cells produce pro-inflammatory chemokines and cytokines and induce the recruitment of immune cells in the lamina propria to the inflamed regions (Tokuhara et al., 2019).

Previous studies have suggested a close link between these intestinal immune cells and dietary fat absorption (Miura et al., 1998; Hara et al., 2003; Tsuzuki et al., 2006; Fujiyama et al., 2007; Ji et al., 2012). In particular, Ji et al. demonstrated that intestinal mucosal mast cells (MMC) in the lamina propria,

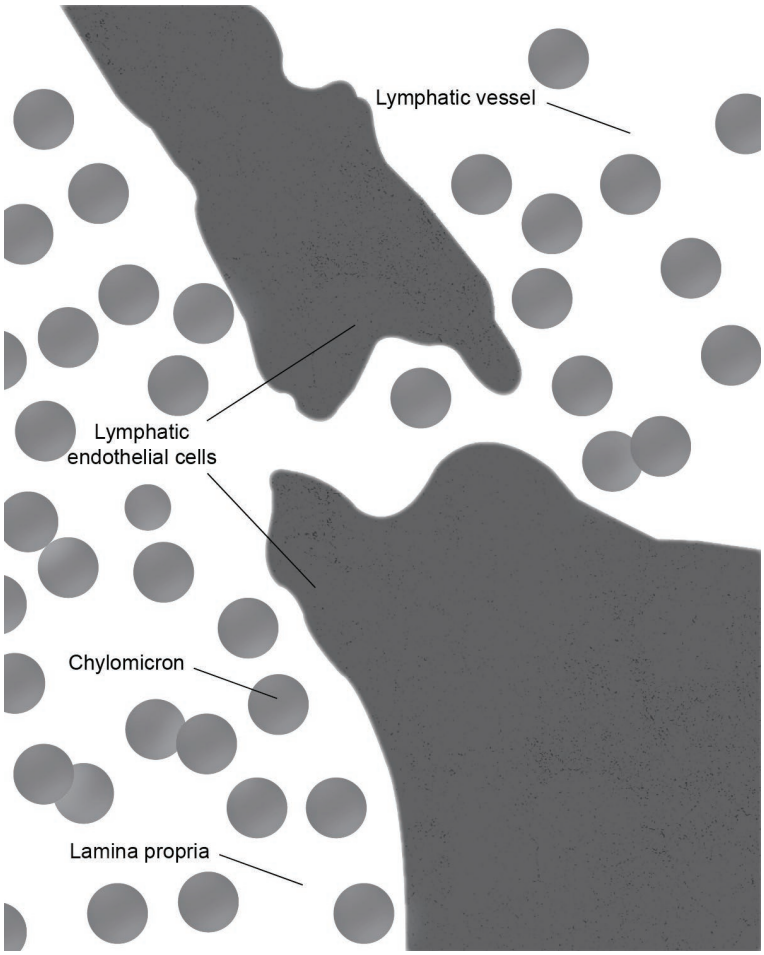

FIGURE 4 | During the active lipid absorption, chylomicrons enter the lymph lacteal through a transcellular gap in the endothelial lining.

key components of the mucosal immune system, are activated during fat absorption to release both pre-formed and *de novo* synthesized mediators in a conscious lymph fistula rat model (Ji et al., 2012). They found that intestinal MMC activation is dependent upon the quantity and type of fat infused intraduodenally and that long-chain fatty acids (LCFA) but not medium-chain fatty acids (MCFA) are effective in activating MMC. It is tempting to speculate that this observation is likely a result of the intestine's handling of fatty acids (Sallee and Dietschy, 1973). MCFAs and short-chain fatty acids (SCFA), due to a relatively higher aqueous solubility than LCFA, are less dependent on luminal micellar solubilization for rapid uptake into enterocytes (Sallee and Dietschy, 1973). Furthermore, MCFAs and SCFAs are preferentially transported in the unesterified form bound to albumin *via* the hepatic portal vein, while LCFAs are preferentially packaged into chylomicrons in the enterocytes and transported through the lymphatic system (Ashbrook et al., 1972; Yang and Kuksis, 1987).

The difference in the intestinal handling of LCFAs and MCFAs and their differential effect on MMC activation suggest that the formation and secretion of chylomicrons is potentially linked to MMC activation. To test this possibility, Ji et al. administered L-81, an inhibitor of chylomicron formation. As expected, lymphatic transport of chylomicrons was completely abolished when L-81 was present in the intestinal lumen. However, eliminating the formation and transport of chylomicrons did not completely abolish the release of mediators by MMC (Ji et al., 2012). It is important to note that L-81 does not inhibit intestinal chylomicron formation instantly. It is possible that once chylomicron formation begins, the activation of MMC starts immediately. It is also entirely possible that there are other factors in addition to chylomicron formation and transport that are responsible for MMC activation by absorption of LCFA. As demonstrated by Chatterjee and Gashev, MMC are distributed in the intestinal lamina propria and are not directly exposed to intraluminal fatty acids (Chatterjee and Gashev, 2012). Thus, it is likely that signals mediated by intestinal epithelial cells, rather than direct interactions with lipid molecules, induce their activation (Ji et al., 2012).

Although results from Ji et al. demonstrated that fat absorption stimulates MMC activation, evidence from previous studies suggest that MMC activation is also important in enhancing the absorption and transport of lipids. It has been reported

that intestinal permeability is increased during fat absorption, which would facilitate uptake of fluid and electrolytes (Kvietys et al., 1991). This could be explained by the finding that mediators released by activated mast cells affect intestinal paracellular permeability (Keita and Soderholm, 2010). Furthermore, *in vitro* studies have shown that mast cell mediator RMCPII directly increases epithelial permeability by decreasing the expression of the tight junction-associated proteins occludin and zonula occludens-1 (Scudamore et al., 1998). It has also been demonstrated that RMCPII can selectively attack collagen IV, which is a key component of intestinal basement membranes (Patrick et al., 1988). Thus, MMC activation may also contribute to the formation of the breakages in the basement membrane mentioned previously that would facilitate the transport of chylomicrons from intercellular space to the lamina propria. Together, these observations suggest that MMC activation may be involved in increasing intestinal permeability and creating discontinuities in the intestinal basement membrane that may facilitate the uptake and transport of fat (Ji et al., 2012). The underlying mechanisms linking fat absorption and MMC activation are not well understood and further studies are warranted. With a better understanding of these complex interactions, we may be able to modify the leaky gut phenomenon often associated with the consumption of a high fat diet.

LINK BETWEEN CHYLOMICRON TRANSPORT, MUCOSAL MAST CELL ACTIVATION, LEAKY GUT, AND THE MICROBIOME

In conscious lymph fistula rats, we demonstrated that treating the animals with antibiotics greatly suppressed the lymphatic transport of chylomicrons as well as the activation of mucosal mast cells normally associated with fat absorption (Sato et al., 2016). The antibiotics treatment resulted in very low bacterial counts that we were unable to get a taxonomic characterization of the remaining microbiome. The reduction in mucosal mast cell activation was determined by the reduction in the release of mucosal RMCPII into the lymph. In addition, we observed that antibiotics treatment reduced the intestinal release of diamine oxidase into the lymph as well as gut permeability, both of which normally increased dramatically as a result of active fat absorption. We found that the decrease in lymphatic lipid transport was also associated with a marked reduction in intestinal apolipoprotein B (apoB) secretion. Because ApoB is critical for the formation and secretion of intestinal chylomicron particles, it is reasonable to conclude that the reduction in apoB secretion may be responsible for the decreased lymphatic lipid transport. The intestinal secretions of ApoA-I and ApoA-IV were also reduced significantly. As far as we know, this is the first time it has been demonstrated that there is a link between intestinal chylomicron transport, mucosal mast cell activation, and the gut microbiota. More experiments are obviously needed to help us understand better the relation between them.

It is not clear how antibiotic treatment affects apolipoprotein output in enterocytes because our dose of penicillin and streptomycin is not known to affect mammalian cells. The question of antibiotics treatment on the transcriptional and the translational production and secretion of apolipoproteins is probably best studied *in vitro*, as there are less variables to content with than in the *in vivo* scenario. Of course, we cannot rule out in our experiment whether there is a direct effect of microbiome on apolipoprotein synthesis and secretion. In our study (Sato et al., 2016), when we allowed the restoration of microbiome in the antibiotics treated animals, there was also a recovery of intestinal lymphatic transport of lipids and apolipoproteins. These are very interesting questions that should be explored in future studies.

AUTHOR CONTRIBUTIONS

PT provided guidance on the overall direction of the manuscript. AZ and PT wrote the manuscript. JQ, ML, and PT edited and proofread the manuscript. All authors critically reviewed the final version of the paper.

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Lymphatic Vasculature in Energy Homeostasis and Obesity

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Obesity is a leading cause of cardiovascular diseases and cancer. Body mass is regulated by the balance between energy uptake and energy expenditure. The etiology of obesity is determined by multiple factors including genetics, nutrient absorption, and inflammation. Lymphatic vasculature is starting to be appreciated as a critical modulator of metabolism and obesity. The primary function of lymphatic vasculature is to maintain interstitial fluid homeostasis. Lymphatic vessels absorb fluids that extravasate from blood vessels and return them to blood circulation. In addition, lymphatic vessels absorb digested lipids from the intestine and regulate inflammation. Hence, lymphatic vessels could be an exciting target for treating obesity. In this article, we will review our current understanding regarding the relationship between lymphatic vasculature and obesity, and highlight some open questions.

Keywords: *Prox1*, vegfc, neuropilin, lymphedema, lipedema, obesity, inflammation

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INTRODUCTION

Obesity is a disease caused by energy imbalance (Hill et al., 2012; Tchernof and Despres, 2013). Appetite, food consumption and exercise are only some of the parameters that regulate energy balance. In fact, obesity is a polygenic disease, and its etiology is determined by multiple factors such as neural circuits, hormones, nutrient absorption, lipid storage, lipid metabolism and inflammation (**Figure 1**). Besides, obesity is a risk factor for cancer and cardiovascular diseases such as type 2 diabetes mellitus, hypertension, hypercholesterolemia, hyperlipidemia, coronary artery disease, and stroke. In the United States, it is estimated that one-third of adults and one-sixth of children are obese (Ogden et al., 2013; Cunningham et al., 2014). The economic impact of obesity is estimated to be more than $100 billion per year in the United States alone (Hammond and Levine, 2010). Hence, better understanding of the mechanisms that regulate obesity and approaches to treat this disease are urgently needed. In this article, we will describe exciting new discoveries regarding the role of lymphatic vasculature in metabolic regulation and obesity.

FUNCTIONS OF THE LYMPHATIC VASCULATURE

Lymphatic vasculature absorbs interstitial fluid and returns it to blood circulation (Tammela and Alitalo, 2010; Geng et al., 2017). Lacteals are specialized lymphatic vessels in the intestine, which absorb digested lipids (Bernier-Latmani and Petrova, 2017; Petrova and Koh, 2018). Lymphatic vessels are also important for clearing low-density lipoprotein particles from the skin and arteries

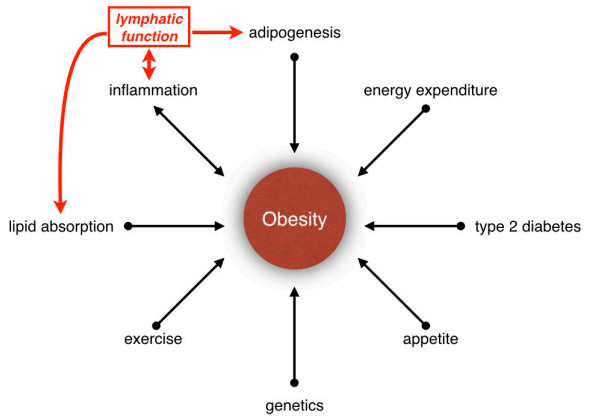

FIGURE 1 | Relationship between obesity and lymphatic vasculature. Obesity is influenced by many parameters, such as inflammation, lipid absorption, adipogenesis, type 2 diabetes, appetite, genetics, exercise, and energy expenditure. Increasing evidences have suggested that the lymphatic vasculature could be participating in the development of obesity by regulating lipid absorption, adipogenesis and inflammation.

by reverse cholesterol transport (Martel et al., 2013). Furthermore, lymphatic vasculature regulates the immune response (Randolph et al., 2005). Lymphatic endothelial cells (LECs) secrete cytokines such as CCL19 and CCL21 to recruit activated dendritic cells and transport them to lymph nodes to promote T- and B-cell activation (Randolph et al., 2005). LECs could directly present antigens to immune cells and promote peripheral immune tolerance (Rouhani et al., 2014). In summary, lymphatic vasculature regulates fluid homeostasis, lipid absorption, and immune response.

LYMPHATIC VASCULAR ARCHITECTURE

Lymphatic vasculature is made of LECs. LECs express unique markers that distinguish them from blood vascular endothelial cells. These markers include prospero-related homeobox 1 transcription factor (PROX1) (Wigle and Oliver, 1999), the tyrosine kinase receptor vascular endothelial growth factor 3 (VEGFR3/Flt4) (Kaipainen et al., 1995), the transmembrane *O*-glycoprotein podoplanin (Breiteneder-Geleff et al., 1999) and neuropilin 2 (NRP2) (Yuan et al., 2002). Endothelial cell junction proteins such as VE-cadherin, claudin-5, and PECAM-1 are expressed in all LECs. However, these junctional molecules are organized in different patterns to generate two functionally distinct structures within the lymphatic vasculature: lymphatic capillaries and collecting lymphatic vessels. Lymphatic capillaries have discontinuous "button-like junctions," which allows immune cells, interstitial fluid, and digested lipids to enter the vessels (Baluk et al., 2007; Yao et al., 2012). Lymphatic capillaries are connected to the surrounding tissues by anchoring filaments, which respond to changes in external fluid pressure by opening the button-like junctions to allow the entry of leukocytes and interstitial fluid into the vessels (Ikomi et al., 1996; Triacca et al., 2017). Unlike lymphatic capillaries, collecting vessels have continuous "zipper-like" endothelial junctions that prevent lymph leakage (Baluk et al., 2007; Yao et al., 2012). Collecting vessels are covered with continuous basement membrane and lymphatic muscle cells with contractile ability (Bridenbaugh et al., 2003; von der Weid and Zawieja, 2004; von der Weid, 2019). Collecting lymphatic vessels contain lymphatic valves (LVs), which regulate the unidirectional flow of lymph. Lymph flows through a series of lymph nodes where the immune cells and antigens are filtered out. Ultimately lymph is returned to blood circulation through two-pairs of lymphovenous valves (LVVs) located bilaterally at the junction of jugular and subclavian veins (Oliver and Srinivasan, 2008; Geng et al., 2017).

In the intestine, cholesterol, long-chain fatty acids, and fat-soluble vitamins are absorbed by intestinal epithelial cells, and repackaged in enterocytes into large (200–1000 nm) triglyceride-loaded particles called "chylomicrons" (Tso and Balint, 1986; Zhang et al., 2018). Each intestinal villus contains a single lacteal, which is a lymphatic capillary with button-like junctions (Zhang et al., 2018). Most chylomicrons are absorbed by the lacteals, from which they are transported to submucosal and mesenteric collecting lymphatic vessels, then into the thoracic duct, and finally to the blood circulation (Oliver and Srinivasan, 2008; Randolph and Miller, 2014). Thus, digested lipids bypass the liver and are accessible to other organs and cells prior to the liver (Ahn and Park, 2016).

LYMPHATIC VASCULAR DYSFUNCTION RESULTS IN LYMPHEDEMA

Structural and functional defects of lymphatic vascular system can lead to lymphedema, which is characterized by localized accumulation of interstitial fluid and tissue swelling (Lohrmann et al., 2009). Although lymphedema is not a life-threatening disease, it could cause functional disability, skin infection, inflammation fibrosis and pain (Mehrara and Greene, 2014; Escobedo and Oliver, 2016; Breslin et al., 2018). Lymphedema is classified into either primary or secondary lymphedema. Primary lymphedema is caused by mutations in genes that regulate lymphatic vascular development. These genetic defects result in structural and functional defects in LECs, LVs, or LVVs. Primary lymphedema is typically present early in life (infancy, childhood, or puberty), and rarely appears in adulthood (older than age of 35) (Grada and Phillips, 2017). Secondary lymphedema is caused by surgery (removal of lymph nodes) or radiation therapy in cancer patients, infection or trauma. Secondary lymphedema is much more prevalent than primary lymphedema, and is an increasing clinical problem due to the improving survival rates of cancer patients (Whelan et al., 2015; Rockson et al., 2019).

CLINICAL CORRELATIONS BETWEEN OBESITY AND LYMPHEDEMA

There is reciprocal relationship between obesity and lymphatic dysfunction. Obesity is one of the risk factors for the development

of secondary lymphedema in breast cancer patients (Werner et al., 1991; Helyer et al., 2010; Mehrara and Greene, 2014). In a study that compared 137 breast cancer patients, those with a body mass index (BMI) greater than 30 showed three times higher risk for developing upper extremity lymphedema compared with patients with lower BMI (Helyer et al., 2010). A similar result was observed in a second independent study involving 282 breast cancer patients (Werner et al., 1991).

Obesity could result in lymphedema in the absence of other risk factors such as primary lymphedema, inguinal lymphadenectomy or radiation treatment (Greene et al., 2012; Arngrim et al., 2013). In a study with a small cohort of 15 obese individuals, the average BMI of the patients with lymphedema (70.1) was much higher than the BMI of obese patients without lymphedema (42.0) (Greene et al., 2012). Another clinical study demonstrated that lean, healthy men (BMI: 22.3) showed better adipose tissue lymphatic drainage when compared with obese, but otherwise healthy men with normal glucose tolerance (BMI: around 35.7) (Arngrim et al., 2013).

Impaired lymphatic function could also lead to adipose tissue accumulation and fibrosis (Brorson, 2003). Secondary lymphedema patients tend to have higher BMI compared to breast cancer survivors without lymphedema (Ahmed et al., 2008). Lymphedema patients have more adipocytes in the edematous tissues. The edematous tissues are infiltrated with macrophages and lymphocytes, which promote the proliferation and hypertrophy of local adipocytes (Brorson, 2012; Ghanta et al., 2015). Adipose tissue-derived stem cells from lymphedema patients are more potent in their ability to undergo adipogenic differentiation *in vitro* when compared to cells derived from control patients (Levi et al., 2013). Taken together, these results suggest that chronic lymphedema creates an inflamed environment, which promotes adipose tissue accumulation (Cucchi et al., 2017). Nevertheless, lymphedema could reduce mobility and thereby reduce energy expenditure. Hence, is adipogenesis a direct outcome of lymphedema or is it indirectly caused by chronic immobility that is associated with lymphedema? This important question was addressed in part by recent studies, which have suggested that exercise (yoga, stretching, strength training) could improve breast cancer-related lymphedema (Baumann et al., 2018; Panchik et al., 2019). These studies must be very encouraging to lymphedema patients as they suggest that lymphedema-associated adipogenesis could be controlled by exercise.

LIPEDEMA: A PAINFUL DISEASE THAT AFFECTS LYMPHATIC VASCULATURE AND ADIPOCYTES

Lipedema is a disease characterized by the swelling of the legs due to the deposition of subcutaneous adipose tissue in the legs, thighs and buttocks (Torre et al., 2018). Most lipedema patients have high BMI, either in the overweight (25 to 30) or obese range (>30) (Fife et al., 2010). However, lipedema patients are less prone to type 2 diabetes mellitus, hypertension and dyslipidemia indicating that lipedema is distinct from obesity (Fonder et al., 2007; Al-Ghadban et al., 2019). Wold et al. proposed a series of criteria to diagnose lipedema in 1940s that is still in use (Child et al., 2010). These criteria include: (1) occurrence almost exclusively in women; (2) bilateral and symmetrical nature of adipose tissue accumulation with minimal involvement of the feet or ankle; (3) minimal pitting edema; (4) pain, tenderness and easy bruising; (5) persistent swelling of lower extremities despite elevation or weight loss (Child et al., 2010). The cause of lipedema is still unclear; however, there is evidence that hormonal and hereditary influence may play a role in the development of lipedema (Fonder et al., 2007). Important for this work, lipedema patients often show abnormal lymphoscintigraphic pattern with slow lymph flow (Bilancini et al., 1995). Ultrasound, MRI, and lymphangiogram are helpful for clinical diagnosis of lipedema (Warren Peled and Kappos, 2016). However, the test results may show "normal lymphatic function" in the early stage of the disorder (Fonder et al., 2007; Lohrmann et al., 2009; Child et al., 2010). Consequently, lipedema is often misdiagnosed as obesity (Fonder et al., 2007; Lohrmann et al., 2009; Godoy Mde et al., 2012; Shavit et al., 2018). Hence, whether lymphatic stasis in these patients cause increased adipogenesis or vice versa remains unknown.

MOUSE MODELS THAT HIGHLIGHT THE ROLE OF LYMPHATIC VASCULATURE ON METABOLISM AND OBESITY

As described in the previous sections, there is strong clinical correlation between lymphatic dysfunction and adipogenesis. In this section we will discuss evidence from mouse models, which allows us to determine causality.

PROX1

Prox1 heterozygous mice develop chylous ascites (leakage of lipid−rich lymph into the peritoneal cavity) soon after birth (Harvey et al., 2005). These $Prox1^{+/-}$ mice develop adipocyte hypertrophy (increase in the size of adipocytes), increased serum free fatty acids, fatty liver and obesity in adulthood. Conditional deletion of one allele of *Prox1* from endothelial cells recapitulated this phenotype. In addition, overexpression of *Prox1* in the LECs rescues the obese phenotype of *Prox1* heterozygous mice (Escobedo et al., 2016). Furthermore, lymph could stimulate adipogenesis *in vitro* (Harvey et al., 2005). Together these results suggest that leakage of lymph triggers the onset of adipogenesis and obesity in $Prox1^{+/-}$ mice (Harvey et al., 2005; Escobedo et al., 2016).

Obesity is mainly defined as a disease of energy imbalance. Young non-obese $Prox1^{+/-}$ mice do not consume more food compared to their control littermates. In contrast, older obese $Prox1^{+/-}$ mice consume less food and exercise less when compared with controls (Harvey et al., 2005). What promotes the transition between these metabolic states is currently unknown. Adipocyte inflammation is known to reduce energy expenditure and lower food consumption (Leibel et al., 1995; Zhao et al., 2018). Hence, it is possible that the adipocyte inflammation

observed in *Prox1*$^{+/-}$ mice (Harvey et al., 2005) might be contributing to the onset of obesity in older mice.

The obese phenotype of *Prox1*$^{+/-}$ mice is strain dependent. *Prox1*$^{+/-}$ and Tie2-Cre; *Prox1*$^{+/f}$ mice bred in the NMRI background develop obesity (Harvey et al., 2005). However, conditional deletion of one allele of *Prox1* using lymphatic vasculature-specific *Lyve1-Cre* in a non-NMRI background does not result in obesity (Escobedo et al., 2016). The reason for this strain-specific onset of obesity remains to be determined. Nevertheless, PROX1 is expressed in many metabolism-related organs (see below). Hence, it is worth investigating whether PROX1 might be playing additional LEC-independent roles in regulating obesity.

PROX1 is necessary for the development of liver (Sosa-Pineda et al., 2000; Seth et al., 2014). Obese *Prox1*$^{+/-}$ mice develop hepatosteatosis (fatty liver disease) (Harvey et al., 2005). This observation is consistent with the fact that PROX1 is important for the maintenance of lipid homeostasis in the liver. In the hepatocytes PROX1 interacts with HDAC3 to down-regulate the expression of genes such as *G0s2*, *Elovl6*, *Mfsd2a*, and *Cidec*, which are involved in lipid synthesis and lipolysis (Charest-Marcotte et al., 2010; Armour et al., 2017). Reducing PROX1 expression by AAV-based shRNA resulted in an increase in triglyceride levels in the mouse liver (Armour et al., 2017). In addition, treatment of mice with rapamycin causes hyperlipidemia by lowering PROX1 expression in the liver (Kwon et al., 2016).

PROX1 is important for the development of pancreatic endocrine cells and glucose metabolism. Conditional deletion of *Prox1* in pancreatic progenitors of mice showed delayed embryonic islet cell genesis and damage of ductal tissue in adulthood (Westmoreland et al., 2012). Overexpression of *Prox1* in immature β-cells promotes acute hyperglycemia (Paul et al., 2016). Consistent with these reports single-nucleotide polymorphisms in *PROX1* are associated with higher fasting glucose levels and type 2 diabetes mellitus (Lecompte et al., 2013; Kretowski et al., 2015; Hamet et al., 2017; Adamska-Patruno et al., 2019). Furthermore, hyperinsulinemia is observed in obese *Prox1*$^{+/-}$ mice (Harvey et al., 2005). However, whether it is due to lymphatic dysfunction or pancreatic defects is currently unknown. Insulin treatment is associated with weight gain in human patients (Russell-Jones and Khan, 2007). Hence, whether increased plasma insulin levels might contribute to obesity in *Prox1*$^{+/-}$ mice could be investigated.

Skeletal muscle is an important tissue for glucose metabolism and fat (mainly free fatty acid) storage (Frontera and Ochala, 2015). Besides, metabolic rate of skeletal muscles is an important index of resting energy expenditure (Zurlo et al., 1990). Skeletal muscles are composed of slow muscle fibers and fast muscle fibers, which generate energy through aerobic and anaerobic mechanisms respectively. Obesity in humans is inversely correlated with proportion of slow muscle fibers (Wade et al., 1990). *Prox1* is expressed in the satellite cells and slow muscle fibers. Conditional deletion of *Prox1* in skeletal muscles switched the slow muscle fibers to fast muscle fibers (Kivela et al., 2016). In contrast, overexpression of PROX1 converted fast muscle fibers to slow muscle fibers. These results suggest that *Prox1* may play a critical metabolic role by regulating the identity of muscle fibers (Kivela et al., 2016).

Prox1 is expressed in brain regions, such as cortex, hippocampus, thalamus, hypothalamus, and cerebellum during embryonic and post-natal stages (Galeeva et al., 2007; Lavado and Oliver, 2007). Importantly, PROX1 is expressed in the paraventricular nucleus and the arcuate nucleus of the hypothalamus (Lavado and Oliver, 2007), which are directly involved in appetite control and feeding behavior (Formolo et al., 2019). *Prox1* is a key factor for granule cell formation, maturation and differentiation during developmental stages (Lavado et al., 2010). *Prox1* is important for adult neurogenesis in dentate gyrus and subventricular zone (Lavado et al., 2010; Bunk et al., 2016). However, whether PROX1 regulates the formation and/or functioning of the appetite centers of the brain remains to be investigated.

PROX1 is not expressed in retroperitoneal adipocytes (Harvey et al., 2005). However, PROX1 is reported to be expressed in subcutaneous and omental adipose tissues (Procino, 2014). Whether PROX1 is expressed in adipocyte progenitors and whether it could regulate adipocyte lineage in an epigenetic manner is unknown.

In summary, based on the known roles of PROX1 in metabolic tissues we are tempted to speculate that PROX1 might be controlling certain aspects of obesity in non-lymphatic tissues. Obesity has not been reported in mice that are heterozygous for *Prox1* specifically in the liver, pancreas, brain, or muscle. Hence, it is possible that obesity arises in *Prox1*$^{+/-}$ mice as a consequence of metabolic defects in multiple tissues as reported for another transcription regulator TRIM28 (Dalgaard et al., 2016). PROX1 regulates the expression of mitochondrial lipid transporter CPT1a in LECs (Wong et al., 2017). Through this metabolic pathway PROX1 promotes epigenetic changes that support lymphangiogenesis. Whether PROX1 regulates CPT1a expression in other tissues, and whether this pathway could contribute to the onset of obesity in *Prox1*$^{+/-}$ mice needs to be evaluated.

VEGF-C, VEGF-D, and VEGFR3

VEGF-C/VEGFR3 signaling is critical for lymphatic vascular development. *Vegfc*$^{-/-}$ mice lack lymphatic vessels due to a failure of LEC budding from the embryonic veins (Karkkainen et al., 2004; Hagerling et al., 2013). Mutations in *VEGFR3* and *VEGF-C* are associated with congenital lymphedema (Ferrell et al., 1998; Brice et al., 2005; Gordon et al., 2013). Subcutaneous fat is observed in *Chy* mice carrying an inactivating point mutation in the kinase domain of VEGFR3 (Karkkainen et al., 2001). However, obesity is not observed in *Chy* mice (Aspelund et al., 2016) indicating that obesity and adipogenesis are not necessarily correlated.

Deletion of *Vegfc* in adult mice caused the regression of intestinal lacteals, reduced lipid absorption and provided resistance to high fat diet-induced obesity (Nurmi et al., 2015). Likewise, K14-VEGFR3-Ig mice in which a soluble form of extracellular VEGFR3 (sVEGFR3) that traps VEGF-C and VEGF-D was expressed from keratinocytes, were also resistant to high fat diet induced obesity, hepatic lipid accumulation

and metabolic dysfunction (Karaman et al., 2015). In contrast, overexpression of VEGF-C in the keratinocytes resulted in weight gain, hepatic lipid accumulation, subcutaneous adipose tissue accumulation and insulin resistance even in chow diet fed mice (Karaman et al., 2016). Furthermore, an increased number of pro-inflammatory macrophages were found in the white adipose tissue of the VEGF-C overexpression mice (Karaman et al., 2016). These results suggest that the pro-lymphangiogenic VEGF-C is also a pro-inflammatory and pro-obesogenic molecule. These results are consistent with the observation that obese patients have elevated levels of VEGF-C in their serum (Silha et al., 2005; Wada et al., 2011).

Gut microbiota are important regulators of metabolism and energy balance (Nicholson et al., 2012; Chevalier et al., 2015). Depletion of gut microbiota leads to morphological defects in lacteals (Suh et al., 2019). Gut microbiota stimulates macrophages of the intestinal villi to produce VEGF-C. Lack of gut microbiota causes the transformation of button-like junctions of lacteals into zipper-like junctions due to reduced VEGF-C signaling (Suh et al., 2019). This junctional transformation reduced lipid absorption in the gut (Suh et al., 2019). Taken together, these results suggest that gut microbiota may play a role in obesity and metabolic syndrome by regulating VEGF-C signaling.

VEGF-D is an additional ligand of VEGFR3. Deletion of *Vegfd* in mice does not result in any obvious lymphatic defects (Baldwin et al., 2005). However, overexpression of VEGF-D can induce lymphangiogenesis and angiogenesis under normal and pathological conditions (Baldwin et al., 2005; Bui et al., 2016). Overexpression of VEGF-D in lungs, kidneys, and adipose tissue induces hyperplasia of lymphatic vessels and lymphangiectasia (Lammoglia et al., 2016). Moreover, long-term overexpression of VEGF-D in adipose tissue of mice caused increased macrophage infiltration and enhanced adipose tissue fibrosis (Lammoglia et al., 2016). However, overexpression VEGF-D did not result in obesity or insulin resistance in chow diet fed mice (Lammoglia et al., 2016). Surprisingly, in high-fat diet fed condition overexpression of VEGF-D in the adipocytes resulted in enhanced glucose clearance, lower insulin levels and reduced liver triglycerides (Chakraborty et al., 2019). Total F4/80^{+} macrophages were reduced in subcutaneous adipose tissue by increased immune trafficking from the tissue. These results suggest that enhanced VEGF-D signaling in the adipose tissue might reduce obesity associated-immune cell accumulation and improve metabolic response (Chakraborty et al., 2019).

These unexpected and somewhat contradictory roles of VEGF-C, VEGF-D, and VEGFR3 call for further investigation of this signaling pathway in metabolic disorders and obesity.

Neuropilin

Neuropilins 1 and 2 (NRP1 and NRP2) were originally identified as molecules that are necessary for the patterning of neurons (Kitsukawa et al., 1995; Kolodkin et al., 1997). NRPs were found to function as co-receptors of VEGF-receptors in endothelial cells (Bouvree et al., 2012). NRP1 is mainly expressed in arteries and NRP2 is expressed in veins and lymphatic vessels (Herzog et al., 2001; Yuan et al., 2002). VEGF-A associates with NRP1/VEGFR1 complex to induce angiogenesis (Soker et al., 1998; Zhang et al., 2018). VEGF-C associates with NRP2/VEGFR2/VEGFR3 complex to regulate lymphangiogenesis (Favier et al., 2006; Bouvree et al., 2012; Deng et al., 2015). NRPs also interact with secreted and transmembrane ligands known as semaphorins (Kolodkin et al., 1997). There are eight classes of SEMA family (SEMA1-7; SEMAV); of which, SEMA3-7 are found in vertebrates (Neufeld and Kessler, 2008). NRPs can interact with members of SEMA3 family. SEMA3/NRP complexes further interact with transmembrane proteins called plexins. Plexins regulate the development of many organs, such as skeleton and kidney, and participate in angiogenesis and vascular patterning (Perala et al., 2012). SEMA3/NRP1/PlexinA1 complex regulates LV morphogenesis (Bouvree et al., 2012; Jurisic et al., 2012).

Nrp1 knockout mice are embryonic lethal at E12.5. *Nrp1*$^{-/-}$ mice exhibited vascular defects such as agenesis and transposition of great vessels, and disorganized and insufficient development of vascular networks in the yolk sac (Kawasaki et al., 1999). Global overexpression of *Nrp1* results in ectopic blood vascular sprouting, dilated vessels, and abnormal heart (Kitsukawa et al., 1995).

Lacteals are made of LECs with discontinuous button-like junctions. These button-like junctions allow the easy passage of large chylomicrons into the lacteals. Deletion of *Nrp1* or *Vegfr1* by inducible endothelial- specific Cre resulted in the transition of button-like junctions of lacteals into zipper-like junctions (Zhang et al., 2018). Knock out of *Nrp1* or *Vegfr1* in blood endothelial cells enhances the bioavailability of VEGF-A and downstream signaling through VEGFR2 in LECs. High VEGFR2 signaling activity promotes zipper-like junctions in lacteals. Increased number of zipper-like junctions in mice lacking NRP1 or VEGFR1 reduced chylomicron uptake and also increased their resistance to high-fat diet-induced obesity (Zhang et al., 2018).

Nrp2-null mice are viable but have hypoplastic lymphatic capillaries compared with littermate controls (Yuan et al., 2002). Whether LEC-specific deletion of *Nrp2* affects metabolism is currently not known.

Neuropilins and their ligands play an additional-complex role in energy homeostasis through the nervous system (van der Klaauw et al., 2019). Several variants of *NRP1*, *NRP2*, and *SEMA3* are identified in severely obese individuals (van der Klaauw et al., 2019). These variants disrupt the secretion of melanocortin and/or signaling downstream of melanocortin-4-receptor (MC4R) in human embryonic kidney 293 cells. *In vivo* melanocortin is released by pro-opiomelanocortin (POMC) or Neuropeptide Y (NPY)/Agouti-related protein (AgRP) positive neurons in hypothalamus. Melanocortin/MC4R signaling reduces food intake and increase energy expenditure (Cowley et al., 2001). Deletion of *Nrp2* in POMC neurons reduces energy expenditure and causes weight gain (van der Klaauw et al., 2019). Hence, the lymphatic and non-lymphatic contribution of NRPs should also be dissected in the context of obesity.

Apelin

Apelin is a peptide and a ligand for the orphan G protein-coupled receptor AJP (Tatemoto et al., 1998). Apelin and AJP

are widely expressed in the brain, intestine, kidney, adipose tissue and vasculature (Lee et al., 2000; Chen et al., 2003). Apelin/AJP signaling is involved in many physiological processes, including glucose homeostasis, lipid metabolism (Trayhurn et al., 2006), obesity (Boucher et al., 2005; Sawane et al., 2013), and diabetes (Li et al., 2006). High fat diet-fed $Apln^{-/-}$ mice were severely obese and had lymphatic and blood vasculature abnormalities (Sawane et al., 2013).

In vitro studies determined that Apelin could stabilize the expression of adherens junction protein VE-Cadherin and reduce fatty acid-induced vascular hyperpermeability (Sawane et al., 2013). These results suggest that Apelin maintains lymphatic vessel integrity, and inhibits dietary fat absorption and accumulation.

In summary, these observations suggest a context dependent role of lymphatic vasculature in regulating obesity. Nevertheless, additional non-LEC roles for these molecules in regulating metabolism cannot be excluded.

MOUSE MODELS OF OBESITY WITH DEFECTIVE LYMPHATIC FUNCTION

As mentioned previously, lymphatic function is abnormal in obese humans (Arngrim et al., 2013). The following mouse studies support those observations and provide mechanistic explanations for obesity-induced lymphatic dysfunction.

Adipokines

Adipocytes secrete signaling molecules such as leptin, adiponectin, and resistin. These signaling molecules are collectively known as adipokines. One of the primary functions of leptin is to activate nerve centers of the brain and promote satiety. *Ob/Ob* mice, which lack leptin develop severe obesity and type 2 diabetes mellitus. Scallan et al. (2015) demonstrated that the lymphatic vessels of *Ob/Ob* mice are dilated and leaky. They further showed that this lymphatic phenotype is due to low nitric oxide (NO) bioavailability (Scallan et al., 2015).

Adiponectin also plays a role in maintaining lymphatic function (Shimizu et al., 2013). Using tail-injury model Shimizu et al. demonstrated that mice lacking adiponectin were deficient in their ability to clear tissue fluid and had severe tail edema. In contrast, administration of adiponectin promoted the regrowth of lymphatic vessels and reduced edema. Mechanistically, adiponectin activated the phosphorylation of AKT and eNOS through AMPK and promoted the survival and proliferation of LECs. These data suggest that adipokines such as leptin and adiponectin act as modulators of lymphatic function.

High-Fat Diet-Induced Obesity

Several studies have investigated the influence of high fat diet induced obesity on lymphatic vascular function (Lim et al., 2009; Blum et al., 2014; Savetsky et al., 2014, 2015; Barton and Husmann, 2016; Garcia Nores et al., 2016; Hespe et al., 2016; Nitti et al., 2016; Torrisi et al., 2016). They overwhelmingly conclude that obesity affects lymphatic vascular formation and function.

High-fat diet aggravates hypercholesterolemia in $ApoE^{-/-}$ mice. Lymphatic vessels of these hypercholesterolemic mice were defective in their ability to take up interstitial fluid and were leaky (Lim et al., 2009). Moreover, collecting lymphatic vessels of these mice lacked smooth muscle cell coverage and had abnormal LVs (Lim et al., 2009). Therefore, hypercholesterolemia could inhibit the maturation of lymphatic vessels.

Weitman et al. (2013) demonstrated that high-fat diet-induced obesity resulted in impaired lymphatic fluid uptake and transport, reduced number of LECs in lymph nodes, dysregulated CCL21 gradient and defective immune cell trafficking. Similarly, Detmar and colleagues showed that high-fat diet impaired the contractility of lymphatic vessels and made them non-responsive to mechanostimulation (Blum et al., 2014). Thus, high-fat diet could inhibit LEC survival or proliferation and could compromise lymphatic vascular function.

How could obesity affect lymphatic function? Using tail-injury model Detmar and colleagues quantified lymphatic function in high-fat diet-fed mice before they became obese (Gousopoulos et al., 2017). They determined that lymph transport is identical between control and high-fat diet-fed mice. This important observation indicated that increased number or size of adipocytes, but not serum lipids, is responsible for lymphatic dysfunction in obese mice. Obesity is associated with chronic, low-grade inflammation (Wellen and Hotamisligil, 2005). Savetsky et al. (2014, 2015) demonstrated that obesity-induced inflammation could compromise lymphatic function. In contrast, inhibition of inflammation increases lymphatic capillary density and restores lymphatic vascular function in high-fat diet-induced obese mice (Torrisi et al., 2016). Taken together, inflammation could play a key role in obesity-induced lymphatic dysfunction. Furthermore, lymphatic dysfunction could also exacerbate inflammation, thus setting up a vicious feedback loop (**Figure 1**). Compression or increased lymph load caused by adipocyte hypertrophy/hyperplasia could also affect lymphatic function.

SUMMARY AND FUTURE DIRECTIONS

Obesity is a complex disease that is influenced by many parameters (**Figure 1**). Evidence is clear that the lymphatic vasculature regulates lipid absorption, adipogenesis, and inflammation (**Figure 1**). It is also clear that obesity could affect lymphatic function. However, whether lymphatic dysfunction could cause obesity is not fully clear. Mouse studies have revealed that while some mutations that cause lymphatic vascular defects could promote obesity, other mutations protect against it. As proteins generally perform multiple functions it is possible that the genes that regulate lymphatic vascular development or functioning could independently regulate metabolism. Additionally, lymphedema causes severe pain and immobility in patients, which in turn could affect energy expenditure and

cause weight gain. Due to these complexities carefully controlled clinical studies and mouse experiments are needed to better understand the relationship between lymphatic function and metabolic diseases. This knowledge could significantly impact the lives of millions of lymphedema, lipedema, and obesity patients worldwide.

AUTHOR CONTRIBUTIONS

Y-CH and RS designed, co-wrote, and coedited the manuscript.

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Emerging Role of Lymphatics in the Regulation of Intestinal Lipid Mobilization

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Intestinal handling of dietary triglycerides has important implications for health and disease. Following digestion in the intestinal lumen, absorption, and re-esterification of fatty acids and monoacylglycerols in intestinal enterocytes, triglycerides are packaged into lipoprotein particles (chylomicrons) for secretion or into cytoplasmic lipid droplets for transient or more prolonged storage. Despite the recognition of prolonged retention of triglycerides in the post-absorptive phase and subsequent release from the intestine in chylomicron particles, the underlying regulatory mechanisms remain poorly understood. Chylomicron secretion involves multiple steps, including intracellular assembly and post-assembly transport through cellular organelles, the lamina propria, and the mesenteric lymphatics before being released into the circulation. Contrary to the long-held view that the intestinal lymphatic vasculature acts mainly as a passive conduit, it is increasingly recognized to play an active and regulatory role in the rate of chylomicron release into the circulation. Here, we review the latest advances in understanding the role of lymphatics in intestinal lipid handling and chylomicron secretion. We highlight emerging evidence that oral glucose and the gut hormone glucagon-like peptide-2 mobilize retained enteral lipid by differing mechanisms to promote the secretion of chylomicrons via glucose possibly by mobilizing cytoplasmic lipid droplets and via glucagon-like peptide-2 possibly by targeting post-enterocyte secretory mechanisms. We discuss other potential regulatory factors that are the focus of ongoing and future research. Regulation of lymphatic pumping and function is emerging as an area of great interest in our understanding of the integrated absorption of dietary fat and chylomicron secretion and potential implications for whole-body metabolic health.

Keywords: lymphatic, triglyceride, intestine, mobilization, glucose, glucagon-like peptide-2

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INTRODUCTION

Intestinal handling of dietary triglycerides (TGs) plays an important role in maintaining whole-body lipid and energy homeostasis and has important implications for health and disease (Abumrad and Davidson, 2012). Improved understanding of intestinal lipid processing may help develop novel therapeutic strategies to treat hyperlipidemia and improve cardiac and metabolic health (Lewis et al., 2015). Dietary fat absorption and transport in the gut through lipoprotein secretion is regulated by many factors. Numerous studies have investigated the regulatory mechanisms of

intestinal lipoprotein particle biosynthesis and assembly (Xiao et al., 2014; Dash et al., 2015). Recently, it is becoming increasingly appreciated that, besides particle production, regulation of post-assembly transport of lipoproteins contributes to the overall lipid delivery through the gut. While the underlying mechanisms remain incompletely understood, intestinal lymphatics are emerging as a potentially important player. This review aims to examine the interplay between lipoprotein secretion and the function of the mesenteric lymphatic system.

DIETARY TG DIGESTION, ABSORPTION, AND CHYLOMICRON SYNTHESIS AND SECRETION

The digestion products of ingested TG, fatty acids and monoacylglycerols, are absorbed at the apical membrane of enterocytes lining the brush border of the small intestinal lumen. Following absorption by enterocytes, fatty acids and monoacylglycerols are re-esterified into TG and form lipid droplets at the ER membrane leaflet. The majority of newly synthesized TGs are packaged into chylomicrons (CMs) for secretion. Lipid droplets bud off into the ER lumen and fuse with a lipid-poor apolipoprotein B48-containing particle to form the pre-chylomicron particle. Pre-chylomicrons are transported in pre-chylomicron transport vesicles (PCTVs) from the ER to the Golgi apparatus where they are further processed. Mature CMs exit enterocytes by exocytosis at the basolateral membrane, pass through the villus lamina propria, enter into the lacteals (a single, blind-ended lymphatic vessel located at the center of each intestinal villus), and move through the mesenteric lymph ducts and then the thoracic duct where they enter the blood circulation at the left subclavian vein.

A portion of lipid droplets are not immediately utilized for pre-chylomicron synthesis. Instead, they bud off the ER into the cytosol, where TGs are utilized for the formation of cytoplasmic lipid droplets (CLDs). CLD synthesis, metabolism, and catabolism are highly dynamic during the fasting and feeding cycle (D'Aquila et al., 2016). CLDs expand in size following a fat meal via either fusion or TG synthesis at the CLD surface, while CLD catabolism during the post-absorptive state occurs through TG lipolysis and redistribution or lipophagy. CLDs may serve as transient storage of TG in enterocytes, which attenuates postprandial TG excursion and provides a continuous TG supply as substrates for late postprandial and post-absorptive CM secretion (Beilstein et al., 2015), enhances the overall efficiency of dietary fat absorption, and prevents cellular and systemic toxicity as a result of lipid overload (Xiao et al., 2018a). It is noted that temporary lipid storage in CLDs is conserved and common in lower species, such as *Caenorhabditis elegans* and reptiles, implicating evolutionary benefits. Dynamic formation and mobilization of CLD lipids are highly regulated and play an important role in determining net CM secretion rate from the intestine. The details of both CM assembly and CLD metabolism have been reviewed elsewhere (Hussain, 2014; Beilstein et al., 2015; D'Aquila et al., 2016). Besides CM secretion and storage in CLDs, dietary fatty acids are also oxidized in enterocytes to generate ketone bodies (Uchida et al., 2011; Schober et al., 2013), a capacity that is well-developed during the suckling period in rodents, disappears at weaning, and can be re-established upon high-fat feeding (Thumelin et al., 1993; Clara et al., 2017; Ramachandran et al., 2018). A high capacity to oxidize dietary fat may translate into a lower risk of weight gain on high fat diets (Kondo et al., 2006). The overall process of dietary fat absorption, CM assembly, and secretion is highly controlled, including regulation of CM biosynthesis (as reviewed Hussain, 2014; Dash et al., 2015) and post-assembly regulation of CM transport prior to entering the circulation (Xiao et al., 2019a).

LIPID RETENTION AND MOBILIZATION

Prolonged Post-absorptive Lipid Retention in the Gut

While the majority of dietary TGs are rapidly secreted in CMs during meal ingestion, evidence supports more prolonged retention of TGs in the intestine during the post-absorptive period, with subsequent release triggered by a number of factors (Xiao et al., 2018a, 2019a). In healthy, lean individuals, postprandial plasma TGs rapidly rise, typically peak at 3–4 h after meal, and gradually return to fasting level after 6–8 h. However, under certain circumstances such as ingestion of a second fat meal or glucose, many hours after lipid ingestion, release of TGs from an intestinal "storage" pool can be demonstrated (Xiao et al., 2019a). Indeed, a stable isotope enrichment study in humans demonstrated that TGs in a fat-rich meal appeared in CM up to 18 h after ingestion (Chavez-Jauregui et al., 2010). In humans, abundant lipid droplets are present in the jejunum and duodenum 5 h after fat ingestion (Robertson et al., 2003; Xiao et al., 2018b). In fact, lipid droplets are observed in human duodenum 10 h after high fat ingestion (Xiao et al., 2018b). In rats, lipid deposits are present in the jejunum up to 12 h after fat ingestion (Hung et al., 2017). The physiological significance of such lipid retention in the gut is not clear. It is plausible that it attenuates postprandial excursion of plasma TG. It is also possible that a readily available intestinal lipid pool functions to "prime" the intestinal CM secretory machinery for the next incoming meal. It remains to be established whether such lipid retention in the gut may be abnormal in compromised metabolic conditions such as hyperlipidemic states, obesity, metabolic syndrome, and diabetes and whether it has implications for metabolic health.

Locations of TG Storage in the Gut

The exact locations of TG storage in the gut remain to be identified; however, several candidate locales appear to host the mobilizable pools. CLDs are one obvious source since they are a transient TG storage pool that undergoes dynamic metabolism during the feeding–fasting cycle (Hung et al., 2017). CLDs quickly expand in number and size following fat ingestion, while during the post-absorptive period, CLDs are catabolized, leading to fewer and smaller CLDs. The exact time frame of CLD synthesis and catabolism in humans has not been fully defined, but as mentioned above, a significant number and size of CLDs are still present many hours after fat ingestion (Robertson et al., 2003; Xiao et al., 2018b). Mature CM particles exit the enterocytes

at the basolateral membrane by exocytosis into intercellular spaces and the lamina propria, before entering the lacteals, larger mesenteric lymphatics, and the thoracic duct, and ultimately enter the blood circulation. The presence of CM particles along this secretory pathway has been identified in the intercellular spaces, the lamina propria, and lymphatic vessels (Robertson et al., 2003; Takahara et al., 2013; Xiao et al., 2018b). These extracellular CMs, which appear to be retained for many hours after food ingestion (Xiao et al., 2018b), may also be a source of mobilized intestinal lipid and CMs in the post-absorptive period. Although not specifically demonstrated, we postulate that CMs within mesenteric lymphatics are also not all rapidly secreted into the blood circulation, likely represent another significant pool of ingested lipid that is retained for many hours following food ingestion, and could be mobilized by various stimuli of lymphatic pumping.

Lipid Mobilization

Intestinal lipid pools are releasable upon receiving certain cues. In earlier studies, ingestion of a fat-rich meal hours after an earlier fat-rich meal that had elicited a typical postprandial TG excursion caused a rise in plasma TG prior to digestion and absorption of the second meal (Evans et al., 1998). In fact, sham fat feeding, i.e., chewing without swallowing of fats, also promoted appearance in CMs of TGs that originated from a previous meal (Mattes, 2009; Chavez-Jauregui et al., 2010). These studies point to a regulatory mechanism of gut lipid mobilization via neural circuits involving lipid sensing in the gut or through the olfactory and taste receptors, eliciting a "cephalic phase response" of CM secretion. Cephalic phase responses have been described for the secretion of hormones, such as insulin (Ahrén and Holst, 2001), ghrelin, and pancreatic polypeptide (Robertson et al., 2002; Simonian et al., 2005; Veedfald et al., 2016) prior to food digestion and absorption, triggered by sensing or perception of food. Cephalic phase secretion of hormones with sham feeding involves vagal efferent stimulation (Simonian et al., 2005), and both cholinergic and non-cholinergic autonomic activations mediate cephalic phase response of insulin secretion (Ahrén and Holst, 2001). Similarly, cephalic phase responses of intestinal lipid mobilization may involve vagal stimulation and increased parasympathetic activity (Robertson et al., 2002).

Oral Glucose and GLP-2 as Stimuli of Intestinal Lipid Mobilization

Ingestion of glucose has been shown to promote gut lipid release. In healthy individuals, drinking a glucose solution 5 h after a high-fat liquid meal elicited rises in plasma and CM TG (Robertson et al., 2003; Xiao et al., 2018b). This was accompanied by depletion of lipid droplets in the enterocytes in the jejunum (Robertson et al., 2003) and shifting toward fewer and smaller CLDs in the duodenum (Xiao et al., 2018b). The underlying mechanism remains to be elucidated, but proteomic analysis of duodenal biopsies suggests differential regulation of several proteins, e.g., syntaxin-binding protein 5 and ethanolaminephosphotransferase, that may be components of the CM biosynthesis and assembly machinery (Xiao et al., 2018b). It is postulated that oral glucose, through yet to be defined mechanisms, mobilized CLD TGs for cycling back to the ER to be utilized as a substrate for CM synthesis, i.e., glucose ingestion triggered the recruitment of the cytosolic transient TG storage pool for CM synthesis and secretion.

Glucagon-like peptide-2 (GLP-2) is a potent stimulus of intestinal lipid mobilization. GLP-2 is a peptide hormone secreted by the intestinal L-cells in response to nutrient ingestion. GLP-2 infusion during meal ingestion potentiated postprandial TG excursion in humans (Meier et al., 2006). In animals, GLP-2 promoted CM secretion when administered with a fat load (Hsieh et al., 2009). More importantly, GLP-2 administered 5 h after a fat load also promoted CM secretion, suggesting mobilization of intestinal lipid stores (Hsieh et al., 2015). In two recent studies in healthy humans, we have examined the effects and regulatory mechanisms whereby GLP-2 affects lipid handling in the gut. We first demonstrated that GLP-2 rapidly and robustly elevated TGs in plasma and CM, which was mostly due to the release of "preformed" CM particles rather than *de novo* synthesis of CM (Dash et al., 2014). GLP-2 receptor (GLP-2R) expression pattern suggests that GLP-2 targets post-enterocyte lipid pools. The GLP-2R is not expressed on enterocytes (El-Jamal et al., 2014; Pedersen et al., 2015) that are responsible for CM intracellular synthesis and assembly. Instead, it is mostly identified on enteric neurons, myofibroblasts, and stromal cells (Yusta et al., 2000, 2019; Ørskov et al., 2005; Guan et al., 2006; Wismann et al., 2017), which are abundantly present in the subepithelial regions of the intestine, including the lamina propria. It has been suggested that GLP-2-induced intestinal wound repair may involve vascular endothelial growth factor (VEGF) secretion from fibroblasts (Bulut et al., 2008). GLP-2R is also expressed in cells positive for nitric oxide synthase (NOS) and vasoactive intestinal peptide, both with roles in regulating vessel dilation and blood flow (Guan et al., 2006). GLP-2 has been shown to stimulate mesenteric blood flow in humans (Bremholm et al., 2010, 2011; Høyerup et al., 2013) and animals (Guan et al., 2003, 2006; Deniz et al., 2007), which may involve nitric oxide (NO) signaling (Guan et al., 2003; Hsieh et al., 2015). Recently, we have examined lipid mobilization by GLP-2 in healthy humans with or without a NOS inhibitor L-NG-monomethyl arginine acetate (L-NMMA) (Xiao et al., 2019b). GLP-2 administration after 7 h of fat ingestion increased TG in plasma, in triglyceride-rich lipoprotein (TRL), and mostly in CM, and increased blood flow in the superior mesenteric artery. L-NMMA co-administration attenuated the effect of GLP-2 on the increase in blood flow, but without a noticeable effect on lipid mobilization. These results suggest that systemic NO does not play a prominent role in mediating GLP-2 mobilization of intestinal lipid stores. In a recent study in mesenteric lymph duct cannulated rats, we also demonstrate that glucose and GLP-2 mobilize intestinal lipid stores via distinct mechanisms, pointing to primarily intracellular and extracellular lipid pools by glucose and GLP-2, respectively (Stahel et al., 2019). Considering the anatomy of the intestinal region, the likely post-enterocyte locations for harboring lipid stores include intercellular spaces, lamina propria, and the mesenteric lymphatic vasculature.

LYMPHATICS IN THE REGULATION OF LIPID MOBILIZATION

Intestinal Lymphatic System

The lymphatic system plays an important role in body fluid homeostasis, dietary fat absorption, inflammation and immune responses, and reverse cholesterol transport (Martel et al., 2013; Randolph and Miller, 2014; Bernier-Latmani and Petrova, 2017). Defects in the lymphatic system have been associated with metabolic disorders, including metabolic syndrome, obesity, diabetes, and atherosclerosis in animal models. Lymphatic function was impaired in rats with metabolic syndrome (Zawieja et al., 2012; Gasheva et al., 2019) and in obese mice (Weitman et al., 2013; Blum et al., 2014; García Nores et al., 2016), and defective lymphatics contributed to obesity and metabolism syndrome (Escobedo and Oliver, 2017; Gasheva et al., 2019). Diabetes in mice was associated with impaired lymphangiogenesis and disrupted lymphatic integrity (Scallan et al., 2015; Wu et al., 2018). The lymphatic system has also been implicated in the pathogenesis of atherosclerosis and cardiovascular disease (Aspelund et al., 2016). Impaired lymphatic function was associated with increased atherosclerosis, while enhancing lymphatic function attenuated development of atherosclerosis in mice (Vuorio et al., 2014; Milasan et al., 2019). Compromised lymphatic function has also been proposed to have systemic consequences for lipid metabolism and transport (Dixon, 2010).

Embryonic development of the lymphatic vessel growth and patterning and postnatal maintenance are under molecular regulation. Recent studies highlight critical roles of VEGF signaling in lymphangiogenesis during development and pathological processes (Alitalo, 2011). VEGF-C, the major lymphangiogenic factor, mediates lymphatic development and functions primarily through binding to VEGF receptor 3 (VEGFR3). In the intestine, VEGF-C is expressed by a subset of smooth muscle cells (SMCs) adjacent to the lacteals in the villus, in the circular smooth muscle layer of the intestinal wall, and in the arterial SMCs (Nurmi et al., 2015). In addition, VEGF-C is also secreted by villus macrophages upon recognition of gut microbes and their products (Suh et al., 2019). VEGF-C is activated by proteolytic cleavage of the full-length protein, a process involving the protease A disintegrin and metalloproteinase with thrombospondin motifs 3 and the secreted factor collagen and calcium binding EGF domains 1 (Bui et al., 2016; Jha et al., 2017). Besides being essential for perinatal lymphangiogenesis in many organs, VEGF-C, through activation of VEGFR3, is required for adult lymphatic vessel maintenance only in the intestine; thus, *Vegfc* deletion in adult mice leads to atrophy of intestinal lymphatic vasculature and lipid malabsorption (Nurmi et al., 2015). A distinct feature of intestinal lacteals is that they undergo continuous remodeling and are in a permanent regenerative and proliferative state in adults. This process is mediated by Notch signaling, and the expression of the Notch ligand delta-like 4 requires activation of VEGFR3 and VEGFR2 (Bernier-Latmani et al., 2015). Besides VEGF-C, adrenomedullin signaling through its receptor complex, calcitonin receptor-like (CRL) receptor, is also an important mediator for lymphangiogenesis during development and maintenance in adult mice (Hoopes et al., 2012; Davis et al., 2017). Lymphatic *Calcrl* (encoding CRL) deletion in adult mice compromises systemic lymphatic function, induces inflammation and lymphatic dilation in the gut, reduces lacteal proliferation, and impairs lipid absorption (Davis et al., 2017). This may be due to downregulation of the Notch signaling pathway that is essential for lacteal regeneration and function.

Collectively, development and maintenance of the intestinal lymphatic vasculature and function have important implications for lipid absorption in the gut (Cifarelli and Eichmann, 2019). We speculate, therefore, that regulation of intestinal lymphatic function plays an active role in the physiological control of the rate of dietary lipid secretion into the circulation, although experimental evidence is currently lacking.

CM Entry Into the Lymphatics

The mesenteric lymphatic vasculature forms a network of lipid drainage. Following exocytosis, CM particles enter the intercellular spaces and the lamina propria. CMs are believed to move through the lamina propria via diffusion, largely influenced by convective movement of fluids (Tso and Balint, 1986). Subsequently, CMs enter the lymphatic system from the lamina propria via the lacteals (Takahara et al., 2013). Although a transcellular pathway has been described, the majority of CMs enter lacteals by a paracellular pathway via large, porous, intercellular junctions at the tip of the lacteal. Entry of CMs into the lacteal is believed to be through size exclusion; thus, the majority of CMs with considerable size enter the lacteal while only a minor fraction of CMs with very small sizes may enter the subepithelial blood capillaries (Takahara et al., 2013). Recent evidence in mice suggests that mechanisms beyond simple size exclusion may underlie the uptake of CMs by the lacteals. First, in mice lacking the transcription factor *PlagL2*, CMs can exit the enterocytes, but cannot enter the lacteals, resulting in accumulation of CMs in the lamina propria, fat malabsorption, and neonatal death (Van Dyck et al., 2007). A small fraction of CMs that do appear in the circulation are not efficiently utilized by peripheral tissues. The exact mechanism for this is not fully understood. *PlagL2* is expressed in the enterocytes and its deficiency reduces the expression of several genes (e.g., sorting nexins and vacuolar sorting proteins) that are candidate regulators of intracellular processing of dietary fat. It seems that particular CM particle properties, such as lipid and protein composition, are required for their successful entry into the lacteals, subsequent metabolism in the circulation, and utilization in peripheral tissues. Second, permeability of intercellular junctions on the lacteal wall is subject to regulatory control. Molecular control of the transition of vascular endothelial-cadherin (VE-cadherin) junctions between zipper (closed) and button (open) states impacts CM uptake into lacteals. Zippering of VE-cadherin junctions through VEGF-A-VEGFR2 signaling and VE-cadherin signaling resulted in CM malabsorption, which was rescued by restoring to buttoning state (Zhang et al., 2018). Third, VEGFR3, the major receptor that

VEGF-C binds to, may play a role in regulating intestinal lipid absorption and CM entry into the lacteals. In the Chy mice that carry an inactivating mutation of the tyrosine kinase domain of VEGFR3, there was TG retention in the enterocytes, decreased postprandial plasma TG levels, and increased fecal excretion of free fatty acids and TGs, which may be mediated through NO in the intestine (Shew et al., 2018). Together, these studies support that passage of CMs through the lacteal endothelial wall involves mechanisms beyond simple size exclusion, requiring specific CM properties, controlled gating of the junctions, and VEGFR3 signaling.

Lymphatic Pumping Functions and Regulation of CM Transport

Flow of lymph in the lymphatic vessel is achieved in part through an active pumping mechanism (Hosoyamada and Sakai, 2005; Kassis et al., 2016; Scallan et al., 2016). The smooth muscles surrounding the lymphatic endothelial wall contract in both tonic (vessel diameter) and phasic (frequency and amplitude) modes. The one-way valves inside the lymphatic vessel prevent lymph backflow. Together, contractile activities and valves provide unidirectional pumping force for active flow of lymph from the intestine to the circulation. Many of the above-mentioned compromised conditions, e.g., metabolic syndrome and obesity, are associated with impaired lymphatic functions, as reflected by reduced lymph drainage and decreased inflammatory cell mobilization and bacterial antigen clearance. For instance, the intrinsic contractility of the mesenteric lymphatics was impaired in a rat model of metabolic syndrome (Zawieja et al., 2012). Further, genetic manipulation to induce zippering (closing) of junctions along the lacteal wall impaired lacteal uptake of CMs and protected against diet-induced obesity in mice (Zhang et al., 2018). Taking advantage of the VEGF signaling in mediating lymphatic growth and function, chronic VEGF-C treatment has been applied to enhance lymphangiogenesis and lymphatic function, with enhanced lymphatic drainage, increased inflammatory cell mobilization, and bacterial antigen clearance in a mouse model of inflammatory bowel disease (D'Alessio et al., 2014).

While most studies demonstrate the importance of pre- and postnatal lymphangiogenesis in normal lymphatic vasculature development and maintenance of functions, studies also point to acute regulation of lymphatic functions. Lipid infusion into the duodenum of rats increased total lymph flow in the mesenteric lymphatic vessels, but decreased both phasic and tonic contractility (Kassis et al., 2016). Besides the major mesenteric lymphatics, intestinal lipid drainage in mice is regulated via contraction of the smooth muscles surrounding each lacteal, a process subject to the control of the autonomic nervous system (Choe et al., 2015). In rats, *in situ* perfusion of the mesenteric lymphatic bed with various VEGF signaling modulating factors acutely affected lymphatic pump activity (Breslin et al., 2007). Both VEGF-C and the VEGFR3-specific activator VEGF-C156S significantly increased contraction frequency, end-diastolic diameter, stroke volume index, and pump flow index. Conversely, inhibition of VEGFR3 caused tonic constriction and decreased contraction frequency and attenuated VEGF-C- and VEGF-C156S-induced lymphatic pump activation. This study supports that VEGFR3 mediates lymphatic pumping in the acute setting. Mechanisms other than VEGF signaling may also play a role in acute modulation of lymphatic functions. Contraction of isolated rat mesenteric lymphatics is reduced with the activation of the σ1-receptor through an NO-dependent mechanism (Trujillo et al., 2017). SMCs surrounding the intestinal lacteals express β-adrenergic receptors and muscarinic receptors (Bachmann et al., 2019), supporting a mediating role of the vagal nerves that provides dense innervation for intestinal lymphatics and surrounding muscles. Collectively, pumping functions of the larger mesenteric lymphatics and contraction of the lacteals actively participate in lipid drainage in the gut. Acute modulation of the functions of mesenteric lymphatics, including the lacteal,

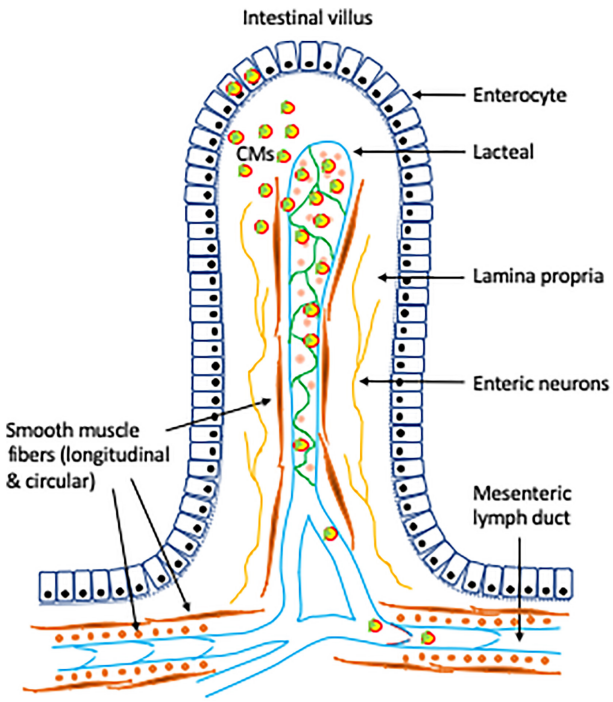

FIGURE 1 | Role of lymphatics in intestinal lipid absorption and mobilization. Following intracellular synthesis and assembly in the intestinal enterocytes, CMs are secreted at the basolateral membrane via exocytosis. CMs move through the lamina propria and enter the lacteals through intercellular junctions. Controlled opening/closing of junctions on the lacteal wall may regulate CM uptake by the lacteals (Zhang et al., 2018). Contractile actions of smooth muscle fibers surrounding the lacteals and the collecting lymphatic vessels confer tonic and phasic pumping activities (Choe et al., 2015; Kassis et al., 2016). Lymphatic pumping and the one-way valves provide unidirectional drainage and transport of lipids through the lymphatic vasculature to the circulation. VEGF-C, expressed in a subset of smooth muscle fibers, plays an important role in intestinal lipid absorption and mobilization via regulation of prenatal lymphangiogenesis, maintenance of intestinal lymphatics in adults (Nurmi et al., 2015), and contraction of smooth muscle fibers surrounding the lymphatic vessels, including the lacteals (Gogineni et al., 2013; Choe et al., 2015). Neural control and σ1-receptor may also play a role in lymphatic functions (Trujillo et al., 2017; Bachmann et al., 2019).

has been documented (Breslin et al., 2007; Choe et al., 2015). New and improved imaging techniques are being developed to specifically assess mesenteric lymphatic functions in relation to lipid drainage (Kassis et al., 2012; Sarimollaoglu et al., 2018). However, challenges remain in this aspect due to technical difficulties for such measurements (Bouta et al., 2018). It is hoped that with further improvement in techniques, the physiological relationship between lymphatic function and intestinal lipid drainage will be better defined.

FUTURE DIRECTIONS

Our research and that of others over the past 5 years have refocused our attention on post-enterocyte regulatory mechanisms of CM secretion. It is becoming evident that the lymphatics are likely to play a much more important regulatory role in this aspect than has traditionally been appreciated. Understanding the role of the lymphatics in lipid mobilization in the gut, as induced by oral glucose or GLP-2 and other stimuli, and in gut lipid handling in general, has important implications for health and disease. The mesenteric lymphatic system has emerged as a potential player in the regulatory control of intestinal TG absorption and mobilization (**Figure 1**). Despite recent advances in the field, many important questions remain to be answered. It is not known whether increased lymphatic pumping activity or induced button transition of lacteal junctions stimulates mobilization of intestinal lipid stores, or what the contribution these have to net CM secretory rate. It also remains to be determined whether increased lymphatic function underlies GLP-2 mobilization of intestinal lipid stores, and if it does, through what mechanisms. Further, it is not known whether lymphatic functions can be modulated, pharmacologically or via dietary means, to improve gut handling of lipids and provide overall health benefits. Future studies addressing these emerging and pressing questions may significantly improve our understanding of the physiological role of lymphatics in gut lipid metabolism. It is expected that advancement in this field may provide novel opportunities for the prevention and treatment of lipid disorders, atherosclerotic cardiovascular disease, and overall metabolic health.

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CX and GL wrote the manuscript. PS and AN contributed and edited the manuscript.

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Lymphatic Programing and Specialization in Hybrid Vessels

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Building on a large body of existing blood vascular research, advances in lymphatic research have helped kindle broader investigations into vascular diversity and endothelial plasticity. While the endothelium of blood and lymphatic vessels can be distinguished by a variety of molecular markers, the endothelia of uniquely diverse vascular beds can possess distinctly heterogeneous or hybrid expression patterns. These expression patterns can then provide further insight on the development of these vessels and how they perform their specialized function. In this review we examine five highly specialized hybrid vessel beds that adopt partial lymphatic programing for their specialized vascular functions: the high endothelial venules of secondary lymphoid organs, the liver sinusoid, the Schlemm's canal of the eye, the renal ascending vasa recta, and the remodeled placental spiral artery. We summarize the morphology and endothelial expression pattern of these vessels, compare them to each other, and interrogate their specialized functions within the broader blood and lymphatic vascular systems.

Keywords: lymphatic, endothelium, hybrid vessel, liver sinusoid, high endothelial venule

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INTRODUCTION

The discovery of new lymphatic markers has been a boon for lymphatic research helping to improve our understanding of the development and maintenance of lymphatics, as well as the identification of new lymphatic vessels. Historically, the distinction between blood and lymphatic vessels has been both categorical and demarcated. However, with new discoveries, we find that endothelial identity is more malleable and impressionable than previously thought. The combined expression of endothelial markers, lymphatic or otherwise, is often indicative of a specific set of functions conferred by the endothelium which possesses these markers (Potente and Makinen, 2017). For example, marker expression on lymphatic capillaries, blunt-ended vessels specialized to support trans-endothelial fluid and cellular transport, differs from that of collecting vessels, which are specialized to support intraluminal transport. The marker expression of these vessels further differ from valves, designed to block or allow intraluminal passage based on pressure gradients, and lymph node lymphatics which are more specialized for the presentation of antigens. Furthermore, the endothelia of these different lymphatic structures are influenced by signals from the local micro- and macro-environment, including from immune cells, extracellular matrix, and organ-specific cells and structures, which is most starkly exemplified by the changes that occur during tumor-associated lymphatic remodeling (Lutter and Makinen, 2014; Petrova and Koh, 2018; Wong et al., 2018; Garnier et al., 2019).

With these discoveries, it has become clear that the endothelium is far more plastic than previously thought and that transitions between blood and lymphatic identities occur (Ma and Oliver, 2017; Azimi et al., 2019). As such, a growing number of endothelial lined vessels which do not fit neatly into a classical vessel type have been identified. Recently, three different vessels,

termed hybrid vessels, have been found to possess a combination of blood and lymphatics markers, presumably necessary for their highly specialized function: the Schlemm's canal (SC) of the eye, the ascending vasa recta (AVR) of the renal medulla, and the remodeled spiral arteries (rSA) of the placental decidua (Aspelund et al., 2014; Park et al., 2014; Truong et al., 2014; Kenig-Kozlovsky et al., 2018; Pawlak et al., 2019). These vessels are in addition to an existing small catalog of highly specialized non-lymphatic vessels with similarly distinct expression patterns, including the liver sinusoid and high endothelial venules (HEV) of secondary lymphoid organs. In this review we compare and contrast the unique functions and expressional programing of these vessels to each other and to the classical features of blood and lymphatic vasculature and discuss the implications of these hybrid vessels within the greater context of physiology and endothelial biology.

BLOOD VASCULATURE

There are three fundamental blood vessel categories within the body: arteries carry blood away from the heart, capillaries exchange gasses and nutrients with local tissue, and veins transport blood back to the heart. Additionally, collateral vessels can shunt blood between vascular beds. Blood vessels are composed of three distinct layers (Mazurek et al., 2017). The tunica intima is the inner most layer with a single layer of endothelial cells (EC), basement membrane, and connective tissue. The endothelium and basement membrane in this layer can morphologically vary to allow for more or less permeability; continuous endothelia and basement membranes are less permeable than discontinuous ones with gaps allowing for passage of larger materials. The middle layer, called tunica media, is thicker in arteries than in veins and contains smooth muscle cells with connective and elastic tissue. The outer tunica adventitia layer, which is often thicker in veins than in arteries, anchors the vessel to the local tissue with elastic and connective fibers. The endothelium of capillaries instead have no smooth muscle, but sometimes have a discontinuous layer of pericytes. The permeability of capillaries vary based on location, but have universally small lumens which allows for gas and nutrient exchange between blood and local tissue.

A variety of versatile endothelial markers exist for the examination of the blood vasculature, including the tyrosine kinase receptors VEGFR1 and TIE2, the blood plasma and Weibel-Palade bodies-bound glycoprotein vWF, and the membrane glycoprotein PLVAP (Jaffe et al., 1973; Sato et al., 1995; Ferrara and Davis-Smyth, 1997; Yamamoto et al., 1998; Stan et al., 1999a,b). Additionally, the platelet endothelial adhesion molecule CD31, and the phosphoglycoprotein CD34, are often used as blood-specific endothelial markers, but can also be weakly expressed on lymphatic endothelium (van Mourik et al., 1985; Kriehuber et al., 2001; Podgrabinska et al., 2002). Distinct markers can differentiate between arterial and venous endothelium. In particular, the receptor tyrosine kinase EphB4 is a venous marker, while its cognate membrane-bound ligand ephrin B2 marks arterial endothelium, though both are expressed in blood capillaries (Wang et al., 1998). Additionally, the VEGFR2 co-receptors NRP1 and NRP2 are differentially expressed, the former is expressed on arterial endothelium and the latter on venous endothelium (Herzog et al., 2001). However, NRP2 is also a co-receptor of VEGFR3 on lymphatic endothelium, making it a more promiscuous venous marker (Yuan et al., 2002). Similarly, the mucin-like sialoglycoprotein, endomucin, preferentially expresses on both venous and lymphatic endothelia (Samulowitz et al., 2002). Pan-endothelial markers are widely used and valuable based on context and application. Commonly used pan-endothelial markers include the tyrosine kinase receptor VEGFR2, and the endothelial junctional protein, VE-Cadherin (Lampugnani et al., 1992; Shalaby et al., 1995; Dellinger et al., 2013; Hagerling et al., 2018).

LYMPHATIC VASCULATURE

Lymphatic vessels are responsible for maintaining fluid homeostasis, fat absorption, and immune cell trafficking. Excess interstitial fluid drains as lymph into blind-ended lymphatic capillaries that contain a permeable, discontinuous basement membrane and overlapping ECs that collectively function like unidirectional valves (Baluk et al., 2007). Lymph is then transported into lymphatic collectors, which have a structure similar to veins. Collectors have a basement membrane, lymphatic muscle cells, pericytes, and are regularly segmented by one-way endothelial valves (Oliver, 2004). Lymph can be pushed though the one-way valves by external pressures and phasic contractile forces generated by collector lymphatic muscle cells, effectively facilitating basal to apical flow (Muthuchamy et al., 2003). Collecting vessels can then move lymph through lymph nodes for antigen presentation to immune cells, and then drain back into the venous circulation (Randolph et al., 2017).

Formation of the mouse lymphatic vasculature begins around embryonic day 9.5 when a subpopulation of ECs on the cardinal vein express PROX1, a master regulator of lymphatic fate, which requires expression of the transcription factors SOX18 and COUP-TFII, the latter of which is considered a master regulator of venous fate (Wigle and Oliver, 1999; You et al., 2005; Francois et al., 2008). The PROX1^{+} cardinal vein ECs, which also express the membrane glycoprotein LYVE1, bud off the vein and acquire the expression of another membrane glycoprotein, podoplanin (PDPN) (Banerji et al., 1999; Breiteneder-Geleff et al., 1999). The ECs coordinately form a primitive lymph sac at embryonic day 11.5 in a process primarily mediated by VEGFR3/NRP2/VEGFC signaling (Karkkainen et al., 2004). Lymphangiogenesis from the lymph sac then forms the peripheral lymphatic network, supported by endothelial proliferation promoted by AM/CLR/RAMP2 signaling (Fritz-Six et al., 2008). Lymphatic valves are formed to help promote unidirectional flow toward the venous circulation, while a lymphovenous valve prevents blood from flowing into the lymphatics (El Zawahry et al., 1983). Notably, both types of valve LECs have distinct expression patterns from each other and more so from vessel wall LECs (Scallan et al., 2016; Janardhan and Trivedi, 2019).

Through derivation from the primitive lymph sac, most of the lymphatic vasculature derives from a venous origin (Srinivasan et al., 2007; Yang et al., 2012; Hagerling et al., 2013; Escobedo and Oliver, 2016). However, newer evidence in mice suggests that a subset of LECs are derived from non-venous sources in the heart (Klotz et al., 2015), dermis (Martinez-Corral et al., 2015), and mesentery (Mahadevan et al., 2014; Stanczuk et al., 2015), as well as LECs derived from the mesenchyme (Buttler et al., 2006; Wilting et al., 2006; Ulvmar and Makinen, 2016). Non-venous LECs were primarily identified by lineage tracing experiments with the venous/endothelial cell marker TIE2. Evidence suggests that LECs lacking TIE2 labeling in the heart and mesentery may instead derive from a hemogenic endothelium population, whereas the origin of non-venous dermal LECs currently remains unknown (Mahadevan et al., 2014; Klotz et al., 2015; Stanczuk et al., 2015).

HIGH ENDOTHELIAL VENULES

The HEV are post-capillary swellings of venous blood vessels that are especially adapted for trafficking of lymphocytes. HEVs are found in secondary lymphoid organs, including lymph nodes and the Peyer's patch of the small intestine, and are required for the function and organogenesis of these organs by recruiting essential lymphocyte populations. HEVs are covered by overlapping pericytes in a thick basement membrane, and HEV ECs are distinct from other blood ECs by their thick (or high) cuboidal shape, from which the vessel's name derives (**Figure 1A**; Ager, 2017).

While the expression pattern of HEV ECs can have some variability by organ, HEV ECs have generally been found to express the lymphatic markers VEGFR3, LYVE1, and CCL21, but not PROX1 (**Table 1**; Gunn et al., 1998; Lacorre et al., 2004; Wrobel et al., 2005). LYVE1 functions as receptor for hyaluronan which is metabolized in the lymph node (Fraser and Laurent, 1989). CCL21, a ligand for CCR7, functions as a chemoattractant for immune cells (Yoshida et al., 1998). VEGFR3 expression on HEVs is controversial, but an abundance of evidence supports that it is expressed in many studies (Lacorre et al., 2004; Farnsworth et al., 2011). HEV ECs also express endothelial markers CD31, CD34, VE-Cadherin, and VEGFR2, and the blood endothelial markers vWF, PLVAP, and VEGFR1 (Pfeiffer et al., 2008; Farnsworth et al., 2011). As found on other venules, HEVs also express the membrane-bound glycoprotein endomucin, where it likely functions as a regulator of cellular adhesion (Samulowitz et al., 2002). Interestingly, HEV ECs do not express PDPN, but fibroblastic reticular cells that surround the HEV do and that expression is required to maintain VE-Cadherin expression and barrier function of HEV ECs (Farr et al., 1992; Herzog et al., 2013).

LIVER SINUSOID

The liver sinusoid is a network of capillaries lined by a discontinuous endothelium. Liver sinusoidal ECs (LSECs) are flat and highly fenestrated (covering ∼20% of the surface), allowing for passage of plasma across the endothelium into the space of Disse located between the endothelium and adjacent hepatocytes (**Figure 1B**). The passage of plasma across LSECs is important for hepatic blood clearance of harmful compounds and drugs from the circulation. This clearance is supported by Kupffer cells within the sinusoid that phagocytose particles too large to pass through LSECs. LSECs have minimal basement membrane with a lack of an organized basal lamina and also lack tight junctions (Wisse, 1972).

LSECs have an interesting expression pattern that includes the lymphatic markers LYVE1, VEGFR3, and integrin α9, but not PROX1 (**Table 1**; Couvelard et al., 1998; Mouta Carreira et al., 2001). Similar to the lymph nodes, the liver metabolizes hyaluronan which its receptor, LYVE1, in LECS likely supports (Vrochides et al., 1996). PDPN has been described as "scantly" expressed on LSECs (Yokomori et al., 2010), but that study did not address if the signal came from PDPN-positive Kupffer cells within the sinusoid (Hitchcock et al., 2015). The pan-endothelial markers TIE2 and VEGFR2 are expressed on LSECs, however, the expression of some other pan-endothelial and blood markers in LSECs are unusually low (vWF, CD31) or absent (VE-Cadherin, CD34) (Lalor et al., 2006). Though it should be noted that the expressions of CD31 and VE-Cadherin are controversial since both have been reported to be either expressed or not expressed on LSECs (Lalor et al., 2006; Ding et al., 2010). The arterial and venous markers Ephrin B2 and EPHB4, respectively, are both expressed in LSECs, which is consistent with function of the liver sinusoid as a capillary plexus since Ephrin B2 and EPHB4 coordinately direct remodeling of capillary networks.

SCHLEMM'S CANAL

The SC of the eye is a channel encircling the periphery of the cornea with a continuous EC monolayer on a discontinuous basement membrane which facilitates bloodless basal-to-apical flow similar to lymphatic capillaries (**Figure 1C**; Ramos et al., 2007; Aspelund et al., 2014). The SC functions as a regulator of intraocular pressure by providing passage for aqueous humor from the trabecular meshwork, draining then into the aqueous and episcleral veins (Tamm, 2009). The aqueous humor outflow is similar between mice and humans. However, only about 20% of total aqueous humor outflow passes through the SC in mice (Aihara et al., 2003), whereas a majority of outflow passes through the SC in humans. Historically, the SC was thought to be a blood vessel due to the expression of blood endothelial markers, including vWF (Dautriche et al., 2015), but more recently the rodent SC has been noted to possess many characteristics of lymphatic vessels.

In 2014, three groups independently identified the expression of PROX1, a transcription factor master regulator of lymphatic fate, in the mouse SC (Aspelund et al., 2014; Park et al., 2014; Truong et al., 2014), and has since been identified in the SC of rats (Jung et al., 2017). Interestingly, only a subset of lymphatic markers are expressed in the SC, including VEGFR3, CCL21, integrin α9, and low or transient expression of SOX18 and

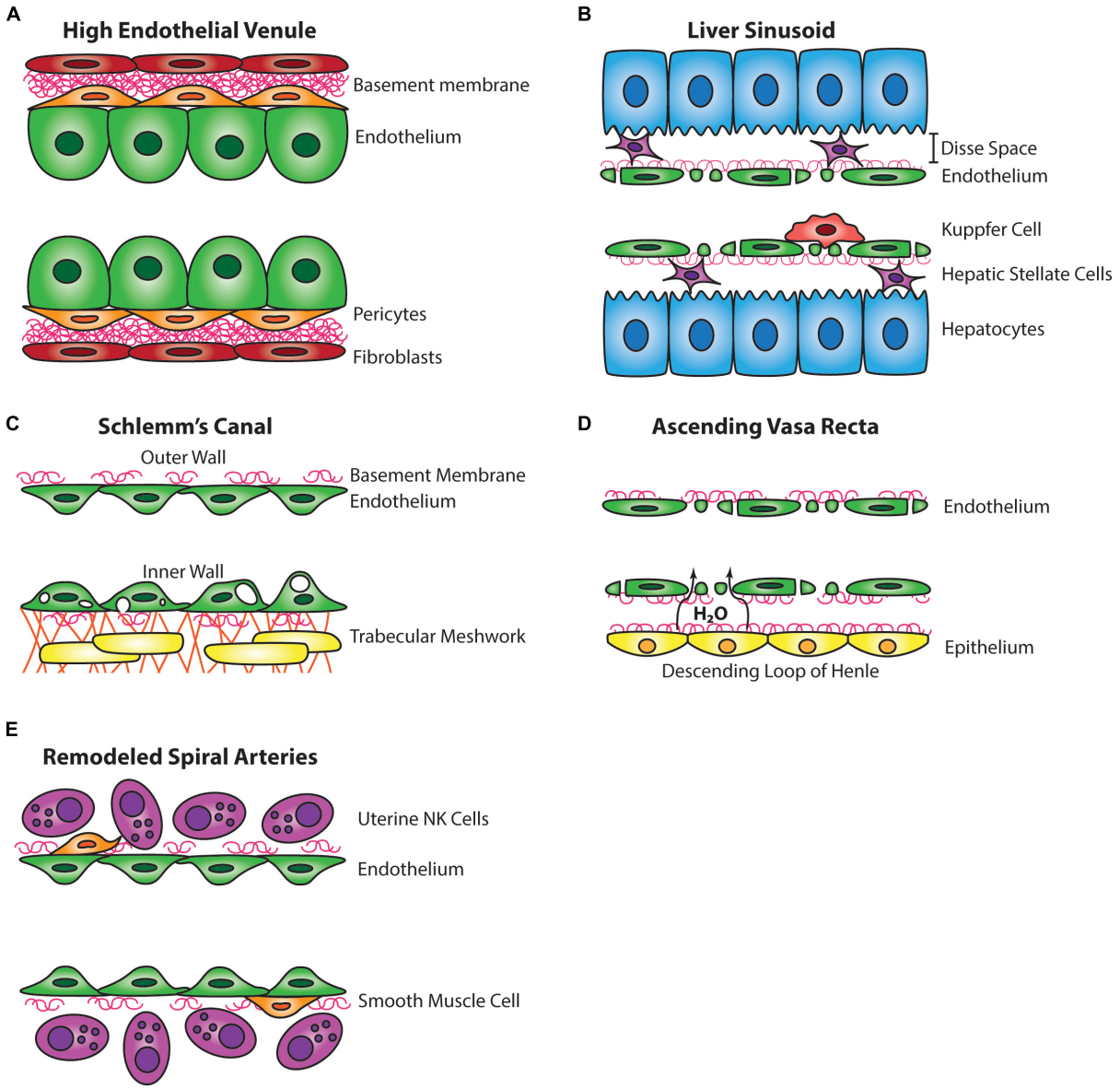

FIGURE 1 | Comparative wall structure of hybrid vessels. **(A)** The endothelial cells of high endothelial venules are tall and cuboidal and are associated with pericytes and a thick basement membrane. **(B)** The endothelium of the liver sinusoid is fenestrated, lacks mural cells, is supported by a thin basement membrane, and is associated with intraluminal Kupffer cells. The Disse space separates the liver sinusoid endothelium from hepatocytes and is inhabited by hepatic stellate cells. **(C)** The Schlemm's canal has a discontinuous basement membrane, lacks mural cells, and the inner wall endothelium forms large vacuoles in response to aqueous humor flow from the trabecular meshwork. **(D)** The endothelium of the ascending vasa recta is fenestrated, lacks mural cells, and takes up water from the descending loop of Henle. **(E)** The endothelium of the remodeled spiral artery has minimal smooth muscle coverage, a discontinuous basement membrane, and closely associates with uterine NK cells.

FOXC2. However, LYVE1 and PDPN expression is not detected in SC ECs. Meanwhile, the blood endothelial markers CD31, vWF, and endomucin are expressed in the SC, as wells as the pan-endothelial markers VE-Cadherin and TIE2, suggesting that these vessels acquire a blood/lymphatic hybrid identity (**Table 1**; Aspelund et al., 2014; Park et al., 2014; Truong et al., 2014). This hybrid identity is hypothesized to help the SC to perform its highly specialized function in a way that neither a classic blood nor lymphatic vessel could perform. Indeed, VEGFR3 signaling is required for SC development (Aspelund et al., 2014), and recent work shows that PROX1 is required for development and maintenance of the SC. Also the blood vessel marker VEGFR2 is required for development but gradually decreases expression from p4 to adulthood during a simultaneous increase in PROX1 and VEGFR3 expression. Interestingly, the lymphatic programing of the SC is flow-mediated; PROX1 and VEGFR3 expression is significantly decreased when aqueous humor outflow is decreased (Park et al., 2014). Since humans rely

TABLE 1 | Expression pattern of hybrid vessels.

Endothelial marker	Common endothelial expression				Schlemm's Canal		Ascending vasa recta		Remodeled spiral arteries		High endothelial venules		Liver sinusoid	
PROX1	L			Wigle and Oliver, 1999	+	Aspelund et al., 2014; Park et al., 2014; Truong et al., 2014	+	Kenig-Kozlovsky et al., 2018	+	Pawlak et al., 2019	–	Lacorre et al., 2004	–	Mouta Carreira et al., 2001
LYVE1	L			Banerji et al., 1999	–	Aspelund et al., 2014; Park et al., 2014; Truong et al., 2014	–	Kenig-Kozlovsky et al., 2018	±	Pawlak et al., 2019	+	Liao and Ruddle, 2006	+	Mouta Carreira et al., 2001
PDPN	L			Breiteneder-Geleff et al., 1999	–	Aspelund et al., 2014; Park et al., 2014	–	Kenig-Kozlovsky et al., 2018	–	Pawlak et al., 2019	–	Herzog et al., 2013	±[3]	Yokomori et al., 2010
CCL21	L			Kriehuber et al., 2001	+	Aspelund et al., 2014	NRF		–	Pawlak et al., 2019	+	Gunn et al., 1998	–	Grant et al., 2002
Integrin α9	L			Huang et al., 2000; Bazigou et al., 2009	+	Aspelund et al., 2014; Park et al., 2014	NRF		NRF		NRF		+	Couvelard et al., 1998
VEGFR3	L	|V|	|A|	Kaipainen et al., 1995	+	Aspelund et al., 2014; Park et al., 2014	+	Kenig-Kozlovsky et al., 2018	+	Pawlak et al., 2019	±	Lacorre et al., 2004; Farnsworth et al., 2011	+	Yamane et al., 1994
SOX18	L	|V|	|A|	Pennisi et al., 2000; Francois et al., 2008	±	Park et al., 2014	NRF		NRF		NRF		+	Matsui et al., 2006
NRP2	L	V		Herzog et al., 2001; Yuan et al., 2002	NRF		NRF		–	Germeyer et al., 2005; Pawlak et al., 2019	NRF		–	Elpek, 2015
Endomucin	L	V		Samulowitz et al., 2002	+	Park et al., 2014	+	Kenig-Kozlovsky et al., 2018	–	Pawlak et al., 2019	+	Samulowitz et al., 2002	+	Walter et al., 2014
CALCRL	L	V	A	Nagaya et al., 2005; Fritz-Six et al., 2008	NRF		NRF		+	Li et al., 2013	NRF		+	MacParland et al., 2018
KLF4	L	V	A	Yet et al., 1998; Dieterich et al., 2015	+	Park et al., 2014	NRF		NRF		NRF		NRF	
VE-Cadherin	L	V	A	Lampugnani et al., 1992; Hagerling et al., 2018	+	Perkumas and Stamer, 2012; Kizhatil et al., 2014	NRF		+	Bulla et al., 2005	+	Pfeiffer et al., 2008	±	Lalor et al., 2006; Ding et al., 2010
VEGFR2	L	V	A	Shalaby et al., 1995; Dellinger et al., 2013	+	Perkumas and Stamer, 2012; Kizhatil et al., 2014	–	Kenig-Kozlovsky et al., 2018	NRF[1]	Hirashima et al., 2003	+	Farnsworth et al., 2011	+	Yamane et al., 1994
FOXC2	L	|V|	A	Dagenais et al., 2004; Seo et al., 2006	+	Aspelund et al., 2014; Park et al., 2014	NRF		NRF		NRF		NRF	

(Continued)

TABLE 1 | Continued

Endothelial marker	Common endothelial expression				Schlemm's Canal		Ascending vasa recta		Remodeled spiral arteries		High endothelial venules			
EPHB4		V		Wang et al., 1998	NRF		NRF		+	Zhang et al., 2008	NRF		+	Das et al., 2010
CD31	/	V	A	van Mourik et al., 1985; Podgrabinska et al., 2002	+	Aspelund et al., 2014; Park et al., 2014	+	Kenig-Kozlovsky et al., 2018	+	Bulla et al., 2005; Pawlak et al., 2019	+	Pfeiffer et al., 2008	±	Lalor et al., 2006
CD34	/	V	A	Kriehuber et al., 2001; Podgrabinska et al., 2002	+	Kizhatil et al., 2014	+	Kenig-Kozlovsky et al., 2018	NRF		+	Wrobel et al., 2005	–	Lalor et al., 2006
TIE2	|/|	V	A	Sato et al., 1995; Shen et al., 2014	+	Perkumas and Stamer, 2012; Kizhatil et al., 2014	+	Kenig-Kozlovsky et al., 2018	+	Goldman-Wohl et al., 2000	+	Hayasaka et al., 2010	+	Poisson et al., 2017
PLVAP		V	A	Stan et al., 1999a,b	+	Herrnberger et al., 2012	+	Pannabecker and Dantzler, 2006; Kenig-Kozlovsky et al., 2018	NRF		+	Rantakari et al., 2015	+	Rantakari et al., 2016
vWF		V	A	Jaffe et al., 1973; Yamamoto et al., 1998	+	Hamanaka et al., 1992; Park et al., 2014	NRF[2]	Pupilli et al., 1997	+	Bulla et al., 2005	+	Lacorre et al., 2004	+	Lalor et al., 2006
VEGFR1		V	A	Ferrara and Davis-Smyth, 1997	+	Perkumas and Stamer, 2012; Fujimoto et al., 2016	+	Young et al., 2018	+	Hirashima et al., 2003	+	Hayasaka et al., 2010	–	Kato et al., 2011
Ephrin B2			A	Wang et al., 1998	+	Kizhatil et al., 2014	NRF		+	Zhang et al., 2008	NRF		+	Das et al., 2010; Mimche et al., 2018
NRP1			A	Herzog et al., 2001	+	Perkumas and Stamer, 2012	NRF		+	Germeyer et al., 2005	–	Lee et al., 2014	+	Elpek, 2015

L = Lymphatic; A = Arterial; V = Venous; |L| |V| |A| = Transiently expressed during development; l v a = Weakly expressed; (+) = Expressed; (–) = Not expressed; (±) = Transiently or Discontinuously Expressed; NRF = No Reporting Found; (±) = Reported both as expressed and not expressed. [1]Hirashima et al. (2003) report that VEGFR2 is not expressed in the SA of the proximal decidua at embryonic day 12.5, but only after the endothelium has been replaced by fetal endovascular trophoblasts. Their data appears to show LacZ reporter staining for VEGFR2 in endothelial lined SAs, but this was not explicitly reported by the authors (Hirashima et al., 2003). This important distinction has not been made in some previous review articles (Rai and Cross, 2014). [2]Staining done by Pupilli et al. (1997) shows low or absent vWF expression in ascending vasa recta, while the descending vasa recta shows clear expression, but the ascending vasa recta vWF staining was not explicitly reported by the authors. [3]Yokomori et al. (2010) described PDPN as "scantly" expressed on LSECs. However, PDPN expression has been described on Kupffer cells which localized to the sinusoids which was not addressed in the study by Yokomori et al. (2010) and Hitchcock et al. (2015).

more on SC-mediated outflow than mice, these findings may have increased relevance to the pathophysiology of the human eye.

More recent work shows that TIE2 (*Tek*) signaling is required for both development and maintenance of the SC, and is otherwise necessary to perform its drainage function (Thomson et al., 2014; Kim et al., 2017). During development, TIE2 is an important regulator of vascular remodeling and stability. In the SC and lymphatics vessels, ANGPT1 and ANGPT2 function as agonists of TIE2, which differs from blood vessels where ANGPT2 acts as an antagonist to ANGPT1 activation of TIE2 (Augustin et al., 2009). Agonist signaling of TIE2 is required for SC development; mice lacking ANGPT1 and ANGPT2 or TIE2 develop glaucoma due to an unformed SC, restricting aqueous humor outflow. Furthermore, inducible deletion of *Angpt1* and *Angpt2* or *Tek* in adult mice demonstrate that agonist TIE2 signaling is also required to maintain SC integrity and PROX1 expression (Kim et al., 2017).

ASCENDING VASA RECTA

The AVR and descending vasa recta (DVR) of the renal inner medulla are blood vessels utilized for concentrating urine along the nephron (Pallone et al., 2003). The AVR in particular is highly fenestrated, lacks mural cell coverage, has a discontinuous basement membrane, and is important for fluid reabsorption back to the vasculature (**Figure 1D**; Schwartz et al., 1976; Takahashi-Iwanaga, 1991). These features are also attributed to lymphatic vessels which are otherwise absent or rare in the renal medulla (Russell et al., 2019). Similar to SC, the AVR expresses both PROX1 and VEGFR3, but not LYVE1 and PDPN. Additionally, the AVR expresses endomucin and blood endothelial markers CD31, CD34, VEGFR1, and PLVAP (**Table 1**; Kenig-Kozlovsky et al., 2018). Interestingly, despite typically being expressed in blood vessels, VEGFR2 expression was not found in the AVR (Kenig-Kozlovsky et al., 2018). VEGFR2 and VEGFR3 are able to hetero-dimerize and activate downstream signaling pathways, such as AKT signaling, that differ from those triggered by homo-dimerized VEGFR3, such as ERK signaling (Deng et al., 2015). Consequently, absence of VEGFR2 in these vessels could lead to increased VEGFR3 homo-dimer signaling (Dixelius et al., 2003). As postulated by Kenig-Kozlovsky et al. (2018) VEGFR3 may be implicated in vessel widening of the AVR, which is significantly wider than the DVR, as VEGFR3 responds to fluid shear stress by promoting outward vessel remodeling (Baeyens et al., 2015).

To date, the developmental origin of the AVR is not well understood. Further, it is unclear whether the AVR derives from a unique set of progenitor cells or undergoes differentiation while under development. It is likely that the acquisition of VEGFR3 is due in large part to PROX1 transcriptional activity (Hong et al., 2002; Petrova et al., 2002), but it is unclear what initiates PROX1 expression in the AVR. Examination of known promoters of PROX1 expression, such as SOX18 and KLF4, may help identify early regulatory mechanisms that push the AVR toward a hybrid identity.

The AVR also expresses TIE2 which is required for development of the AVR, but not the DVR (Kenig-Kozlovsky et al., 2018). Constitutive deletion of *Tek* or simultaneous deletion of *Angpt1* and *Angpt2* leads to early embryonic lethality (Dumont et al., 1994; Sato et al., 1995), but Kenig-Kozlovsky et al. (2018) overcame this lethal phenotype by inducing deletion of *Tek* or *Angpt1* and *Angpt2* simultaneously at embryonic day 16.5. These mice develop renal cysts and have reduced urine concentrating ability which is attributed to the absence of the AVR, despite the normal formation of the DVR (Kenig-Kozlovsky et al., 2018).

SPIRAL ARTERIES

The spiral arteries (SA) of the decidual placenta are tortuous maternal blood vessels that transport maternal blood to the fetal side of the placenta where gasses and nutrients are exchanged with the fetal vasculature. With the support of local uterine NK cells, SAs must remodel during midgestation to increase blood flow and nutrient delivery to support the growing fetus (Moll et al., 1978). In humans, poor SA remodeling is associated with pregnancy complications, including fetal growth restriction, preterm birth, and preeclampsia (Lyall, 2002; Pijnenborg et al., 2006), and can otherwise lead to long-term health complications for mother and child (Barker et al., 1989; Gastrich et al., 2010; Geelhoed and Jaddoe, 2010). Prior to remodeling, the SA is supported by smooth muscle coverage, extracellular matrix, and a basement membrane, but during SA remodeling smooth muscle coverage is shed, extracellular matrix degrades, and basement membrane is diminished (**Figure 1E**; Sweeney et al., 2006; Whitley and Cartwright, 2010; Robson et al., 2012). These changes help facilitate an increase in lumen diameter and reduced tortuosity to promote increased blood flow.

Our group recently discovered that rodent SAs acquire expression of a subset of lymphatic markers, similar to the SC and AVR (Pawlak et al., 2019). We found that the SA acquires these lymphatic markers during the remodeling period between embryonic day 11.5 to embryonic day 13.5 when smooth muscle coverage is shed and the lumen diameter expands. Similar to the SC and AVR, the remodeled SA (rSA) expresses PROX1 and VEGFR3, but not PDPN, as well as TIE2 and CD31 (**Table 1**; Goldman-Wohl et al., 2000). VEGFR3 expression in particular persists throughout pregnancy. Our work suggests that the rSA utilizes lymphatic expression to help expand the lumen diameter and modify the vascular tone to allow for increased blood flow, and that VEGFR3/VEGFC signaling in particular plays a central role in that function. The expression of VEGFR3 allows the SA to become responsive to locally secreted VEGFC which is required to promote remodeling. However, unlike the aforementioned hybrid vessels, the rSA expresses LYVE1, but only in a non-continuous subset of ECs. Also unlike the SC, the rSA does not express CCL21, which may be related to the immune privileged nature of the placenta intended to prevent an immunological maternal response to fetal antigens (Weber et al., 2013). Additionally, the rSA is not a fenestrated vessel and does not express endomucin, which is often associated with vessels that help perform fluid homeostasis.

Fundamentally, the differences in expression in the rSA compared to the other hybrid vessels likely relates to the physiological differences in function and origin of these vessels. The rSA regulates blood transport, the SC regulates aqueous humor fluid homeostasis, and the AVR serves as both a transport and homeostasis vessel. Though one key difference between rSAs and the other hybrid vessels and lymphatics is that rSAs are arterial while the others are of a venous origin (Srinivasan et al., 2007). It is important to note that some LECs are derived from a non-venous origin (Ulvmar and Makinen, 2016), and while VEGFR3 is regarded as a lymphatic marker in adult vessels, it is also expressed in fetal blood vessels, including arterioles. Furthermore, it should be appreciated that SAs acquire transient expression of the venous blood vessel marker EPHB4 prior to lymphatic expression, suggesting a shift from arterial to venous to lymphatic identity that may help facilitate the full transition and remodeling process (Zhang et al., 2008).

CLOSING REMARKS

The molecular profile of hybrid vessels is related to the unique microenvironments in which they are located and the highly specialized functions they perform, and cell-autonomous mechanisms may also contribute to their heterogeneity. Notably, the molecular profiles of mature hybrid vessels appear to be plastic, as evident by expressional changes to HEVs in response to immunization (Liao and Ruddle, 2006). Indeed, this vessel plasticity under aberrant signaling is presumed to contribute to certain pathologies, such as poor spiral artery remodeling in preeclampsia and poor aqueous humor drainage via the SC in glaucoma (Karpinich and Caron, 2014; Pawlak et al., 2019).

It is worth commenting that some of the newly characterized hybrid vessels are found in regions that are either currently or historically believed to be devoid of lymphatic vessels (Dickinson and Gausas, 2006; Red-Horse et al., 2006; Castro et al., 2011; Lee et al., 2011). Certainly, the expansion of diverse and reliable lymphatic markers has enabled more precise characterization of these structures. It is also evident that the field is moving away from overreliance on only one or two lymphatic markers in *in vivo* studies, not only because expression patterns on vessels may be altered by local or organ-specific factors, but also because no single marker identified to date is completely exclusive to lymphatic vessels. Finally, instances of programmed and pathological endothelial mimicry could further complicate molecular profiling, particularly in a tumor environment.

It is likely that more uncharacterized hybrid vessels still remain to be identified. As with the aforementioned hybrid vessels, vascular beds with highly specialized functions may be ripe for the examination of lymphatic markers which may have been coopted to promote the specialized morphology that serves their function. Indeed, understanding how the lymphatic vasculature responds to pathological conditions may be informative in the identification and characterization of potential hybrid vessels (see the comprehensive review on this topic by Padera et al. (2016). Ultimately, what qualifies as a hybrid vessel may be debatable or evolve over time. Nevertheless, this can be considered a natural and positive outcome of our expanding appreciation for the breadth of endothelial plasticity and heterogeneity.

AUTHOR CONTRIBUTIONS

JP researched the literature and wrote the manuscript. KC provided direction on, reviewed, and edited the manuscript.

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REVIEW
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The Unresolved Pathophysiology of Lymphedema

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Lymphedema is the clinical manifestation of impaired lymphatic transport. It remains an under-recognized and under-documented clinical condition that still lacks a cure. Despite the substantial advances in the understanding of lymphatic vessel biology and function in the past two decades, there are still unsolved questions regarding the pathophysiology of lymphedema, especially in humans. As a consequence of impaired lymphatic drainage, proteins and lipids accumulate in the interstitial space, causing the regional tissue to undergo extensive and progressive architectural changes, including adipose tissue deposition and fibrosis. These changes are also associated with inflammation. However, the temporal sequence of these events, the relationship between these events, and their interplay during the progression are not clearly understood. Here, we review our current knowledge on the pathophysiology of lymphedema derived from human and animal studies. We also discuss the possible cellular and molecular mechanisms involved in adipose tissue and collagen accumulation during lymphedema. We suggest that more studies should be dedicated to enhancing our understanding of the human pathophysiology of lymphedema to pave the way for new diagnostic and therapeutic avenues for this condition.

Keywords: lymphedema, pathophysiology, inflammation, adipose tissue, fibrosis

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INTRODUCTION

The lymphatic system consists of a network of vessels connecting lymphoid organs such as lymph nodes, tonsils, thymus, and spleen. Running parallel to the venous circulation, the primary function of the lymphatic system is to drain excess interstitial fluid leaking out from blood capillaries into the tissue spaces. Other functions include fat absorption in the intestine, immune surveillance, and resolution of inflammation. Lymph fluid transports various antigens and antigen-presenting cells into lymph nodes for immune response. Lymph containing lipids, immune cells, macromolecules, and fluid is first collected by blind-ending initial or capillary lymphatic vessels which in turn empty into larger lymphatic vessels, i.e., the collecting vessels or collectors. Unlike the initial lymphatic vessels, the collecting lymphatics exhibit circumferential smooth muscle cell coverage and luminal valves that propel and maintain unidirectional flow (Tammela and Alitalo, 2010).

Lymphedema is a chronic and progressive disease arising from impaired lymphatic drainage causing the accumulation of interstitial fluid which results in tissue swelling (Rockson, 2001).

Lymphatic dysfunction can be caused by genetic abnormalities affecting the lymphatic development and/or function and it typically becomes apparent during infancy, childhood, or adolescence, a condition known as primary lymphedema. Less frequently, primary lymphedema can appear after age 35 and is known as lymphedema tarda (Rockson, 2001; Greene and Maclellan, 2013). The incidence of primary lymphedema is low, affecting 1 in 100,000 people worldwide (Smeltzer et al., 1985). Lymphedema may occur secondary to damage or obstruction of lymphatic vessels due to infectious diseases such as filariasis or trauma, including radiotherapy and surgical removal of lymph node in cancer treatments (Rockson, 2001; Witte, 2001; Greene and Maclellan, 2013). Today, secondary lymphedema is more common, due to increasing cancer rates, affecting 1 in 1,000 persons, whereby 24–49% of cancer patients develop secondary lymphedema after receiving cancer treatment (Karaca-Mandic et al., 2015; Kurt et al., 2016). However, this rate of incidence and prevalence are likely underestimated because lymphedema remains under-recognized and under-documented. Breast cancer-associated lymphedema is the most common form of lymphedema in developed countries followed by sarcoma, gynecologic cancers and malignant melanoma (Rockson, 2018). Tissue swelling in extremities due to interstitial fluid accumulation causes discomfort, restricted range of motion, and decreased quality of life for lymphedema patients. Increased susceptibility to infections, recurrent infections, psychological morbidity, functional disability, skin changes, and malignant transformation are known complications associated with human lymphedema (Greene and Maclellan, 2013).

PATHOPHYSIOLOGY OF LYMPHEDEMA

Research in the past decades has led to the understanding that the factors causing lymphedema are not solely attributed to lymph and fluid accumulation in the interstitial tissue. It is proposed that its pathophysiology involves a chain of complex and progressive events affecting different tissue compartments. These events are described below although their exact chronological order remains unresolved.

Adipose Tissue Expansion and Remodeling in Lymphedema

Substantial evidence exists that tissue swelling in lymphedema is due to fat deposition and not just the accumulation of fluid. The presence of excess adipose tissue in the affected limb has been well documented in patients with chronic non-pitting arm lymphedema following breast cancer (Brorson and Svensson, 1997). Our clinical observations show that hypertrophic fat lobules compress and collapse their feeding lymphatic capillaries, resulting in a vicious cycle of fluid and lipid transport disruption, ultimately leading to further fat accumulation in the periphery. A recent study using magnetic resonance imaging (MRI) revealed that fat deposition is not limited to the epifascial compartment (between skin and muscle cells) of the lymphedematous compartment, but is also present in the subfascia (between muscle cells or within muscle cells) which did not change following liposuction (Hoffner et al., 2018b). However, from a clinical perspective, the subcutaneous compartment is the main area of fat accumulation and this fat is amenable to removal with liposuction. Adipose tissue accumulation has also been described in mouse models with lymphatic stasis (**Table 1**). For example, in mice whose skin lymphatic vessels have been surgically resected, subcutaneous fat deposition and fat thickness increased (Aschen et al., 2012). Similarly, *Chy* mice, a mouse model of lymphedema, due to heterozygous inactivating mutations in the vascular endothelial growth factor receptor 3, developed abnormal subcutaneous fat deposition, especially in edematous subcutaneous adipose tissue close to dysfunctional hypoplastic lymphatic vessels (Karkkainen et al., 2001). In addition, high levels of fat in the tail skin was observed (Rutkowski et al., 2010). In Prox1 heterozygous mice, lymphatic malfunction resulted in lymph leakage and accumulation of adipose tissue, culminating in adult-onset obesity (Harvey et al., 2005). Importantly, *in vivo* restoration of the Prox1 level, specifically in the lymphatic endothelial cell, was sufficient to reverse the lymphatic defects in *Prox1*$^{\pm}$ mice and ameliorate their obesity phenotype (Escobedo et al., 2016). Together, these mouse and human findings support a relationship between lymphatic dysfunction and adipose tissue accumulation. A detailed histological analysis of the adipose tissue from healthy and patients with lower extremities lymphedema revealed significant differences between the two groups (Tashiro et al., 2017). Macroscopic and ultrasonic examination demonstrated that lymphedema adipose tissues have larger lobules, which are surrounded by thicker collagen matrix and lymph fluid compared to healthy adipose tissue. Healthy adipocytes are non-ruptured and uniformed in size, whereas adipocytes in lymphedema are hypertrophic and highly variable in size (Tashiro et al., 2017).

Adipose tissue functions as an energy reservoir either by storing excess nutrients or supplying nutrients to other tissues (Birsoy et al., 2013). It also plays an important endocrine function whereby adipocytes secrete hormones and cytokines, known as adipokines, to regulate energy homeostasis (Rondinone, 2006). In obesity, pathological adipose tissue remodeling occurs when excess nutrients cause adipocytes to expand in size (hypertrophy) or increase in number (hyperplasia) once they have reached their maximum lipid storage capacity. This adipose tissue remodeling leads to dysregulation of adipokine production, metabolic stress, and a low-grade local inflammation through the increased secretion of pro-inflammatory cytokines (TNF-α, IL-6, MCP-1, IL-8) that promote immune cell infiltration and their pro-inflammatory polarization (de Ferranti and Mozaffarian, 2008; Drolet et al., 2008). Adipose tissue hypertrophy in lymphedema is accompanied by adipose remodeling, similar to what occurs in obesity. Increased serum levels of adiponectin and leptin were found in lymphedema patients, likely reflecting expansion of adipose tissue (Zaleska and Olszewski, 2017). Increased adiponectin expression, which correlates with fat accumulation caused by lymph stasis, was also observed in mouse tail surgical model of lymphedema (Aschen et al., 2012) (**Table 1**). Moreover, IL-6 expression which correlates with adipose tissue depots in obese patients (Mohamed-Ali et al., 1997; Fried et al., 1998)

TABLE 1 | Animal models of lymphedema.

	Species	Models	Pathophysiology observed	References
Surgical models	Mouse	Tail incision	Increase adipose deposition and adipocyte hypertrophy Macrophages surrounding subcutaneous fat deposits Collagen deposition within subcutaneous fat & collecting lymphatic vessels Changes in immune cell infiltrate Enlarged lymphatic capillaries Loss of function of both initial & collecting lymphatic vessels Increase in VEGF-C locally and systemically	Rutkowski et al., 2010; Zampell et al., 2012a; Cuzzone et al., 2014; Gousopoulos et al., 2016b, 2017; Jun et al., 2017
	Dog Mouse Rat Monkey Pig	Lymph node dissection/removal & radiation	Sustained tissue inflammation Decreased number of regeneration lymphatics Worsened swelling & lymph drainage Reduced fluid transport Dilated lymphatic vessels Increase volume in affected limb Lymph backflow	Knight et al., 1987; Blum et al., 2010; Lahteenvuo et al., 2011; Cuzzone et al., 2014; Wu et al., 2014; Yang et al., 2014; Triacca et al., 2019; Wang et al., 2020
	Mouse Rat	Hind limb incision	Swelling & impaired lymph drainage Insufficient wound healing Reduced lymph transport Dilated lymph vessels Increased fibrosis	Lynch et al., 2015; Jun et al., 2017; Jørgensen et al., 2018
	Rabbit	Ear incision	Dermal backflow Disrupted lymphatic flow Fibrosis Lymphatic vessels dilated and increased in number	Fu et al., 1998; Szuba et al., 2002; Yoon et al., 2003; Fernández Peñuela et al., 2018
Transgenic mouse models	Mouse	*Chy Chy-3*	Swelling in forepaw and hindpaw Higher levels of collagen and fat in skin Devoid of initial lymphatics in dermis Defective lymphatic vessels Decreased lymph flow	Karkkainen et al., 2001; Karlsen et al., 2006, 2012; Dellinger et al., 2007; Rutkowski et al., 2010
	Mouse	*Prox1*$^{\pm}$	Baseline lymphatic dysfunction Impaired lymphatic transport & leaky lymphatics Impaired immune cell migration Increased inflammation after lymph node removal Defective lymphatic vasculature	Srinivasan et al., 2014; Hespe et al., 2019
	Mouse	K14-VEGFR-3-Ig (sR3)	Lack dermal lymphatics Smaller adipocytes Increased M2 macrophages Lack of podoplanin positive vessels in tail & back skin Regression of developing lymphatics	Mäkinen et al., 2001; Karaman et al., 2015
		FLT4-DTR	Mimic chronic lymphedema Initial swelling resolved temporarily, followed by late onset of lymphedema CD4 + infiltration Inhibition of lmyphagiogenesis Sclerosis of collecting lymphatic vessels	Gardenier et al., 2016

has also been shown to be increased in human and mouse lymphedematous tissues as well as in serum of lymphedema patients (Olszewski et al., 1992; Cuzzone et al., 2014). Increased IL-6 expression in lymphedematous murine tissues is associated with fat deposition, and it is postulated that its role is to regulate adipose tissue homeostasis since blocking its activity limits the expansion of adipose tissue (Cuzzone et al., 2014).

Fibrosis in Lymphedema

Fibrosis, which is the excessive deposition of extracellular matrix in various organs, potentially leads to their dysfunction. This condition occurs in the extremity lymphedema and is an important pathological change in lymphedema. Histological and immunohistochemical examinations of skin tissues from clinical and experimental lymphedema revealed increased amounts of collagen fibers in the edematous skin (Schirger et al., 1962; Ryan, 1995; Rutkowski et al., 2010; Zampell et al., 2012c; Gardenier et al., 2016; **Table 1**). Fibrosis in lymphedema is not confined to the dermis, but has also been detected in the subcutaneous tissue including the adipose tissue. Hypertrophic adipocytes exhibit thick fibrous matrix between lobules (Tashiro et al., 2017) in human lymphedema. Collagen accumulation within subcutaneous fat in mouse models of lymphedema was observed (Zampell et al., 2012c; Gardenier et al., 2016). This causes lymphedematous tissue to harden, resulting in non-pitting edema.

Collecting lymphatic vessels have been shown to play a role in lymphedema onset, and studies of lymphedema patients and animal models demonstrated morphological and structural changes in collecting lymphatic vessels including collagen deposition (Mihara et al., 2012; Gardenier et al., 2016). In lymphedema, lymph fluid stasis results in an increase in the pressure within lymphatic vessels. When this process perpetuates, the smooth muscle cells in the lymphatic vessels become slimmer and flattened (Koshima et al., 1996; Ogata et al., 2015) and dermal capillary lymphatic vessels become hypertrophic (Tashiro et al., 2017), causing dermal back flow of lymph fluid. Mihara et al. (2012) elegantly investigated four types of collecting lymphatic vessel changes throughout disease progression in lymphedema patients that began before the onset of lymphedema. Normal type of collecting lymphatic vessels has collagen fibers and smooth muscle cells present in medial layer. Ectasis type is characterized by the dilation of the lymphatic vessel wall, with long and elongated collagen fibers. Contraction type shows the deposition of thick collagen fibers mixed with smooth muscle cells in the medial layer. The thick collagen fibers impair vessel contraction, resulting in loss of function in the collecting lymphatic vessels. Sclerosis type vessels exhibit increased smooth muscle cells and collagen fibers and a loss in their ability to transport lymph fluid, causing excessive lymph leakage (Mihara et al., 2012). These changes in collecting vessels are consistent with previous findings showing decreased lymphatic vessels contractility in human lymphedema (Olszewski, 2002; Modi et al., 2007). In addition, fibrosis in the skin and subcutaneous tissue may worsen lymphatic vessel dysfunction by directly inhibiting lymphatic endothelial cell proliferation and preventing the sprouting and branching of new lymphatic vessels. This is supported by findings in mice and rat models demonstrating that fibrosis negatively regulates lymphatic flow and lymphangiogenesis, which in turn aggravate swelling, fluid transport and lymph drainage (Lynch et al., 2015). However, when fibrosis was inhibited, lymphatic vessel repair and transport were improved, slowing down the progression of lymphedema (Avraham et al., 2009, 2010; Savetsky et al., 2014).

In contrast to our knowledge on the deleterious effect of increased collagen deposition on lymphedema, little information is available on the collagen fiber structure and spatial organization in lymphedema. Two recent studies on human and mouse lymphedema tissues using multi-photon microscopy revealed changes in the spatial organization of collagen network, leading to irreversible structural damages (Wu et al., 2011; Kistenev et al., 2019). However, the possible consequences of these changes on lymphatic function and lymphedema remains to be elucidated.

Skin Changes

In the later stages of lymphedema progression, skin changes such as hyperkeratosis may occur together with fibrosis of dermis, subcutaneous tissue, and muscular fascia (Daroczy, 1995; Domaszewska-Szostek et al., 2016). As the disease progresses, skin indurated developing a leathery texture and is more prone to recurrent infections, wart formation, cellulitis, warts, ulceration, fissures, and in rare cases, cutaneous angiosarcoma (Sinclair et al., 1998; Grada and Phillips, 2017).

POTENTIAL MECHANISMS FOR TISSUE TRANSFORMATION IN LYMPHEDEMA

The mechanism of adipose tissue and fibrosis in lymphedema remains unclear, and several studies especially in mouse model of lymphedema have revealed potential factors in these processes.

Role of Lymph Stasis and Deposited Lipids

In lymphedema, the affected tissue becomes suffused with lymph as a result of lymphatic obstruction or insufficiency. Studies by G. Oliver's group in *Prox1*$^{\pm}$ mice provided the first evidence that lymphatic leakage can promote adipose tissue hypertrophy and adipogenesis (Harvey et al., 2005). They showed that fat accumulates near leaky mesenteric lymphatic vessels in *Prox1*$^{\pm}$ mice, and the egressed fluid induces *in vitro* the differentiation of adipocyte (Harvey et al., 2005). One indication of adipogenesis is the increased expression of fat differentiation markers demonstrated in a tail model of lymphedema (Aschen et al., 2012). Stagnant lymph contains various factors including proteins and lipids. In a follow-up study, G. Oliver's group demonstrated that the lipid fraction within the leaking fluid is the adipogenic factor, although no significant differences in lipid composition of lymph from WT and *Prox1*$^{\pm}$ mice were noted. Lymph from both groups promoted the differentiation of preadipocytes into mature adipocytes (Escobedo et al., 2016). This finding is consistent with a previous work showing that mesenteric lymph, or more specifically, chylomicron isolated from the lymph, supports the differentiation of adipocyte precursors (Cambon et al., 1998). Furthermore, free fatty acids, which are abundant in lymph, promoted adipogenesis *in vitro* (Escobedo et al., 2016). Cholesterol is another lipid component that may deposit in tissue from lymph stasis. Lipoproteins including low-density (LDL) and high-density (HDL) lipoproteins play a critical role in lipid transport in lymph, as well as in blood (Randolph and Miller, 2014). The transport of cholesterol conjugated to HDL from peripheral tissues back to the systemic circulation is known as reverse cholesterol transport (RCT) and critically depends on efficient lymphatic transport. In experimental models, the surgical disruption of lymphatic channels impairs the return of cholesterol to the systemic circulation from a transplanted artery (Martel et al., 2013) and the skin (Lim et al., 2013). Notably, adipose tissue is the major site of cholesterol storage and alterations in cholesterol balance in adipocytes can modulate metabolic and pro-inflammatory adipose tissue functions (Chung and Parks, 2016). This raises the possibility that RCT may be compromised in lymphedema as a consequence of impaired lymphatic drainage. The resultant cholesterol accumulation in the affected limb may in turn contribute to adipose remodeling. This phenomenon needs to be demonstrated experimentally. From a clinical perspective, the above lipid factors in lymphedematous

tissue, and correspondingly, their levels in the blood have not yet been clearly studies.

Traditionally, it is believed that high protein content in the interstitial tissue induces fat deposition and fibrosis. A study in the K14-VEGFR3-Ig transgenic lymphedema mouse model revealed that lymph stasis *per se* may not be sufficient to induce these tissue changes (Markhus et al., 2013). Clearly, other factors and events evolving around lymph stasis trigger these pathological changes in the lymphedematous tissues, explaining why the majority of the patients develop lymphedema months to years after the initial injury (Petrek et al., 2001).

Role of Inflammation

It is clear from both experimental and clinical studies that inflammation is a critical player in the pathophysiology of lymphedema (Ly et al., 2017). The first demonstration that lymphedema in rats leads to chronic inflammatory response (Gaffney and Casley-Smith, 1981), together with the later identification of inflammatory genes associated with several symptoms in human lymphedema (Fu et al., 2016), has fueled more research this area. One of the well-characterized manifestations of the inflammatory reaction associated with lymphedema is the infiltration of inflammatory cells in the edematous tissues. Studies by the group of B. J. Mehrara and M. Detmar showed in clinical and experimental lymphedema that the majority of the cells that accumulate chronically in lymphedematous tissues are $CD4^+$ T cells and that they contribute to the pathological changes including fibrosis (Avraham et al., 2010, 2013; Zampell et al., 2012c; Savetsky et al., 2014; Gousopoulos et al., 2016b). Further phenotypic characterization of the infiltrating $CD4^+$ T cells revealed that there is a mix of Th1, Th2, and regulatory T cells (Avraham et al., 2013; Gousopoulos et al., 2016a). Notably, the blockade of Th2 differentiation but not Th1 differentiation was effective in preventing the development of lymphedema, and in established cases, treating it (Avraham et al., 2013; Ly et al., 2019). Similar results were obtained when the pro-fibrotic cytokines and growth factors i.e., IL-4, IL-13, and TGF-β, produced by Th2 cells were blocked (Avraham et al., 2013; Savetsky et al., 2015). In contrast to the pathogenic role of Th2 cells in lymphedema, T regulatory (Treg) cells seem to limit the pathological changes in lymphedema. Indeed, the depletion of Treg cells exacerbates edema and fibrosis and is associated with increased infiltration of immune cells with a mixed Th1/Th2 cytokine profile (Gousopoulos et al., 2016a). Conversely, expansion of T regulatory cells significantly reduced lymphedema development by attenuating the tissue inflammation in lymphedema (Gousopoulos et al., 2016a). However, these cells may also participate to the local immune suppression observed in lymphedema, which is consistent with the recurrence of soft tissue infections observed in this disease (Sharkey et al., 2017; Garcia Nores et al., 2018). Collectively, these findings suggest targeting T cells as a potential novel therapeutic strategy for lymphedema.

Macrophages can serve multiple functions including regulation of lymphatic vessels (Kataru et al., 2009), inflammation, immunity, and tissue repair (Ginhoux and Jung, 2014), which are all functions relevant to lymphedema progression. Accumulation of macrophages has been detected in lymphedema. However, macrophages have been shown to serve opposing functions. Macrophages have been commonly classified into alternatively activated macrophages (M2) or classical (M1) phenotype, with repair and pro-inflammatory functions, respectively. However, this classification is simplistic and may not represent the entire spectrum of macrophage phenotypes and their corresponding functions *in vivo* (Ginhoux and Jung, 2014). Depletion of macrophages in the mouse tail surgery model significantly promotes fibrosis (Zampell et al., 2012b; Ghanta et al., 2015). Macrophages that exhibit a M2 phenotype may mediate this anti-fibrotic function through the regulation of $CD4^+$ T cell accumulation and Th2 differentiation (Ghanta et al., 2015; Savetsky et al., 2015; Shin et al., 2015). In healthy adipose tissue, M2 macrophages are dominant. In adipose tissue of obese individuals, the number of M2 macrophages decreased while M1 macrophages appear to be more frequent. In line with these observations in obesity, flow cytometry analysis of adipose tissue-derived cells from healthy and lymphedema subjects showed that there is an imbalance between M1 and M2 macrophages, where M2 macrophages decreased in number in lymphedema adipose tissues compared to healthy controls (Tashiro et al., 2017). Notably, M1 macrophages in obese adipose tissue have been shown to localize predominantly to dead adipocytes to form crown-like structures and to scavenge residual lipid and debris from necrotic adipocytes (Cinti et al., 2005). The report by Tashiro et al. reveals that the accumulation of M1 macrophages in adipose tissue of lymphedema patient was rarely associated with crown-like structures (Tashiro et al., 2017). In the mouse tail model, lymph stasis is associated with the infiltration of $F4/80^+$ macrophages, which accumulate around the expanded subcutaneous fat (Zampell et al., 2012c). Whether this infiltration is a prelude to crown-like structure formation can only be confirmed by the presence of adipocyte necrosis and the M1 macrophage phenotype. Therefore, further investigations are warranted to study the formation of crown-like structures in lymphedema-associated adipocyte remodeling and its significance. Macrophages may also control adipose tissue remodeling through the production of IL-6 that is a key factor in chronic inflammation and adipose metabolism (Scheller et al., 2011). In addition, macrophages may play a role in lymphedema by controlling lymphangiogenesis through the production of vascular endothelia growth factor-C, since depletion of macrophage, in established lymphedema, decreases lymphatic transport activity and VEGF-C expression (Zampell et al., 2012b; Ghanta et al., 2015). Finally, macrophages may improve lymph stasis through the upregulation of lymphatic pumping activity by modulating the expression of inducible nitric oxide synthase (Liao et al., 2011; Scallan et al., 2016). Altogether, these findings suggest a complex role for macrophages in the pathophysiology of lymphedema. This diversity of functions may depend on the stage of the disease, its anatomical location (upper versus lower extremity), and the type of macrophage population.

Clinical and animal studies show that inflammatory genes are upregulated in lymphedema (Foldi et al., 2000;

Lin et al., 2012; Leung et al., 2014). Importantly, the expression of pro-inflammatory genes, such as TNF-α and IFN-γ, were decreased after complete decongestive treatment. Transcriptional profiling of lymphedematous tissues in a mouse tail model revealed the upregulation of genes involved in acute inflammation, immune response, fibrosis, and wound healing (Tabibiazar et al., 2006). The authors hypothesize that leukotrienes produced by 5-lipoxygenase (5-LO) have a potential role in the pathogenesis of the disease. Human lymphedema patients exhibit increased levels of plasma leukotriene B4 (LTB4)(Tian et al., 2017). In mice treated with LTB4 antagonist, edema is reversed, together with improvement in lymphatic function and skin pathological changes (Tian et al., 2017). Ketoprofen is a NSAID drug with a dual anti-inflammatory mechanism of action, including inhibition of the 5-LO pathway (Rajic et al., 2010). Interestingly, the systemic treatment with ketoprofen of mice with established lymphedema reverses the disease and histopathological changes (Nakamura et al., 2009). The inhibition of the 5-LO pathway account for the therapeutic effect of ketoprofen. Together these preclinical results led to the clinical pilot study to evaluate the potential therapeutic effect of ketoprofen to ameliorate human lymphedema. This exploratory study demonstrated the beneficial effect of targeted anti-inflammatory therapy with ketoprofen in lymphedema patients, as shown by the reduction of skin thickness and amelioration of histological changes (Rockson et al., 2018). It remains to be seen whether this treatment reverses fibrosis, adipose tissue deposition and is long-lasting.

DIAGNOSIS AND ASSESSMENT OF LYMPHEDEMA AND ASSOCIATED TISSUE CHANGES

Lymphedema is diagnosed clinically and classified in four stages according to International Society of Lymphology (ISL). Stage 0 is latent, and despite impairment of lymph transport, swelling is not evident. Stage I is characterized by early accumulation of fluid, and elevating the affected limb may subside swelling. Pitting (indentation remains when a finger is pressed onto affected area) may also occur in Stage I. Pitting is more evident in Stage II, as swelling increases and will not subside from limb elevation alone. As it progresses to late Stage II, pitting may or may not occur due to onset of fibrosis. Stage III lymphedema, also known as lymphostatic elephantiasis, is advanced lymphedema with pitting absent.

Methods to diagnose lymphedema have primarily focused on the detection of edema, lymphatic vessel transport, and lymph flow, until the recent advances in our knowledge of disease pathophysiology prompted the development of methods to assess tissue transformation including fibrosis and fat deposition (O'Donnell et al., 2017). These novel approaches may be more effective modalities to monitor the progression of the disease and the response to treatments that are, to our knowledge, still lacking in the clinic. Analysis of lymphatic vessel structure and transport is carried out by direct or indirect lymphography. Direct lymphography is the injection of contrasting agents into lymphatic vessels (Kinmonth, 1952). Because of the risk of damaging lymphatic vessels, this method has been replaced overtime by indirect lymphography based on the introduction of radiolabeled contrast agents injected into soft tissue that will penetrate the lymphatic vessels allowing their analysis (Cambria et al., 1993). However, there is a lack of standardization, due to the different isotopes used. More recently, infrared fluorescence imaging of lymphatic vessels using indocyanine green dye has enabled the visualization of fine lymphatic vessels and has been used to diagnose and grade lymphedema (Unno et al., 2010). The latest imaging modality is photoacoustic lymphangiography, which provides high resolution imaging of lymphatic vessels and veins (Kajita et al., 2020).

Methods aiming at measuring and recording increase in limb volume include water displacement, optoelectronic perometry, bioelectrical impedance and circumferential measurements (Rincon et al., 2016). However, these methods are not ineffective in detecting early lymphedema.

Non-invasive methods such as ultrasonography, MRI, computerized tomography, and dual-energy X-ray absorptiometry are able to detect skin tissue changes such as tissue density variations, fluid accumulation, fibrosis, and fat components. Although these methods have been used and are able to provide information on the lymphoedematous tissue, they are expensive and complex and present some potential hazards (Brorson et al., 1998; Ward, 2011). Lately, SkinFibroMeter has shown promising results for the assessment of skin stiffness in human lymphedema using a special three-dimensional computational finite element to analyze the biomechanical response of skin tissue to external force (Sun et al., 2017). As discussed above, multi-photon microscopy allows the analysis of collagen structure and may also be used for lymphedema diagnosis as this modality has been used for *in vivo* assessment of human skin aging and photoaging (Lin et al., 2005; Koehler et al., 2006).

MANAGEMENT OF LYMPHEDEMA BY MULTI-MODALITIES TREATMENT

There is yet no cure for lymphedema. The current treatments are multi-modality and aim to reduce the swelling and discomfort of the affected extremity in lymphedema patients. One treatment option to manage early stage lymphedema is decongestive therapy, which includes manual lymphatic drainage (MLD), compression bandaging, exercise, skin care, and compression garments. Surgical therapies are indicated for stage I onward, with the modalities being broadly classified into lymphatic reconstructive procedures and excisional procedures (Raju and Chang, 2015). Excisional procedures can produce drastic reductions in limb girth, but may be complicated by unstable scars and poor aesthetic appearance. Lymphatic reconstructive procedures such as lymphovenous bypass and lymphatico-lymphatic anastomosis are useful acute surgical decongestive therapies and may be accomplished stage-wise under local anesthesia. The creation of a peripheral connection between lymphatic and venous systems to treat lymphedema was

described as early as the 1960s (Olszewski et al., 1968), but it was not until the 2000s (Koshima et al., 2000), with the introduction of refined instruments and techniques, that lymphovenous bypass gained traction worldwide. The advantages of the technique are that it is minimally invasive and is effective in draining lymphatic fluid immediately. Lymphovenous bypass is coupled with compressive therapy. However, fibrosis at late stages of lymphedema may limit the efficiency of this surgical intervention by compromising the function of the remaining lymphatic vessels that become no longer suitable (Suami and Chang, 2010). Timing of intervention is therefore important, and it is proposed that surgical interventions have better outcomes when performed at earlier stages of the disease (Becker et al., 2006).

In recent years, vascularized lymph node transfer has become a rapidly emerging method of lymphatic reconstruction shown to lead to lymphatic regeneration (Tan et al., 2016). The implanted lymph nodes create new channels and pathways through which fluid drains. Lymphangiogenesis is mediated by vascular endothelial growth factor C (Viitanen et al., 2013). When new channels sprout from the transferred lymph node connected to a peripheral artery and vein functions, it becomes a vascularized lymphaticovenous bypass "relay station." With better understanding of the vascular anatomy of lymph nodes, surgeons are able to safely harvest lymph nodes from the groin, head, and neck region and abdomen for transfer as lymph node flaps (Gould et al., 2018). From clinical observations, it takes about 2 years for new lymphatic channels to form and be functional. The disadvantage of the technique is donor site morbidity resulting in lymphedema.

The observations that tissue swelling in lymphedema is due to fat deposition have led to the development of liposuction for the treatment of this disease (Brorson et al., 2006, 2009; Damstra et al., 2009). Our clinical observations show that hypertrophic fat lobules compress and collapse their feeding lymphatic capillaries, resulting in a vicious cycle of fluid and lipid transport disruption, ultimately leading to further fat accumulation in the periphery. Conservative forms of surgery such as lymphaticovenous bypass promote clearance of lymphatic fluid and the lipids therein, but are ineffective for large volume fat clearance. Today, with better understanding of lymphatic anatomy and the path of lymphatic channels, surgeons perform selective liposuction where fat is removed with minimal disruption of lymphatic channels (Brorson, 2016). The indication for liposuction is fat hypertrophy in the affected extremities as shown on MRI. Proponents of liposuction demonstrate good volume reduction and no recurrence after 5 years (Hoffner et al., 2018a). The risks of liposuction include blood loss, hematoma, contour irregularity, and skin necrosis (Chen et al., 2019). These patients need to be on lifelong compression garments.

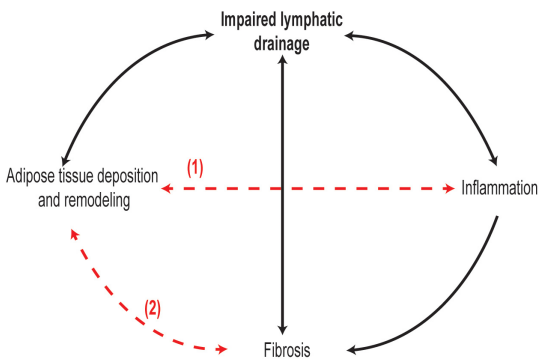

FIGURE 1 | Interplay and chronology between lymphedema pathological changes are not well understood. It is evident in clinical and animal models of lymphedema that inflammation, adipose tissue remodeling, and fibrosis in skin and lymphatic vessels occur as lymphedema progresses. In particular, chronology of adipose tissue remodeling between lymphedema stages remains unexplored. Moreover, it is not known if adipose tissue remodeling (1) and inflammation are two separate or synchronized events, and whether adipose tissue remodeling in lymphedema can promote fibrosis (2) as observed in obesity.

CONCLUSION AND FUTURE DIRECTIONS

In conclusion, lymphedema is characterized by several pathophysiological events, including lymph stasis, lymphatic vessel remodeling and dysfunction, inflammation, adipose tissue deposition, and fibrosis. However, the exact sequence of these events and their interplay during the development and progression of lymphedema are far from being well described (**Figure 1**). Much of the recent knowledge in the pathophysiology of lymphedema is derived from animal models of lymphedema, especially mouse models. Most lymphedema animal models are acute, whereby swelling occurs immediately after lymphatic injury and resolves within weeks, with few exceptions such as the FLT4-DTR mouse which develops a more prolonged state of lymphedema with human pathological features (Gardenier et al., 2016; **Table 1**). In contrast, lymphedema in humans is chronic and lifelong, developing a few months or years after surgery. Therefore, animal models poorly stimulate the onset human lymphedema development and progression. Due to this limitation, not all observations in animal models of acute lymphedema may be extrapolated to humans. More human studies are needed with particular focus on the types of tissue changes across the stages of lymphedema. A better understanding of the pathophysiology of lymphedema and its cellular and molecular mediators will pave the way for novel therapeutic approaches for this chronic and debilitating condition.

AUTHOR CONTRIBUTIONS

VA, HL, B-KT, and SA contributed to the manuscript. SA and HL generated the figure and table.

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Characterizing Lymphangiogenesis and Concurrent Inflammation in Adipose Tissue in Response to VEGF-D

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The metabolic consequences of obesity arise from local inflammation within expanding adipose tissue. In pre-clinical studies targeting various inflammatory factors, systemic metabolism can be improved through reduced adipose inflammation. Lymphatic vessels are a critical regulator of inflammation through roles in fluid and macromolecule transport and immune cell trafficking and immunomodulation. Lymphangiogenesis, the expansion of the lymphatic network, is often a necessary step in restoring tissue homeostasis. Using Adipo-VD mice, a model of adipocyte-specific, inducible overexpression of the potent lymphangiogenic factor vascular endothelial growth factor-D (VEGF-D), we previously identified that dense *de novo* adipose lymphatics reduced immune accumulation and improved glucose homeostasis in obesity. On chow diet, however, Adipo-VD mice demonstrated increased adipose tissue immune cells, fibrosis, and inflammation. Here, we characterize the time course of resident macrophage accumulation and lymphangiogenesis in male and female Adipo-VD mice fed chow and high fat diets, examining multiple adipose depots over 4 months. We find that macrophage infiltration occurs early, but resolves with concurrent lymphatic expansion that begins robustly after 1 month of VEGF-D overexpression in white adipose tissue. In obesity, female Adipo-VD mice exhibit reduced lymphangiogenesis and maintain a more glycolytic metabolism compared to Adipo-VD males and their littermates. Adipose lymphatic structures appear to expand by a lymphvasculogenic mechanism involving lymphatic endothelial cell proliferation and organization with a cell source we that failed to identify; hematopoietic cells afford minimal structural contribution. While a net positive effect occurs in Adipo-VD mice, adipose tissue lymphangiogenesis demonstrates a dichotomous, and time-dependent, inflammatory tissue remodeling response.

Keywords: lymphangiogenesis, VEGFR-3, lymphatic, obesity, VEGF-D, metabolic syndrome

INTRODUCTION

Tissue inflammation, fibrosis, and immune cell accumulation are all characteristic of adipose tissue expansion in obesity (Sun et al., 2011; Rutkowski et al., 2015). This results in metabolic dysfunction locally, and a spill-over of cytokines and lipids to the system, thus driving the metabolic syndrome (Sun et al., 2013). Genetically altering adipose tissue immune populations, fibrosis, or inflammation has demonstrated efficacy at correcting overall metabolic health (Sun et al., 2011, 2013; Card et al., 2014; Rutkowski et al., 2015). Lymphangiogenesis, the expansion of the lymphatic vessel network, is a common process during tissue inflammation. Lymphatic vessel roles in regulating interstitial fluid balance, macromolecule and immune cell clearance, and the peripheral immune response makes lymphangiogenesis an important part of inflammation resolution in a variety of tissue pathologies (Wiig and Swartz, 2012; Abouelkheir et al., 2017).

The lymphangiogenic proteins vascular endothelial growth factor C (VEGF-C) and –D (VEGF-D), ligands for VEGFR-3, are elevated in adipose tissue during obesity (Karaman et al., 2015; Chakraborty et al., 2019). In humans, lymphatic density varies greatly from adipose depot to depot, but local lymphatic vessel function may still impact local adipose health (Redondo et al., 2020; Varaliova et al., 2020). In mice, lymphatics are absent in brown adipose tissue, rare in gonadal white adipose tissue, and sparse in subcutaneous adipose depots. Tackling the VEGFR-3 signaling axis in obesity has resulted in dichotomous effects on adipose inflammation with some studies worsening and others improving metabolism (Karaman et al., 2015, 2016; Chakraborty et al., 2019). We recently demonstrated that overexpressing VEGF-D specifically in adipose tissue resulted in *de novo* lymphatics in subcutaneous adipose tissues and improved overall metabolism in mice (Chakraborty et al., 2019). The interplay of adipose inflammation and lymphangiogenesis upon elevated VEGFR-3 ligand availability thus remains unclear.

Since lymphatic expansion is naturally absent in murine adipose tissue during obesity, the source of new lymphatic structures upon VEGF-D induction in Adipo-VD mice is unknown. Lineage tracing studies have identified tissue specific venous and non-venous progenitors of lymphatic endothelial cells (LECs) contributing toward lymphatic network expansion during development, particularly in the skin (Srinivasan et al., 2007; Martinez-Corral et al., 2015). In the adult, new lymphatics arise by both sprouting lymphangiogenesis and from several potential progenitor pools, including resident PDGFRβ$^+$ cells and infiltrating hematopoietic or immune cells (Maruyama et al., 2005; Klotz et al., 2015; Martinez-Corral et al., 2015; Ulvmar et al., 2016). The mechanisms, and cell source, of adipose lymphatic expansion may provide insight into the potential for these newly present tissue LECs to modulate the immune response.

In this study, we utilized the Adipo-VD mouse with adipose-specific VEGF-D induction under a doxycycline-controllable transgene (Lammoglia et al., 2016; Chakraborty et al., 2019) to identify mechanisms of adipose lymphangiogenesis. We hypothesized that macrophage chemotaxis precedes lymphatic structure formation and that these macrophages may play a role in lymphangiogenesis. Comparison were made between male and female Adipo-VD mouse lymphatic densities and metabolic outcomes on chow and high fat diets and to determine if gender or obesity impacts new lymphatic growth or metabolic response. Lymphangiogenic activity was assessed over time and potential sources of new LECs were tested. This time-course characterization helps elucidate the interplay between lymphatic expansion and inflammation and provides some clues about the potential origins of the cell populations contributing toward induced adipose lymphangiogenesis.

MATERIALS AND METHODS

Animals

Genetic adipose-specific VEGF-D expression in Adipo-VD mice was accomplished as previously described (Lammoglia et al., 2016; Chakraborty et al., 2019). Prox1-tdTomato mice were derived at the Texas Institute for Genomic Medicine by *in vitro* fertilization from sperm obtained from the Mutant Mouse Resource & Research Centers (stock 036531). Mice were backcrossed 4 generations to C57Bl6/J before crossing to C57Bl6/J Adipo-VD mice, then further backcrossed 3-4 generations to C57Bl6/J. Donor constitutive tdTomato mice were made by selecting for Jackson Rosa26STOP-tdTomato mice (stock 007909) demonstrating spontaneous germline excision following a cross with TRE-Cre mice (stock 006234); ubiquitous expression, clearly identified visually, was confirmed genetically by lack of the loxP-flanked neomycin "stop" cassette.

All Adipo-VD mice were used as hemizygous for the TRE-VEGF-D transgene and either wildtype or hemizygous for the *AdipoQ*–rtTA transgene as non-functional and functional mice indicated as –rtTA and +rtTA throughout the text and figures. Age matched male and female mice were used in experiments and housed with 12-h light-dark cycles and ad libitum access to water and food in Association for Assessment and Accreditation of Laboratory Animal Care International. Starting at 6 weeks of age, all mice were fed custom chow diet (Bio-Serv F4107; Bio-Serv, Flemington, NJ) or 60% kcal from fat [D16042102, lard based (supplemented D12492); Research Diets, Inc., New Brunswick, NJ], each containing 600 mg/kg doxycycline to control for its potential effects on –rtTA littermate metabolism. Studies were performed at 1, 2, 3, and 4 months of feeding (mice thus aged up to approximately 24 weeks). Mice raised on standard facility chow were used as a normalization control for qPCR measurements as described. All animal study protocols were approved by the Institutional Animal Care and Use Committee at Texas A&M University (College Station, TX) or UT Southwestern Medical Center (Dallas, TX).

Tissue and RNA Preparation

Following exsanguination under isoflurane, mouse hair was removed from the mouse inguinal skin area. The entire subcutaneous inguinal white adipose tissue depot (with and without skin attached) along with intrascapular brown adipose depot (surrounding white adipose tissue trimmed), gonadal white adipose, and inguinal white adipose depots were harvested and weighed. Tissues were flash frozen immediately in liquid N_2 for RNA extraction or fixed in 10% buffered zinc formalin.

TABLE 1 | Quantitative Real-Time RT-PCR mouse primers utilized for gene expression analysis.

Target	Forward	Reverse
Cd206	5′-CAGGTGTGGGCTCAG GTAGT-3′	5′-TGTGGTGAGCTGAAA GGTGA-3′
F4/80	5′-CTTTGGCTATGGGCTTC CAGTC-3′	5′-GCAAGGAGGACAGAGTTTA TCGTG-3’
Tgfb	5′-GGGCCTCTTCTGCG ATTTC-3′	5′-ATCCAGGCAAGTGCAT TGGTA-3′
Tnfa	5′-GAGAAAGTCAACCTCCT CTCTG-3′	5′-GAAGACTCCTCCCAGGT ATATG-3′
Ubc	5′-GCCCAGTGTTACCACCA AGAAG-3′	5′-GCTCTTTTTAGATACTGT GGTGAGGAA-3’
Vegfr3	5′-ATCAGAAGATCGGGCGCTG TTGTA-3′	5′-TGTGTCATGTCCGCCCTTC AGTTA-3′
Il6	5′-GAGGATACCACTCCCAAC AGACC-3′	5′-AAGTGCATCATCGTTGT TCATA-3′
Il10	5′-GCTCTTACTGACTGG CATGAG-3′	5′-CGCAGCTCTAGGAGC ATGTG-3′

RNA was extracted using Zymo Direct-zol RNA Miniprep Plus, according to the manufacturer's instructions (Zymo Research, Irvine, CA). Reverse transcription of 1 μg RNA was performed using the iScript cDNA Synthesis kit instructions (Bio-Rad Laboratories, Inc., Hercules, CA).

Quantitative Real-Time RT-PCR

Adipose tissue cDNAs were amplified using BioRad iTaq universal SYBR Green supermix (Bio-Rad Laboratories, Inc.) in 5 μL reactions on a 384-well 7900T quantitative PCR machine (Applied Biosystems, Foster City, CA). Analyses in adipose tissues utilized *Ubc* as the most reproducible universal control, with expression represented as $2^{\Delta\Delta CT}$ compared to untreated control mouse adipose RNA. Primer sequences are listed in **Table 1**.

Histology and Immunofluorescence

Twenty-four-hours post fixation, tissues were thoroughly rinsed in deionized water and stored in 50% ethanol:water until processed for paraffin embedding. 3-4 μm thick sections were cut to maximum area (lymph node included for inguinal depot). For whole mount imaging, Prox1-tdTomato x Adipo-VD adipose depots were tissue cleared using the PEGASOS immunolabeling and clearing protocol (Jing et al., 2018). Immunofluorescence was detected using single track Zeiss Lightsheet Z1 microscope using an EC Plan-Neofluar 5x/0.16 objective. For widefield immunofluorescence, sections were deparaffinized, rehydrated and labeled for LYVE1, CD11b, tdTomato and Ki-67 with fluorophore- conjugated secondary detection (All antibodies are listed in **Table 2**). Immunofluorescence was imaged using an Axio-Observer fluorescence microscopy system and MRc camera (Zeiss, Thornwood, NY) or an Eclipse E600 microscope (Nikon, Melville, NY), and images were captured using cellSens Standard version 1.18 imaging software (Olympus, Waltham, MS). Several thick (20–30 μm) sections or hand-trimmed whole mount tissues were prepared and imaged as thin sections, above, but with composite z-stack maximal projections rendered for an in-focus 2D image using a Zeiss Stallion Digital Imaging Workstation or Olympus Fluoview FV3000 Confocal microscope.

TABLE 2 | List of primary antibodies along with RRID and catalog number used for immunofluorescence and lightsheet imaging.

Antigen	Source	Catalog number	RRID
CD11b	Abcam	ab133357	AB_2650514
Ki-67	Abcam	ab15580	AB_443209
LYVE1	R&D Systems	AF2125	AB_2297188
Mac2	Cedarlane	CL8942AP	AB_10060357
Podoplanin	R&D Systems	AF3244	AB_2268062
VEGFR3	R&D Systems	MAB3491	AB_2105112
tdTomato	Mybiosource	MBS448092	AB_2827808

Image Analysis

Image analysis of the histology sections was performed using ImageJ software version 1.52 (NIH, Bethesda, MD; http://imagej.nih.gov/ij) to determine the positive immunolabeled areas for $LYVE1^+$ and $Mac2^+$. For each quantified tissue, four to five random fields were captured at 10X magnification as the tissue area permitted at the same exposure settings for each fluorophore. Larger blood vessel regions and tissue defects were purposefully avoided. In ImageJ, positive labeling was identified by a fluorescence threshold, selected to differentiate lymphatic structures from the background, and measured as area/total area. Images were first averaged per animal, normalized to –rtTA chow-fed controls at 1-month, and then averaged per group. Whole mount images of the adipose were quantified using volume analysis in IMARIS software. The volume of the labeled lymphatics was calculated as [positive volume—(lymph node + afferent/efferent lymph duct volumes)] and reported as the percentage of total image volume.

Metabolic Measurements

To assess metabolic activity, food intake, respiratory exchange rate (RER), and heat production were measured. Mice were housed for 72 h (24-h acclimatization, 48-h collected measures) in TSE PhenoMaster cages (TSE Systems, Inc., Chesterfield, MO) with recorded ad libitum access to their respective diets and water. Cumulative values are reported for the final 12-h light and dark cycles. Serum glucose levels were measured using colorimetric reagents as before (Chakraborty et al., 2019) following a 4 h fast and 30 min post an intraperitoneal injection of 0.75 U/kg insulin.

Bone Marrow Transplant

Adipo-VD mice were exposed to 1,400 rads in an Ullman 113, Shepherd Mark I Irradiator. One week prior to irradiation, mice were provided sterilized pH 2.6 drinking water containing 0.1 mg/ml neomycin (Sigma). Bone marrow cells were sterilely isolated from mice constitutively expressing tdTomato and 5×10^6 were delivered intravenously to mice 2 h after irradiation. One mouse per cage was not reconstituted as a lethal irradiation

control; these mice died within 1 week. Mice were maintained on acidic water with neomycin for 2 weeks following irradiation then switched to acidic water only for 4 weeks. After 6 weeks of recovery, mice were returned to facility drinking water and started on 600 mg/kg dox-containing chow as above. Mice were terminated following 2 months of VEGF-D induction as above for tissue assessment.

Statistical Analysis and Data Presentation

All mice carry a single copy of the TRE-VEGF-D transgene (VD). The lack or the presence of the *Adipoq*–rtTA transgene designates Adipo-VD vs. littermates, respectively. Time course studies used 8 vs. 8 mice for histology studies and all subsequent measures started with minimally five vs. five mice per group based on the cage distribution at weaning. Mice were re-genotyped for the rtTA transgene at termination for confirmation, which altered some planned group numbers. Metabolic cages mice ranged from 3–8 mice across the groups. Final group numbers are reported in the Figure Legends for each experiment. Statistical significance for column data was tested by unpaired *t*-test comparisons, assuming unequal variance between the Adipo-VD and littermate groups using GraphPad Prism software version 7.05 (GraphPad Software, La Jolla, CA). For grouped column data multiple comparison two-way ANOVA followed by Holm-Šídák test were performed in GraphPad Prism software. Standard deviation is displayed throughout except standard errors are displayed for grouped graphical time courses for visual clarity. Significance across the +rtTA and –rtTA groups have been represented at *. Significance from month 1 for the subsequent months of each genotype has been represented as #.

RESULTS

Lymphatic Expansion and Reduced Crown-Like Structures in Male Adipo-VD Mice

Adipose expansion is accompanied by obesity-associated inflammation, metabolic dysregulation, and potentially reduced lymphatic density (Nitti et al., 2016; Escobedo and Oliver, 2017). To test the interplay between lymphvasculogenesis and inflammation, Adipo-VD male mice were put on a 60% kcal from fat diet containing 600 mg/kg doxycycline or custom chow diet containing 600 mg/kg doxycycline for 1-4 months. We observed marked lymphatic expansion in the +rtTA inguinal adipose tissue after 4-months of VEGF-D induction (**Figure 1A**). Three-dimensional rendering of the Prox1-tdTomato+ adipose lymphatic network revealed increased vessels and dense lymphatic regions in +rtTA mice compared to –rtTA mice (**Supplementary Videos 1, 2**, respectively). Percentage volume quantification of tdTomato positive structures confirmed more lymphatic structures in the +rtTA mice (**Figure 1B**). LYVE1+ lymphatic area expansion along 1–4 month of HFD feeding demonstrated prolific lymphatic expansion in the white inguinal adipose depots of +rtTA mice (**Figure 1C**). Comparisons across the –rtTA and +rtTA groups for each month demonstrated significant lymphatic expansion at month 2 &3 for male HFD +rtTA mice compared to –rtTA mice and significantly more LYVE1+ area at 4 months (**Figure 1D**). Under chow fed conditions, despite the presence of LYVE1+ lymphatic structures early, image quantitation revealed a significant increase in +rtTA white inguinal adipose only at 4 months (**Supplementary Figures 1A,B**). In both feeding conditions, lymphatic structures were also immunolabeled with podoplanin (**Figure 1E**, **Supplementary Figure 1C**). Image quantitation demonstrated a significant increase in podpoplanin+ area under obese conditions at 4 months (**Figure 1F**), but no significant difference under chow feeding (**Supplementary Figure 1D**). Quantitative real time PCR was used to confirm these image analyses. On HFD, there was no significant difference in *Lyve1* between groups or over time (**Figure 1G**), while *Pdpn* expression was significantly elevated in 4 month +rtTA mice, mirroring the image analyses (**Figure 1H**). Under chow fed conditions, the trends in *Lyve1* and *Pdpn* were similar to the image analysis with no significance identified (**Supplementary Figures 1E,F**).

Antibodies against Mac2 are used to identify adipose "crown-like structures," or resident macrophages that surrounding unhealthy adipocytes, in inflamed, obese adipose tissue (Asterholm et al., 2012a; Murano et al., 2013). Mac2+ labeling was visualized (**Figure 1I**) and quantified by image analysis (**Figure 1J**) on HFD. Adipo-VD mice (+rtTA) demonstrated significantly higher Mac2+ area early at 1 month compared to their –rtTA littermates, indicating increased crown-like structure formation. This elevation was significantly reduced by month 4, when adipose lymphatics were present (**Figure 1J**). Mac2+ area in chow feed males appeared to be higher (**Supplementary Figure 1G**), but their area quantification (**Supplementary Figure 1H**) was not significant.

While the *AdipoQ* promoter should be active in all adipocytes (Wang et al., 2010), we observed no LYVE1+ lymphatic structures in the epididymal depot of either HFD or chow fed +rtTA mice suggesting that chronic, low-level VEGF-D overexpression is insufficient to expand lymphatics in visceral depots of Adipo-VD mice (**Supplementary Figure 2**).

Despite increased lymphatics and reduced crown-like structures, qPCR measurement of inflammation-associated genes in +rtTA mice under HFD were largely unchanged compared to –rtTA mice over the times analyzed (**Figure 1K**). Only *Il10* was significantly reduced at 3 months, likely resulting from an odd increased expression in –rtTA mice. No significant differences were identified between groups or over time for *Il6*, *Tnfa*, *Tgfb*, *Cd206*, or *F480*, nor was the ratio of *Cd206*:*F480* changed (**Figure 1K**). These null results mirrored our previous work that found that despite reduced immune cell numbers, inflammatory markers were largely equivalent to wildtype mice during HFD-induced obesity (Chakraborty et al., 2019). Under chow feeding, despite early macrophage accumulation gene expression levels of the same markers were not increased in +rtTA Adipo-VD mice and were, surprisingly, less than –rtTA mice for *Il6* and *Il10* at 3 months and *Tnfa* at 4 months (**Supplementary Figure 1I**). Again, these negative results over time mirrored the lack of differences seen previously at 4 months in this model (Chakraborty et al., 2019).

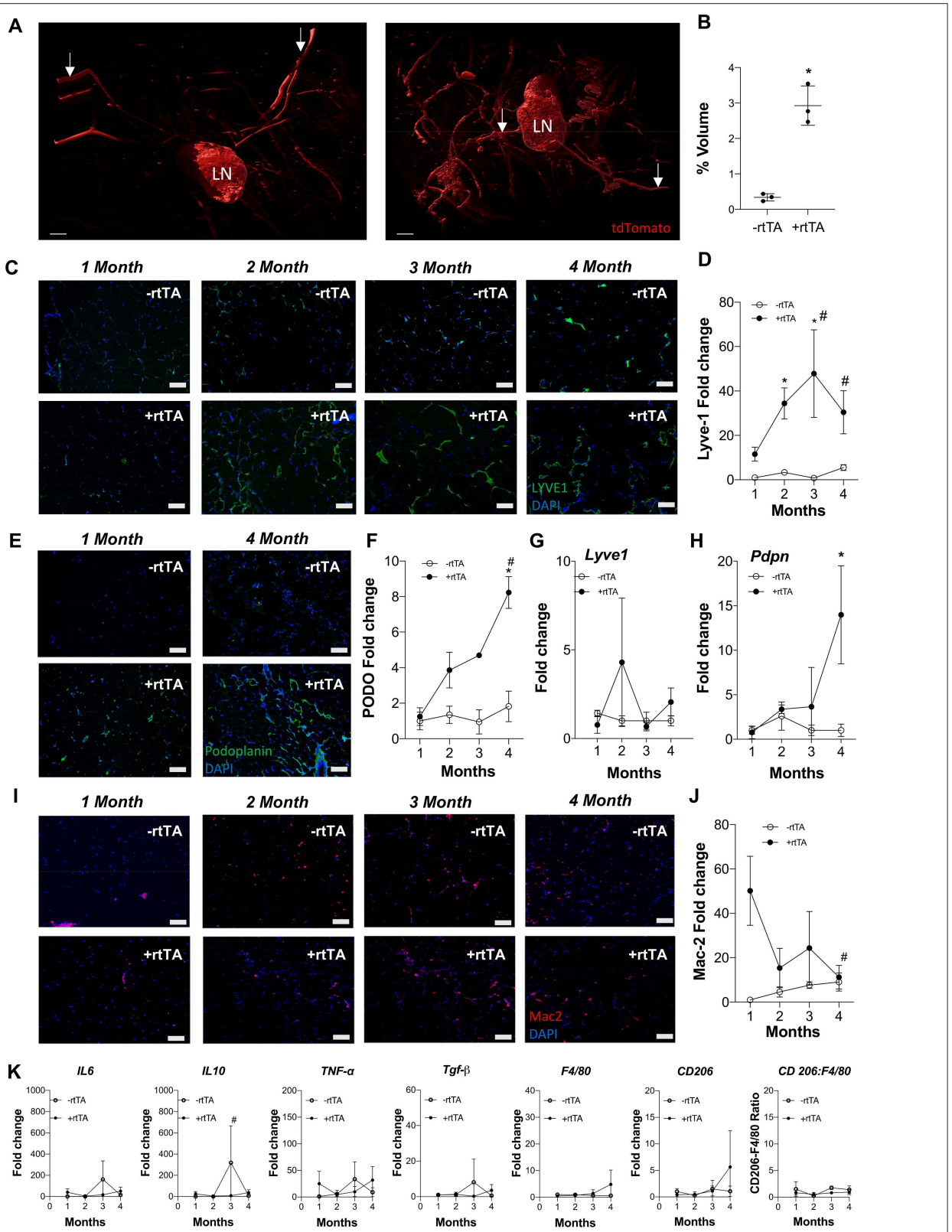

FIGURE 1 | Subcutaneous inguinal adipose lymphatics and inflammation in high fat diet fed male Adipo-VD mice. **(A)** Gradient imaging of subcutaneous adipose lymphatic structures in Adipo-VD x Prox1-tdTomato mice (Red = tdTomato); White arrows: presumed afferent and efferent lymph ducts; lymph node (LN).Scale

(Continued)

FIGURE 1 | bar = 500 μm. **(B)** Percentage positive volume of lymphatics within the tissue (LN and lymphatic ducts excluded) **(C)** LYVE1 (green) immunofluorescence of lymphatic structures in male –rtTA and +rtTA subcutaneous inguinal adipose following 1, 2, 3, and 4-month high fat diet feeding. **(D)** LYVE1 pixel area fold change comparison between –rtTA vs. +rtTA mouse inguinal depots quantified from random imaging and all values normalized to –rtTA tissues at 1 month. **(E)** Podoplanin immunofluorescence (green) of lymphatic structures in male –rtTA and +rtTA inguinal adipose depot following 1 and 4-month high fat diet feeding. **(F)** Podoplanin pixel area fold change comparison between –rtTA vs. +rtTA inguinal depot quantified from random imaging and all values normalized to –rtTA tissues at 1 month. **(G,H)** QPCR time course relative expression of *Lyve1* and *Pdpn* between –rtTA and +rtTA inguinal depot normalized to untreated control mouse adipose. **(I)** Macrophage $Mac2^+$ crown like structures (red) in –rtTA and +rtTA male subcutaneous inguinal adipose depot following 1, 2, 3, and 4-month high fat diet feeding. **(J)** Mac2+ pixel area fold change comparison between –rtTA vs +rtTA inguinal depot quantified from random imaging and all values normalized to –rtTA tissues at 1 month. **(K)** QPCR immune profile of *IL6, IL10, TNF-α, TGF-β, CD206, F4/80*, and ratio of *CD206:F4/80*-fold change across the time course normalized to untreated control mouse adipose. Images **(C–I)**, blue = DAPI and scale bars = 20 μm. **(B)** $n = 3,3$ **(D,J)** $n = 8,8$. **(F–H,K)** $n = 5,5$. $^*P < 0.05$ vs. –rtTA; $^{\#}P < 0.05$ vs. 1 month.

Lymphangiogenesis was robust in the interscapular brown adipose tissue of +rtTA mice on HFD (**Supplementary Figure 3A**). Image quantitation revealed this to be highly variable between mice, but significantly increased by 2 months (**Supplementary Figure 3B**). Chow fed +rtTA mice also demonstrated adipose lymphangiogenesis in interscapular brown adipose as early as 2 months following VEGF-D induction (**Supplementary Figure 3C**). Image quantitation revealed this to also be highly variable between mice and surprisingly not significant (**Supplementary Figure 3D**).

In total, male Adipo-VD +rtTA mice exhibited variable, but significant lymphatic expansion in multiple adipose depots within 2 months of VEGF-D overexpression with more profound increases in lymphatic structures measured during HFD feeding.

Female Adipo-VD Mice Exhibit Adipose Lymphatic Expansion and Reduced Crown-Like Structures

Females demonstrate an increased propensity for diseases of lymphatic overgrowth, such as lymphangioleiomyomatosis, and lymphatic dysfunction as in lymphedema (Moir, 2016; Morfoisse et al., 2018), yet are protected from the metabolic syndrome in obesity (Karastergiou et al., 2012). We thus sought to test if lymphangiogenesis and inflammation in female Adipo-VD tissues were equivalent to our findings in males. Following a similar protocol and analysis as above, we identified an increase in LYVE1+ lymphatic structures in +rtTA female white inguinal on HFD that was consistent with the males with structures forming by month 2 (**Figure 2A**). Image quantitation of LYVE1+ pixel area confirmed this with comparisons across the –rtTA and +rtTA groups for each month demonstrating significant lymphatic expansion at months 2 and 3 for female +rtTA mice (**Figure 2B**). The LYVE1+ pixel area at 2, 3, and 4 months in females was less significantly less than in males (by *post-hoc* analysis; $P < 0.01$, $P < 0.01$, and $P = 0.048$, respectively). While structures were visualized by podoplanin immunolabeling (**Figure 2C**), no significant difference in podoplanin+ pixels was measured in female HFD inguinal adipose tissue over time (**Figure 2D**). Under chow diet feeding, while LYVE1+ structures were visually present after 2 months of VEGF-D induction (**Supplementary Figure 4A**), quantification of LYVE1+ pixel area confirmed a pattern of progressive lymphatic structure expansion only after 4 months of VEGF-D induction (**Supplementary Figure 4B**). Podoplanin immunolabeling also identified increased structures in female +rtTA inguinal adipose tissue (**Supplementary Figure 4C**) that was quantified and found to be significant after 4 months (**Supplementary Figure 4D**). Confirmation of these image analyses by qPCR identified that on HFD, *Lyve1* expression was significantly increased in +rtTA mice at 2 and 4 months (compared to –rtTA) (**Figure 2E**), while *Pdpn* expression changes were not significant, mirroring the image findings (**Figure 2F**). Under chow fed conditions, the trends in *Lyve1* and *Pdpn* were highly variable (**Supplementary Figures 4E,F**).

Adipose depot resident macrophages, "crown like structures," were identified by $Mac2^+$ immunofluorescence in female mice after only 1 month of VEGF-D overexpression in both HFD and chow fed conditions (**Figure 2G**, **Supplementary Figure 4G**). Subsequent area quantification demonstrated that on chow diet these areas were significantly reduced from this early peak at 2, 3, and 4 months (**Supplementary Figure 4H**). This trend was present during HFD, but statistically not significant (**Figure 2H**).

Similar to the findings in males, gene expression of inflammatory markers *Il6*, *Il10*, *Tnfa*, *Tgfb*, *Cd206*, and *F480* were mostly unchanged over time and between –rtTA and +rtTA female mice on HFD (**Figure 2I**). An increased ratio of *Cd206*:*F480*, potentially an indicator of macrophage anti-inflammatory polarization, was significant at 4 months, but would require flow cytometry to confirm. Gene expression in chow feeding largely mirrored the findings in males **(Supplementary Figure 4I**).

In brown adipose tissue, sporadic expansion of LYVE1+ structures were observed in +rtTA mice as demonstrated by area quantification under both HFD (**Supplementary Figures 5A,B**) and chow conditions (**Supplementary Figures 5C,D**). Visually present at 1 month regardless of diet, image analysis demonstrated a significant increase in LYVE1+ structure area at 1 month under chow feeding and 2 months with HFD. In both diets, these trends or significant increases were maintained through 4 months.

Female +rtTA Adipo-VD mice thus exhibit a similar, though somewhat reduced, propensity for adipose tissue lymphangiogenesis compared to males and an early increase in Mac2+ structures in white adipose tissue that significantly resolves over time.

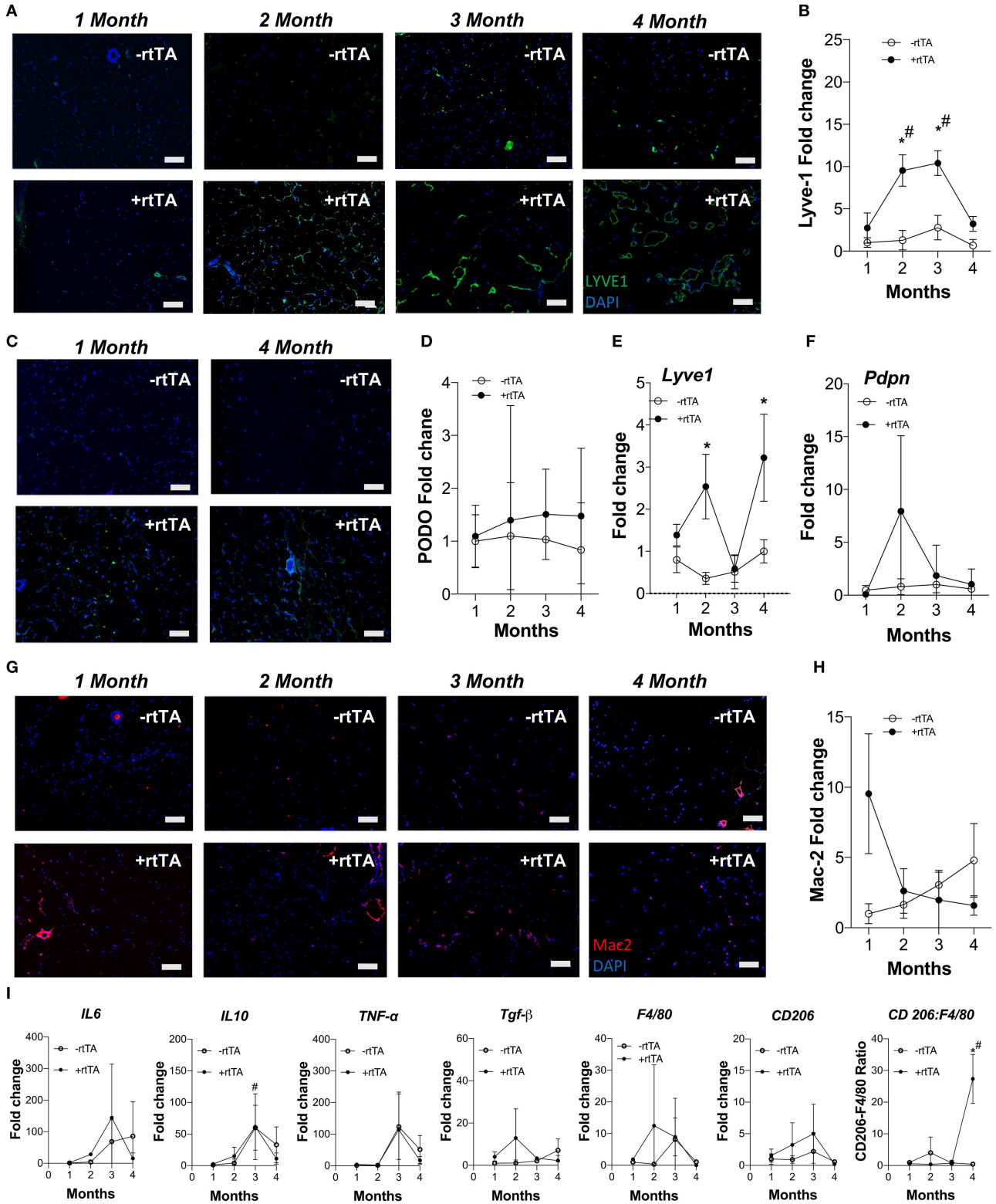

FIGURE 2 | Subcutaneous inguinal adipose lymphatics and inflammation in high fat diet fed female Adipo-VD mice. **(A)** LYVE1 (green) immunofluorescence of lymphatic structures in female –rtTA and +rtTA subcutaneous inguinal adipose following 1, 2, 3, and 4-month high fat diet feeding. **(B)** LYVE1 pixel area fold change comparison between –rtTA vs +rtTA mouse inguinal depots quantified from random imaging and all values normalized to –rtTA tissues at 1 month. **(C)** Podoplanin

(Continued)

FIGURE 2 | immunofluorescence (green) of lymphatic structures in female –rtTA and +rtTA inguinal adipose depot following 1 and 4-month high fat diet feeding. **(D)** Podoplanin pixel area fold change comparison between –rtTA vs +rtTA inguinal depot quantified from random imaging and all values normalized to –rtTA tissues at 1 month. **(E,F)** QPCR time course relative expression of *Lyve1* and *Pdpn* between –rtTA and +rtTA inguinal depot normalized to untreated control mouse adipose. **(G)** Macrophage Mac2$^+$ crown like structures (red) in –rtTA and +rtTA female subcutaneous inguinal adipose depot following 1, 2, 3, and 4-month high fat diet feeding. **(H)** Mac2+ pixel area fold change comparison between –rtTA vs +rtTA inguinal depot quantified from random imaging and all values normalized to –rtTA tissues at 1 month. **(I)** QPCR immune profile of *IL6, IL10, TNF-α, TGF-β, CD206, F4/80* and ratio of *CD206:F4/80*-fold change across the time course normalized to untreated control mouse adipose. Images **(A–G)**, blue = DAPI and scale bars = 20 μm. **(B,H)** $n = 8,8$. **(D–F,I)** $n = 5,5$. $^*P < 0.05$ vs. –rtTA; $^\#P < 0.05$ vs. 1 month.

Metabolic Assays Identify Changes in Male and Female Adipo-VD Mice Across High Fat and Chow Diet Conditions

We hypothesized that adipose lymphatic expansion was necessary for the metabolic improvements in glucose handling that we previously described (Chakraborty et al., 2019). Weight gain over time for HFD fed male and female mice was equivalent across genotype over time (**Figure 3A**) as previously reported (Chakraborty et al., 2019). Similarly, no differences between male –rtTA and +rtTA mice weight gain over time was measured (**Figure 3B**); there was no difference in genotype in females on chow either (**Figure 3B**). The body weight and adipose tissue depot weight:body weight ratios were largely unchanged between –rtTA and +rtTA male and female mice on both chow and HFD in the final groups studied (**Table 3**). Only the gonadal white adipose depots increased significantly over time, but did so under both diets. As a rapid test of insulin sensitivity, all mice were fasted for 4 h prior to a bolus of insulin and glucose levels measured 30 min later. No significant differences were identified across the time groups or between genotypes. While this indicated that our mice were metabolically similar, it suggested that 4 months of HFD feeding had not made any group particularly insulin resistant for this study.

To identify potentially more subtle metabolic differences, mice were housed and monitored in Phenomaster metabolic cages. Adipo-VD male mice on a HFD demonstrated significantly reduced respiratory exchange (RER: VCO_2/VO_2) during the dark cycle (**Figure 3C**), suggesting increased fatty acid utilization, with no change in heat production or food intake. During chow feeding, +rtTA Adipo-VD mice also demonstrated a lower RER, though during the day cycle (**Figure 3D**). Heat production and food intake were again unchanged. Conversely, HFD female +rtTA Adipo-VD mice demonstrated a significantly elevated RER during the dark cycle and ate significantly more food throughout the day (**Figure 3E**) suggesting increased carbohydrate utilization. Interestingly, under chow feeding, +rtTA females demonstrated a reduced dark cycle RER, like their male counterparts, and heat production and food intake were not significantly different (**Figure 3F**). In total, the mice used in this study were largely similar in their basic body phenotype and metabolism, with Adipo-VD mice generally utilizing more oxidative respiration than –rtTA mice.

Lymphatic Proliferation and Lymphvasculogenesis in Adipose Tissue of Adipo-VD Mice

Proliferation, migration, and organization of LECs via VEGFR-3 signaling is part of the lymphangiogenic process (Makinen et al., 2001; Goldman et al., 2007; Karpanen and Alitalo, 2008). When adipocyte VEGF-D expression was induced in high fat diet fed mice, Adipo-VD mice demonstrated lymphatic structure expansion in subcutaneous inguinal adipose tissue at 2 and 4 months (**Figure 4A**). However, few lymphatics in the adipose tissue were positive for the proliferation marker Ki67 (**Figure 4A**). At 2 months, Ki-67+ cells were limited to the adjacent dermis (not shown). At 4 months, however, lymphatic structures in the adipose tissue were found to contain Ki67+ cells. The number of Ki-67+ LYVE1+ cells was significantly greater on sections of +rtTA compared to –rtTA tissues (though few lymphatic structures were identified in –rtTA mice), indicative of continued proliferation with chronic VEGF-D overexpression (**Figure 4B**). Lack of proliferative markers earlier at 2 months suggests inclusion of LECs that may have proliferated elsewhere or earlier or other cell types that newly express LEC markers.

We crossed Prox1-tdTomato mice with Adipo-VD mice to visualize expanded lymphatics in adipose tissue. As demonstrated above, a Prox1-tdTomato+ network of lymphatic structures forms (**Figure 1A**) in Adipo-VD mice. We identified many LYVE1-expressing cells and cell clusters in thick sections of 2-month adipose tissues of –rtTA and +rtTA mice that were Prox1-tdTomato negative (**Figure 4C**). These cells have been previously reported by others as macrophages in adipose tissues (Cho et al., 2014). In +rtTA mice, we found that new lymphatic structures demonstrated varying degrees of tdTomato and LYVE1 immunolabeling, co-localized in some places, but independent in others (**Figure 4C**). Organization of these structures, and lack of early proliferation, suggests a lymphvasculogenic mechanism as previously reported (Chakraborty et al., 2019).

Macrophages reportedly contribute to lymphatic vessel structure in corneal inflammation (Maruyama et al., 2005). In the adipose tissue, this does not appear to occur as CD11b$^+$ cells were identified independent of LYVE1+ structures at month 2 of VEGF-D induction, and rarely associate with lymphatic structures at month 4 (**Figure 4D**). Mac-2+ crown-like structure macrophages were sometimes VEGFR-3+ (**Figure 4E**). To test the contribution of hematopoietic cells to lymphvasculogenesis, irradiated Adipo-VD mice were reconstituted with constitutive Rosa26-tdTomato bone marrow. Interestingly, lymphangiogenesis did not occur in the inguinal adipose tissue of these mice after 2 months of VEGF-D overexpression; tdTomato+ cells were identified in the lymph node and throughout white and brown adipose (not shown, **Figure 4F**). The only lymphatic structures found were identified in the brown adipose tissue of one mouse. In that tissue several tdTomato+ cells were identified near

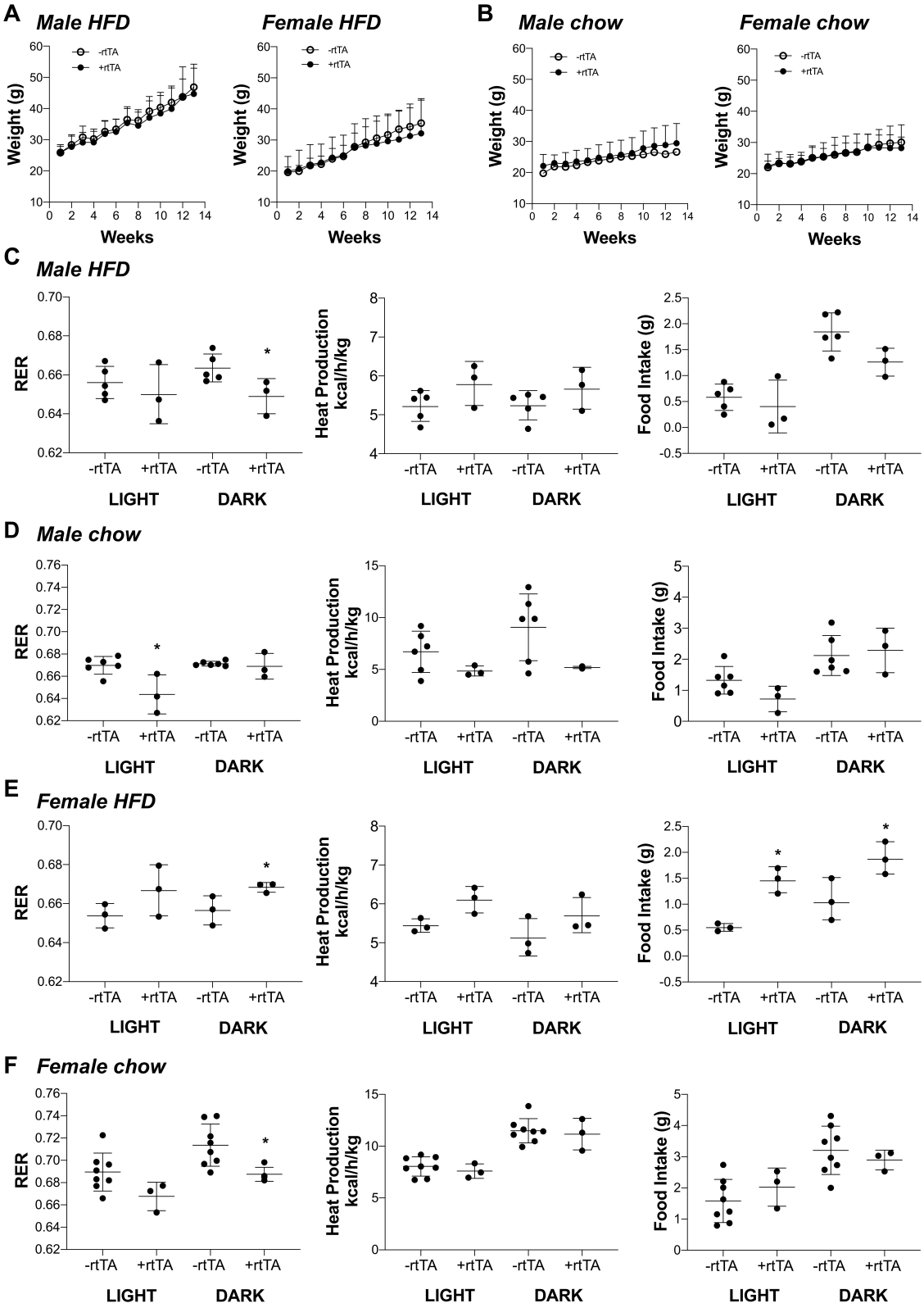

FIGURE 3 | Metabolic analyses of male and female Adipo-VD mice during high fat and chow diet conditions. **(A)** Weight gain curve for high fat diet fed –rtTA and +rtTA male ($n = 5,3$) and female ($n = 3,3$) Adipo-VD mice over 4 months. **(B)** Weight gain curve for chow diet fed –rtTA and +rtTA male ($n = 6,3$) and female ($n = 8,3$)

(Continued)

FIGURE 3 | Adipo-VD mice over 4 months. Metabolic cage data were obtained during the light and dark cycles following over 24 h of acclimatization. **(C)** Cumulative average respiratory exchange ratio [RER: VCO_2 (generation)/VO_2 (consumption)], average heat production, and cumulative 12-h food intake for high fat diet fed –rtTA and +rtTA male Adipo-VD mice. n=5,3. **(D)** Cumulative average respiratory exchange ratio (RER), average heat production, and cumulative 12-h food intake for chow fed –rtTA and +rtTA male Adipo-VD mice. *n* = 6,3. **(E)** Cumulative average respiratory exchange ratio (RER), average heat production, and cumulative 12-h food intake for high fat diet fed –rtTA and +rtTA female Adipo-VD mice. n=3,3. **(F)** Cumulative average respiratory exchange ratio (RER), average heat production, and cumulative 12-h food intake for chow fed –rtTA and +rtTA female Adipo-VD mice. *n* = 8,3.

TABLE 3 | Characteristics of the mice used in this study.

Months	Sex	Genotype	Mass (g)	IWAT:bw (x10^2)	GWAT:bw (x10^2)	BAT:bw (x10^2)	GLUCOSE (MG/DI)
HIGH FAT DIET							
1	M	–rtTA	47.2 ± 5.4	1.7 ± 0.50	1.2 ± 0.70	0.7 ± 0.10	135.1 ± 41.3
	M	+rtTA	37.3 ± 4.7	0.8 ± 0.30	1.2 ± 0.13	0.7 ± 0.06	84.60 ± 16.1
	F	–rtTA	24.4 ± 5.7	0.9 ± 0.28	1.3 ± 0.12	0.9 ± 0.32	156.2 ± 22.5
	F	+rtTA	24.2 ± 6.0	0.9 ± 0.35	1.3 ± 0.10	0.8 ± 0.10	141.5 ± 22.9
2	M	–rtTA	35.8 ± 5.8	0.8 ± 0.20	1.7 ± 0.37#	0.1 ± 0.05	117.5 ± 30.9
	M	+rtTA	30.7 ± 1.8	1.3 ± 0.40	1.3 ± 0.08	0.9 ± 0.10	94.60 ± 28.5
	F	–rtTA	31.0 ± 5.9	1.0 ± 0.10	1.3 ± 0.13	0.8 ± 0.60	88.00 ± 2.40
	F	+rtTA	35.6 ± 2.4	0.7 ± 0.07	1.5 ± 0.42	1.0 ± 0.60	99.50 ± 14.2
3	M	–rtTA	48.8 ± 3.1	2.8 ± 0.10	1.7 ± 0.01#	1.6 ± 0.23*#	172.0 ± 43.3
	M	+rtTA	54.2 ± 1.7	2.0 ± 0.70	1.6 ± 0.03	0.9 ± 0.32	193.5 ± 19.1
	F	–rtTA	29.6 ± 4.7	0.3 ± 0.05	1.4 ± 0.30	0.9 ± 0.02	94.60 ± 4.90
	F	+rtTA	28.4 ± 4.3	0.9 ± 0.30	1.4 ± 0.20	0.9 ± 0.40	117.6 ± 17.8
4	M	–rtTA	42.4 ± 4.7	1.7 ± 0.80	1.7 ± 0.70#	1.6 ± 0.10#	98.00 ± 21.1
	M	+rtTA	43.6 ± 8.1*	1.5 ± 0.50	1.6 ± 0.12	1.3 ± 0.32#	88.20 ± 1.70
	F	–rtTA	35.4 ± 5.4	1.6 ± 0.10	1.9 ± 0.20#	1.3 ± 0.45	97.30 ± 11.4
	F	+rtTA	33.3 ± 7.6	0.9 ± 0.20	1.9 ± 0.09#	1.3 ± 0.44	86.80 ± 44.5
CHOW DIET							
1	M	–rtTA	32.2 ± 5.2	1.2 ± 1.1	1.1 ± 0.44	1.3 ± 0.13	144.1 ± 28.5
	M	+rtTA	34.2 ± 0.9	0.9 ± 0.04	1.3 ± 0.60	0.9 ± 0.30	117.3 ± 30.9
	F	–rtTA	21.4 ± 1.6	0.5 ± 0.10	1.1 ± 0.86	0.8 ± 0.30	118.2 ± 19.6
	F	+rtTA	22.2 ± 0.6	0.5 ± 0.19	1.3 ± 0.08	1.1 ± 0.11	116.2 ± 12.6
2	M	–rtTA	43.4 ± 10	1.2 ± 0.30	1.3 ± 0.09	0.9 ± 0.2.1	104.4 ± 21.3
	M	+rtTA	36.6 ± 17	1.5 ± 0.01	1.4 ± 0.77	0.1 ± 0.30	123.6 ± 23.7
	F	–rtTA	21.2 ± 2.4	0.5 ± 0.20	1.2 ± 0.03	0.8 ± 0.02	87.20 ± 25.9
	F	+rtTA	23.0 ± 1.4	0.6 ± 0.20	1.4 ± 0.1.1	0.9 ± 0.04	93.20 ± 22.7
3	M	–rtTA	34.2 ± 3.7	0.6 ± 0.05	1.7 ± 0.40#	1.2 ± 0.01	129.6 ± 15.9
	M	+rtTA	34.2 ± 5.5	1.1 ± 0.34	1.4 ± 0.60	0.1 ± 0.12	142.6 ± 31.4
	F	–rtTA	22.3 ± 2.6	0.5 ± 0.20	1.5 ± 0.01	0.7 ± 0.43	93.40 ± 27.7
	F	+rtTA	23.9 ± 3.4	0.6 ± 0.20	1.4 ± 0.02	1.1 ± 0.90	101.8 ± 18.2
4	M	–rtTA	22.1 ± 2.3	1.4 ± 0.76	1.8 ± 0.30#	1.3 ± 0.20	126.3 ± 4.10
	M	+rtTA	27.6 ± 6.1	1.4 ± 0.70	1.5 ± 0.01*	1.0 ± 0.12	136.3 ± 2.50
	F	–rtTA	28.2 ± 5.0	1.0 ± 0.60	1.5 ± 0.20	0.7 ± 0.40	94.90 ± 19.0
	F	+rtTA	29.1 ± 3.9	1.4 ± 0.20*	1.6 ± 0.20	1.2 ± 0.32	107.6 ± 27.4

*Values represent the mean ± SD. IWAT, inguinal white adipose tissue; GWAT, gonadal white adipose tissue; BAT, brown adipose tissue; bw, body weight. Glucose levels were measured in the fasted state, 30 min following insulin injection. *indicates significant difference between genotypes, # indicates significant difference from 1-month.*

lymphatic structures, but did not appear to make any significant structural contribution to the vessels (**Figure 4F**). Absent further lineage tracing, the precise cell source of expanding adipose lymphatics remains unclear. Lymphatic proliferation and potentially other non-hematopoietic cell types may therefore contribute toward lymphvasculogenesis in Adipo-VD mouse adipose tissues.

Discussion

Obesity presents a chronic, inflammatory remodeling process in adipose tissue. Inflammation-associated lymphangiogenesis is often necessary to ameliorate inflammation, but despite increased expression of VEGF-C and VEGF-D reported in obesity, lymphatic vessels remain sparse in adipose tissue and those present demonstrate reduced function (Arngrim et al.,

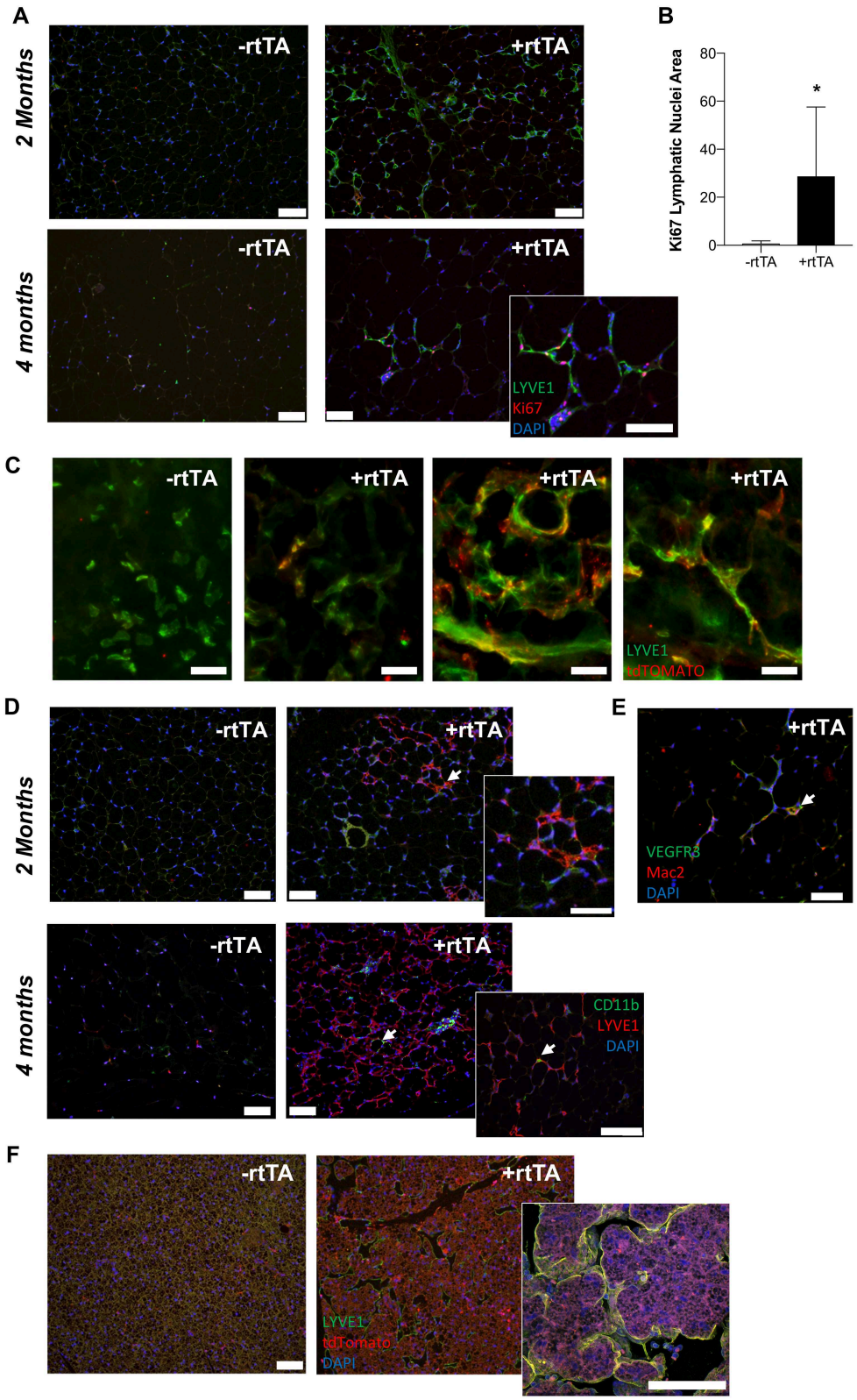

FIGURE 4 | VEGF-D induces LEC proliferation within the inguinal adipose depot. **(A)** Month 2 and 4 immunolabeling of proliferating (Ki-67, red) lymphatics (LYVE1, green) in the inguinal adipose depot of chow fed male –rtTA and +rtTA mice. Blue = DAPI and scale bars = 20 μm. **(B)** Number of Ki-67 positive lymphatic nuclei per

(Continued)

FIGURE 4 | 4 cm^2 section of subcutaneous inguinal adipose of male and female mice. $n = 5, 5$. **(C)** Whole mount images of $LYVE1^+$ (green) and Prox1-tdTomato (red) cells and structures in –rtTA and several +rtTA Adipo-VD mouse subcutaneous adipose tissues at 4 months. Scale bars = 50 μm. **(D)** Month 2 and 4 immunofluorescence for lymphatics (LYVE1, red) and $CD11b^+$ macrophages (green) in inguinal adipose of –rtTA and +rtTA mice at 2 months. Arrows = potential interaction. Blue = DAPI and scale bars = 20 μm. **(E)** Some Mac2 crown-like structure macrophages (red) can be found to be VEGFR-3 (green) positive (arrow). Blue = DAPI and scale bars = 20 μm. **(F)** Immunofluorescence of LYVE1 (green) and Prox1-tdTomato (red) bone marrow-derived cell expression in –rtTA and +rtTA brown adipose tissues at 2 months. $^*P < 0.05$ vs. –rtTA.

2013; Redondo et al., 2020). In this study we demonstrate that locally increasing VEGF-D levels specifically in the adipose tissue of Adipo-VD mice augmented lymphatic vessel structure formation in subcutaneous adipose depots. Obesity does not impair this expansion and male mice demonstrated greater lymphatic densities than females. Despite increased numbers of Mac2+ crown-like structures present in Adipo-VD inguinal adipose depots early, these are significantly reduced over time. Male Adipo-VD mice exhibit a more oxidative metabolism, while female Adipo-VD mice are more glycolytic in obesity than their respective –rtTA littermates. Finally, we identified that the lymphvasculogenesis process of new lymphatic structures in Adipo-VD adipose tissue includes LEC proliferation and other cells of unconfirmed identity.

Lymphatic vessels and adiposity have an intimate relationship. Dysfunctional lymphatics may cause adipose tissue expansion and obesity; conversely, conditions of expanding adipose tissue—obesity, lymphedema, and lipedema—appear to inhibit lymphatic function (Lim et al., 2009; Garcia Nores et al., 2016; Gousopoulos et al., 2017; Al-Ghadban et al., 2019; Gasheva et al., 2019). Reduced lymphatic density has also reported in obese mice (Garcia Nores et al., 2016). In studying the time course of lymphangiogenesis in Adipo-VD mice, we have found that high fat diet feeding and obesity do not impair lymphatic growth. Lymhpangiogenesis occurs quickly and is visually present at 1 month and quantitatively significant at 2 months in obese Adipo-VD mice, faster than the 4 months it required under chow conditions. It is possible that obesity simply does not slow an already rampant process, with induced VEGF-D synergizing with already elevated VEGFR-3 ligands, rather than inhibiting existing lymphatics once truly obese. VEGFR-3 signaling may also improve downstream collecting lymphatic vessel contractility (Breslin et al., 2007), or help to maintain progenitor cells in a lymphatic-like Prox-1+VEGFR-3+ phenotype (Srinivasan et al., 2014), potentially increasing lymphatic function and enhancing lymphangiogenesis in Adipo-VD adipose tissues. Lack of noticeable lymphangiogenesis in the gonadal adipose depot is surprising. The adiponectin promoter is active there with a demonstrated ability to overexpress VEGF-C and VEGF-D (Nitschke et al., 2017; Chakraborty et al., 2019). The depot, particularly in obesity, may demonstrate lower promoter activity as adiponectin levels decline (Sun et al., 2014) or the adult depot may harbor fewer of the cell pools that contribute to induced lymphangiogenesis discussed later. Higher levels or earlier VEGF-D expression, when the tissue is first forming, may still promote visceral lymphangiogenesis.

Adipose tissue inflammation is a hallmark of the metabolic syndrome. Murine studies targeting various immune cell, inflammatory cytokines, fibrotic matrix components, or the vasculature have demonstrated that by limiting the inflammatory response in adipose tissue, systemic health may be protected (Rutkowski et al., 2015). Previous studies manipulating VEGFR-3 signaling in obesity have demonstrated that mice overexpressing VEGF-C in the skin have impaired metabolism in obesity and that systemic VEGFR-3 blockade reduces VEGFR-3+ pro-inflammatory M1 macrophage numbers in adipose tissue (Karaman et al., 2015, 2016). In both male and female Adipo-VD mice, under both chow and high fat diet feeding, adipose VEGF-D induction significantly increases macrophage numbers, judged by Mac2 immunolabeling. This accumulation at 1 month, however, was reduced over time, coinciding with significant lymphatic expansion in each condition. We previously reported that increasing lymphatics in adipose tissue increased immune cell migration from the inguinal subcutaneous adipose tissue to the inguinal lymph node (Chakraborty et al., 2019), so these macrophages may migrate away. Alternatively, as Mac2 is indicative of adipose crown-like structures that form around dysfunctional adipocytes, improved adipose tissue health may necessitate fewer of these cells. The inflammatory markers tested were largely unchanged between –rtTA and +rtTA Adipo-VD mice. Few differences between –rtTA and +rtTA mice in protein products of these genes was reported previously (Chakraborty et al., 2019), but to find no elevation over time in obesity is surprising. It could be that these mice never fully developed the metabolic syndrome or that these markers were measured in the metabolically responsive inguinal depot that saw little increase in mass over time; therefore it is possible inflammatory genes were more upregulated in the gonadal depot that demonstrated the most weight gain in obesity.

Local lymphatic endothelium may serve as an immunomodulatory site, dampening the immune response within the tissue through innate and acquired mechanisms that were not examined in this study (Maisel et al., 2017; Lane et al., 2018). It is also possible that dense lymphatics in Adipo-VD adipose may act like fat associated lymphoid clusters (FALCs) in the tissue (Benezech et al., 2015; Camell et al., 2019). Resident B cells in aging mouse FALCs were recently identified to be negative regulators of adipose health compared to splenic B cells (Camell et al., 2019). While a significant increase in B cell recruitment was previously identified in Adipo-VD adipose (Chakraborty et al., 2019), we failed to identify any co-localization of larger number of B220+ cells with new lymphatic structures by immunofluorescence (not shown); increased B cells may still play a role in the tissue. Obesity also reduces lymph node tissue structure, which may be prevented with lymphangiogenic signaling in Adipo-VD mice (Solt et al., 2019). Our study and others' demonstrate that elevated VEGF-D is likely chemotactic toward pro-inflammatory cells, but chronic overexpression in

Adipo-VD mice reduces immune accumulation through several potential mechanisms.

Following the same VEGF-D induction protocol, we previously demonstrated the Adipo-VD mice are protected from obesity's metabolic syndrome, with improved glucose handling and reduced liver lipid deposition (Chakraborty et al., 2019). The mice used in this timecourse characterization were largely equivalent across genotype from a body weight, adiposity, and fasting glucose level. While HFD fed mice did gain more weight than those fed on chow they were not overly obese (averaging <50 g), which makes identifying changes in adipose inflammation and the metabolic syndrome more challenging. Both male and female Adipo-VD mice were more oxidative, judged by a lower RER, on chow diet than their littermates. Males maintained this difference on HFD, while females were more glycolytic. This could be due to overall "healthier" adipose tissue. An alternative interpretation of this data may be how readily Adipo-VD mice adapt to a different fuel source: RER should be reduced once HFD is introduced (Asterholm et al., 2012b). Female Adipo-VD RER was low at ∼0.67 under both feeding conditions. Adipo-VD mice may thus be primed for fatty acid utilization in contrast to VEGF-D knockout mice that exhibit higher lipid levels (Tirronen et al., 2018). The roles of lymphatics in lipid utilization are an active area of study in the field.

Both the sprouting of pre-existing lymphatic vessels and circulating transdifferentiating cells have been identified to contribute toward late developmental and adult lymphangiogenesis. Several studies found that non-venous cells, or non-LECs, contribute to lymphatic expansion in the dermis, heart, and mesentery (Ulvmar and Makinen, 2016). Several potential cell sources were proposed in these studies, including circulating angioblasts or hemogenic endothelial cells (Klotz et al., 2015; Martinez-Corral et al., 2015; Stanczuk et al., 2015). Hematopoietic, Vav1$^+$/Tie2- cells contributed to cardiac lymphatics (Martinez-Corral et al., 2015). Past studies in the cornea identified CD11b$^+$ macrophages contributing to new lymphatics (Maruyama et al., 2005). Hyperplasia of existing vessels and sprouting lymphangiogenesis was previously reported with high levels of VEGF-C overexpression in adipose (Nitschke et al., 2017). Characterization of lymphatics in healing mouse hearts identified two separate mechanisms or sub-populations of cells, sprouting lymphangiogenesis and isolated LECs, contributing toward expanding lymphatics; the cell source of the new LECs was unclear (Gancz et al., 2019). Recently, an imaging study identified LYVE1+ cells from both endothelial and hematopoietic lineage within human adipose tissue (Redondo et al., 2020). This study reinforced that hematopoietic LYVE1+CD45+ cells associate with adipose lymphatics, but they do not form their structure. While LYVE1+ adipose macrophages have been previously implicated in angiogenesis (Cho et al., 2007), our data does not confirm their role in lymphangiogenesis. Rather, we identified proliferation of tissue LECs (once present) and a lymphvasculogenesis mechanism that includes potentially LYVE1$^+$ Prox1$^-$ cell clusters, but largely excluded cells of hematopoietic origin. Interestingly, lymphatics failed to populate Adipo-VD white adipose tissue following irradiation suggesting that the cell source may have been lost or transformed. Bone marrow adipocytes survive irradiation and do have an impact on the hematopoietic microenvironment (Naveiras et al., 2009): it is possible that expressing VEGF-D during a period of reconstitution changes the marrow populations or their differentiation. There are also tissue-resident and non-hematopoietic endothelial cell precursor pools that demonstrate importance in adipose tissue vascularization (Gavin et al., 2016; Panina et al., 2018). More rigorous lineage tracing experiments, potentially targeting macrophages or PDGFβ+ cells (Ulvmar et al., 2016), would be necessary to truly identify these cells. These experiments in Adipo-VD mice may provide future understanding of the adult lymphvasculogensis process and these new vessels' immunomodulatory functions.

In conclusion, overexpression of VEGF-D in adipose tissue induces macrophage crown-like structure formation and a lymphvasculogenesis process of increased lymphatic vessel density. Increased lymphatics reduce the number of resident adipose tissue macrophages over time and increase systemic fatty acid utilization. The results of local VEGFR-3 activation within adipose tissue thus represent a dichotomous, and time-dependent inflammatory tissue remodeling response.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee at Texas A&M University (College Station, TX) or UT Southwestern Medical Center (Dallas, TX).

AUTHOR CONTRIBUTIONS

AC, CS, KR, and JR performed experiments and analyzed data. TM assisted with lineage tracing. AC and JR prepared the manuscript. All authors have read and approve the work.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2020.00363/full#supplementary-material

Supplementary Figure 1 | Subcutaneous inguinal adipose lymphatics and inflammation in chow fed male Adipo-VD mice. **(A)** LYVE1 (green) immunofluorescence of lymphatic structures in male –rtTA and +rtTA subcutaneous inguinal adipose following 1, 2, 3, and 4-month chow diet feeding. **(B)** LYVE1 pixel area fold change comparison between –rtTA vs. +rtTA mouse inguinal depots quantified from random imaging and all values normalized to –rtTA tissues at 1 month. **(C)** Podoplanin immunofluorescence (green) of lymphatic structures in male –rtTA and +rtTA inguinal adipose depot following 1 and 4-month chow diet feeding. **(D)** Podoplanin pixel area fold change comparison between –rtTA vs +rtTA inguinal depot quantified from random imaging and all values normalized to –rtTA tissues at 1 month. **(E,F)** QPCR time course relative expression of *Lyve1* and *Pdpn* between –rtTA and +rtTA inguinal depot normalized to untreated control mouse adipose. **(G)** Macrophage Mac2^{+} crown like structures (red) in –rtTA and +rtTA male subcutaneous inguinal adipose depot following 1, 2, 3, and 4-month chow diet feeding. **(H)** Mac2+ pixel area fold change comparison between –rtTA vs +rtTA inguinal depot quantified from random imaging and all values normalized to –rtTA tissues at 1 month. **(I)** QPCR immune profile of *IL6, IL10, TNF-α, TGF-β, CD206, F4/80*, and ratio of *CD206:F4/80*-fold change across the time course normalized to untreated control mouse adipose. Images **(A–G)**, blue = DAPI and scale bars = 20 μm. **(B,H)** n = 8,8. **(D–F,I)** n = 5,5. $^{*}P < 0.05$ vs. –rtTA; #$P < 0.05$ vs. 1 month.

Supplementary Figure 2 | Gonadal adipose tissue lymphatics and crown-like structures. Representative images of LYVE1 (green) and Mac2 (red) immunofluorescence find no lymphatics within the gonadal adipose tissue depot of chow and HFD –rtTA and +rtTA mice. Blue = DAPI and scale bars = 20 μm.

Supplementary Figure 3 | Interscapular brown adipose lymphatic expansion in high fat diet and chow fed male Adipo-VD mice. **(A)** LYVE1 (green) immunofluorescence of lymphatic structures in male –rtTA and +rtTA interscapular brown adipose tissue following 1, 2, 3, and 4-month high fat diet feeding. **(B)** LYVE1 pixel area fold change comparison between high fat diet fed –rtTA vs +rtTA mouse brown adipose depots quantified from random imaging and all values normalized to –rtTA tissues at 1 month. **(C)** LYVE1 (green) immunofluorescence of lymphatic structures in male –rtTA and +rtTA interscapular brown adipose tissue following 1, 2, 3, and 4-month chow diet feeding. **(D)** LYVE1 pixel area fold change comparison between chow fed –rtTA vs +rtTA mouse brown adipose depots quantified from random imaging and all values normalized to –rtTA tissues at 1 month. Images **(A,C)** blue = DAPI and scale bars = 20 μm **(B,D)** n = 8. $^{*}P <$ 0.05 vs. –rtTA.

Supplementary Figure 4 | Subcutaneous inguinal adipose lymphatics and inflammation in chow fed female Adipo-VD mice. **(A)** LYVE1 (green) immunofluorescence of lymphatic structures in female –rtTA and +rtTA subcutaneous inguinal adipose following 1, 2, 3, and 4-month chow diet feeding. **(B)** LYVE1 pixel area fold change comparison between –rtTA vs +rtTA mouse inguinal depots quantified from random imaging and all values normalized to –rtTA tissues at 1 month. **(C)** Podoplanin immunofluorescence (green) of lymphatic structures in female –rtTA and +rtTA inguinal adipose depot following 1 and 4-month chow diet feeding. **(D)** Podoplanin pixel area fold change comparison between –rtTA vs +rtTA inguinal depot quantified from random imaging and all values normalized to –rtTA tissues at 1 month. **(E,F)** QPCR time course relative expression of *Lyve1* and *Pdpn* between –rtTA and +rtTA inguinal depot normalized to untreated control mouse adipose. **(G)** Macrophage Mac2^{+} crown like structures (red) in –rtTA and +rtTA female subcutaneous inguinal adipose depot following 1, 2, 3, and 4-month chow diet feeding. **(H)** Mac2+ pixel area fold change comparison between –rtTA vs +rtTA inguinal depot quantified from random imaging and all values normalized to –rtTA tissues at 1 month. **(I)** QPCR immune profile of *IL6, IL10, TNF-α, TGF-β, CD206, F4/80*, and ratio of *CD206:F4/80*-fold change across the time course normalized to untreated control mouse adipose. Images **(A–G)**, blue = DAPI and scale bars = 20 μm. **(B,H)** n = 8,8. **(D–F,I)** n = 5,5. $^{*}P < 0.05$ vs. –rtTA; #$P < 0.05$ vs. 1 month.

Supplementary Figure 5 | Interscapular brown adipose lymphatic expansion in high fat diet and chow fed female Adipo-VD mice. **(A)** LYVE1 (green) immunofluorescence of lymphatic structures in female –rtTA and +rtTA interscapular brown adipose tissue following 1, 2, 3, and 4-month high fat diet feeding. **(B)** LYVE1 pixel area fold change comparison between high fat diet fed –rtTA vs. +rtTA mouse brown adipose depots quantified from random imaging and all values normalized to –rtTA tissues at 1 month. **(C)** LYVE1 (green) immunofluorescence of lymphatic structures in female –rtTA and +rtTA interscapular brown adipose tissue following 1, 2, 3, and 4-month chow diet feeding. **(D)** LYVE1 pixel area fold change comparison between chow fed –rtTA vs +rtTA mouse brown adipose depots quantified from random imaging and all values normalized to –rtTA tissues at 1 month. Images **(A,C)** blue = DAPI and scale bars = 20 μm **(B,D)** n = 8. $^{*}P < 0.05$ vs. –rtTA.

Supplementary Video 1 | Three-dimensional rendering of –rtTA mouse inguinal adipose tissue lymphatics. Prox1-tdTomato+ lymphatic vessels immunolabeled in –rtTA inguinal adipose with an anti-tdTomato antibody then visualized by light sheet microscopy following PEGASOS tissue clearing.

Supplementary Video 2 | Three-dimensional rendering of +rtTA mouse inguinal adipose tissue lymphatics. Prox1-tdTomato+ lymphatic vessels immunolabeled in +rtTA Adipo-VD inguinal adipose with an anti-tdTomato antibody then visualized by light sheet microscopy following PEGASOS tissue clearing.

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frontiers in Physiology

REVIEW
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The Role of Lymphatic Vascular Function in Metabolic Disorders

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In addition to its roles in the maintenance of interstitial fluid homeostasis and immunosurveillance, the lymphatic system has a critical role in regulating transport of dietary lipids to the blood circulation. Recent work within the past two decades has identified an important relationship between lymphatic dysfunction and patients with metabolic disorders, such as obesity and type 2 diabetes, in part characterized by abnormal lipid metabolism and transport. Utilization of several genetic mouse models, as well as non-genetic models of diet-induced obesity and metabolic syndrome, has demonstrated that abnormal lymphangiogenesis and poor collecting vessel function, characterized by impaired contractile ability and perturbed barrier integrity, underlie lymphatic dysfunction relating to obesity, diabetes, and metabolic syndrome. Despite the progress made by these models, the contribution of the lymphatic system to metabolic disorders remains understudied and new insights into molecular signaling mechanisms involved are continuously developing. Here, we review the current knowledge related to molecular mechanisms resulting in impaired lymphatic function within the context of obesity and diabetes. We discuss the role of inflammation, transcription factor signaling, vascular endothelial growth factor-mediated signaling, and nitric oxide signaling contributing to impaired lymphangiogenesis and perturbed lymphatic endothelial cell barrier integrity, valve function, and contractile ability in collecting vessels as well as their viability as therapeutic targets to correct lymphatic dysfunction and improve metabolic syndromes.

Keywords: obesity, lymphatic, inflammation, nitrous oxide, vascular endothelial growth factors, transcription factor, metabolic syndrome

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INTRODUCTION

The growing epidemic of obesity is characterized predominately by the accumulation of excess adipose tissue, which is due to an array of causes including the transition to an increasingly sedentary lifestyle and diets containing processed foods high in calorie content. The progression of this epidemic presents a major challenge to chronic disease prevention, including predisposal to the development of disability, depression, certain cancers, type 2 diabetes (T2D), metabolic syndrome, and cardiovascular disease (Grundy, 2004; De Pergola and Silvestris, 2013; Hruby and Hu, 2015; O'Neill and O'Driscoll, 2015; Ortega et al., 2016). In the United States, severe estimates predict that 86.3% of adults will be overweight or obese by 2030 (Wang et al., 2008), thus underscoring the critical need for development of novel therapeutic treatments in addition to preventative measures and lifestyle changes. Emerging evidence generated within the past

two decades has identified key mechanisms relating lymphatic vascular dysfunction with the pathogenesis of obesity, T2D, abnormal lipid metabolism and metabolic syndrome. In particular, the development and progression of metabolic syndrome, obesity, and T2D in some cases is associated with abnormal lymphangiogenesis and impaired function of collecting vessels during lipid transport and immune surveillance (Harvey, 2008; Dixon, 2010; Scallan et al., 2016; Escobedo and Oliver, 2017; Cifarelli and Eichmann, 2019). This review summarizes our current knowledge of the mechanisms by which lymphatic dysfunction contributes to pathogenesis of obesity, diabetes, and metabolic syndrome; focusing on the role of inflammation, transcription factors, vascular endothelial growth factor (VEGF)-signaling and nitric oxide (NO) signaling. Additionally, we discuss recent therapeutic developments targeting lymphatic function for the treatment of diabetes, obesity, and metabolic syndrome.

THE LYMPHATIC VASCULAR SYSTEM

Unlike the closed blood circulatory system, the lymphatic system consists of a blind-ended, unidirectional network of absorptive vessels and secondary lymphoid organs, which include lymph nodes, spleen, Peyer's patches, adenoids, and tonsils, in which lymph collected from the interstitial space is eventually drained into the blood vasculature via transport through the thoracic duct into the subclavian vein (Ruddle and Akirav, 2009; Choi et al., 2012). This unidirectional transport mechanism serves to support the critical physiological roles of the lymphatic vasculature in maintaining interstitial fluid and protein homeostasis, developing an immune surveillance response by transporting antigen and antigen-presenting cells to lymph nodes, and facilitating the uptake of vitamins and dietary lipids, transported by lipoprotein particles known as chylomicrons, into the mesenteric lymphatic vessels from the digestive tract via the intestinal lacteals (Schulte-Merker et al., 2011; Randolph et al., 2017; Santambrogio, 2018; Cifarelli and Eichmann, 2019). During lymph formation, interstitial fluid containing water, solutes, and immune cells is initially transported into the lumen of highly permeable lymphatic capillary vessels. These vessels consist of lymphatic endothelial cells (LECs) arranged in an oak-leaf pattern characterized with discontinuous, overlapping, "button-like" cell-cell junctions (Baluk et al., 2007). Hydrostatic pressure gradients acting across the lymphatic wall facilitate the opening or closure of these overlapping cell-cell junctions, thus functioning as a primary valve system (Schmid-Schonbein, 2003). Additionally, capillary LECs are also directly attached to the extracellular matrix (ECM) by anchoring filaments (Leak and Burke, 1968). High interstitial fluid pressure also facilitates the opening of overlapping junctions by stretching these anchoring filaments to promote the transport of interstitial fluid into the capillary lumen. Increased intraluminal pressure then promotes the closure of overlapping junctions to inhibit lymph leakage (Schmid-Schonbein, 2003; Sabine et al., 2016). Lymph formed in the capillaries is then passively transported to collecting vessels where LECs are arranged into contractile segments, known as lymphangions, which are separated by intraluminal, bi-leaflet lymphatic valves. These lymphangion regions are additionally coated by basement membrane and lymphatic muscle cells (Muthuchamy et al., 2003). In contrast to the lymphatic capillaries, LECs in collecting vessels form continuous "zipper-like" cell-cell junctions to prevent lymph-leakage during transport (Sabine et al., 2016). Coordination of several forces then promotes the propulsion of lymph within the collecting vessels and maintenance of unidirectional transport. This includes both intrinsic pump forces, derived from contraction of lymphatic muscle and extrinsic forces generated from surrounding tissues (e.g., respiration and contraction of cardiac and skeletal muscle). Additionally, regulation of the opening and closure of intraluminal valves supports unidirectional transport of lymph and prevents backflow. Increases in luminal lymph pressure within lymphangions drives the closure of valve leaflets as they are pushed together. As lymph pressure continues to increase within the lymphangion, the lymphatic endothelium stretches and activates the lymphatic pump, which opens valves to transport lymph to sequential lymphangions (Zawieja, 2009; Bertram et al., 2011; Sabine et al., 2016).

During murine embryonic development, formation of the early lymphatic vascular system is initiated at embryonic day (E) 9.5 as a subset of endothelial cells in the cardinal vein begin to express the Prospero-related homeobox 1 (PROX1) transcription factor, that acts as a master lymphatic regulator. These cells then differentiate into LECs, and migrate to establish primitive lymphatic vessels, identified as the jugular lymph sac, by E11.5 (Wigle and Oliver, 1999). However, more recent evidence has also demonstrated the contribution of other tissue-associated vascular progenitor cells as well as non-endothelial cells to early lymphatic vasculature morphogenesis and the development of the primitive lymphatic vascular network (Bernier-Latmani et al., 2015; Klotz et al., 2015; Martinez-Corral et al., 2015; Stanczuk et al., 2015; Kazenwadel and Harvey, 2016). Several critical signaling pathways are involved in this process including VEGF-C/-D activation of the VEGFR-3 receptor tyrosine kinase, regulation of key downstream gene expression by SOX18, PROX1, GATA2, and FOXC2 transcription factors, as well as Notch1, FGF2/FGFR1, ANG2/TIE2, and EFNB2/EPHB4 signaling, which have been previously reviewed in great detail in articles we refer to the reader (Tammela and Alitalo, 2010; Schulte-Merker et al., 2011; Yang and Oliver, 2014; Zheng et al., 2014). We discuss the progression of metabolic syndrome derived from lymphatic dysfunction as a result of impaired signaling in several of the pathways mentioned, and in contrast the effect of chronic inflammation derived from obesity and metabolic syndrome on several of these signaling pathways, resulting in lymphatic dysfunction, in greater detail below.

THE RELATIONSHIP OF METABOLIC SYNDROME, INFLAMMATION, AND LYMPHATIC DYSFUNCTION

Numerous studies have demonstrated a role for obesity in the induction of a chronic low-grade inflammatory response

(Lumeng and Saltiel, 2011; Saltiel and Olefsky, 2017). In human obese and T2D patients, evidence has shown that several inflammatory markers are elevated, including white blood cell counts and plasma levels of coagulation factors (PAI-1 and fibrinogen), acute-phase proteins (C-reactive protein and serum amyloid A), pro-inflammatory cytokines (tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6), as well as proinflammatory chemokines (Pickup et al., 1997; Yudkin et al., 1999; Bastard et al., 2000; Haffner et al., 2005; Bruun et al., 2006; Belalcazar et al., 2013; Esser et al., 2014). Furthermore, in most cases increases in these pro-inflammatory markers also positively correlated with insulin resistance and metabolic syndrome independent of the degree of obesity (Esser et al., 2014). Both clinical data and experimental data from mouse models have demonstrated that liver, muscle, pancreas, and adipose tissue are sites of both ectopic lipid deposition (in non-adipose tissues) (Mittendorfer, 2011; Snel et al., 2012) and inflammation present in metabolic syndrome including obesity, T2D, and non-alcoholic steatohepatitis (NASH) as a result of infiltration of pro-inflammatory macrophages, which secrete pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 into these tissues (Chawla et al., 2011; Walenbergh et al., 2013; Esser et al., 2014; Martin-Murphy et al., 2014; McGettigan et al., 2019). During obesity, the adipose tissue in particular has been associated with an accumulation of immune cells in its stromovascular fraction (Chawla et al., 2011) and importantly, increased intra-abdominal visceral fat accumulation is more strongly correlated with metabolic syndrome compared to subcutaneous fat accumulation, suggesting that the pathogenic role of adipose tissue is related to its anatomic location (Koster et al., 2010; Esser et al., 2014). Of critical importance, several clinical studies within the past three decades have demonstrated that obesity and elevated body mass index (BMI) presents a significant risk to lymphatic dysfunction, particularly to female breast cancer patients who are at risk for the development of lymphedema postoperatively (Werner et al., 1991; Meek, 1998; Petrek et al., 2001; Johansson et al., 2002; Meric et al., 2002; Goffman et al., 2004; Ozaslan and Kuru, 2004; Clark et al., 2005; Wilke et al., 2006; McLaughlin et al., 2008; Swenson et al., 2009; Helyer et al., 2010; Vasileiou et al., 2011; Greene et al., 2012; Paskett et al., 2012). Moreover, clinical studies utilizing non-invasive imaging techniques such as near-infrared lymphatic imaging, magnetic resonance imaging, and bioimpedance spectroscopy have characterized lymphatic dysfunction associated with diseases characterized by abnormal adipose tissue accumulation in addition to obesity, such as lipedema and Dercum's disease (Lohrmann et al., 2009; Rasmussen et al., 2014; Sevick-Muraca et al., 2014; Crescenzi et al., 2018; Crescenzi et al., 2019). Numerous studies within the past two decades using both genetic and diet-manipulated mouse models have now identified key mechanisms underlying the cross-talk of obesity, diabetes, NASH, and metabolic syndrome with lymphatic vascular dysfunction (**Table 1**), of which several models are discussed in additional detail in this review.

Several studies utilizing non-genetic, diet-induced obesity mouse models, in which WT mice have been fed a prolonged high-fat diet compared to control WT mice that are fed normal chow, have demonstrated that increased inflammation induced in obese mice is associated with impaired lymphatic function (Savetsky et al., 2014; García Nores et al., 2016; Hespe et al., 2016; Nitti et al., 2016). Diet-induced obesity in mice was shown to impair lymphatic function as uptake of ^{99m}Tc-labeled sulfur colloid into sacral lymph nodes after tail injections was decreased in obese mice compared to lean controls and this was exacerbated in obese mice exhibiting lymphedema after surgical tail lymphatic excision. In subcutaneous tissue of the mouse tail, $CD4^+$ cell infiltration was significantly increased in obese mice at baseline and the presence of lymphedema in obese mice greatly increased both $CD45^+$ and $CD4^+$ cell infiltration as well as subcutaneous adipose deposition (Savetsky et al., 2014). Importantly, evidence has shown that impaired lymphatic function and reduced lymphatic density in obese mice is associated with obesity-induced perilymphatic accumulation of inflammatory cells, such as $CD3^+$ T cells and $CD11b^+$ macrophages, and toxic lipid by-products contributing to the chronic inflammatory environment. Furthermore, comparison of obesity-prone and obesity-resistant mice fed a prolonged high-fat diet demonstrated that dietary changes alone were not sufficient to induce lymphatic dysfunction, thus underscoring the role of obesity-induced inflammation in lymphatic dysfunction (García Nores et al., 2016).

The observation that increased inflammation associated with obesity is correlated with lymphatic dysfunction and reduced vessel density may seem contradictory provided that several studies have shown that under an inflammatory environment, lymphangiogenesis is stimulated as a mechanism for antigen clearance and inflammation resolution in response to pro-lymphangiogenic factors, such as VEGF-C, VEGF-D, and VEGF-A, that are secreted from infiltrating macrophages that respond to chemoattractants expressed by LECs (Kataru et al., 2009; Rahier et al., 2011; Kim et al., 2014). However, other studies have demonstrated that anti-lymphangiogenic cytokines, including IFN-γ and TGF-β1 (Shao and Liu, 2006; Clavin et al., 2008; Oka et al., 2008; Kataru et al., 2011), are elevated in chronic inflammatory responses (Zampell et al., 2012b; Savetsky et al., 2015b; Shin et al., 2015; Kataru et al., 2019) and elevated IFN-γ and TGF-β1 levels were previously detected in obese mice (Winer et al., 2009; Hespe et al., 2016). Additionally, elevated TGF-β1 levels and BMI were observed to have significant partial correlation in human subjects (Yadav et al., 2011). A key observation in studies utilizing both mouse and rat models of obesity and metabolic syndrome is that the lymphatic vessels are leaky and collecting vessel contraction is impaired in subcutaneous and mesenteric collecting vessels (Zawieja et al., 2012; Weitman et al., 2013; Blum et al., 2014; Savetsky et al., 2014, 2015a; García Nores et al., 2016; Hespe et al., 2016; Nitti et al., 2016). While the specific mechanism of lymphatic leakiness in obesity is unclear at this time, it has been proposed that increased perilymphatic accumulation of inflammatory cells, increased production of inflammatory cytokines, and the observation that perilymphatic inducible nitric oxide synthase (iNOS) expression is increased in obese mice may contribute to this phenotype (Hespe

TABLE 1 | Genetic and diet-induced animal models relating obesity, diabetes, and metabolic syndrome with lymphatic dysfunction.

Animal model	Definition	Metabolic and lymphatic phenotypes	References
apoE$^{-/-}$ mice	Deficiency of lipoprotein receptor ligand apolipoprotein E	• Delayed lipoprotein clearance • Development of hyper- and dyslipoproteinemia • Development of severe hypercholesterolemia • Prone to development of atherosclerotic lesions • Enlarged initial lymphatic vessels, with decreased lymphatic muscle cell coverage and abnormal distribution of LYVE-1 • Lymphatic leakiness and decreased transport of fluid and dendritic cells • Impaired lymphatic drainage required for reverse cholesterol transport	Lim et al., 2009, 2013; Martel et al., 2013; Lo Sasso et al., 2016
Ldlr$^{-/-}$	Deficiency of low-density lipoprotein receptor	• Prone to accelerated atherosclerosis progression with incorporation of a high-fat diet • Pre-treatment with VEGF-C 152S before incorporation of a pro-atherosclerotic diet regimen stimulates lymphangiogenesis of initial vessels, improves lymphatic collecting vessel transport and contractile frequency, and reduces inflammatory cell accumulation	Daugherty et al., 2017; Milasan et al., 2019
db/db mice	Mutation resulting in deficiency of the leptin receptor	• Dyslipidemia • Susceptibility to development of obesity-induced type 2 diabetes • Increased lymphatic collecting vessel permeability associated with reduced NO bioavailability • Decreased expression of VEGFR-3 at protein level • Exogenous VEGF-C administration improves wound healing and enhances lymphangiogenesis response • Abnormal lymphatic proliferation in renal cortex and medulla	Kobayashi et al., 2000; Saaristo et al., 2006; Scallan et al., 2015; Wu et al., 2018; Kim et al., 2019
APN-KO mice	Deficiency of adipose-specific plasma adipokine adiponectin, which is down-regulated in association with various obesity-related diseases	• Development of insulin resistance and neointimal formation • Greater susceptibility to microsurgically induced lymphedema associated with reduced lymphatic vascular density	Kubota et al., 2002; Shimizu et al., 2013
Apelin KO mice	Deficiency of apelin ligand, which signals through the G-protein coupled receptor, apelin receptor (APJ)	• Increased susceptibility to weight gain and obesity on high-fat diet • Enlarged lymphatic vessel diameter and increased vessel leakiness in mice on high-fat diet compared to individuals fed normal chow	Sawane et al., 2013
Prox1$^{+/-}$ mice	Haploinsufficiency mutation in the transcription factor Prospero-related homeobox 1 (*Prox1*)	• Subset of mice survive to adulthood but develop adult-onset obesity associated with elevated triglyceride levels • Exhibit abnormal, dilated lymphatics and vascular mispatterning • Characterized by chlyous ascites, chylothorax, and defective lymph transport	Harvey et al., 2005; Escobedo et al., 2016
Chy mutant mice	Heterozygous inactivating mutation in *Vegfr3*	• Characterized by defective lymphatic vessels and appearance of chylous ascites • Display abnormal subcutaneous adipose deposition in the edematous subcutaneous adipose layer • Have increased tissue lipid and collagen content in the skin	Karkkainen et al., 2001; Rutkowski et al., 2010
K14-VEGFR-3-Ig mice	Express a soluble, ligand-binding extracellular portion of VEGFR-3 in the mouse epidermis under the keratin 14 (K14) promoter	• Inhibits dermal lymphatic vessel formation and lymphangiogenesis and exhibits lymphedema phenotype in limbs • As opposed to Chy mice with a similar lack of dermal lymphatics, heterozygous K14-VEGFR-3-Ig mice did not display abnormal collagen or lipid deposition • Protected against high-fat diet-induced obesity and improved systemic insulin sensitivity by suppressing infiltration of pro-inflammatory macrophages via scavenging of VEGF-C and VEGF-D	Makinen et al., 2001; Rutkowski et al., 2010; Karaman et al., 2015

(Continued)

TABLE 1 | Continued

Animal model	Definition	Metabolic and lymphatic phenotypes	References
High-fat diet-induced obesity	Increased weight gain and progression to obesity by incorporation of a larger percentage of calories acquired from fats	• Development of insulin resistance, impaired glucose tolerance, dyslipidemia, ectopic lipid accumulation, hepatic steatosis • Decreased lymphatic pumping frequency • Reduction of initial lymphatic vessel density • Increased leakage from lymphatic vessels • Increased perilymphatic inflammation	Weitman et al., 2013; Blum et al., 2014; Savetsky et al., 2014, 2015a; García Nores et al., 2016; Hespe et al., 2016; Nitti et al., 2016
Non-alcoholic steatohepatitis (NASH)	Chronic liver disease and metabolic syndrome characterized by hepatic steatosis, inflammation, and fibrosis. Can be induced by a combination of high-fat diet and oxidized low-density lipoprotein (oxLDL) administration in mice	• NASH-related cirrhosis can result in the development of ascites • Human NASH patients show increased lymphatic vessel density in liver and induction of IL-13 pathway in hepatic LECs • Mice treated with oxLDL show increased hepatic LEC IL-13 production • oxLDL treatment of human LECs reduced *PROX1* expression *in vitro*.	Yimin et al., 2012; Chung and Iwakiri, 2013; Tamburini et al., 2019

et al., 2016). This hypothesis is supported by previous studies demonstrating that inflammatory cytokine signaling enhanced LEC monolayer permeability *in vitro* (Cromer et al., 2014) and increased NO production elevated lymphatic permeability of collecting vessels *in vivo* (Scallan et al., 2015). Furthermore, it was demonstrated that perilymphatic inflammatory cell accumulation may contribute to lymphatic dysfunction and the pathogenesis of obesity via loss of LEC identity as gene expression of *VEGFR-3*, *Prox1*, and *CCL21* was reduced in LECs isolated from sedentary, obese mice compared to lean controls (Hespe et al., 2016). Of note, impaired lymphatic function and leakiness in a subset of $Prox1^{+/-}$ pups results in the effusion of lipid-rich chyle into the abdominal cavity from the mesentery lymphatics and evidence has shown that free fatty acids enriched in lymph promote adipogenesis *in vitro*, thus offering a possible mechanism relating lymphatic dysfunction and adipose tissue accumulation (Escobedo and Oliver, 2017). Support of the existence of cross-talk between the lymphatic system and adipose tissue also comes from recent studies demonstrating that weight loss in mice and reduction of adipose tissue in postmastectomy patients is able to improve lymphatic function and reduce tissue volume accumulation associated with lymphedema (Brorson, 2016; Nitti et al., 2016). Weight loss in a diet-induced obesity mouse model was shown to reverse both perilymphatic inflammation and iNOS expression and improvements were observed in lymphatic vessel density, lymphatic barrier integrity, and collecting vessel contractility (Nitti et al., 2016). Reduction of adipose tissue by longitudinal liposuction treatment with respect to limb was also shown to improve lymphatic function and the reduction of tissue fluid accumulation in patients diagnosed with postmastectomy arm lymphedema (Brorson, 2016) as well as patients diagnosed with primary lymphedema (Schaverien et al., 2018). Together, this data emphasizes the critical role the lymphatic vascular system has as a mediator in the pathogenesis of obesity. As obesity initially contributes to the development of a chronic inflammatory environment, this in turn results in impairment of lymphatic function which can then exacerbate the pathological consequences of obesity and present a significant risk to patients with elevated BMI and the development of lymphedema.

IMPAIRED TRANSCRIPTION FACTOR SIGNALING RELATED TO LYMPHATIC DYSFUNCTION, OBESITY, AND DIABETES

The PROX1 transcription factor is critical for LEC fate and differentiation during lymphatic development as well as the formation of the lymphatic vasculature during lymphangiogenesis and maturation into adulthood (Wigle and Oliver, 1999; Wigle et al., 2002; Ma and Oliver, 2017). *Prox1* null mutant mice die between embryonic (E) stage 14.5 and E15.0, and are characterized by severe edema due to the complete absence of the superficial lymphatic vascular network while normal development of the blood vasculature occurs (Wigle and Oliver, 1999). In contrast, $Prox1^{+/-}$ pups also display edema at E14.5 yet maintain formation of a superficial lymphatic vascular plexus. However, many individuals die shortly after birth as a result of severe lymphatic dysfunction and accumulation of chyle in the peritoneal and thoracic cavities (Wigle and Oliver, 1999; Harvey et al., 2005). Surprisingly, a small proportion of $Prox1^{+/-}$ mice generated on the NMRI genetic background are capable of surviving to adulthood (Wigle et al., 1999; Harvey et al., 2005), but develop adult-onset obesity with weight gain noticeable at nine weeks of age (Harvey et al., 2005). These $Prox1^{+/-}$ individuals are characterized by both lymphatic mispatterning in the intestine and mesentery and impaired lymphatic transport as ingested fluorescent lipid leaked from the mesenteric lymphatic collecting vessels, indicative of perturbed vessel integrity and barrier function (Harvey et al., 2005; Escobedo et al., 2016). Therefore, this evidence provides a direct link of lymphatic dysfunction to the development and pathogenesis of obesity. Mechanistically, chyle containing lipid-rich lymph from $Prox1^{+/-}$ mice promoted differentiation of 3T3-L1 preadipocytes into adipocytes.

Differentiation was synergistically enhanced by the addition of insulin at a concentration of 10 μg/mL to chyle suggesting the presence of a factor in chyle that cooperates with insulin to promote differentiation. Thus, it was proposed that disruption of lymphatic vascular integrity promotes the ectopic growth of fat in lymphatic-rich regions due to stimulation of preadipocyte differentiation in addition to increased lipid storage in adipocytes (Harvey et al., 2005). Importantly, the development of obesity was directly linked to LEC maintenance of *Prox1* expression in this model as conditional, endothelial cell-*Prox1*$^{+/-}$ mice similarly were characterized by impaired lymphatic vascular function and the development of obesity (Harvey et al., 2005). Furthermore, restoration of *Prox1* expression specifically within the lymphatic vasculature was capable of rescuing the obese phenotype in *Prox1*$^{+/-}$ mice (Escobedo et al., 2016).

Several studies in humans have now linked *PROX1* expression to the development of hyperlipidemia, obesity, and T2D (Horra et al., 2009; Kim et al., 2013; Kretowski et al., 2015; Adamska-Patruno et al., 2019). Familial combined hyperlipidemia (FCHL) is a complex genetic dyslipidemia characterized by elevated apolipoprotein B, small, dense LDL particles, triglycerides, and total cholesterol (Ayyobi and Brunzell, 2003). Assessment of *PROX1* expression, as well as the critical lymphatic transcription factor *FOXC2* (Norrmen et al., 2009; Sabine et al., 2012, 2015; Fatima et al., 2016), in subcutaneous adipose tissue of a subset of FCHL patients and healthy control individuals identified a reduction of *PROX1, FOXC2*, or expression of both in the subset of FCHL subjects compared to controls, suggesting that lymphatic dysfunction may contribute to the FCHL phenotype (Horra et al., 2009). Furthermore, a genome-wide association study was performed in families from a Mongolian population that identified the single nucleotide polymorphism (SNP) rs1704198 on chromosome 1q32 associated with larger waist circumference that is located 251 kb away from the *PROX1* gene. Additionally, this SNP was also associated with waist circumference in a replicate study using samples from Korean families and the 1q32 locus was previously reported in a linkage study using an American-Indian diabetes sample (Franceschini et al., 2008; Kim et al., 2013). Recent evidence has identified another SNP in the 5′ UTR of *PROX1* (rs340874) that is a genetic risk factor for T2D (Dupuis et al., 2010; Lecompte et al., 2013; Kretowski et al., 2015; Hamet et al., 2017; Adamska-Patruno et al., 2019). Previous analysis of allelic variants (high-risk CC-genotype carriers and low risk allele T carriers) of the rs340874 SNP demonstrated that carriers of the PROX1 CC genotype presented elevated free fatty acid levels after high-fat meal intake and lower glucose utilization after high-carbohydrate meal intake in comparison to subjections with other PROX1 genotypes. Likewise, carriers of the PROX1 CC genotype were found to have higher visceral fat accumulation despite reduced daily food consumption (Kretowski et al., 2015). Moreover, analysis of non-diabetic PROX1 CC genotype carriers postprandially after challenges with high-carbohydrate (89%) and normo-carbohydrate (45%) meal intake showed altered metabolite profiles compared to low-risk allelic variant carriers including differences in glycerophosphocholine, glycerophosphoethanolamine, sphingolipid, leukotriene, fatty acid, oxidized fatty acid, amino acid, carnitine, bile acids, and amide levels, which may be associated with insulin resistance and T2D development (Adamska-Patruno et al., 2019).

The forkhead-box transcription factor FOXC2 is critical for regulation of lymphangiogenesis during embryonic development (Fatima et al., 2016) and for the formation and maturation of lymphatic valves and collecting vessels postnatally (Norrmen et al., 2009; Sabine et al., 2012, 2015). While inactivating mutations in FOXC2 are predominately associated with the development of a form of hereditary lymphedema, lymphedema-distichiasis syndrome (Fang et al., 2000; Dagenais et al., 2004), previous studies have also linked genetic variation in FOXC2 to obesity and diabetes and identified FOXC2 function as protective against obesity and insulin resistance (Cederberg et al., 2001; Kovacs et al., 2003; Carlsson et al., 2005). In mice, *Foxc2* is found expressed in both white and brown adipose tissue and transgenic mice with increased expression of *Foxc2* in adipose tissue were characterized by reduced cell size in intrabdominal white adipose tissue (Cederberg et al., 2001). Moreover, serum triglyceride, free fatty acids, plasma insulin and glucose content, weight, and lipid content were reduced in *Foxc2* transgenic mice compared to control mice on a high fat diet. Of note, *Foxc2* mRNA levels were also increased in epididymal white adipose tissue of WT mice fed a high-fat diet compared to individuals fed a standard diet, suggesting that *FOXC2* acts as a metabolic regulator in protection against diet-induced obesity and insulin resistance (Cederberg et al., 2001). Clinically, a SNP in the putative *FOXC2* promoter region, a C-512T transition, was identified in a population of Pima Indians which was associated with BMI and percentage of body fat in both male and female subjects as individuals homozygous for the C-512T allele had lower BMI than homozygous C/C or heterozygous C/T individuals. Additionally, the C-512T variant appeared to be involved in female subjects' regulation of basal glucose turnover and plasma triglyceride levels (Kovacs et al., 2003). Furthermore, an additional study from a Finnish population of T2D patients and control subjects did not identify a significant difference in the C-512T allele and genotype distribution between the study subjects, but the C/C genotype was associated with elevated BMI in T2D (Carlsson et al., 2005). From the patient studies above, it is not known whether the identified mutations are associated with lymphatic dysfunction and in actuality may primarily be related to alterations in hepatocyte function or adipogenic potential. However, the growing evidence supporting the relationship of metabolic syndrome and lymphatic dysfunction warrants further investigation of lymphatic function associated with these described patient mutations. Additionally, individuals suffering from hereditary lymphedema associated with mutations in transcription factors such as *FOXC2* contributing to the development of lymphedema-distichiasis, or *GATA2* contributing to the development Emberger syndrome (Mansour et al., 2010; Ostergaard et al., 2011), may be at risk for abnormal adipose deposition and development of obesity due to proliferation and hypertrophy of adipocytes in lymphedematous tissues (Zampell et al., 2012a; Mehrara and Greene, 2014). Nonetheless,

continued studies of these patient populations are required for risk assessment.

THE VEGF-C/VEGF-D/VEGFR-3 SIGNALING AXIS AND LYMPHATIC DYSFUNCTION IN THE PATHOGENESIS OF OBESITY AND DIABETES

Vascular endothelial growth factors signaling through their respective VEGF receptor (VEGFR) tyrosine kinases provide a critical driving force for the growth of new vessels during angiogenesis and lymphangiogenesis (Karaman et al., 2018). In the lymphatic vasculature, VEGF-C-induced activation of VEGFR-3 predominately stimulates lymphangiogenesis (Karkkainen et al., 2004). In contrast, VEGF-D, which is capable of activation of both VEGFR-2 and VEGFR-3 in humans (Achen et al., 1998) but only VEGFR-3 in mice (Baldwin et al., 2001), was found to be dispensable for developmental lymphangiogenesis in mice (Baldwin et al., 2005) but its deficiency led to smaller, dysfunctional initial lymphatic vessels in the skin of adult mice (Paquet-Fifield et al., 2013). Independent of its function in the vasculature however, it was recently shown that VEGF-D deficiency significantly elevated cholesterol and triglyceride levels in an atherogenic mouse model via reduction of hepatic expression of syndecan 1, a receptor for chylomicron remnant uptake (Tirronen et al., 2018). In two studies of obese subjects, serum concentrations of VEGF-C were significantly elevated in overweight and obese individuals in addition to VEGF-A (Silha et al., 2005; Gomez-Ambrosi et al., 2010), but elevated VEGF-D was only detected in one report in women after gender segregation (Silha et al., 2005) whereas it was found to be reduced in obese individuals in a separate study using a similar population (Gomez-Ambrosi et al., 2010). It has also been shown in human patients that plasma VEGF-A levels were more strongly correlated with BMI and waist circumference than serum VEGF-C, but VEGF-C levels were more strongly correlated with dyslipidemia (Wada et al., 2011). Importantly, the growth of visceral fat and expansion of white adipose tissue associated with obesity results in a hypoxic environment that activates increased levels of HIF1-α which in turn increases VEGF-A to stimulate angiogenesis and support rapid growth of adipose tissue, but also leads to an upregulation of inflammatory adipokines and promotes tissue fibrosis leading to adipose tissue dysfunction (Rutkowski et al., 2009). Moreover, blocking angiogenesis in young individuals of the obese, leptin deficient *ob/ob* mouse strain via treatment with several inhibitors acting on the endothelium, including TNP-470, angiostatin, and endostatin, prevented expansion of adipose tissue and returned mice to normative metabolic function in adulthood (Rupnick et al., 2002).

More recently, evidence has shown that lymphatic dysfunction associated with inactivation of VEGFR-3, which is classically associated with lymphedema in Milroy's disease (Butler et al., 2007), is linked to the pathogenesis of obesity and adipose tissue accumulation (Rutkowski et al., 2010) and that targeting this signaling pathway to improve lymphatic function may provide resolution of metabolic syndrome. Two separate mouse models of impaired VEGFR-3 signaling, the Chy (Karkkainen et al., 2001) and K14-VEGFR-3-Ig (Makinen et al., 2001), are both characterized by lack of dermal lymphatic capillaries and presence of lymphedema, but the skin of Chy mice consisted of higher deposition of collagen and fat (Rutkowski et al., 2010). The increase in collagen and lipid content in the tail dermis of Chy mice was attributed to remodeling of collagen and fat density to decrease dermal elasticity and normalize tissue hydraulic conductivity to make tissue easier to swell within the context of edema. Alternatively, fluid accumulation in the K14-VEGFR-3-Ig mice, which expresses a soluble VEGFR-3-Ig construct in the skin under the keratin 14 promoter to trap VEGF-C/D, led to increased hydraulic conductivity to compensate for changes in interstitial fluid pressure, thus highlighting important pathological and functional differences although the lymphedema phenotype is similar in both strains (Rutkowski et al., 2010).

In addition to its role in the lymphatic vasculature, VEGFR-3 is also expressed by a subset of macrophages and VEGF-C and VEGF-D are able to induce their chemotactic migration (Skobe et al., 2001a,b; Schoppmann et al., 2002; Stepanova et al., 2007; Zhang et al., 2014). A more recent study utilized K14-VEGFR-3-Ig mice to investigate the effects of VEGFR-3 signaling inhibition on adipose tissue growth and insulin sensitivity in mice fed a high-fat diet. VEGFR-3 signaling inhibition improved systemic insulin sensitivity and protected against high-fat diet induced fatty liver disease as adipocyte size and adipose tissue inflammation were reduced in K14-VEGFR-3-Ig mice compared to controls (Karaman et al., 2015). In contrast, infiltration of pro-inflammatory M1 macrophages, which express higher levels of *Vegfr3*, was stimulated in control mice fed high-fat diet, which was attributed to elevated adipocyte expression of VEGF-C and VEGF-D. However, blockage of VEGF-C and VEGF-D in K14-VEGFR-3-Ig mice on high-fat diet, or by utilization of a VEGFR-3 blocking antibody in leptin deficient *db/db* mice, protected against the development of insulin resistance and infiltration of pro-inflammatory macrophages. Thus, inhibition of VEGFR-3 signaling suppressed a shift to increased M1/M2 macrophage ratio associated with obesity (Lumeng et al., 2007; Karaman et al., 2015). Furthermore, by utilizing K14-VEGF-C mice that overexpress human VEGF-C in the skin it was shown that these mice more rapidly gained weight, became insulin resistant, had increased ectopic lipid accumulation in adulthood compared to WT littermates on normal chow, and pro-inflammatory macrophage infiltration into adipose tissue was significantly increased (Karaman et al., 2016). Conversely, by stimulating adipose-specific lymphangiogenesis via VEGFR-3 to resolve metabolic syndrome in high-fat diet fed mice, Chakraborty et al. (2019) recently demonstrated that adipose-specific VEGF-D overexpression induced *de novo* lymphangiogenesis and these transgenic mice exhibited improved metabolic responsiveness and reduced obesity-associated macrophage accumulation as immune trafficking was increased from adipose tissue. Furthermore, stimulation of the VEGF-C/VEGF-D/VEGFR-3 signaling axis was shown

to improve clearance of contact hypersensitivity-induced inflammation in obese mice as injections of recombinant VEGF-C improved lymphatic function (Savetsky et al., 2015a). Additionally, treatment of atherosclerotic prone, high-fat diet fed $Ldlr^{-/-}$ mice with VEGF-C 152S resulted in improved lymphatic molecular transport and reduction of both plaque formation and macrophage accumulation, which was associated predominately with improved lymphatic contraction frequency (Milasan et al., 2019).

Complications from defects in the lymphatic vasculature in diabetic individuals are associated with perturbed wound healing, which can manifest in secondary lymphedema arising from disruption of the lymphatics (Rutkowski et al., 2006; Lin et al., 2013). However, improving VEGFR-3 availability at the cell surface of LECs by inhibition of epsin-mediated degradation was recently shown to improve lymphangiogenesis in diabetic mice, which improved resolution of wound healing and secondary lymphedema in a tail edema model (Wu et al., 2018). Epsins 1 and 2 are ubiquitin-binding adaptor proteins involved in endocytosis and regulate VEGFR-3 bioavailability at the surface of LECs during lymphatic collecting vessel maturation and valve formation (Liu et al., 2014). In the context of adult diabetic mice generated by treatment with streptozotocin and fed a high-fat diet, hyperglycemia-induced oxidative stress increased epsin expression, mediated by phosphorylation of c-Src and the transcription factor AP-1, which promoted degradation of newly synthesized VEGFR-3. Inducible, LEC-specific deletion of epsins was then able to attenuate VEGFR-3 degradation and thus maintained VEGFR-3 bioavailability at the LEC cell surface to improve lymphangiogenesis and restore lymphatic function (Wu et al., 2018). Collectively, these results demonstrate a critical role for the VEGF-C/VEGF-D/VEGFR-3 signaling axis in lymphatic dysfunction associated with the pathogenesis of obesity and metabolic syndrome. Yet from the studies discussed, it is clear that there are underlying contextual factors contributing to the differences observed in Chy and K14-VEGFR-3-Ig models as well as differences in tissue-specific activation of VEGFR-3 signaling. Although both the Chy and K14-VEGFR-3-Ig models are characterized by the lack of dermal lymphatics, the differences in tissue remodeling exhibited by both models in response to changes in interstitial pressure may be attributable to the background strains used, CH3 for Chy mice and C57BL/6 for K14-VEGFR-3-Ig mice, or individual genetic manipulation (Rutkowski et al., 2010). Variations between different mouse strains in immune cell activity, inflammation, vascular remodeling and response, and wound healing have previously been characterized (Bendall et al., 2002; Ryan et al., 2002; Rajnoch et al., 2003; Chan et al., 2005; Marques et al., 2011) as well as the lymphangiogenic response in the mouse cornea micropocket assay and suture-induced corneal neovascularization model (Regenfuss et al., 2010). Furthermore, the differences observed between activation of VEGFR-3 signaling in several of these models is attributable to the physiological responses induced. It was noted that adipose-specific overexpression of VEGF-D resulted in adipose tissue fibrosis, macrophage accumulation, and elevated cytokine levels similar to what was observed in mice with overexpression of VEGF-C under the keratin 14 promoter. However, *de novo* lymphangiogenesis was observed in mice with adipose-specific overexpression of VEGF-D but not mice with overexpressed VEGF-C in the skin (Karaman et al., 2016; Lammoglia et al., 2016; Chakraborty et al., 2019). Therefore, it has been proposed that localization of the initiation of the lymphangiogenic gradient from the adipose tissue, and not the skin, has a role in supporting new lymphatic growth and the resolution of inflammatory cell accumulation in the context of obesity (Chakraborty et al., 2019). Thus, the localization of VEGFR-3 signaling has a critical role in determining physiological responses in the context of obesity and chronic inflammation and the role of adipose-specific VEGFR-3 expression needs to be further elucidated.

While stimulation of lymphangiogenesis is an attractive therapeutic target in obesity and metabolic syndrome to resolve insulin resistance, ectopic lipid deposition, and local immune cell accumulation, careful consideration is needed for the use of therapies stimulating the VEGF-C/VEGF-D/VEGFR-3 signaling axis. Notably, lymphatic vessels become largely independent of VEGF-C/VEGFR-3 signaling after early postnatal development (Karpanen et al., 2006) with the exception of intestinal lacteals, which have been shown to be dependent on VEGF-C/VEGFR-3 signaling for maintenance in adult mice (Nurmi et al., 2015). Furthermore, the effects of tissue-specific activation of VEGFR-3 signaling need to be taken into consideration. As noted previously, there are differences in the lymphangiogenic response between skin-specific and adipose-specific activation of VEGFR-3 (Karaman et al., 2016; Lammoglia et al., 2016; Chakraborty et al., 2019). Additionally, the roles of VEGFR-3 signaling activation beyond the vasculature, such as in regulation of chylomicron remnant removal by hepatocytes and lipoprotein metabolism (Tirronen et al., 2018), need to be carefully considered in the development of therapeutic strategies. Although the development of targeted therapeutics for tissue specific activation of VEGFR-3 is currently a limitation, increasing evidence has shown that targeting this pathway to improve lymphatic function within this pathological context warrants further investigation. Thus, strategies to specifically stimulate adipose-specific lymphangiogenesis may be viable options for therapy to improve inflammatory cell clearance, similar to strategies targeting lymphatic expansion in an inflammatory site-specific manner within the context of a model of chronic skin inflammation (Schwager et al., 2018).

ABNORMAL NO SIGNALING AND IMPAIRED LYMPHATIC FUNCTION IN OBESITY AND METABOLIC SYNDROME

Contributing to its pathogenesis, numerous studies utilizing both genetic and non-genetic, diet-induced obesity and metabolic syndrome mouse models (**Table 1**) have demonstrated that lymph and cholesterol transport, as well as migration of antigen-presenting dendritic cells, is impaired in obese mice compared to lean controls (Lim et al., 2009, 2013; Chakraborty et al.,

2010; Dixon, 2010; Martel et al., 2013; Weitman et al., 2013). Underlying this impaired ability of the lymphatic vasculature to support functional transport, several studies have now implicated the role of iNOS and perturbed NO signaling as a mechanism related to poor lymphatic contraction observed in obesity, diabetes, and other models of metabolic syndrome (Zawieja et al., 2012; Blum et al., 2014; Scallan et al., 2015; Hespe et al., 2016; Torrisi et al., 2016). NO is a gaseous free radical that is known to have strong regulatory effects on vasodilation and permeability in the blood vasculature (Kubes and Granger, 1992; Yuan, 2006; Duran et al., 2010) and it is produced by three isoforms of NO synthase, including neuronal NOS (nNOS, NOS I), inducible NOS (iNOS, NOS II), and endothelial NOS (eNOS, NOS III) (Förstermann and Sessa, 2012). In the lymphatic endothelium, eNOS expression, and thus NO production, is regulated by both intracellular calcium levels and vessel shear stress (Leak et al., 1995; Gashev et al., 2002; Tsunemoto et al., 2003). Here, NO signaling serves dual roles as NO produced by LECs, and in particular from the valve/sinus region (Bohlen et al., 2011), functions to control relaxation after a contraction (diastole) in lymphatic collecting vessels (Gashev et al., 2002; Tsunemoto et al., 2003; Gasheva et al., 2006) and exogenous sources of NO have been shown to slow lymphatic contraction frequency (von der Weid et al., 2001; Liao et al., 2011; Zawieja et al., 2016). Thus, NO signaling serves a key role in regulating lymph transport and lymphatic vessel function in a controlled, responsive manner to various stimuli.

As described above, perilymphatic iNOS expression is increased in obese mice associated with an increased inflammatory response (Savetsky et al., 2015a; Hespe et al., 2016; Nitti et al., 2016) and it has been previously shown that under an acute inflammatory response in the mouse, iNOS-expressing inflammatory cells attenuate lymphatic contraction via increased NO production (Liao et al., 2011). Supporting the role of iNOS signaling in the development of impaired lymphatic function and the pathogenesis of obesity, utilization of the selective iNOS inhibitor 1400W was able to improve lymphatic contractile function in obese mice (Torrisi et al., 2016). Moreover, diet-induced weight loss in obese mice also decreased perilymphatic inflammation and iNOS expression, which was also associated with decreased lymphatic leakiness and improved lymphatic contractile function (Nitti et al., 2016). Work from Scallan et al. (2015) has however identified a paradoxical role for NO signaling in the lymphatics in the context of either healthy collecting vessels or those from the *db/db* mouse model of T2D. Here, increased NO production was shown to increase lymphatic permeability in collecting vessels of healthy mice, but restoration of NO signaling in leptin receptor-deficient *db/db* mice was shown to reduce permeability and rescue lymphatic barrier dysfunction. To activate signaling mechanisms functioning downstream of NO signaling in *db/db* mice, the authors demonstrated that pharmacological inhibition of phosphodiesterase 3 (PDE3) was able to rescue lymphatic barrier dysfunction by suppressing PDE3 mediated degradation of cyclic AMP, which activates PKA/Epac1 to maintain endothelial barrier function. It was then proposed that low levels of cGMP from reduced NO bioavailability in diabetic mice relieves the inhibition of PDE3 and in turn, high insulin or leptin levels, as occur in T2D, promote phosphorylation and activation of PDE3 which results in loss of cAMP and impaired signaling through PKA/Epac1 (Scallan et al., 2015). In support of reduced NO bioavailability contributing to an impaired lymphatic function in the context of metabolic syndrome, it was also shown that the thoracic ducts of Sprague-Dawley rats under a high-fructose-fed diet, a model of metabolic syndrome, were characterized by a reduction in eNOS expression coupled to impaired flow responses (Zawieja et al., 2016). *In vitro*, acute insulin signaling activated eNOS phosphorylation mediated by PI3K/Akt signaling in LECs, but prolonged hyperinsulinemia and hyperglycemia promoted insulin resistance, impaired PI3K/Akt/eNOS signaling, mitochondrial function, and NO bioavailability, and also increased lymphatic permeability. The increased lymphatic permeability promoted by hyperglycemia and hyperinsulinemia was then associated with a significant increase in NF-κB nuclear translocation (Lee et al., 2018). While the role of NO signaling within the context of obesity and metabolic syndrome remains incompletely understood, the current data demonstrates that both lymphatic barrier function and contractility are susceptible to elevated levels of NO production, while barrier function is also susceptible to markedly impaired NO production. Evidence from the current studies then suggests that reduction of exogenous NO donors and preservation of endogenous NO synthase function within the lymphatic endothelium may prove as a promising therapeutic strategy.

IMPROVING LYMPHATIC FUNCTION FOR THERAPEUTIC TREATMENT OF METABOLIC SYNDROME

Provided the growing evidence that the relationship between lymphatic dysfunction and the pathogenesis of metabolic syndrome, and obesity in particular, is bi-directional and reciprocating (Mehrara and Greene, 2014; Escobedo and Oliver, 2017), methods to improve lymphatic function have become attractive for therapeutics, including previously proposed VEGF-C related therapies. Several recent studies have proposed other various methods to improve lymphatic function, including both the use of pharmacological agents as well as behavioral and lifestyle changes (**Table 2**).

In a model of diet-induced obesity, Torrisi et al. (2016) demonstrated that treatment with topical tacrolimus, an inhibitor of T cell differentiation, improved lymphatic vessel density and lymphatic pumping frequency, reduced perilymphatic accumulation of leukocytes, macrophages, T cells, and perilymphatic iNOS expression in the hindlimbs of mice, whereas direct inhibition of iNOS with the small molecule inhibitor 1400W did not improve lymphatic function. Importantly, adipose tissue volume was not significantly changed at the site of tacrolimus application, suggesting that increased lymphatic density resulted from capillary LEC proliferation

TABLE 2 | Therapeutics targeting lymphatic function improvement for treatment of obesity, diabetes, and metabolic syndrome.

Therapeutic	Target	Effect on lymphatic function	References
Tacrolimus	Macrolide calcineurin inhibitor that suppresses T cell proliferation/differentiation	•Reduces perilymphatic inflammation in skin tissue of obese mice •Improves lymphatic transport capacity from distal injection site to the popliteal lymph node •Improves dendritic cell migration capacity and lymphatic contractile function	Torrisi et al., 2016
Cilostamide	Phosphodiesterase 3 (PDE3) inhibitor	•Reduces lymphatic permeability and leakage in collecting vessels of *db/db* mice	Scallan et al., 2015
Y-27632	Rho-associated protein kinase (ROCK) inhibitor	•Induces intestinal lacteal junction "zippering" and conversion of "button-like" cell-cell junctions to linear junctions •Reduces chylomicron uptake into lacteals and transport to mesenteric lymphatics	Zhang et al., 2018
Exercise	Behavioral/lifestyle change with improvements in mediating chronic inflammation, glucose intolerance and endothelial dysfunction in obesity	•Improves lymphatic contractile function •Reduces perilymphatic immune cell accumulation in skin tissue •Reduces expression of inflammatory, anti-lymphangiogenic cytokines including TNF-α, IFN-γ, and IL-1β in skin tissue •Reduces lymphatic leakiness and improves dendritic cell migration capacity •Improves expression of LEC gene expression of *Vegfr3*, *Prox1*, and *Ccl21*	Hespe et al., 2016
Ketogenic diet	Behavioral/lifestyle change associated with incorporation of high-fat, low-carbohydrate diet to improve weight loss	•Increases circulating levels of β—hydroxybutyrate ketone body *in vivo*, which promotes LEC proliferation, migration, and sprouting *in vitro* •Stimulated corneal lymphatic growth after injury •Improved resolution of lymphedema in model of tail microsurgical excision of lymphatic vessels	Keith et al., 2017; García-Caballero et al., 2019

with inhibition of obesity-mediated chronic inflammation (Torrisi et al., 2016). In support of targeting perilymphatic inflammation to improve lymphatic function in the context of obesity, it was also shown that behavioral changes to introduce aerobic exercise training, independent of weight loss, were effective in decreasing perilymphatic inflammatory cell accumulation and improving lymphatic function (Hespe et al., 2016). Additional evidence adapting behavioral and lifestyle changes has suggested that long-term incorporation of a high-fat, low-carbohydrate ketogenic diet improves weight loss and limb volume reduction in patients with lymphedema and obesity (Keith et al., 2017). Evidence in mice has demonstrated that LECs rely on fatty acid β-oxidation (FAO) for proliferation, migration, and sprouting, and generation of acetyl-coenzyme A (acetyl-CoA) by FAO facilitates LEC differentiation via acetylation of histone at lymphangiogenic genes, which promotes their expression (Wong et al., 2016). Prolonged incorporation of a high-fat, low-carbohydrate ketogenic diet results in the elevated production of ketone bodies secreted from the liver, which are then able to be oxidized in mitochondria into two molecules of acetyl-CoA in extrahepatic tissues (Puchalska and Crawford, 2017). To characterize the role of ketone body oxidation in the lymphatic vasculature, García-Caballero et al. (2019) recently demonstrated that elevation of lymph ketone bodies in mice, by either a ketogenic diet or administration of the ketone body β-hydroxybutyrate, stimulated lymphangiogenesis after corneal injury and myocardial infarction. Studies investigating high-fat diet induced obesity typically utilize a high-fat diet chow consisting of 60% kcal obtained from fat, 20% from protein, and 20% from carbohydrates resulting in accelerated weight gain and progression to obesity (Weitman et al., 2013; Savetsky et al., 2014; García Nores et al., 2016; Hespe et al., 2016; Nitti et al., 2016; Torrisi et al., 2016). In contrast, high-fat, low-carbohydrate diet (ketogenic) chow generally consists of >70% of kcal obtained from fats, <5% obtained from carbohydrates, and the remaining ∼25% obtained from proteins to induce a ketogenic state and the higher production of ketone bodies as a result of activated FAO (Kennedy et al., 2007; Nilsson et al., 2016; Puchalska and Crawford, 2017). Supporting a stimulated lymphangiogenesis response, ketone body oxidation was shown to increase acetyl-CoA generation, which was incorporated into the TCA cycle to support LEC nucleotide synthesis. Additionally, reducing equivalents generated from oxidation were incorporated for mitochondrial respiration and ATP production. Moreover, it was also shown in a mouse model of secondary lymphedema, via microsurgical ablation of lymphatic vessels in the tail, that a ketogenic diet improved lymphatic function and growth, reduced accumulation of anti-inflammatory $CD4^+$ and $CD8^+$ T cells, and reduced edema (García-Caballero et al., 2019).

In addition to therapeutically targeting the inflammatory environment to improve lymphatic function, several studies have proposed methods of modifying lymphatic barrier function within the context of obesity and diabetes to improve outcome.

As described previously, metabolic syndrome and insulin resistance is associated with impaired NO synthase expression in LECs, resulting in leaky lymphatic collecting vessels and impaired contractile activity. However, Scallan et al. (2015) demonstrated that lymphatic barrier function could be improved in a mouse model of T2D by inhibiting PDE3 with the pharmacological inhibitor cilostamide, thereby providing a method to improve interstitial fluid clearance during inflammation. Additionally, it was also recently proposed that induction of intestinal lacteal junction "zippering" could protect against diet-induced obesity (Zhang et al., 2018). Zhang et al. (2018) recently demonstrated that inducible, pan-endothelial-specific deletion of the VEGF-A receptors VEGFR-1/FLT1 and neuropilin 1 (NRP1) protected mice from high-fat diet-induced obesity. Mice lacking endothelial *Flt1* and *Nrp1* were characterized by lacteals consisting of more linear, "zipper-like" junctions compared to control mice characterized by lacteals with discontinuous "button-like" junctions. This conversion from discontinuous to linear junctions in mutants then prevented absorption of chylomicrons from the intestine. Endothelial-specific deletion of both receptors increased the bioavailability of VEGF-A to activate pathways downstream of LEC VEGFR-2 signaling, which includes inhibition of LEC VE-cadherin cytoskeletal anchoring. Interestingly, short-term treatment of WT mice with the Rho-associated protein kinase (ROCK) inhibitor Y-27632 disrupted cytoskeletal VE-cadherin anchoring in lacteals and induced the formation of linear junctions, mimicking the effect of endothelial-specific deletion of *Flt1* and *Nrp1*, which also resulted in reduced chylomicron absorption (Zhang et al., 2018). Therefore, targeting cytoskeletal regulation of lymphatic junctions may prove promising for therapeutic development to suppress dietary lipid absorption in prevention of and resolution of obesity, but further studies assessing the effects such drugs that regulate cytoskeletal activity are warranted. Collectively, these studies demonstrate that pharmacological or behavioral therapeutics focused on improving lymphatic function may function to improve outcomes in obese and metabolic syndrome patients, including those who also have or are at risk for lymphedema. However, further studies are required to assess how combined therapies may be adapted and to specifically determine what molecular mechanisms underlie perilymphatic inflammatory cell accumulation and lymphatic dysfunction.

CONCLUDING REMARKS

The growing obesity epidemic presents a worldwide health concern as excess weight gain is associated with elevated risk for development of several diseases, but most notably cardiovascular disease and diabetes (Engin, 2017). As the population of obese and metabolic syndrome individuals continues to grow, novel therapeutic developments are required to meet this need. The studies summarized in this review underscore the importance of the lymphatic vascular system in the pathogenesis of obesity, diabetes, and metabolic syndrome, yet our understanding of the molecular mechanisms and signaling pathways involved in the cross-talk between these diseases and the lymphatic system is wholly incomplete. The field of developmental lymphatic biology is continuing to grow as new mechanisms related to LEC differentiation, morphogenesis, maintenance and function continue to be identified, but additional comprehensive studies are required to understand the role of the lymphatic system in pathological states. Moreover, it is important to consider that the lymphatic vasculature interfaces and communicates with numerous tissues, including the blood vasculature, adipose tissue, and inflammatory cells associated with pathological obesity and metabolic syndromes. Therefore, studies investigating combined therapies to target multiple aspects of this pathological environment, such as inhibition of inflammatory cell accumulation, modulation of lymphatic barrier function, and behavioral changes, such as diet modifications and incorporation of exercise regimens, should be of future focus.

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PN contributed to the writing and editing of the manuscript, and creation of tables. TK contributed to the concepts, editing, and final formatting of the manuscript.

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REVIEW
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Regulation of Lymphatic Function in Obesity

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The lymphatic system has many functions, including macromolecules transport, fat absorption, regulation and modulation of adaptive immune responses, clearance of inflammatory cytokines, and cholesterol metabolism. Thus, it is evident that lymphatic function can play a key role in the regulation of a wide array of biologic phenomenon, and that physiologic changes that alter lymphatic function may have profound pathologic effects. Recent studies have shown that obesity can markedly impair lymphatic function. Obesity-induced pathologic changes in the lymphatic system result, at least in part, from the accumulation of inflammatory cells around lymphatic vessel leading to impaired lymphatic collecting vessel pumping capacity, leaky initial and collecting lymphatics, alterations in lymphatic endothelial cell (LEC) gene expression, and degradation of junctional proteins. These changes are important since impaired lymphatic function in obesity may contribute to the pathology of obesity in other organ systems in a feed-forward manner by increasing low-grade tissue inflammation and the accumulation of inflammatory cytokines. More importantly, recent studies have suggested that interventions that inhibit inflammatory responses, either pharmacologically or by lifestyle modifications such as aerobic exercise and weight loss, improve lymphatic function and metabolic parameters in obese mice. The purpose of this review is to summarize the pathologic effects of obesity on the lymphatic system, the cellular mechanisms that regulate these responses, the effects of impaired lymphatic function on metabolic syndrome in obesity, and the interventions that may improve lymphatic function in obesity.

Keywords: lymphatic vessels, lymphatic function, obesity, inflammation, metabolic syndrome

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INTRODUCTION

Lymphatic Anatomy and Physiology

The lymphatics are a component of the vascular system and are present only in vertebrate animals. The lymphatic system is comprised of open-ended initial or capillary lymphatic vessels that drain successively into larger collecting lymphatics. Capillary lymphatic vessels comprise of single layer of overlapping oak-leaf shaped lymphatic endothelial cells (LECs) with interendothelial gaps, discontinuous button-like junctions that enable interstitial fluid absorption and leukocyte entry. Collecting lymphatic vessels (CLVs) are surrounded by basement membrane, covered with lymphatic muscle cells (LMC) and contain bileaflet intraluminal valves that prevent lymph back flow. Unlike capillary lymphatic vessels CLVs exhibit zipper-like interendothelial junctions to prevent lymph leakage. The contractile activity of LMCs of CLVs help actively pump interstitial fluid and drain into lymph nodes that then filter the fluid for antigens and bacteria

(Alitalo et al., 2005; Baluk et al., 2007; Tammela and Alitalo, 2010; Wang et al., 2017). Efferent lymphatics drain from lymph nodes into larger CLVs that eventually return the interstitial fluid back into the blood circulation (Petrova and Koh, 2018; Liu and Oliver, 2019). In this manner, the lymphatic system plays a key role in regulating fluid homeostasis, immune cell migration and antigen presentation, resolution of inflammatory responses, and regulation of peripheral tolerance to self-antigens (Angeli and Randolph, 2006; Kim et al., 2012; Betterman and Harvey, 2016; Randolph et al., 2017).

The earliest observation of lymphatic vessels was made based on the ability of intestinal lymphatic vessels to uptake lipids after a fat-rich meal. In 300 B.C., ancient Greeks observed milk-filled intestinal lymphatics and provided the first anatomic description of the lymphatic system (Lord, 1968). In the 17th Century, Gaspero Aselli identified these milk-filled vessels as components of the vascular system (Asellius, 1627). In the last few decades, the discovery of lymphatic-specific markers helped to differentiate lymphatic vessels from the blood vasculature, and to identify the vital role of the lymphatic system in absorption of dietary fat and cholesterol transport. The intestinal lymphatics are known as lacteals and are located centrally within each intestinal villus. Dietary fats are converted by enterocytes into triglyceride-rich lipoproteins enveloped by proteins and cholesterol, which are called chylomicrons, and absorbed and transported by the intestinal lacteals by both passive and active contractile mechanisms (Choe et al., 2015; Escobedo and Oliver, 2017; Suh et al., 2018; Cifarelli and Eichmann, 2019; Hokkanen et al., 2019). Intestinal lacteals can also absorb fat-soluble vitamins. Once taken up by the lacteals, chylomicrons are transported by lymphatic vessels and drained into the systemic venous circulation, bypassing the portal venous system. This is important since bypassing the liver decreases first-pass metabolism of dietary compounds and is, as a result, an active area of research in the pharmaceutical industry.

Peripheral lymphatics also play a role in cholesterol transport by a mechanism known as reverse cholesterol transport (RCT). In this process, cholesterol molecules deposited in peripheral tissues are released by cells facilitated by lipid free apolipoprotein A1 (APOA1) or lipidated high-density lipoprotein (HDL). This extracellular cholesterol is transported by the lymphatic system back to the blood stream and liver, where they are excreted or processed. This mechanism is thought to play an important role in regulating pathological processes such as atherosclerosis. Thus, understanding how the lymphatics regulate this process may facilitate development of novel therapies for treatment of cardiovascular diseases (Lim et al., 2013; Martel et al., 2013; Huang et al., 2015).

Defects in lymphatic development or function resulting from a variety of genetic defects result in adipose deposition, changes in cholesterol metabolism, metabolic changes, and, in some cases, adult-onset obesity depending on the degree of lymphatic structural and functional abnormality (Karkkainen et al., 2001; Gale et al., 2002; Harvey et al., 2005; Dellinger et al., 2007, 2008). These findings suggest that the lymphatic system and obesity are related, and that this interaction is bi-directional.

Previous reviews covered several aspects of obesity and its implication on lymphatic vessels in relation to different pathologies like lymphedema and metabolic disorders (Escobedo and Oliver, 2017; Jiang et al., 2019; Ho and Srinivasan, 2020). This article emphasizes on the bidirectional interaction between obesity and lymphatic function. We highlighted how inflammatory pathologic changes of obesity in the form of peri-lymphatic inflammation adversely affect lymphatic structure, function and how these changes predispose obese individuals to lymphedema and metabolic complications. We also discussed how life style and pharmacological interventions mitigate inflammation to improve lymphatic function in obesity.

OBESITY CAUSES LYMPHATIC DYSFUNCTION AND INCREASES RISK OF LYMPHEDEMA

The etiology of obesity is, in most cases, related to excess calorie consumption and limited caloric expenditure, however, in rare cases, obesity may also be caused by genetic abnormalities. Obesity is a systemic disease affecting virtually every organ system and increases the risk of developing metabolic syndrome and a variety of malignancies (Barton et al., 2012; Barton, 2013; Flegal et al., 2013; Segula, 2014; Kim et al., 2016).

Recent studies have also shown that obesity and adipose related disorders have significant negative effects on the lymphatic system. For example, clinical studies have demonstrated that obese individuals have impaired subcutaneous clearance of macromolecules (Arngrim et al., 2013). Patients with Dercum's disease, a poorly understood disorder characterized by accumulation of subcutaneous adipose tissue and painful lipomas, have structural and functional lymphatic defects (Rasmussen et al., 2014). Lipedema, another subcutaneous adipose tissue disorder with excessive fat deposition under skin, exhibits lymphatic vessel microaneurysms causing leakage of lipids from damaged and leaky lymphatic vessels causing excessive adipogenesis (Stallworth et al., 1974; Herbst, 2000; Amann-Vesti et al., 2001; Al-Ghadban et al., 2019). Reports indicate that lymphatic transport defects are more prominent in late stage lipedema compared to early stages (Gould et al., 2020). In similar lines, research indicates that lymphedema a chronic, morbid disease characterized by tissue swelling, accumulation of fluids, fibro-adipose tissue spontaneously develops in severely obese individuals. Early research showed the occurrence of massive localized lymphedema in morbidly obese individuals (Farshid and Weiss, 1998). Furthermore, several studies show that obesity is a well-recognized risk factor and body mass index (BMI) has a direct correlation with development of spontaneous as well as secondary lymphedema following lymph node dissection for cancer treatment (Swenson et al., 2009; Greene et al., 2012; Greene and Maclellan, 2013; Mehrara and Greene, 2014; Maclellan et al., 2017). This is supported and likely related to the fact that baseline lymphatic function is a predictor of lymphedema development and severity of disease (Hespe et al., 2019). As a result, obese patients are 2- to 3-fold more likely to develop lymphedema after surgery (Clark et al., 2005; Arrault and Vignes, 2006; Helyer et al., 2010). Even postoperative weight gain (and, presumably, the altered lymphatic function that ensues) increases the risk of

lymphedema development (McLaughlin et al., 2008). Finally, recent studies have demonstrated that weight loss is an effective means of treating lymphedema and decreasing its symptoms (Shaw et al., 2007; Ridner et al., 2011), however, not in the case of dercum's disease, in which lipomas still exist despite weight loss (Herbst and Asare-Bediako, 2007).

Animal Models Reveal That Obesity Results in Structural and Functional Changes in the Lymphatic System

Animal models of obesity have been used by several groups to study the cellular mechanisms that regulate development of obesity-induced lymphatic dysfunction. A commonly used mouse model is high fat diet (HFD)-induced obesity. This model is thought to reflect changes that occur in spontaneous obesity (in contrast to genetically induced models of obesity) and requires feeding adult animals a diet in which 60–70% of the total calories are derived from fat. Typically, adult male C57BL/6J mice are maintained on an HFD for 10–12 weeks and are compared to littermates fed a normal chow diet (30% calories from fat) (Savetsky et al., 2014). Male mice are much more prone to HFD-induced obesity and are used by most researchers. Female mice also gain weight following prolonged exposure to HFD, but to a lesser extent than male mice with over all less body fat levels; however, this process can be accelerated by ovariectomy. Ovariectomized female mice gained weight similar to male mice indicating that ovarian hormones might have a protective effect on female mice in weight gain (Hong et al., 2009). Report indicates that, 10–12 weeks of HFD feeding regime showed higher the blood glucose, insulin, liver enzymes, plasma lipids, cholesterol, adipose tissue macrophages, and circulating leukocytes in male mice compared to female (Singer et al., 2015; Ingvorsen et al., 2017). However, long term HFD regime (24 weeks) results in no differences between male and female in any of the parameters mentioned above indicating duration of high fat feeding is critical in exhibition of sexual dimorphism in HFD induced obesity (Bruder-Nascimento et al., 2017). Most common mice strains like C57BL/6J, FVB/N, 129 × 1, DBA/2 are prone for obesity upon HFD feeding but BALB/c mice strain is found to show certain degree of resistance to obesity even after HFD feeding (Montgomery et al., 2013). Other studies have used genetically induced models of obesity such as leptin deficient mice (Zhang et al., 1994). These animals become obese spontaneously, regardless of the diet, but are less representative of obesity clinically, since only a small percentage of obese patients have genetic abnormalities.

Studies in HFD-induced obese mice have consistently shown that obese animals have decreased lymphatic density in subcutaneous tissues, reduced LEC proliferation, increased lymphatic leakiness of both initial and collecting lymphatics, decreased collecting-vessel pumping capacity, and impaired clearance of macromolecules (Weitman et al., 2013; Blum et al., 2014; Garcia Nores et al., 2016; Torrisi et al., 2016). Not surprisingly, obese mice have significantly decreased trafficking of dendritic cells (DCs) from tissues to regional lymph nodes, have structurally abnormal and dilated lymphatic vessels, and have lymph node abnormalities consisting of decreased lymph node size, irregular architecture and loss of chemokine gradients (Weitman et al., 2013; Blum et al., 2014). Reports also indicated that obese mice lymph nodes showed loss of follicular pattern, T-cell zones and impaired *ex vivo* T-cell recall ability after *in vivo* sensitization with 1-fluoro-2,4-dinitrobenzene (DNFB) (Savetsky et al., 2015a; Hespe et al., 2016).

Obese Animals Have Decreased Expression of Lymphatic Genes by Isolated LECs

How does obesity cause lymphatic vessel abnormalities and decrease LEC proliferation? Several lines of evidence suggest that HFD-induced obesity results in marked alterations in LEC gene expression. Using fluorescence-activated cell sorting to isolate LECs from dermal samples, studies in our lab have shown that obese mice have downregulated expression of LEC genes including lymphatic vessel endothelial hyaluronan receptor 1 (LYVE-1), prospero-related homeobox 1 (PROX-1), podoplanin, and vascular endothelial growth factor receptor 3 (VEGFR-3) (Garcia Nores et al., 2016; Hespe et al., 2016; Nitti et al., 2016). It is well known that, the transcription factor PROX-1 is the master regulator of LEC differentiation and VEGFR-3 is the main receptor for vascular endothelial growth factor C (VEGF-C) and vascular endothelial growth factor D (VEGF-D), the most important lymphangiogenic growth factors. Binding of VEGFR-3 by VEGF-C/D results in activation of downstream signaling pathways that are key regulators of LEC proliferation, differentiation, and protection from apoptosis. Thus, decreases in VEGFR-3 transcription and cell surface expression in LECs of obese animals are likely important mechanisms by which obesity decreases lymphatic function. This hypothesis is supported by the fact that expression of VEGF-C is increased in the tissues and serum of obese mice and patients (Silha et al., 2005; Garcia Nores et al., 2016; Zafar et al., 2018). This increased expression of the receptor ligand VEGF-C may represent a homeostatic effect to maintain VEGFR-3 activation. Such a homeostatic regulation of insulin secretion by pancreatic β-cells in response to decreased insulin receptors or vice versa is observed in diabetic individuals (Zick, 2005; Rhodes et al., 2013). Additional research is clearly needed to determine how obesity modulates the VEGFR-3/VEGF-C signaling axis, and how these changes translate to lymphatic abnormalities *in vivo*.

Obesity-Induced Inflammation and Lymphatic Function

Chronic, low-grade inflammation is a key cellular mechanism that regulates the pathophysiology of obesity in a variety of organ systems. This phenomenon also appears to hold true for the effects of obesity on the lymphatic system. For example, several studies from our lab have shown that HFD-induced obesity results in peri-lymphatic accumulation of leukocytes ($CD45^+$ cells) (Garcia Nores et al., 2016; Torrisi et al., 2016). Interestingly, we found that not only do obese mice have increased generalized low-grade inflammation in their subcutaneous fat, but that these inflammatory cells tended to accumulate around lymphatic

vessels (both capillary and collecting lymphatics) (Savetsky et al., 2015a; Torrisi et al., 2016; Ariyagunarajah and Chen, 2017). This peri-lymphatic inflammatory response was mixed in nature consisting of $CD11b^+$/inducible nitric oxide synthase $(iNOS)^+$ macrophages and $CD4^+$ T-lymphocytes. Although the mechanisms regulating the peri-lymphatic clustering of inflammatory cells remain unknown, some authors have shown changes in expression patterns of chemokines such as C-C motif chemokine ligand 21 (CCL21) (Weitman et al., 2013). Gradients of CCL21 regulate inflammatory cell migration into lymphatics, suggesting that the loss of these gradients in obese mice may result in trapping of inflammatory cells in tissues (Johnson and Jackson, 2010; Tal et al., 2011; Russo et al., 2016). In addition, CLV permeability is reported to facilitate peri-nodal adipose tissue inflammation. CLVs leak lymph born antigens enabling sampling by adipose tissue DCs and T cell recruitment during recall responses (Kuan et al., 2015). Furthermore, leaky CLVs facilitate severe adipose deposition, chronic inflammation and tertiary lymphoid organ formation in mesentery of Crohn's disease patients aggravating the disease pathology (Randolph et al., 2016). Another mechanism that may contribute to white adipose tissue inflammation is increased expression of growth factors such as vascular endothelial growth factor A (VEGF-A) and VEGF-C in obese individuals (Miyazawa-Hoshimoto et al., 2003; Tinahones et al., 2012; Zafar et al., 2018). VEGF-C is highly chemotactic for myeloid-derived cells such as macrophages (Skobe et al., 2001; Nurmi et al., 2015; Shew et al., 2018). High levels of VEGF-C can also increase blood and lymphatic leakiness, macrophage infiltration and may impair lymphatic function in a feed forward manner (Jeon et al., 2008; Gousopoulos et al., 2017). An interesting study demonstrated that blockade of VEGF-C is an effective means of preventing development of insulin resistance by decreasing macrophage chemotaxis to subcutaneous tissues (Karaman et al., 2015).

A recent study reported that enhancing adipose tissue-specific lymphangiogenesis not only improves adipose tissue lymphatic function but also decreases metabolic abnormalities (Chakraborty et al., 2019). In this study, a novel mouse model of adipose tissue-specific overexpression of VEGF-D (Adipo-VD mice) was created, resulting in abundant functional lymphangiogenesis in white adipose tissue. Adipo-VD mice fed an HFD had improved glucose clearance, lower insulin levels, and reduced triglycerides from the liver as compared to controls. Functionally, these mice exhibited higher glycerol flux from adipose tissue and decreased number of macrophage-positive crown-like structures as compared to controls suggesting that these animals had improved immune cell trafficking from adipose tissue. This study therefore suggests that the effects of lymphangiogenic growth factor expression are context dependent and variable depending on the target cell (i.e., adipocyte versus inflammatory cells).

Several lines of evidence suggest that peri-lymphatic inflammatory responses play a role in the development of obesity-induced lymphatic dysfunction. For example, mice deficient in $CD4^+$ cells (CD4 knockout), athymic nude mice (deficient in T cells), or Rag-1 mice (deficient in both T and B cells) have decreased peri-lymphatic inflammation after prolonged exposure to HFD and have improved lymphatic function as compared with similarly fed wild-type controls (Weitman et al., 2013; Torrisi et al., 2016). Topical application of tacrolimus, an IL-2 inhibitor that decreases T cell proliferation and differentiation, results in decreased lymphatic leakiness and improved collecting lymphatic pumping capacity in obese mice (Torrisi et al., 2016). Tacrolimus applied topically poorly absorbed into systemic circulation and the improvements in lymphatic function were only limited to the site of drug delivery, suggesting that local changes in inflammation rather than systemic effects of obesity were responsible for obesity-induced lymphatic leakiness and abnormal pumping. This hypothesis is supported by the fact that there was no difference in total body weight or adipose tissue weights in animals treated with topical tacrolimus versus controls. However, topical tacrolimus treatment significantly reduced the number of $CD4^+$ T cells and related inflammation locally, correlating with the increased lymphatic function. Thus, neutralizing T cell inflammation seems to have a strong therapeutic value to enhance lymphatic function in obesity. In fact, previous research demonstrated that neutralization of $CD3^+$ T cells with systemic antibody therapy decreases metabolic syndrome and insulin resistance in obese mice (Winer et al., 2009). It will be interesting to see how systemic $CD3^+$ cell neutralization effects lymphatic function in obesity.

Behavioral modifications that decrease inflammation also improve lymphatic function. It is well established that lifestyle modifications such as caloric restriction or aerobic exercise training significantly decrease white adipose tissue, skeletal muscle inflammation, iNOS, and release of inflammatory cytokines (Bruun et al., 2006; Bradley et al., 2008; Kawanishi et al., 2010; Baynard et al., 2012; Kawanishi et al., 2013). These interventions also improve glucose intolerance and insulin sensitivity, decrease adipose tissue inflammation and adipokine production, and improve cardiac function (Sjostrom et al., 2000; Poirier et al., 2003; Clement et al., 2004; Sjostrom et al., 2004; Moschen et al., 2010). More recently, it has become clear that behavioral modifications also improve lymphatic function. For example, HFD-induced obese mice that underwent a 6-week course of aerobic exercise training that did not cause weight loss but did decrease white adipose tissue inflammation had significantly improved lymphatic function (decreased leakiness, improved pumping) as compared with sedentary obese controls (Hespe et al., 2016). These findings are interesting, as they suggest that lymphatic dysfunction in obesity is regulated by paracrine responses rather than direct effects of dietary toxins or physical compression of lymphatic vessels by enlarged adipocytes. In another study, caloric restriction of obese mice not only resulted in weight loss but also decreased white adipose tissue inflammation and restored lymphatic function to near normal levels (Nitti et al., 2016). This study also demonstrated a threshold effect from weight gain (weight > 40 g) beyond which lymphatic functional deficits become more pronounced and measurable. These findings are important because they suggest that obesity-induced lymphatic dysfunction is reversible (at least partially), and that behavioral modifications or pharmaceutical interventions that decrease inflammation may aid in this process.

Peri-lymphatic inflammatory cells, particularly macrophages, strongly express iNOS (Mattila et al., 2013; Xue et al., 2018). It has been reported that, collecting lymphatic pumping is regulated by gradients of NO and, under normal circumstances, NO production around lymphatic vessels is secondary to the expression of endothelial nitric oxide synthase (eNOS) (Berg and Scherer, 2005; Lahdenranta et al., 2009; Liao et al., 2011; Mauricio et al., 2013; Iantorno et al., 2014). Valvular and tubular lymphatic segments increase NOS expression during phasic contractions in turn regulate lymphatic contraction and relaxation (Bohlen et al., 2009). These findings suggest that the loss of NO gradients around collecting lymphatics due to high levels of iNOS expression by perilymphatic inflammatory cells impairs lymphatic pumping and results in collecting lymphatic dilatation (a phenotype that is commonly observed). This conclusion is supported by the fact that *in vivo* inhibition of iNOS in obese mice using a small molecule inhibitor, 1400 W, significantly improves lymphatic pumping and overall lymphatic function (Torrisi et al., 2016), and increased levels of iNOS are known to cause abnormalities in lymphatic contractile function in a variety of pathologic settings (Liao et al., 2011; Scallan et al., 2016). On the other hand, *ex vivo* experimental reports suggests basal NO causes decreased contractile function and lymph flow (Scallan and Davis, 2013).

High concentrations of NO decrease lymphatic contraction frequency and amplitude of contractions (Gashev et al., 2002; Gasheva et al., 2006, 2007). High levels of iNOS and NO may also regulate lymphatic function by other mechanisms. For example, NO is an oxygen donor, and high local concentrations of this molecule may result in generation of free oxygen and free nitrogen radicals and LECs are sensitive to free radical injury (Kasuya et al., 2014). Free radicals and iNOS itself also regulate inflammatory cell migration and may promote chronic inflammatory reactions (Liang et al., 2016). Obese iNOS knockout mice have improved metabolic parameters, decreased insulin resistance, and, most importantly, decreased tissue inflammation (Perreault and Marette, 2001). Whether or not these animals also have improved lymphatic function remains to be seen, and this is a topic of active study in our laboratory.

Inflammatory cells are also a major source of cytokines that may have important effects on LECs. For example, T cells are major sources of cytokines including interferon gamma [IFN-γ, interleukin 4 (IL-4), IL-13, and transforming growth factor beta (TGF-β)]. These cytokines have potent anti-lymphatic effects, and downregulate LEC proliferation and function *in vivo* and *in vitro* (Clavin et al., 2008; Kataru et al., 2011; Savetsky et al., 2015b; Shin et al., 2015). Thus, subcutaneous tissue inflammatory responses may directly inhibit lymphangiogenesis and lymphatic function by increasing the expression of anti-lymphangiogenic cytokines.

Is It Obesity or HFD?

Is it possible that some of the lymphatic abnormalities noted in HFD-induced obese mice may be related to toxic compounds in their diet or perhaps generated by high levels of dietary fat? To study this question, we compared lymphatic function in genetically obesity-resistant [myostatin (MSTN) knockout and BALB/c mice] and obesity-prone (C57BL/6J) mice that were fed on a HFD for prolonged periods of time (Garcia Nores et al., 2016). Not surprisingly, obesity-prone mice fed an HFD progressively increased in weight and became obese after 12–14 weeks of HFD feeds. In contrast, obesity-resistant mice gained only modest amounts of weight and did not become obese. Obesity-prone mice fed an HFD had severely abnormal lymphatic function, leaky lymphatics, impaired collecting vessel pumping, and decreased LEC mRNA expression of lymphatic-specific markers, including VEGFR-3. These mice also had abnormal metabolic parameters, including hyperinsulinemia and increased serum leptin as well as accumulation of crown-like structures (macrophages engulfing enlarged adipocytes) (Boi et al., 2016). In contrast, obesity-resistant mice fed an HFD had essentially normal lymphatic function, preserved lymphatic architecture/anatomy, and normal metabolic parameters. More importantly, these mice had limited white adipose tissue inflammation and very few peri-lymphatic inflammatory cells. Taken together, these findings suggest that obesity and inflammation are both important regulators of lymphatic dysfunction, and that dietary modifications alone are insufficient to induce this effect.

Role of Adipokines and Free Fatty Acids

In addition to inflammation, obesity induces the expression of a variety of adipokines and accumulation of free fatty acids (FFAs) in adipose tissues, and several studies have suggested that these products can negatively impact lymphatic function. For example, high levels of leptin, as noted in obese individuals, strongly inhibits LEC tubule formation/proliferation, and leptin treatment results in aberrant morphological changes in human lymphatic ducts (Leal-Cerro et al., 1996; Rose et al., 2002; Sato et al., 2016). Adiponectin, a cytokine with a positive effect on obesity, is reported to be found in low levels in obese patients (Shibata et al., 2009; Nigro et al., 2014). Interestingly, adiponectin is found to have a protective effect on lymphatic vessels by LEC differentiation and viability. This is also supported by the finding that adiponectin-knockout mice show reduced lymphangiogenesis and exacerbated lymphedema phenotype (Shimizu et al., 2013).

FFAs are abundant in tissues of obese subjects, and their role in insulin resistance and inflammation in target tissues is well studied (Boden, 2008). Reports indicate that FFAs, especially stearic acid, induced significantly more blood endothelial cell apoptosis and necrosis (Harvey et al., 2010). Treatment with oleic acid caused hyperpermeability of LECs *in vitro* (Sawane et al., 2013). Recent studies on cultured LECs show that some FFAs are highly toxic to LECs even in low concentrations by causing dose-dependent increase in apoptosis, decreased proliferation and downregulation of PROX-1 and VEGFR-3 expression, (Garcia Nores et al., 2016). These reports indicate that increased tissue concentration of FFAs in obese subjects can directly injure the lymphatic endothelium. In addition to direct toxic effects on LECs, FFAs may interact with other molecules that regulate lymphatic vessel stability. For example, apelin, a well-known endogenous ligand orphan G protein-coupled receptor (APJ),

is an important regulator of lymphatic vessel stability and integrity (Sawane et al., 2011). Apelin-knockout mice exhibit hyperpermeability and abnormal skin lymphatic function under HFD-fed conditions, due to elevated plasma FFAs and their negative effects on LECs. The lymphatic defective phenotype in Apelin-knockout mice is reversed in Apelin-transgenic mice with skin-specific overexpression of apelin (K14-Apelin), indicating the protective role of apelin against FFA-induced damage (Sawane et al., 2013).

LYMPHATIC DYSFUNCTION CAUSES ADIPOSE DEPOSITION

Lymphatic Fluid Causes Adipocyte Maturation and Lipid Accumulation

While it is clear that obesity causes lymphatic dysfunction, it is equally clear that lymphatic dysfunction can cause alterations in adipocyte biology and contribute to local adiposity (**Figure 1**). Indirect evidence for this hypothesis can be derived from adipose deposition in patients with lymphedema. Lymphedema is a disease that occurs as a result of genetic or acquired abnormalities of the lymphatic system secondary to infections, trauma, or cancer treatment. While early stage lymphedema is characterized by accumulation of interstitial fluid and pitting edema, late stages of the disease result in chronic and progressive adipose tissue deposition, often resulting in massive tissue distortion (Rockson, 2001, 2018; Warren et al., 2007; Mehrara and Greene, 2014). Lipedema, a less well-defined disease resulting in abnormal adipose deposition, has also been shown to be associated with functional alterations in the lymphatic system (Harwood et al., 1996). Lymphatic injury results in expression of IL-6, a broad inflammation marker and an important regulator of adipose tissue homeostasis in lymphedema (Cuzzone et al., 2014). Lymphatic injury in mouse models results in remodeling of adipose tissues and increased expression of adipocyte differentiation genes such as CCAAT/enhancer-binding protein-alpha (CEBP-α), adiponectin, peroxisome proliferator-activated receptor-gamma (PPAR-γ) (Rutkowski et al., 2010; Aschen et al., 2012; Zampell et al., 2012; Tashiro et al., 2017). Other experiments using a rat model in which collecting lymphatics are ligated have shown that insufficient lymphatic drainage results in abnormal lipid accumulation along the vein walls, causing chronic venous insufficiency (Tanaka et al., 2016). These findings are supported by *in vitro* experiments demonstrating that addition of lymphatic fluid (chylous fluid) to culture media of preadipocytes results in cellular differentiation, mature adipocyte gene expression (e.g., PPAR-γ, GLUT-4), and intracellular lipid accumulation (Harvey et al., 2005). More recent studies suggest that FFAs including oleic acid, α-linoleic acid, and palmitic acid are the components of lymphatic fluid that induce adipocyte maturation and differentiation (Escobedo et al., 2016). Taken together, these studies suggest that lymphatic injury causes activation of adipocytes and accumulation of adipose tissues.

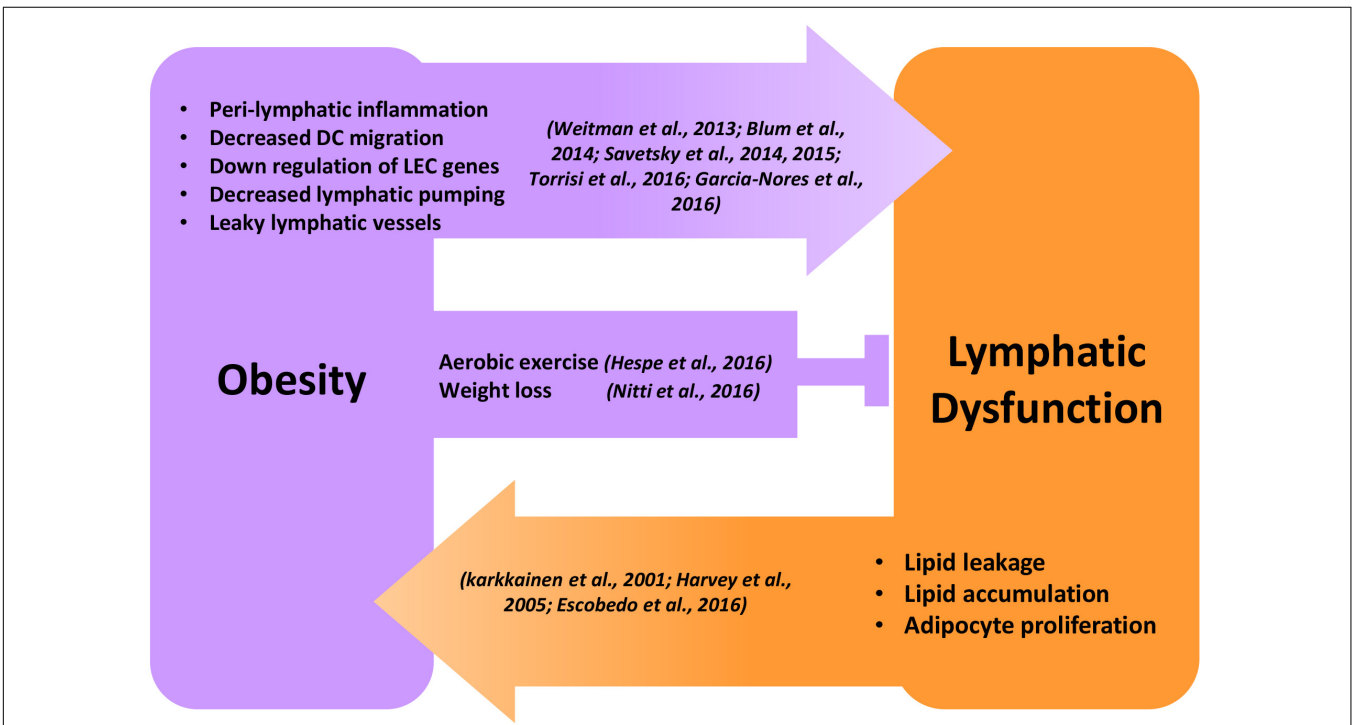

FIGURE 1 | Reciprocal regulation of obesity and lymphatic dysfunction. Schematic illustration showing the reciprocal regulation of obesity and lymphatic dysfunction. Peri-lymphatic inflammation in obesity causes decreased lymphatic pumping, DC migration, LEC gene expression, and increased lymphatic leakage leading to lymphatic dysfunction. Behavioral interventions like aerobic exercise and weight loss inhibit and reverse obesity-induced lymphatic dysfunction. Genetic or surgical lymphatic damage or dysfunction causes malabsorption of lipids, adipocyte proliferation, and progressive accumulation of adipose tissue, leading to obesity.

Lymphatics Are Anatomically Co-localized With Adipose Tissues

Anatomic considerations also suggest that the interaction between the lymphatics and adipose tissues is bidirectional. Throughout the body, lymphatic structures are physically located in close proximity to adipose tissues. The open-ended initial dermal lymphatic vessels are located just above the adipose tissue layer in the skin. Major CLVs and trunks are always surrounded by adipose tissues, and lymph nodes are maintained in thick fat pads even in highly lean individuals (Harvey, 2008). These fat pads serve as an energy reservoir that helps to sustain immune responses in lymph nodes (Pond and Mattacks, 1995, 1998, 2002). Surgical removal of lymph nodes postnatally causes failure of fat pad development, suggesting that there is a 2-way communication between adipocytes and the lymphatic system during development (Eberl et al., 2004).

Congenital Abnormalities of the Lymphatic System Result in Adult-Onset Obesity in Mice

Experiments using transgenic animals with lymphatic defects provide even more direct evidence that lymphatic function can regulate adipose tissue deposition. The homeobox transcription factor Prox-1 is the master regulator of lymphatic specification and identity (Wigle et al., 2002). Prox-1 null mutations are lethal, with death occurring around embryonic day 14.5. However, heterozygous mutations (Prox-$1^{\pm}$) as well as conditional Prox-1 knockouts survive to adulthood despite anatomic and functional lymphatic abnormalities. These mice have leaky lymphatics and chylous ascites that partially resolve (Harvey et al., 2005). Interestingly, mice with heterozygous Prox-1 mutations become obese as adults (by 4 months of age) even when fed a normal chow diet. These mice display no differences in food intake but nevertheless gain significantly more weight, accumulate adipose tissues in both intra-abdominal and subcutaneous tissues, and develop metabolic abnormalities, including insulin resistance when compared with littermate controls. Restoration of lymphatic Prox-1 signaling not only rescues lymphatic function but also prevents abnormal adult-onset weight gain and metabolic abnormalities (Escobedo et al., 2016). Interestingly, although the most common clinical cause of obesity is over-nutrition rather than genetic abnormalities, recent genome-wide association studies have identified single-nucleotide polymorphisms in the Prox-1 that are linked with increased waist circumferences and obesity (Kim et al., 2013; Lecompte et al., 2013). However, more in-depth research is warranted attributing these results to lymphatic phenotype as Prox-1 is also expressed by liver and skeletal muscle which significantly contributes to metabolism and energy homeostasis.

Other mouse models with genetic abnormalities of the lymphatic system that cause lymphatic dysfunction also display abnormal adipose deposition. For example, mice with heterozygous inactivating mutations in Vegfr-3 (also known as Chy mice) develop chylous ascites post-natally that resolve spontaneously. However, these mice develop swollen feet and increased subcutaneous adipose tissue deposition as compared to wild-type mice (Karkkainen et al., 2001; Dellinger et al., 2007; Rutkowski et al., 2010). Similar findings have been reported in angiopoietin-2 (Ang-2) knockout mice. These mice have abnormal embryonic lymphatic patterning and develop adult-onset foot pad swelling due to adipose deposition (Dellinger et al., 2008).

REGULATION OF METABOLIC ABNORMALITIES BY THE LYMPHATIC SYSTEM

Lymphatic vessel function plays a key role in regulation of wide variety of biological phenomenon, and, as a result, lymphatic abnormalities are associated with many pathologic conditions, including cancer, autoimmune disorders, transplant rejection, atherosclerosis, diabetes, myocardial infarction (MI), and hypertension, to name a few. However, in the context of this review, we will briefly discuss those pathologies that are concerned with lymphatic function and metabolic disorders.

Lymphatic Function in Hypercholesterolemia and MI

Obesity is frequently associated with hypercholesterolemia. However, many obese patients exhibit normal blood lipids, and not all hypercholesterolemia patients are obese (Miettinen, 1971; Guerrero-Romero and Rodriguez-Moran, 2006). These irregularities indicate that some other common factor might play a key role in hypercholesterolemia pathology. Accumulation of lipids in the extracellular tissues is the key feature of hypercholesterolemia. Recent studies have shown that the lymphatic system plays an important role in cholesterol metabolism by transporting cholesterol from the peripheral tissues to the liver via a process called RCT (Lim et al., 2013; Martel et al., 2013; Veronique and Ying, 2013; Huang et al., 2015). Studies using Chy mice or surgical ligation of lymphatic channels results in decreased RCT and cholesterol clearance from the tissues (Lim et al., 2013; Martel et al., 2013). The efflux of cholesterol from peripheral tissues to systemic circulation through lymphatic vessels is not passive, but an active process could dependent on uptake and transcytosis of HDL by scavenger receptor class B type 1 (SR-B1) expressed on LECs (Lim et al., 2013). This concept is supported by the fact that patients with primary lymphedema often display xanthomas (cholesterol-rich deposits in the skin) (Berger et al., 1972; Romani et al., 2012). Poor lymphatic function in primary lymphedema causing increased cholesterol accumulation might contribute to fat deposition, a major symptom of lymphedema. Impaired lymphatic function is also linked to lipoprotein metabolism, increased plasma cholesterol levels, and enhanced atherogenesis (Vuorio et al., 2014). Other studies using apolipoprotein E knockout (ApoE$^{-/-}$), an important protein in fat metabolism, or low-density lipoprotein receptor knockout (Ldlr$^{-/-}$) models of hypercholesterolemia have shown that these mice also have severe lymphatic dysfunction, suggesting the interaction between elevated cholesterol levels and lymphatic function

may be bidirectional (Lim et al., 2009; Milasan et al., 2019). Based on these findings, RCT by lymphatic vessels from the arterial wall or atherosclerotic plaques has been discussed as a potential anti-atherogenic therapeutic target in patients with cardiovascular diseases (Milasan et al., 2015; Zheng et al., 2018; Csanyi and Singla, 2019).

The most common complication of obesity and atherosclerosis is MI (Zhu et al., 2014). It is caused by obstruction of coronary circulation, leading to cardiomyocyte loss and scar tissue accumulation culminating in heart failure. The primary event post MI is a potent innate inflammatory reaction for phagocytosis of the dead cell debris and clearance. Reports indicate that after an MI incident, there is a profound remodeling of cardiac lymphatic vessels, and that augmentation of this response by VEGF-C treatment can lead to improved cardiac function (Klotz et al., 2015). These findings are also supported by data from soluble VEGFR-3 decoy transgenic mice (sVEGFR-3) and Chy mice, which reveal that downregulation of VEGFR-3 signaling structurally alters the cardiac lymphatic vasculature and increases mortality after MI (Vuorio et al., 2018). In addition to VEGF-C, adrenomedullin, a known cardioprotective peptide, is also shown to induce cardiac lymphatic growth and function after MI (Trincot et al., 2019). Mechanistically, after MI, cardiac lymphatic vessels clear acute inflammatory immune response, help in resolution of cardiac inflammation, and inhibit scaring and fibrosis and this process is dependent on cardiac lymphatic vessel expression of LYVE-1 (Vieira et al., 2018). Taken together, improving both cardiac lymphatic regeneration and function has the promise to be a therapeutic strategy for infracted myocardium recovery.

Lymphatic Function and Diabetes

Obesity is highly associated with diabetes and chronic hyperglycemia (Al-Goblan et al., 2014). The role of lymphatic function on diabetes, and how hyperglycemia regulates lymphatic vessels, is still an active topic of investigation. Impaired wound healing is a severe complication of diabetes, and lymphatic regeneration is an integral part of wound healing; therefore, it is imperative to understand how lymphatic function influences diabetes and vice versa (Saaristo et al., 2006; Brem and Tomic-Canic, 2007; Clavin et al., 2008). Using a mouse corneal suture model, it is reported that lymphangiogenesis is significantly suppressed in a diabetic mouse model of leptin receptor deficiency (db/db). These mice also showed impaired wound healing due to defective lymphatic vessel proliferation and collateral lymphatic vessel formation (Maruyama et al., 2007). Uptake of dextran tracer from peripheral tissues to lymph nodes was decreased in alloxan-induced diabetic rats, and insulin treatment rescued this phenomenon, indicating possible decreased lymphatic function in diabetes (Moriguchi et al., 2005). Other studies analyzing gene expression pathways in LECs isolated from patients with diabetes have shown that pro-inflammatory, pro-lymphangiogenic, and lipid-shuttling gene expression pathways are increased in diabetes, while genes related to immune defense, apoptosis mediation, and small-compound transporters are downregulated (Haemmerle et al., 2013). Using cultured LECs, Lee et al. showed that prolonged hyperglycemia induced insulin resistance and these LECs showed disruption of adherent junction proteins, indicating negative effects of hyperglycemia on LECs (Lee et al., 2018). Using lymphatic muscle cells (LMCs), the same group found that hyperglycemia strongly inhibits LMC contractile function by alteration of cellular bioenergetics and activation of inflammatory signaling in lymphatic muscle. These results were supported by an elegant *ex vivo* study by Scallan et al. (2015) that used db/db diabetic mice to study CLV function. They found that collecting lymphatic vascular integrity is disrupted in Type 2 diabetes due to low NO bioavailability. Scallan et al. (2015) further showed that inhibition of the phosphodiesterase 3 (PDE3) enzyme, that is normally inhibited by NO signaling, restored lymphatic vascular integrity and improved lymphatic function (Alexander and Becker, 2015; Scallan et al., 2015). Taken together, these findings indicate that diabetes impairs lymphangiogenesis, lymphatic permeability, and contractile function. However, the molecular events leading to impaired lymphangiogenesis during diabetes were unknown known recently. Using a streptozotocin-induced model of diabetes in mice fed an HFD, Wu et al. (2018) found that lymphangiogenesis and lymphatic function were impaired in diabetes due to downregulation of VEGFR-3 expression on LECs. This VEGFR-3 downregulation is mediated by epsin-mediated endocytosis (Wu et al., 2018). The specific deletion of epsin in LECs not only improved lymphatic growth but also enhanced lymphatic function in HFD-fed diabetic mice. These findings reveal that diabetes-induced lymphatic dysfunction is mainly mediated by endocytosis of VEGFR-3 by epsins and depletion of epsins in a potential therapeutic target to improve lymphatic function in diabetes.

Intestinal Lacteal Vascular Endothelial-Cadherin (VE-Cadherin) Zippering

Lymphatic leakiness causes chylus leakage and obesity. Lacteals are the intestinal villi lymphatic vessels with button-like VE-cadherin junctions. Vascular factors such as VEGF-C, angiopoietin-2, and DLL4 are reported to maintain VE-cadherin junctional integrity and regeneration of lacteals (Zheng et al., 2014; Bernier-Latmani et al., 2015; Suh et al., 2019). These VE-cadherin intercellular button-like junctions allow fluid and chylomicrons into lacteals and, eventually, into blood circulation (Cifarelli and Eichmann, 2019; Hokkanen et al., 2019). Converting the lacteal LEC button junctions to zipper-like junctions prevents chylomicron entry into lacteals thus reducing lipid absorption and protection against diet-induced obesity. A study by Zhang et al. (2018) reported that endothelial-specific genetic deletion of Neurophilin-1 (Nrp-1) and vascular endothelial growth factor receptor 1 (Vegfr-1) induced lacteal VE-cadherin junction zippering and chylomicron malabsorption. Deletion of Nrp-1 and Vegfr-1 on endothelial cells increased signaling through VEGFR-2 due to increased VEGF-A bioavailability, causing zippering of the lacteal junctions. This resulted in malabsorption of the chylomicrons in these transgenic mice and turning them resistant to diet-induced obesity. As an alternative pharmacologic approach, Rho-associated protein kinase (ROCK) inhibitor Y27632 was used to induce straightening lacteal junctions,

causing reduced chylomicron absorption into lacteals of wild-type mice. This novel intervention of closing lymphatic junctions by transformation of buttons to zippers is quite intriguing. However, detailed research is needed to determine the adverse effects of lacteal zippering on overall intestinal health.

FUTURE DIRECTIONS

Although considerable progress has been made in understanding the reciprocal relationship between obesity and lymphatic function, many questions still remain. One of the major topics that requires addressing is identification of the mechanisms by which obesity injures the lymphatic system. While it is clear that inflammation plays a key role in this process, the cellular pathways that mediate lymphatic dysfunction in obesity, both clinically and in mouse models, remain largely unknown. Equally important in this discussion are studies analyzing the reversibility of these changes clinically. Thus, while preclinical models indicate that anti-inflammatory medications and behavioral modifications can reverse the pathologic effects of obesity on the lymphatic system, it remains to be seen whether these abnormalities can be reversed in patients with longstanding obesity. Understanding the cellular mechanisms that regulate lymphatic function in obesity and understanding how these effects are normalized with targeted treatments, will have a profound impact on a variety of metabolic diseases, and is an urgent biomedical need.

AUTHOR CONTRIBUTIONS

RK, BM, HP, JB, and CL performed the literature search, compiled and wrote the manuscript. HP, JB, and JS proofed the manuscript. RK and HP created the figures. All authors contributed to manuscript revision and read and approved the submitted version.

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REVIEW
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Extracellular Vesicles as Potential Prognostic Markers of Lymphatic Dysfunction

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Despite significant efforts made to treat cardiovascular disease (CVD), more than half of cardiovascular events still occur in asymptomatic subjects devoid of traditional risk factors. These observations underscore the need for the identification of new biomarkers for the prevention of atherosclerosis, the main underlying cause of CVD. Extracellular vesicles (EVs) and lymphatic vessel function are emerging targets in this context. EVs are small vesicles released by cells upon activation or death that are present in several biological tissues and fluids, including blood and lymph. They interact with surrounding cells to transfer their cargo, and the complexity of their biological content makes these EVs potential key players in several chronic inflammatory settings. Many studies focused on the interaction of EVs with the most well-known players of atherosclerosis such as the vascular endothelium, smooth muscle cells and monocytes. However, the fate of EVs within the lymphatic network, a crucial route in the mobilization of cholesterol out the artery wall, is not known. In this review, we aim to bring forward evidence that EVs could be at the interplay between lymphatic function and atherosclerosis by summarizing the recent findings on the characterization of EVs in this setting.

Keywords: lymphatic function, lymph, extracellular vesicles, atherosclerosis, cardiovascular disease, circulating marker

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INTRODUCTION

In the 1620s, two important networks of vessels were discovered: the blood and the lymphatic circulation (Suy et al., 2016). Whereas tools to study the blood circulation have been developed at a greater pace, exploring the thin and translucent vessels that characterize the lymphatic network had its load of hurdles. Considered as white blood-containing vessels in the early beginnings, the lymphatic system is now recognized as one of the most crucial supporters of the immune response (Jones and Min, 2011). It is generally known to perform three main functions (Cueni and Detmar, 2008). First, the lymphatic system defends the body against infections. The vessels displace lymph throughout the body, and the lymph nodes act as a filter to get rid of debris, bacteria, viruses, and other foreign bodies (Butler et al., 2009). Second, as lymphatic vessels called lacteals are located within the digestive tract, it helps absorb fat-soluble vitamins and dietary fat, which will in turn reach the bloodstream and be used as needed (Iqbal and Hussain, 2009). Third, it is critical in maintaining tissue homeostasis. Excess fluid that escapes from the bloodstream is collected by the lymphatic system, thus preventing the formation of edema (Phelps, 1990).

With the development of genetic mouse models and imaging tools adapted to fit both animals and humans, new functions of the lymphatic system have been brought forward, including the central role of functional adventitial lymphatic vessels the in removal of cholesterol from the atherosclerotic lesion (Lim et al., 2013; Martel et al., 2013; Vuorio et al., 2014; Milasan et al., 2016a, 2019; Rademakers et al., 2017). Atherosclerosis, the major cause of cardiovascular disease (CVD), is characterized by the accumulation of cholesterol and an intensified inflammatory reaction within the artery wall (Libby et al., 2011). The triggered cell activation and apoptosis plays a central role in the disease progression and results in the formation and accumulation of submicron particles called extracellular vesicles (EVs) within the blood vessel intima (Leroyer et al., 2007; Bobryshev et al., 2013). As EVs interact with surrounding cells to transfer their cargo that comprises messengerRNA (mRNA), microRNA (miRNA), proteins and lipids, they are suspected to be key players in the exacerbation of atherosclerosis. The lymphatic network is a crucial route in the mobilization of cholesterol out the artery wall (Martel et al., 2013), and we hypothesize that the massive accumulation of EVs in the artery wall could also be due to a poor clearance by the lymphatic vessels located in the affected blood vessels. Albeit the advances in analytical technologies combined with improved lymph collection techniques have led to the detection of EVs in mouse lymph (Milasan et al., 2016b), the fate of EVs within the lymphatic vessels is only emerging. In this review article, we discuss the association between lymphatic function and CVD, provide an overview of the biogenesis, functions and role of EVs in atherosclerosis, and bring forward evidence of a causal relationship between EVs and lymphatic dysfunction associated with atherosclerosis.

THE ROLE OF THE LYMPHATIC SYSTEM IN ATHEROSCLEROSIS

Hoggan and Hoggan (1882) reported that lymphatics are present in the arterial wall. A century later, Dr. Gerald Lemole suggested that the accumulation of interstitial fluid in the artery wall may contribute to the development of atherosclerosis due to factors present in the intimal edema, and instigated the concept of lymphstasis in atherogenesis (Lemole, 1981). The following decade revealed that disruption of cardiac lymphatic drainage in allogenic transplanted hearts could be at the origin of the enhanced atherosclerosis observed (Miller et al., 1992). Lymphatic vessels are present in the adventitial layer of atherosclerotic arteries (Kholova et al., 2011), and Martel et al. (2013) have reported that functional adventitial lymphatic vessels are essential to first mobilize cholesterol out of the vessel wall before it reaches the thoracic lymphatic duct and the blood circulation at the level of the subclavian vein, bringing front stage the role of lymphatics in macrophage reverse cholesterol transport (mRCT). Using a surgical model of aortic transplant from a hypercholesterolemic $apoe^{-/-}$ donor mouse to a hypercholesterolemic $apoe^{-/-}$ receiver mouse in which an apoE vector was subsequently injected to induce cholesterol efflux, the authors revealed that the newly formed lymphatic vessels facilitated cholesterol removal from advanced plaque (Martel et al., 2013). Mice that were given an anti-VEGFR3 antibody to prevent the development of lymphatic connections between the transplanted aorta and the receiver's artery had enhanced cholesterol accumulation compared to the control mice when the apoE vector was given. Subsequently, it had been shown that treatments known to reduce lipid and immune cell accumulation within the aortic root of hypercholesterolemic mice (Wilhelm et al., 2010) were potentially mediating their beneficial effects through the enhancement of lymphatic function (Milasan et al., 2017). In their manuscript, Milasan and collaborators injected lipid-free apoA-I intradermally in mice bearing mature atherosclerosis lesion and observed that the reduction in plaque size was associated to an improved molecular and cellular lymphatic transport and to a significant drop in the atherosclerosis-associated collecting lymphatic vessel leakage. ApoA-I appeared to strength junctions between lymphatic endothelial cells (LECs) through an upregulation of the VEGFR-3 pathway in LECs. Furthermore, *ex vivo* experiments revealed that apoA-I also acts upon the enhancement of platelet adhesion on the lymphatic endothelium and on the reduction of platelet aggregation induced by either thrombin or podoplanin (Milasan et al., 2017). The authors envisioned that by limiting platelet aggregation, apoA-I would clear the way for platelet adhesion on LECs, which would in turn exert a shielding effect on the lymphatic endothelium, just like macrophages are exercising a "bridge effect" between adjacent blood endothelial cells (EC) (He et al., 2016). By enhancing the adherence of pseudopodia-shaped platelets that able to reach and pull several LECs together, apoA-I might reinforce the lymphatic endothelial barrier and thus contribute to the preservation of the lymphatic endothelium integrity in atherosclerotic subjects.

The prerequisite role of the lymphatic system in onset of atherosclerosis was demonstrated using atherosclerosis-prone mice ($ldlr^{-/-}$; $hapoB100^{+/+}$) (Milasan et al., 2016a). At 3 months of age, these mice displayed an impaired lymphatic function, even before the onset of atherosclerosis. This early dysfunction that was not cholesterol-dependant was associated to a defect in the collecting lymphatic vessels, rather than a defect in the absorptive capacity of the initial lymphatic vessels (also called lymphatic capillaries) (Milasan et al., 2016a). Collecting vessels are contractile lymphatics that propel lymph in a unidirectional manner, with the help of intraluminal bi-leaflet valves and lymphatic muscle cells (LMCs) that cover sporadically the functional pumping unit of a collecting lymphatic vessel called the lymphangion (Zawieja, 2009). LMCs enable the spontaneous contraction of the lymphatics. The follow-up study reported that early treatment with a mutant form of VEGF-C (VEGF-C152S), a selective agonist of VEGFR-3, rescues the contractile capacity of collecting lymphatic vessels, delays plaque onset and limits its progression (Milasan et al., 2019). Whereas the exact mechanisms responsible for the prompt defect in lymphatic function observed prior to the atherosclerosis plaque onset remain to be elucidated, these findings strongly suggest that targeting lymphatic function in patients at risk of developing coronary artery disease (CAD) may constitute a novel therapeutic target for the prevention and treatment of atherosclerosis.

THE DIVERSITY OF EXTRACELLULAR VESICLES DRIVES THEIR FATE AND FUNCTION IN ATHEROSCLEROSIS

EVs are released by cells under physiological and pathological conditions and express markers pertaining to their cell of origin (Yuana et al., 2013). Due to their diversity and prominent presence in tissues and fluids all over the body (Freyssinet, 2003), they are considered important potential markers in health and disease, including in rheumatoid arthritis (Boilard et al., 2010; Fu et al., 2018), tumor progression (O'Loghlen, 2018), angiogenesis (Todorova et al., 2017), metastasis (Peinado et al., 2017; Zhao et al., 2018), diabetes (Freeman et al., 2018), hypertension (Manakeng et al., 2019), metabolic syndrome (Diamant et al., 2002; Martinez and Andriantsitohaina, 2017), hypercholesterolemia and CVD (Jansen et al., 2017b). EVs that are derived from diverse cell types such as leukocytes, erythrocytes, smooth muscle cells (SMCs) and endothelial cells (ECs), are found in atherosclerotic lesions as a result of cell activation or death (Leroyer et al., 2007). Whereas red blood cell (RBC)- and platelet-derived EVs are the most abundant EV subsets in the blood vasculature, platelets, leukocytes and vascular cells are also known to release EVs in the circulation. Notwithstanding, CVD is associated to increased concentrations of EVs, regardless of the cell of origin. Numerous studies have explored and confirmed that circulating and tissue EVs originating from diverse cell types exert different roles in key steps of atherosclerosis and the subsequent clinical outcomes (reviewed in Boulanger et al., 2017). All EVs are surrounded by a lipid bilayer like that of a cell plasma membrane, in contrast to the single-layered high-density lipoprotein (HDL) and low-density lipoprotein (LDL) (Boulanger et al., 2017). However, the cargo they transfer, namely proteins, lipids, miRNAs, non-coding RNAs and surface receptors and antigens, depends on the cell they originate from Diamant et al. (2004) and therefore alters the functional state of the recipient cells in a different manner (Silva and Melo, 2015). In addition of differing in morphology, cellular origin, number, antigenic composition and functional properties, EVs are also heterogenous in size, even if they are originating from the same cell type (Boulanger et al., 2017).

EVs are classified according to their size and mechanism of formation, and it is possible to distinguish these diverse released populations: exosomes, exocytosed from multivesicular bodies; microvesicles (MVs) that bud directly from the plasma membrane; and apoptotic bodies which result from apoptotic blebbing following cell death (Raposo and Stoorvogel, 2013). The release of EVs is a process conserved through evolution, which indicates how essential their role is physiologically (Boilard et al., 2015). To better understand the impact of the release of each subset in CVD, we will herein explore in greater details these submicron vesicles individually.

Exosomes

Exosomes constitute the smallest subpopulation of EVs with a size ranging between 40 and 120 nanometers (nm) (Zaborowski et al., 2015). Secreted by various types of cells, exosomes are formed from multivesicular bodies (MVB), specialized endosomes containing intraluminal vesicles (Hessvik and Llorente, 2018). The MVB are involved in several functions related to endocytosis and protein trafficking such as sorting, recycling, transport, storage and release (Borges et al., 2013; Shao et al., 2018). Exosome formation can be divided into several stages. First, microdomains rich in lipids and membrane-associated proteins are formed on the membrane limiting the MVB. In this process, cytosolic cargo destined for inclusion in EVs are recruited to the microdomains (van Niel et al., 2018). Consequently, intraluminal vesicles are generated by inward budding from the endosomal membrane into the MVB lumen. These MVB can thereafter fuse with the plasma membrane and be released into the extracellular medium to form exosomes (Raposo and Stoorvogel, 2013). Exosome biogenesis involves different molecular mechanisms and can be either dependent or independent of the endosomal sorting complex required for transport (ESCRT) (van Niel et al., 2018).

Given their origin, exosomes contain endosome-associated proteins, such as GTPase Rab, SNAREs, Annexins and flotillin (Raposo and Stoorvogel, 2013). They also harbor a set of evolutionally conserved proteins, including Tsg101 and Alix, and are rich in lipids, such as cholesterol and ganglioside GM121 (Borges et al., 2013; Osteikoetxea et al., 2015; Kowal et al., 2016). Additionally, they contain heat shock proteins HSP60, HSP70, HSPA5, CCT2, and HSP90 (Hong et al., 2018). Tetraspanins, such as CD9, CD63, and CD81 are the most frequently identified proteins and by now, they are considered good general markers of exosomes (Andreu and Yanez-Mo, 2014). However, they may be expressed by other subtypes of EVs emerging from the plasma membrane due to their presence on the surface of various cell types and have already been identified on the surface of MVs as well (Willms et al., 2018). Exosomes contain more sphingomyelins, gangliosides, and desaturated lipids, while their phosphatidylcholine and diacylglycerol proportion is decreased when compared to the membranes of the cells from which they originate (Laulagnier et al., 2004). Furthermore, they are enriched with nucleic acids, such as mRNA and miRNA, further supporting the hypothesis that they are a biological vehicle with the ability to modulate protein synthesis of the target cell and can confer it new functions (Valadi et al., 2007). Exosomes have been identified in several body fluids, such as plasma, urine, saliva, bile, breast milk, sperm, amniotic fluid, cerebrospinal fluid, ascites fluid (reviewed in Yanez-Mo et al., 2015) and most recently, in lymph (Milasan et al., 2016b). They can be released by practically any cell type following activation or apoptosis, including ECs, platelets, RBCs, SMCs, dendritic cells, monocytes and macrophages, and cardiomyocytes (Golchin et al., 2018). Importantly, the microenvironment features, such as hypoxia, also exert important effects on the properties of the origin cell-derived exosomes (Willms et al., 2018). Exosomes produced by cells exposed to oxidative stress can mediate protective signals, thus reducing oxidative stress and cell death in recipient cells (Eldh et al., 2010). Exosomes can also contain cytokines that induce inflammation via numerous different pathways (Distler et al., 2005), and can contribute to

cell aggregation following neutrophil and leukocyte recruitment (Camussi et al., 2010). Therefore, exosomes, and especially their cargoes, play different key roles in various normal physiological instances and pathological responses to disease.

Microvesicles

Microvesicles bud directly from the plasma membrane, measure approximately 100 nm to 1 μm in size and contain cytoplasmic cargo (Zaborowski et al., 2015). They are formed by the remodeling of the cytoskeleton, and their release is increased under inflammatory conditions, hypoxia or activation (Morel et al., 2011; Boulanger et al., 2017). Following cell stimulation, a cytosolic influx of calcium ions (Ca^{2+}) may disrupt the asymmetric distribution of phospholipids in the membrane bilayer by activation of the scramblase involved in the translocation of membrane phospholipids. This results in redistribution of phospholipids. Subsequently, this leads to phosphatidylserine (PS) translocation, creating an imbalance between the internal and external leaflets that leads to budding of the plasma membrane and MVs release. The cytoskeleton degradation caused by the Ca^{2+}-dependent proteolysis promotes the budding of these vesicles which can then express at their surface phospholipids such as PS, that normally constitute the inner membrane layer (Hugel et al., 2005). This translocation can then be used to identify them (Shao et al., 2018). However, it has already been observed in plasma that not all MVs externalize PS (Arraud et al., 2014; Boulanger et al., 2017), but they contain flotillin-2, selectins, integrins, and metalloproteinases (Borges et al., 2013). Highly discussed is the fact that circulating vesicles seem to be composed of both exosomes and MVs. Therefore, currently available purification methods do not yet allow to fully discriminate beyond a reasonable doubt between these two entities.

Apoptotic Bodies

Apoptotic bodies are released as a result of apoptotic cell disassembly and consist of apoptotic material surrounded by a permeable membrane. Apoptotic bodies typically range in size between 1 and 5 μm (Atkin-Smith and Poon, 2017), under certain conditions can become more abundant than exosomes and MVs, and vary greatly in content between biofluids (El Andaloussi et al., 2013). Furthermore, the rapid clearance of apoptotic body fluids by phagocytic cells complicates their characterization (Pitt et al., 2016).

Although exosomes and MVs have been more thoroughly studied to date, as they are significantly and differentially involved in diverse pathologies, apoptotic bodies have similar functional importance with respect to immunomodulatory effects. Exosomes represent an attractive mean of cargo transportation, and many studies to date focused on understanding the precise functions of these smaller entities. Interestingly, apoptotic cells are also suspected to release exosomes, but it remains to be confirmed (Caruso and Poon, 2018). Apoptotic bodies are created to aid in apoptotic cell clearance, as well as a means of intercellular communication. They are involved in the horizontal transfer of DNA including tumor DNA that can result in the induction of a tumorigenic phenotype, in the presentation of epitopes to T cells via internalization by phagocytic cells and in the presentation of autoantigens to B lymphocytes (Bergsmedh et al., 2001; Gyorgy et al., 2011). Apoptosis is an important process in different immunological disease settings such as inflammation, infection, autoimmunity, and cancer (Zitvogel et al., 2010; Hochreiter-Hufford and Ravichandran, 2013; Poon et al., 2014).

INTERNALIZATION OF EXTRACELLULAR VESICLES

Atherosclerosis onset and progression is associated with the accumulation of several subsets of EVs that are internalized differently and thus harbor distinct functions in several stages of the disease. An abundance of mechanisms can be involved in the internalization of EVs inside target cells (Borges et al., 2013). Currently, an increasing number of specific protein-protein interactions that seem to differentially mediate EVs uptake/internalization are on the rise (Mulcahy et al., 2014). Tetraspanins are abundantly expressed by EVs in raft-like structures within their plasma membrane and are generally involved in adhesion, displacement, fusion, activation and proliferation (Hemler, 2005; Andreu and Yanez-Mo, 2014). Inhibition of tetraspanin expression with antibodies was shown to decrease the internalization of EVs in target cells (Rana et al., 2012). Integrins and immunoglobulins, involved in leukocyte adhesion and transmigration, as well as intracellular signaling, also seem to be involved in EVs uptake (Mulcahy et al., 2014). Blockade of CD11a, its ligand ICAM-1, CD51 and CD61 each caused a decrease in the internalization of EVs by dendritic cells (Morelli et al., 2004a; Mulcahy et al., 2014). Proteoglycans are entities that contain a significant amount of carbohydrate components, such as heparin sulfate proteoglycans. Treating bladder cancer cells with a heparin sulfate mimetic reduced cancer cell exosomes uptake thus showcasing their role as key receptors of macromolecular cargo (Christianson et al., 2013; Franzen et al., 2014). Lectins such as DC-SIGN, DEC-205 and Galectin-5, which can trigger phagocytic entry, have also been associated with the internalization of EVs (Hao et al., 2007; Blanc and Vidal, 2010; Naslund et al., 2014).

Endocytosis is another mechanism largely involved in the internalization of EVs. Uptake of EVs within the endosome can occur in a matter of minutes and this internalization was significantly reduced at 4°C, which demonstrates that endocytosis of EVs is an active process that requires energy (Morelli et al., 2004b; Mulcahy et al., 2014). Cytochalasin D, an actin depolymerizing agent that alters endocytosis in mammalian cells, has also contributed to a decrease in EVs internalization in several cell types (Mulcahy et al., 2014). Clathrin-mediated endocytosis involves the progressive formation of clathrin-coated vesicles expressing several transmembrane receptors, as well as their ligands. This allows them to integrate the target cell, undergo clathrin un-coating and fuse with the endosome where it can release its contents (Kaksonen and Roux, 2018). Alternatively, caveolin-dependent endocytosis involves the invagination of caveolae,

which are subdomains of lipid rafts, formed by the action of caveolin (Doherty and McMahon, 2009). Several studies demonstrate that both types of endocytosis are involved in the internalization of EVs. Blocking dynamin 2, a highly conserved GTPase involved in endocytosis and vesicle transport, significantly impaired exosome entry into cells (Yao et al., 2018). Since dynamin 2 is relied upon by both types of endocytosis, further confirmation of caveolin-dependent endocytosis-specific implication requires knockdown of the *Cav1* gene (Nanbo et al., 2013). EVs can also be internalized via phagocytosis which is induced by physical contact with receptors on the surface of specialized phagocytic cells, such as macrophages (Zent and Elliott, 2017). The use of wortmannin and LY294002, PI3-kinase inhibitors that prevent phagosome formation, caused dose-dependent inhibition of the internalization of exosomes within macrophages (Feng et al., 2010; Mulcahy et al., 2014). PS, important in phagocytosis of apoptotic bodies, is frequently externalized on EVs outer membrane (Fomina et al., 2003) and seems to be involved in their internalization. Treatment with inhibitors that bind TIM4, present on macrophages and involved in PS-dependent phagocytosis, or that directly bind PS, such as annexinV, significantly reduced EVs uptake within macrophages and natural killer cells (Nolte-'t Hoen et al., 2009; Feng et al., 2010; Yuyama et al., 2012).

Recently, EVs were shown to depend mainly on macropinocytosis and clathrin-independent endocytosis to enter cells (Costa Verdera et al., 2017). Macropinocytosis involves the invagination of the cell membrane ruffles and its retraction into the intracellular compartment (Mulcahy et al., 2014). Clathrin-independent endocytosis, alternatively called raft-dependant endocytosis, requires functional lipid rafts within the plasma membrane and depends on cholesterol (Teissier and Pecheur, 2007). Lipid rafts are found within invaginations formed by caveolin-1 or in planar regions of the plasma membrane that associate with flotillins (Hooper, 1999). As cholesterol reducing agents like filipin and simvastatin have been shown to prevent EVs uptake, lipid rafts are suspected to play a role in EVs internalization (Costa Verdera et al., 2017; Pfrieger and Vitale, 2018). Furthermore, inhibition of lipid rafts prevented the release of platelet-derived EVs (PEVs) that expose PS at their surface (Mulcahy et al., 2014; Wei et al., 2018). Lastly, EVs membranes can directly fuse with the cell plasma membrane. Increased exosome uptake at low pH by fusion with melanoma cells was observed *in vitro* potentially due to EVs lipid content and ionic charge, as assessed by pre-treatment with proton pump inhibitors (Parolini et al., 2009).

Since the precise proportions of each mechanism involved in EVs internalization are incompletely defined, the consensus is that endocytosis is primarily involved through surface binding. Overall, studies suggest that despite the fact that EVs can be taken up by virtually every cell, through a variety of mechanisms, specificity to certain target cells is not to be neglected, as the engulfment of certain EVs is more effective in one cell type than another (Mulcahy et al., 2014).

THE ROLE OF LYMPH EXTRACELLULAR VESICLES IN INTERCELLULAR COMMUNICATION

Beyond classical signaling through cell–cell contact and soluble factors, such as cytokines, inflammatory mediators, metabolites, and hormones, EVs are now recognized by the scientific community as important mediators of both local and systemic cellular communication (Huang-Doran et al., 2017; Hutcheson and Aikawa, 2018). EVs have several functions depending on the number of bioactive molecules, surface receptors, and genetic information they carry, as well as the cell type of origin and the particular physiological and pathological condition at the time of their packaging and secretion (Montecalvo et al., 2012). Their membrane bilayer gives them the ability to protect their cargo from the enzymes that could degrade them, such as ribonuclease or trypsin (Koga et al., 2011). It has been demonstrated that the mRNAs and miRNAs contained within EVs, once transferred to target cells, are translated into proteins, or regulate gene expression via *de novo* translation or post-transcriptional regulation (Valadi et al., 2007), and may even favor specific signaling cascades in order to induce phenotypic changes (Al-Mayah et al., 2012). Several examples showcase how EVs can differentially control the function of specific cell types, including types distinct of those from which they originated (Borges et al., 2013).

The critical role of plasma EVs derived from platelets, leukocytes, ECs and others in activating immune, endothelial and vascular SMCs, have been extensively described (reviewed in Oggero et al., 2019). However, studies decrypting the role of the lymphatic circulation in the transport of EVs are only starting to emerge. New insights are available into how lymphatics could contribute to the clearance of these inflammatory mediators and support their role in cell-cell communication. The inflammatory response occurring after trauma/hemorrhagic shock (T/HS) is now believed to be driven by the affluence of mesenteric lymph. In a study conducted in rats that underwent T/HS, Kojima et al. (2017) demonstrated that the exosomes contained in mesenteric lymph are the major component triggering inflammatory responses in monocytes and macrophages after T/HS. Cancer-related studies also revealed that exosomes, herein derived from melanoma, can travel through the lymphatic vessels to accumulate in the lymph nodes and promote tumor metastasis (Hood et al., 2011). These nanosized vesicles can be transported by the lymphatic vessels from peripheral tissues to draining lymph nodes where they can be observed for up to 2 days. The internalization of exosomes by macrophages and B cells appears to play an important role in this process (Srinivasan et al., 2016). A study confirmed in mice revealed that postoperative lymphatic exudate of metastatic melanoma patients provides a rich source of extracellular vesicles containing melanoma-associated proteins and miRNAs enabling the differentiation between early and advanced metastatic spread (Broggi et al., 2019). Similarly, Maus et al. (2019) reported that lymph EVs aid in pre-metastatic niche formation in sentinel lymph nodes in human. Earlier this year, Tessandier et al. (2020) unraveled the route followed by EVs

that are accumulating in the synovial fluid during rheumatoid arthritis (RA) and identified lymph as the main path involved in the drainage of EV from an inflamed joint. The group thus hypothesized that the transport of the EVs in lymph during RA may represent a way for platelets to transfer their cargo to tissue locations outside blood vessels. Albeit the exact fate of these PEVs in the context of RA remain unstudied, these observations may be transposable to other vascular inflammatory conditions in which platelets play a major role.

As of today, most of the effects mediated through the internalization of EVs has been thought to be caused by the transfer of their protein and nucleic acid components. Lipids have been implicated in multiple aspects of EV biogenesis and function. Studies aiming at characterizing the EV lipidome should be insightful to further understand the role of EVs in several disease onset and progression (Ouweneel et al., 2019).

EXTRACELLULAR VESICLES ARE ASSOCIATED WITH SEVERAL STAGES OF ATHEROSCLEROSIS

EVs are thought to contribute to vascular endothelial dysfunction, which is considered an early stage of atherosclerosis. For instance, studies have reported that EVs can alter the protective function of the vascular endothelium by interfering with nitric oxide (NO) synthesis (Amabile et al., 2005; Agouni et al., 2008) or by directly increasing its permeability (Densmore et al., 2006). EVs are also believed to play an active role in plaque destabilization, as they can contain various proteolytic factors that promote matrix degradation (van der Vorst et al., 2018). Treatment with EVs derived from human atherosclerotic lesions enhanced EC proliferation *in vitro* and induced angiogenesis *in vivo* (Jansen et al., 2017a). These effects were more pronounced when EVs were isolated from the plaques of symptomatic patients rather than those without any symptoms, pointing out an important determinant of plaque vulnerability (Leroyer et al., 2008). Whereas still debatable, other studies report that the different subsets of EVs display distinct effects on thrombosis. For instance, in human atherosclerotic lesions, the presence of exosomes appear to have antithrombotic effects (van der Vorst et al., 2018) while MVs have been associated with thrombogenic effects (Leroyer et al., 2007). Along with tissue factor (TF) and exposure of PS on the outer membrane layer, MVs are suspected by these teams to contribute to the coagulation pathway (Mallat et al., 1999; Boulanger et al., 2017).

Another study showed that patients with stable CAD but high levels of circulating $CD31^+$/annexinV^+ EVs were at higher risk for coronary revascularization and cerebral events (Sinning et al., 2011). Patients that are at high risk of developing a major cardiovascular event have been shown to have higher levels of $CD3^+$/$CD45^+$ EVs and SMA-α^+ EVs (Chiva-Blanch et al., 2016). In a group of patients with diverse cardiovascular risk factors for CAD, levels of circulating $CD144^+$ EC-derived EVs, measured by flow cytometry as an indicator of endothelial dysfunction, predicted future cardiovascular events (Nozaki et al., 2009). The presence of subclinical atherosclerosis in asymptomatic patients was shown to be associated with increased levels of leukocyte derived MVs (Chironi et al., 2006). In patients with acute stroke, the levels of EC-derived EVs correlated with lesion volume and clinical outcome (Simak et al., 2006). The accumulation of EVs within the artery wall can undoubtedly be detrimental, raising the need for a better control of EVs homeostasis.

LYMPH EXTRACELLULAR VESICLES AND THEIR POTENTIAL CONTRIBUTION TO ATHEROSCLEROSIS

Clearance of EVs is thought to occur through different mechanisms that include annexin V-PS receptor-dependent mechanisms (Jansen et al., 2012), phagocytosis by splenocytes (Al Faraj et al., 2012), internalization by endothelial cells, macrophages (Dasgupta et al., 2009, 2012; Imai et al., 2015) or Kupffer cells (Willekens et al., 2005). Nevertheless, our understanding of the mechanisms underlying the clearance of EVs from the peripheral tissues such as the atherosclerotic lesion, the interstitial space or the blood circulation is still in an embryonic stage. **Figure 1** depicts the new insights described herein on the different roles of the lymphatic system, including the dissemination of EVs.

Plasma is continuously filtered to the extracellular space by a semipermeable layer of blood endothelial cells. The majority of the extravasated interstitial fluid and macromolecules are reabsorbed by the lymphatic capillaries, whereas venules are responsible for transient reabsorption (Levick and Michel, 2010; Aspelund et al., 2016). Overall, an average of 3 l of plasma extravasates from the blood circulation every 9 h and is returned in its majority to the systemic circulation by the lymphatic system (Levick and Michel, 2010). Considering the crucial role of the lymphatic system in the clearance of cells and molecules from peripheral tissues, it is not surprising that circulating EVs can also be found in lymph. A study by our group reported for the first time the presence of PEVs and RBC-derived EVs, inclusively but not exclusively, in the lymph of healthy mice and at higher concentrations in atherosclerotic mice (Milasan et al., 2016b). EVs can thus easily travel from the plasma ultrafiltrate to the lymphatic circulation through initial lymphatics. Additionally, our preliminary data suggest that EVs could act on LECs, modulate their function and alter lymphatic vessel integrity (Jean et al., 2018). These observations lead us to believe that EVs could initially be absorbed adequately by initial lymphatics, while subsequently reaching and affecting the contractile capacity of collecting vessels (**Figure 2**).

Whereas our data suggest that EVs circulating within the lymphatic vessels are responsible for the various effects on the lymphatic endothelium, we do not exclude that EVs contained in the surrounding environment of the lymphatics can also affect the lymphatic contraction capacity. Collecting lymphatic vessels are generally embedded in adipose tissue (Harvey, 2008; Escobedo and Oliver, 2017), such as in the heart (epicardial adipose tissue) (Montani et al., 2004), around the aorta (Martel et al., 2013), in the skin (Tavakkolizadeh et al., 2001) or

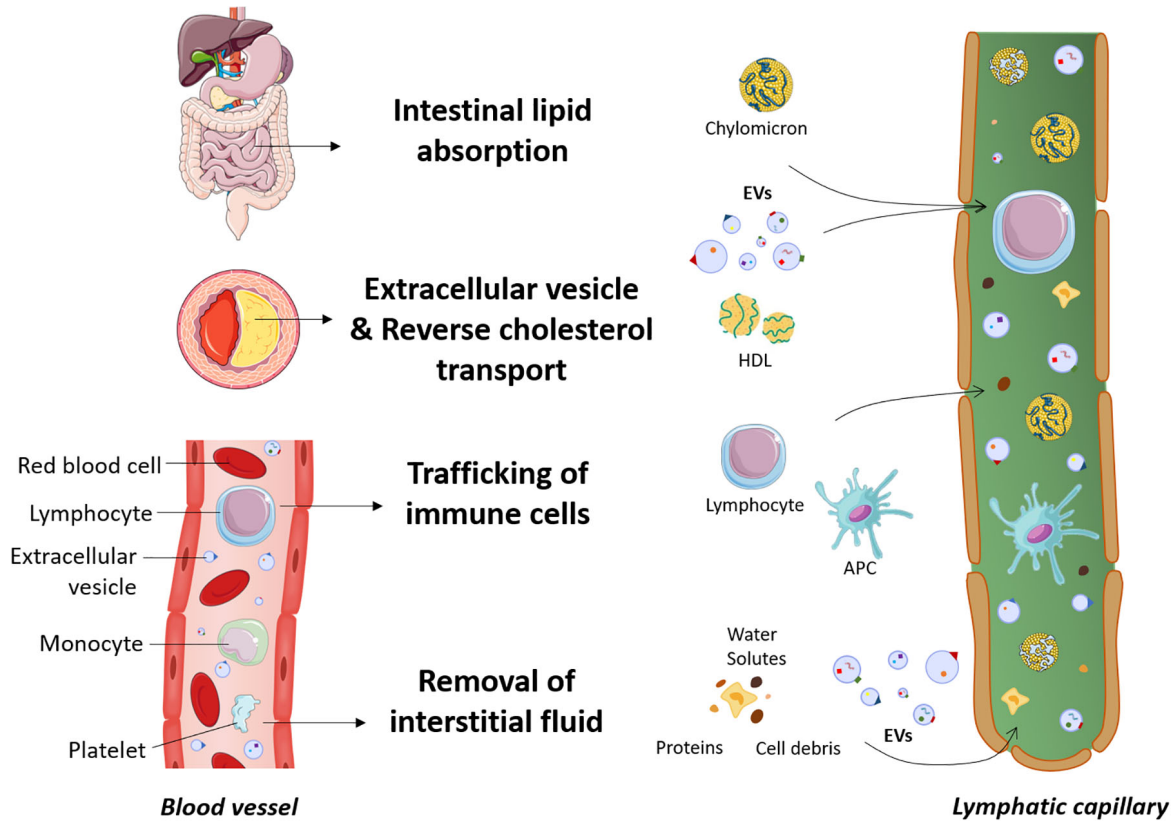

FIGURE 1 | New insights into the roles of the lymphatic system. The lymphatic system is involved in dietary lipid absorption, preservation of fluid balance and host defense. In the past years, more far-reaching effects on several diseases, including cardiovascular disease, have been discovered. Lymphatic vessels are now known to play a prerequisite role in macrophage reverse cholesterol transport. Cholesterol is transported by cholesterol acceptors such as HDL particles through the adventitial lymphatics before reaching the bloodstream. As extracellular vesicles are also a constituent of the atherosclerotic lesion, we hypothesize that these cell fragments also preferentially travel through the lymphatics to be mobilized out of the artery wall. Extracellular vesicles are abundant in the blood circulation, and since plasma ultrafiltrates are collected by the lymphatic system after escaping from the bloodstream, we envision that extracellular vesicles could easily access the lymphatic circulation along with proteins, cells debris and other macromolecules. APC, antigen presenting cells; EVs, extracellular vesicles; HDL, high-density lipoprotein.

in the intestines (Bernier-Latmani et al., 2015). EVs that are derived from various cell types, such as adipocytes (Durcin et al., 2017; Clement et al., 2020) or macrophages (Ying et al., 2017) have been found in peripheral fat and associated with inflammation (Wadey et al., 2019; Dini et al., 2020). Fat and the immune cells it contains are known to affect lymphatic function (Kuan et al., 2015; Escobedo and Oliver, 2017). We thus also suggest that adipose EVs derived from these cells upon activation or death might in turn be able to interact directly with the collecting lymphatic vessels by transferring their cargo to lymphatic muscle or endothelial cells and thus modulate their integrity and affect lymph transport. Regardless of how EVs would get in lymph, they could disturb lymphatic function and therefore modify lymph flow. This could result in the accumulation of EVs within the lymphatic vessels, which would enhance the effect of EVs on LECs and create a feedback loop (**Figure 2**). Altogether, these sequential events would potentially contribute to the instigation of the lymphatic transport impairment that precedes the onset of atherosclerosis (Milasan et al., 2016a).

Since cholesterol preferentially travel through the lymphatic vessels to get out of the artery wall (Martel et al., 2013), a defect in collecting lymphatic vessels could mirror a decrease in cholesterol transport and promote plaque build-up. Furthermore, the massive accumulation of EVs in the atherosclerotic lesion could also be due to a poor clearance by the adventitial lymphatic, favoring disease progression and exacerbation. Based on these findings, EVs can potentially be considered as the missing link between atherosclerosis and lymphatic dysfunction. However, whether and how specific subsets of EVs could control lymphatic function requires further attention (Raposo and Stoorvogel, 2013; van Niel et al., 2018).

POTENTIAL ROLE OF EXTRACELLULAR VESICLES ON LYMPHATIC FUNCTION

As mentioned before, LECs are exposed to the cargo of numerous subsets of EVs. But what is the direct effect of lymph EVs on lymphatic endothelial and muscle cells? To

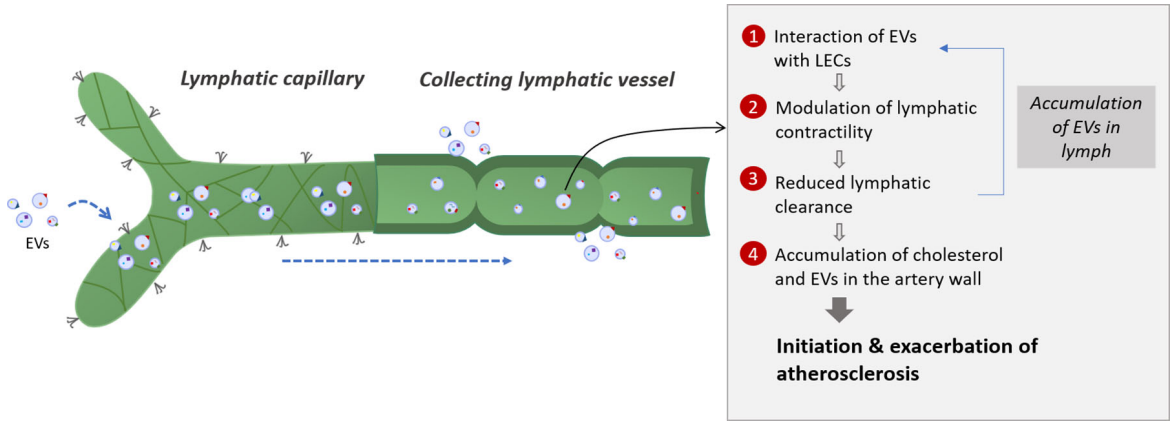

FIGURE 2 | Potential interplay between lymphatic function and extracellular vesicles in atherosclerosis. Extracellular vesicles are first taken up by lymphatic capillaries and then transported through the lymph. Once they reach the collecting lymphatic vessels, extracellular vesicles are thought to modulate the lymphatic contractility by interacting with lymphatic endothelial cells (LECs). Adipose EVs could also interact directly with the collecting lymphatic vessels by affecting the lymphatic muscle or endothelial cells integrity. The potential reduced lymphatic clearance could then result in the accumulation of extracellular vesicles in the lymphatic vessels which will enhance the effect of extracellular vesicles on LECs, thus creating a feedback loop. A defect in collecting lymphatic vessels could also mirror a decrease in the reverse cholesterol transport from the artery wall and promote plaque build-up. Several subsets of extracellular vesicles will then accumulate in the atherosclerotic lesion due to a poor clearance by the adventitial lymphatic vessels, thus modulating the disease progression according to the EVs subsets involved. EVs, extracellular vesicles; LEC, lymphatic endothelial cell.

better understand whether and how EVs might affect lymphatic vessel function, a thorough review of the fate of EVs on the blood endothelium is requisite. We will herein focus on subsets that are also confirmed to be found abundantly in lymph (Milasan et al., 2016b).

Extracellular Vesicles Released by Red Blood Cells

Red blood cells are found in the largest quantity in the blood, accounting for nearly 83% of total cells (Nemkov et al., 2018), and are one of the main vesicle-secreting cells in the blood circulation (Harisa et al., 2017) as they shed most of their damaged content by vesiculation to prolong their lifespan (Leal et al., 2018). EVs derived from RBCs exert a procoagulant activity as they can generate thrombin by a factor XIIa126-dependent mechanism and stimulate thrombus formation or erosion of the atherosclerotic plaque in proportion to their exposure to TF (Biro et al., 2003; Leroyer et al., 2007). The presence of PS on the surface of RBC-derived EVs provides a site for the assembly of prothrombinase promoting a thrombin clot. Additionally, PS-exposed RBC-derived EVs provide sites for adhesion of platelets and neutrophils localized in the subendothelium, further aggravating CVD progression (Noh et al., 2010).

The main function of RBCs is the transport of oxygen from the lungs to body tissues, and of CO_2 as a waste product, away from tissues and back to the lungs (Harisa et al., 2017). RBCs are also recognized to supply ATP, which in turn contributes to vessel dilation by stimulating NO production in the endothelium (Bakhtiari et al., 2012). They also typically contain enzymes and molecules with antioxidant activities (Harisa et al., 2017). Although RBCs are mostly absent in lymph under normal physiological conditions, RBC-derived EVs were reported to be present in lymphatic circulation (Milasan et al., 2016b). RBC-derived EVs measure between 100 and 300 nm (Arraud et al., 2014), contain hemoglobin and are surrounded by a lipid bilayer rich in acetylcholinesterase (Harisa et al., 2017). EVs derived from RBCs are associated with increased oxidative stress, including free heme transfer to endothelial cells (Camus et al., 2015), and thus they can interfere with NO signaling and promote initiation of endothelial dysfunction (Boulanger et al., 2017). They were also reported to scavenge NO faster than intact erythrocytes (Herring et al., 2013), causing vasoconstriction, increased erythrocyte adhesion and enhanced endothelial damage (Camus et al., 2015; Harisa et al., 2017).

Lymphatic contractions are tightly regulated by endothelium-derived relaxation factors such as NO (Gasheva et al., 2006) and histamine (Nizamutdinova et al., 2014). NO is released in a shear-dependent manner to limit contractions in periods of high lymph velocity while ensuring proper diastolic relaxation and lymphatic filling (Bohlen et al., 2011). However, NO bioavailability critically depends on the delicate balance between its production and degradation by reactive oxygen species (ROS). Disruption in this finely tuned balance can alter the lymphatic pumping and endothelial permeability (Zawieja et al., 1991; Gasheva et al., 2007).

Based on the findings stated above, RBC-derived EVs are likely to be involved in atherosclerosis-related lymphatic dysfunction given their capacity to increase oxidative stress and scavenge NO. Preliminary studies are in line with the results reported on the blood endothelium. RBC-derived EVs were found to increase oxidative stress on LEC and thus increase lymphatic endothelial permeability *in vitro* (Jean et al., 2018).

Extracellular Vesicles Released by Platelets

Platelets are essential during embryogenesis (Uhrin et al., 2010a) and throughout life (Hess et al., 2014) in the maintenance of a proper lymphatic function, and Milasan et al. (2017) have reported that promising treatments in atherosclerosis are acting through platelet adhesion on LECs to exert their beneficial effects. Platelets regulate the blood/lymphatic vessel separation by inhibiting the proliferation, migration, and tube formation of LECs upon the interaction of C-type lectin-like receptor 2 (CLEC-2) with podoplanin (Osada et al., 2012). In the blood circulation, platelets have been shown to act through their secreted active releasates and extracellular vesicles (Arraud et al., 2014) to instigate (Massberg et al., 2002) and exacerbate (Huo et al., 2003) atherosclerosis. Albeit they do not form a normal constituent of lymph, our laboratory has demonstrated that PEVs are abundant in mouse lymph (Milasan et al., 2016b), and we now suspect that presence of these submicron particles in lymph may be critical in maintenance of a proper lymphatic function during atherosclerosis.

Platelets are versatile blood cells involved in thrombosis and hemostasis but are increasingly recognized as key players in innate and adaptive immune responses (Semple et al., 2011) as well as lymphatic vessel development (Hess et al., 2014). They are the major source of circulating EVs, releasing preferentially exosomes and MVs (Heijnen et al., 1999). PEVs have been extensively studied in various settings including vascular inflammation, atherosclerosis and hemostasis. The amount and cargo of PEVs are determined by whether their release is spontaneous or induced (Aatonen et al., 2014). They could be thus either beneficial or deleterious depending on the composition of the membrane and biological cargo contained in the vesicle. For instance, several studies suggest that PEVs increased blood endothelial permeability (Marcos-Ramiro et al., 2014; Edrissi et al., 2016; Boulanger et al., 2017). This effect could be due to enhanced apoptosis through delivery of caspase 3 from PEVs to ECs (Edrissi et al., 2016). However, a recent study reported that PEVs protect the microvasculature from factors, such as thrombin, capable of disrupting endothelial permeability (Miyazawa et al., 2019).

PEVs may also play a dual role in inflammation as they can induce either a pro- or anti-inflammatory response (Zaldivia et al., 2017). PEVs can activate the release of pro-inflammatory cytokines, including IL-1 and IL-6, and the expression of ICAM-1 by ECs (Barry et al., 1998). In the setting of atherosclerosis, PEVs activate ECs, promote monocyte adhesion and plaque recruitment. PLT-derived MVs can also contribute to atherogenesis by inducing hyperplasia of vascular SMCs (Weber et al., 2000). Alternatively, MVs shed by stored human platelets suppressed proinflammatory differentiation of monocytes to macrophages, as well as maturation of DCs (Sadallah et al., 2011). Another study has demonstrated that PLT-derived MVs hampered the differentiation of peripheral regulatory T cells into a pro-inflammatory phenotype (Dinkla et al., 2016). Moreover, PEVs are able to stimulate thrombosis (Mallat et al., 1999). Their procoagulant activity is mediated by the exposure of the anionic phospholipid PS or the expression of TF, main activator of the extrinsic pathway (Mackman et al., 2007).

Given their diverse biological effects, predicting the effect of PEVs on LECs seem quite challenging. However, platelets have been well recognized for their crucial role in lymphangiogenesis and lymphatic function throughout life (Suzuki-Inoue et al., 2007; Bertozzi et al., 2010; Hess et al., 2014). During embryogenesis, separation of lymphatic and blood circulation depends on platelet activation (Uhrin et al., 2010b). In fact, platelets prevent blood-lymphatic mixing at the lymphovenous junction by inhibiting the proliferation and migration of LECs, through CLEC-2/podoplanin interaction (Osada et al., 2012). Furthermore, a study has shown that platelets enhance the lymphatic endothelial integrity *in vitro* as they seem to exert a bridging effect between LECs (Milasan et al., 2017). Since platelets are absent from lymph, this protective effect could be mediated through PEVs. Preliminary work indicates that when PEVs are incubated on a lymphatic endothelium *in vitro*, they tend to be associated with a decrease in ROS production by LECs (Jean et al., 2018), which is known to cause cellular damage and alter DNA (Bhattacharyya et al., 2014). As opposed to RBC-derived EVs, PEVs could maintain lymphatic pumping by reducing the oxidative stress. Further deciphering the role of lymph PEVs (Milasan et al., 2016b) in the maintenance of lymphatic function and integrity would be of great interest.

Extracellular Vesicles Released by Endothelial Cells

Blood ECs are an important source of EVs known to be involved in crosstalk between ECs, SMCs and immune cells in both normal and atherosclerotic conditions. EC-derived EVs are released in response to extracellular stimuli that trigger changes in phenotype and tissue remodeling (Danielson and Das, 2014). Similar to other cell types, they contain a multitude of proteins and RNAs (de Jong et al., 2012). Various stressful conditions such as hypoxia or tumor necrosis factor alpha (TNF-α), both simulating an inflammatory environment as observed in atherosclerosis, affected the proteome and transcriptome of EVs secreted by cultured ECs (de Jong et al., 2012; Chistiakov et al., 2015). In hypoxic EVs, proteins involved in stress response and proapoptotic function were observed (Melotte et al., 2010). In TNF-α induced EVs, significant changes in the amount of mRNA were observed, especially proinflammatory ones such as IL-8, MCP-1, IL-32, and VCAM-1 (Chistiakov et al., 2015). In another study, EVs released by cultured serum-starved human ECs that underwent advanced apoptosis and autophagy were shown to contain autophagosomes, as well as mitochondria, and delivered various danger signals including ATP release which is involved in autophagy regulation (Pallet et al., 2013). As such, detection of these EC-derived apoptotic EVs in blood may suggest endothelial dysfunction (Chistiakov et al., 2015).

Exposure to higher shear stress is another factor that predisposes to atherosclerosis, and in human umbilical vein endothelial cells, leads to formation of EC-derived EVs that contain miR-143/145 clusters (Boon and Horrevoets, 2009). The latter were shown to prevent hyperplasia and maintain the

contractile phenotype of co-cultured SMCs (Cheng et al., 2009; Hergenreider et al., 2012).

Taken together, all these findings allow us to consider LECs-derived EVs as an attractive new tool to assess lymphatic vessel function. LECs are now known to also be active players in the production of EVs. Recent findings suggest that LECs *per se* can release exosome-rich endothelial vesicles to guide the migration of cancer cells and promote their metastasis, a phenomenon that was enhanced after exposure to the inflammatory cytokine TNFα (Brown et al., 2018). While lymphatic exosomes appear to aid with cellular transport through the lymphatics, all types of LEC-derived EVs can also be used as biological particles reflective of lymphatic integrity. Since lymphatic dysfunction occurs even before the onset of atherosclerosis (Milasan et al., 2016a), characterizing LECs-derived EVs in blood circulation could potentially become an early diagnostic tool of lymphatic dysfunction, while assessing the risk of atherosclerosis.

CONCLUDING REMARKS

Extracellular vesicles and lymphatic vessel function are emerging biomarkers for the prevention of atherosclerosis. Whereas past studies focused on role EVs in the onset and progression of atherosclerosis, the interaction between EVs and the lymphatic system during atherosclerosis is understudied. We herein sought to concatenate evidence that EVs could be at the interplay between lymphatic function and atherosclerosis. The field of EVs is quickly growing, however, several factors must be considered when assessing their function. One major necessity in the field is to improve and standardize methods for EVs isolation and analysis (Thery et al., 2006). Their small size adds up to the challenge of performing lymph puncture and complexifies the proper identification of the specific EVs subtypes with the imaging tools available to date. A multitude of isolation methods that produce distinct populations of EVs do exist, and new ones emerge relatively often, which makes data comparability difficult. Once EVs have been isolated from their respective media, the biggest problem to date remains EVs purity, which is crucial when evaluating EVs dosage for functional studies and efficient therapies (Xu et al., 2016). Currently, no method allows an entire and accurate phenotyping, characterization and sizing of all types of EVs. Thus, the EVs community acknowledges the need for a standardized, feasible and cost-effective method to isolate and analyze EVs properly. With emerging research, some consensus has been achieved and continues to evolve (van der Vorst et al., 2018). The International Society for Extracellular Vesicles (ISEV) attempts to provide clear guidelines in an effort to standardize EVs procedures (Thery et al., 2018). To further improve reliability, the EV-TRACK (transparent reporting and centralizing knowledge in EV research) platform was developed (Consortium et al., 2017; van der Vorst et al., 2018). Its purpose is to encourage scientists to integrate all collected data in a uniform matter with associated details so that studies can be accurately replicated and compared (van der Vorst et al., 2018). The involvement of EVs in chronic inflammatory diseases such as atherosclerosis is reputable. Refining and standardizing the characterization of these small vesicles in lymph will enable the discovery of new prognostic markers bridging the onset and progression of several pathologies to an impairment in lymphatic function.

AUTHOR CONTRIBUTIONS

AM, MF, and CM contributed to the concepts, writing, and editing of the manuscript.

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REVIEW
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Lymphatic Endothelial Cell Junctions: Molecular Regulation in Physiology and Diseases

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Lymphatic endothelial cells (LECs) lining lymphatic vessels develop specialized cell-cell junctions that are crucial for the maintenance of vessel integrity and proper lymphatic vascular functions. Successful lymphatic drainage requires a division of labor between lymphatic capillaries that take up lymph via open "button-like" junctions, and collectors that transport lymph to veins, which have tight "zipper-like" junctions that prevent lymph leakage. In recent years, progress has been made in the understanding of these specialized junctions, as a result of the application of state-of-the-art imaging tools and novel transgenic animal models. In this review, we discuss lymphatic development and mechanisms governing junction remodeling between button and zipper-like states in LECs. Understanding lymphatic junction remodeling is important in order to unravel lymphatic drainage regulation in obesity and inflammatory diseases and may pave the way towards future novel therapeutic interventions.

Keywords: lymphatic vessel, endothelial junction, button-like junction, zipper-like junction, VE-cadherin, VEGFR2 signaling

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INTRODUCTION

The lymphatic system plays pivotal roles in fluid balance, immune cell trafficking and lipid uptake (Petrova and Koh, 2018; Cifarelli and Eichmann, 2019; Jackson, 2019b), and lack or malfunction of lymphatic vessels leads to edema, disturbed immune responses and lipid malabsorption (Alitalo, 2011; Venero Galanternik et al., 2016; Escobedo and Oliver, 2017). The lymphatic vasculature is present in most organs, and consists of a complex branched network of capillaries (also called initial lymphatics), collecting vessels and lymph nodes. The blind-ended initial lymphatics are

Abbreviations: ANGPT1, angiopoietin-1; ANGPT2, angiopoietin-2; Calcrl, calcitonin receptor-like receptor; CCL21, chemokine (C-C motif) ligand 21; CCR7, C-C chemokine receptor type 7; DLL4, delta-like 4; ESAM, endothelial-specific adhesion molecule; FAK, focal adhesion kinase; FLT1, Fms-related tyrosine kinase 1; FOXC2, forkhead Box C2; ICAM-1, intercellular adhesion molecule 1; JAM-A, junctional adhesion molecule-A; LYVE-1, lymphatic vessel endothelial receptor 1; MTP, microsomal triglyceride transfer protein; NRP1, neuropilin1; NRP2, neuropilin2; PECAM-1, platelet and endothelial cell adhesion molecule 1; PROX1, prospero homeobox 1; RAMP2, receptor activity-modifying protein 2; RASIP1, Ras-interacting protein 1; ROCK, Rho-associated kinase; TAZ, tafazzin; TIE1, TEK receptor tyrosine kinase1; TIE2, TEK receptor tyrosine kinase2; TNFα, tumor necrosis factor α; VE-cadherin, vascular endothelial cadherin; VCAM-2, vascular cell adhesion molecule 2; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; VE-PTP, vascular endothelial protein tyrosine phosphatase; YAP, yes-associated protein 1; ZO-1, zonula occludens-1.

lined by a single layer of oak leaf-shaped endothelial cells, which are characterized by a sparse and highly perforated basement membrane, numerous anchoring filaments and lack of perivascular cell coverage (Gerli et al., 2000; Pflicke and Sixt, 2009; Tammela and Alitalo, 2010; Schulte-Merker et al., 2011). Fluids, macromolecules and immune cells that extravasate from blood capillaries are drained from the interstitium by the initial lymphatics. The absorbed fluid, known as lymph, flows towards collecting lymphatics, which are characterized by an intact basement membrane and are covered with smooth muscle cells. The collecting lymphatic vessels contain intraluminal valves to prevent the backflow of lymph, which is transported through progressively larger collecting vessels and lymph nodes, and eventually returns back to the blood circulation via lymphatic connections to major veins (Tammela and Alitalo, 2010; Schulte-Merker et al., 2011; Petrova and Koh, 2018). Of note, the lymphatic system has been known since the mid-1660s to transport dietary lipids from the intestine to the blood circulation (Suy et al., 2016). Dietary fats are absorbed by intestinal enterocytes and assembled into triglyceride-enriched particles called chylomicrons. Thereafter, chylomicrons are taken up by initial intestinal lymphatics called lacteals and transported into the blood circulation via the lymphatic system (Randolph and Miller, 2014; Bernier-Latmani and Petrova, 2017; Petrova and Koh, 2018; Cifarelli and Eichmann, 2019).

Blood and lymphatic vessels are both lined with endothelial cells, which are connected by cell-cell junctions. Blood endothelial cell (BEC) junctions include adherens junctions expressing VE-cadherin, tight junctions expressing claudins and gap junctions characterized by connexin expression. BEC junctions are involved in a range of blood vascular processes, such as vasculogenesis, angiogenesis, vessel leak and leukocyte extravasation (Dejana et al., 2009b; Muller, 2015; Okamoto and Suzuki, 2017; Szymborska and Gerhardt, 2018). LEC junctions, on the other hand, have unique structural characteristics reflecting the specific demands of the lymphatic vasculature. In lymphatic capillaries, discontinuous button-like junctions (buttons) (**Figure 1**) endow these vessels with high permeability to allow the entry of interstitial fluid, macromolecules, lipids and immune cells, while collecting lymphatics have continuous zipper-like junctions (zippers) that strengthen the vascular barrier to allow lymph transport. Buttons and zippers are named based on their distinct morphology to distinguish focal sites of intercellular adhesions (buttons) in lymphatic capillaries, and mature junctions (zippers) in lymphatic collectors that are also present between endothelial cells of other blood vessels (Baluk et al., 2007).

An increasing number of studies have now shown that lymphatic drainage requires properly organized LEC junctions that are dynamically remodeled; yet, the molecular basis of LEC junction remodeling in development and disease is at an early stage of investigation. Here, we discuss the current state-of-the-art on the molecular organization of LEC junctions in physiological and pathological contexts.

GENERAL HISTOLOGY OF LEC JUNCTIONS

The oak leaf-shaped LECs of initial lymphatics have overlapping flaps that have long been recognized to be sites for the intercellular entry of fluids (Casley Smith, 1965; Trzewik et al., 2001; Schmid-Schonbein, 2003). A transcellular fluid transport mechanism has been also reported (Lim et al., 2013; Triacca et al., 2017; Yazdani et al., 2019). When interstitial pressure increases due to local fluid accumulation and periodic tissue stress, the filaments that anchor LECs to the extracellular matrix pull on the flaps and open them, and the pressure gradients drive fluids and solutes to flow into initial lymphatics through the openings (Casley Smith, 1965; Leak, 1968; Swartz et al., 1999; Breslin, 2014). The overlapping flaps are believed to serve as primary microvalves between LECs to prevent backflow of fluids from the lymphatic lumen into the interstitial space (Schmid-Schonbein, 1990, 2003; Trzewik et al., 2001; Breslin, 2014). However, how initial lymphatics maintain their integrity while taking up fluids has been a mystery. Initial electron microscopy studies revealed that lymphatic capillaries in the ears of mice and guinea pigs possess open intercellular junctions that drained colloidal carbon, and described the existence of attachment plaques between membranes of adjacent LECs, with characteristic structural features of adherens junctions (zonula adherens) and tight junctions (zonula occludens) (Leak and Burke, 1966; Leak, 1970). A number of studies using gene profiling and cell biology approaches have also identified major junctional molecules expressed in primary and cultured LECs (Podgrabinska et al., 2002; Hirakawa et al., 2003; Johnson et al., 2006; Sironi et al., 2006; Amatschek et al., 2007; Kakei et al., 2014). The *in vivo* organization of LEC junctions, however, was unknown until high-resolution confocal microscopy studies identified discontinuous buttons in the endothelium of initial lymphatics and continuous zippers in collecting lymphatics (Baluk et al., 2007; Dejana et al., 2009a; Yao et al., 2012).

Buttons are discontinuous junctions at the sides of interdigitated membrane flaps of neighboring endothelial cells of initial lymphatics. They are enriched with adherens junction- and tight junction-associated proteins and establish anchorage points for adjoining LECs, with an orientation parallel to the cell borders. Between buttons, the flap tips overlap loosely, thereby enabling unimpeded drainage into initial lymphatics driven by interstitial-to-intraluminal pressure gradients (Baluk et al., 2007). This unique button structural organization allows for fluid entry via the tips of the LEC flaps, without the need for LEC junction dissolution, thus maintaining vessel integrity. Current evidence also supports that immune cell transmigration into initial lymphatics relies on the same button-flap structures (Pflicke and Sixt, 2009; Johnson et al., 2017), although this process is actively controlled by lymphatic endothelial chemokines and their corresponding receptors on leukocytes (Jackson, 2019b). Similar specialized buttons are observed in the initial lymphatics of many body tissues, including the trachea, dermis, diaphragm, bladder and small intestine (Baluk et al., 2007; Dejana et al., 2009a; Yao et al., 2012; Bernier-Latmani et al., 2015; Hagerling et al., 2018; Zhang et al., 2018).

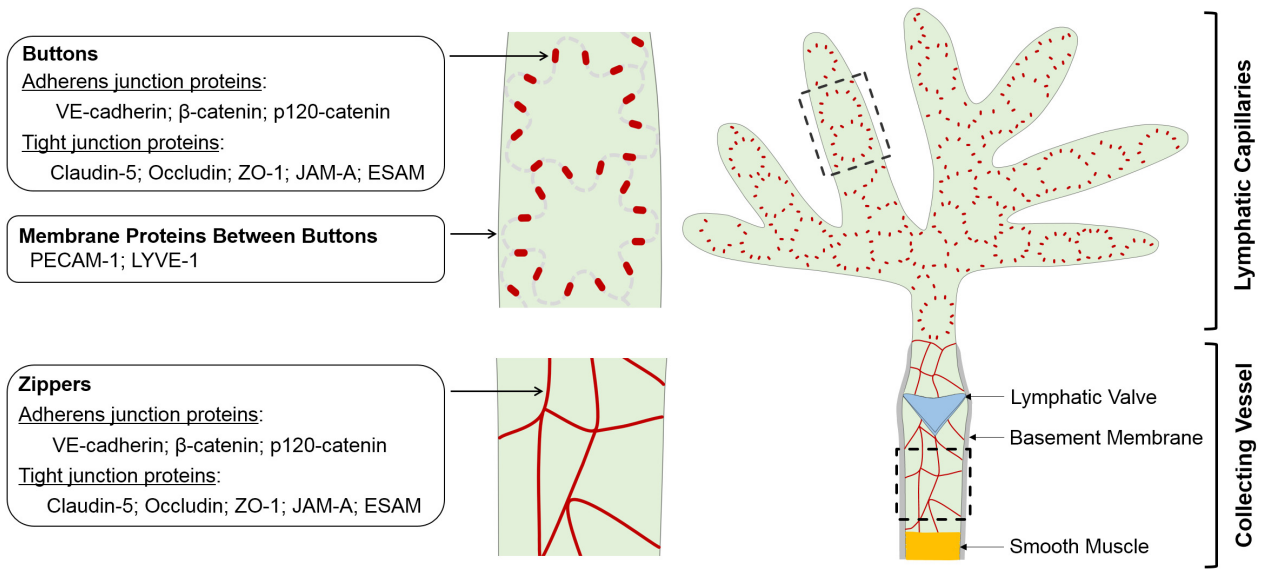

FIGURE 1 | Schematic of morphology, localization and protein components of LEC junctions. LECs lining initial and collecting lymphatics develop different cell-cell junctions to execute uptake and transport of lymph, respectively. The specialized discontinuous buttons in initial lymphatics serve as anchoring sites at the sides of interdigitated flaps of adjacent oak leaf-shaped LECs, and the loosely apposed border regions between buttons are entry sites for fluids, chylomicrons and immune cells. The endothelium of collecting vessels has continuous zippers that prevent vascular leak and allow transport of lymph, a process that is aided by smooth muscle cell coating and intraluminal valves. Adherens junction- and tight junction-associated proteins are enriched in both buttons and zippers. In mature initial lymphatics, most PECAM-1 and LYVE-1 are distributed at the tips of flaps between buttons. LEC, lymphatic endothelial cell.

It should be noted that discontinuous junctions can also occur in BECs, in response to growth factors and inflammatory mediators. However, these hyperpermeable junctions are structurally and functionally different from the buttons of initial lymphatics (Dejana et al., 2009b; Trani and Dejana, 2015). Interestingly, airway epithelium can also form intercellular openings for plasma and leukocytes to traverse from the tissue into the lumen without disrupting the integrity of the epithelial lining (Erjefalt et al., 1995; Fischer and Widdicombe, 2006).

The larger collecting lymphatic vessels are specialized for lymph transport, thus leakage should be restricted in those structures. The endothelial cells lining collecting lymphatics therefore have continuous junctions (zippers) without openings at the borders of adjacent LECs, which are similar in appearance to those seen in the endothelium of blood vessels (Baluk et al., 2007). This feature, together with the continuous basement membrane of the LECs, endows collecting lymphatic vessels with low permeability that limits lymph leakage. In addition, LECs lining lymphatic valves appear to be joined by zippers as well (Wang et al., 2016).

JUNCTIONAL PROTEINS IN LYMPHATICS

Despite the striking differences in their morphology, buttons and zippers are composed of the same junctional proteins, including the adherens junction components VE-cadherin, β-catenin and p120-catenin, the tight junction components occludin, claudin-5 and ZO-1, and tight junction-associated JAM-A and ESAM (Baluk et al., 2007; Dejana et al., 2009a; Yao et al., 2012). These proteins are also expressed by BECs, where they participate in adherens and tight junction complexes at cell-cell contacts. LECs seem to organize these proteins into adherens and tight junction complexes at junctional points as well, as suggested by the ultrastructure of adherens junction and tight junction in electron microscopic images of lymphatic capillaries (Leak and Burke, 1966; Leak, 1970). In mature initial lymphatics, PECAM-1 and LYVE-1 are concentrated at the tips of LEC flaps and are spatially segregated from VE-cadherin-containing buttons at LEC borders (Baluk et al., 2007; Yao et al., 2012). Some connexins, which are components of gap junctions, are also expressed in lymphatics (Kanady et al., 2011; Meens et al., 2014, 2017; Geng et al., 2016; Munger et al., 2016, 2017; Castorena-Gonzalez et al., 2018). The expression pattern and function of these junctional proteins in the lymphatic vasculature are discussed below. Highlighted in particular are *in vivo* results documented by recent knockout mouse studies (**Table 1**).

Adherens Junction Proteins

In blood endothelial adherens junctions, VE-cadherin mediates adhesion through homophilic interactions *in trans* and associations with an intracellular protein network including α-, β-, and γ- and p120-catenin *in cis*. The intracellular proteins β- and p120-catenin stabilize endothelial adhesion by linking VE-cadherin to the actin cytoskeleton (Dejana et al., 2009b; Dorland and Huveneers, 2017; Szymborska and Gerhardt, 2018). In the lymphatic endothelium on the other hand, VE-cadherin is present at all junction types, and its distribution

TABLE 1 | Lymphatic vascular phenotypes after genetic deletion of junctional proteins.

Genes	Mouse models	Phenotypes
Adherens junction proteins		
VE-cadherin (Cdh5)	*Cdh5*$^{-/-}$	Lethality at ∼E9.5–E10.5 due to undeveloped vascular structures (Carmeliet et al., 1999; Gory-Faure et al., 1999)
	Cdh5$^{f/f}$*;Tie2Cre*	Lethality at ∼E10.5 due to undeveloped vascular structures (Yang et al., 2019)
	Cdh5$^{f/f}$*;Lyve-1Cre*	Lethality at ∼E10.5 probably due to defects in cardiovascular development (Yang et al., 2019)
	Cdh5$^{f/f}$*;Prox1CreER*^{T2}	Deletion at E10.5/E11.5/E12.5: severe edema and lymphatic hyperplasia at E14.5, and lethality at E14.5–E18.5. Deletion at P2/P4 and analysis at 6 week: hyperplasic and fragmentized mesenteric lymphatics and lacteals, deteriorated lymphatic valves, mildly dilated dermal initial lymphatics with button junctions maintained, unregulated expression of tight junction proteins. Adult deletion: hyperplasia and disintegration of mesenteric lymphatics and lacteals, lymphatic valve deterioration, unaltered structure of dermal initial lymphatics (Hagerling et al., 2018)
	Cdh5$^{f/f}$*;Prox1CreER*^{T2}*;Prox1-GFP*	Deletion at E10.5: edema and loss of lymphovenous valve formation at E16.5. Deletion at E14.5: loss of lymphatic valve formation at E18.5. Deletion at P1/P3 and analysis at P14: chylous ascites, regression of lymphatic valves (Yang et al., 2019)
β-catenin (Ctnnb1)	*Ctnnb1*$^{f/f}$*;Lyve-1Cre*	Severe edema; Dilated lymphatic vessels with reduced sprouting capacity; Loss or impaired lymphovenous valve and lymphatic valve formation; Lethality before birth (Cha et al., 2016)
Tight junction proteins		
Claudin-5	*Claudin-5*$^{-/-}$	Size-selective impairment of blood-brain barrier; Lethality within 10 h of birth; Lymphatic vascular phenotypes were not reported (Nitta et al., 2003)
	Claudin-5$^{+/-}$	Dilated and leaky lymphatic vessels and exacerbated edema and inflammation following ultraviolet B exposure (Matsumoto-Okazaki et al., 2012)
JAM-A	*JAM-A*$^{-/-}$	Differentially altered leukocyte trafficking (Cera et al., 2004, 2009; Woodfin et al., 2007)
	JAM-A$^{f/f}$*;Tie2Cre*	Unaltered dendritic cell migration across lymphatic endothelium (Cera et al., 2004)
Other junction-related proteins		
PECAM-1	*Pecam1*$^{-/-}$	Partially dilated and mis-branched mesenteric lymphatics and abnormal lymphatic valve formation; Unaltered leukocyte *trans*-lymphatic migration (Baluk et al., 2007; Wang et al., 2016)
	Pecam1$^{-/-}$*;Syndecan4*$^{-/-}$	Increased mural cell coverage and more severe lymphatic abnormalities than *Pecam1*$^{-/-}$ single mutants, which cause blood-filled lymphatic and partial lethality before birth (Wang et al., 2016)
LYVE-1	*Lyve-1*$^{-/-}$	Delayed lymphatic trafficking of dendritic cells; Unaffected lymphatic development and drainage function (Gale et al., 2007; Luong et al., 2009; Torzicky et al., 2012; Vieira et al., 2018)
Connexins	*Connexin37*$^{-/-}$	Lymphatic reflux; Enlargement of the jugular lymph sac; Defective lymphovenous valve and lymphatic valve formation; Unaffected lymphatic contractile capacity (Kanady et al., 2011; Sabine et al., 2012, 2015; Geng et al., 2016; Munger et al., 2016; Castorena-Gonzalez et al., 2018)
	Connexin43$^{-/-}$	No mesenteric lymphatic valves; Abnormally patterned thoracic duct; Perinatal lethality due to heart defects (Kanady et al., 2011; Munger et al., 2016)
	Connexin43$^{f/f}$*;Lyve-1Cre*	Often sudden lethal chylothorax; Impaired lymphatic valve formation; Leaky or disrupted thoracic duct; Increased lymphatic capillary branching; Unaltered lymphatic contractile capacity (Munger et al., 2017; Castorena-Gonzalez et al., 2018)
	Connexin47$^{-/-}$	Normal lymphatic development and function (Munger et al., 2016; Meens et al., 2017; Castorena-Gonzalez et al., 2018)
	Connexin37$^{-/-}$*;Connexin43*$^{-/-}$	Severe lymphedema; Blood-filled lymphatics; Abnormal thoracic duct development; Dilated jugular lymph sac and dermal lymphatics; Perinatal lethality (Kanady et al., 2011)
	Connexin37$^{-/-}$*;Connexin43*$^{+/-}$	Lymphatic reflux and life-threatening chylothorax (Kanady et al., 2011)
	Connexin43$^{-/-}$*;Connexin47*$^{-/-}$	Mild edema; Loss of lymphovenous valve and mesenteric lymphatic valve formation; Lethality around birth (Munger et al., 2016)
	Connexin45$^{f/f}$*;NestinCre*	Impaired entrainment of spontaneous lymphatic contraction (Castorena-Gonzalez et al., 2018)
	Connexin45$^{f/f}$*;SmmhcER*^{T2}	Reduced lymphatic contraction capacity 6–11 days after gene deletion at adult age (Castorena-Gonzalez et al., 2018)
	Connexin26$^{f/f}$*;Keratin5Cre*	Lymphedema; Dilated vessel diameters and reduced networking in dermal lymphatics; Lethality at E16.5–E18.5 (Dicke et al., 2011)

is particularly enriched in buttons (Baluk et al., 2007; Dejana et al., 2009a; Yao et al., 2012). β- and p120-catenin colocalize with VE-cadherin at both buttons and zippers between LECs (Yao et al., 2012), but how adherens junction complexes are organized at these junctions is unclear. In BECs, VE-cadherin phosphorylation, internalization and degradation in response to differential vascular cues regulate the assembly and stability of adherens junctions and vascular permeability (Dejana et al., 2009a; Orsenigo et al., 2012; Conway et al., 2017). Whether these mechanisms are responsible for button and zipper junction organization in LECs remains to be further investigated.

Constitutive loss of VE-cadherin causes embryonic lethality at approximately embryonic day (E) 9.5–E10.5, before the development of lymphatic vasculature (Carmeliet et al., 1999; Gory-Faure et al., 1999; Yang et al., 2019). Studies using tamoxifen-inducible genetic models have revealed that prenatal

LEC-specific deletion of VE-cadherin leads to an aberrant primitive lymphatic plexus, loss of lymphatic valves, edema and fetal death (Hagerling et al., 2018; Yang et al., 2019). Similarly, mice with constitutive deletion of β-catenin in LECs exhibit significantly dilated lymphatic sacs, lack of lymphatic valves, severe edema and prenatal lethality (Cha et al., 2016). Postnatal or adult deletion of VE-cadherin also impairs the integrity of lacteals and mesenteric lymphatic collectors and the maintenance of lymphatic valves, whereas mature adult dermal lymphatics are resistant to VE-cadherin loss (Hagerling et al., 2018; Yang et al., 2019). Interestingly, VE-cadherin loss causes chylous ascites, possibly resulting from lymph leakage from mesenteric collecting vessels due to endothelial barrier dysfunction, as well as valve deterioration, which enables retrograde flow of lymph from larger collecting vessels into lymphatic tributaries where chyle is prone to leak (Nitschke et al., 2017). Growing *in vivo* evidence (Hagerling et al., 2018; Yang et al., 2019) supports that the lymphatic vasculature uses VE-cadherin as a mechanotransducer at junctional sites, similarly to the blood vasculature (Tzima et al., 2005; Coon et al., 2015). Due to the high flow shear at valve sinuses, lymphatic valves may be particularly susceptible to defective cell-cell adhesion and mechanotransduction; this could explain why lymphatic valves deteriorate upon VE-cadherin deletion. At the molecular level, VE-cadherin regulates a number of signaling pathways that are crucial for LEC junction maturation, proliferation and mechanotransduction, including the β-catenin, VEGFR/AKT, YAP/TAZ, PROX1 and FOXC2 pathways (Sabine et al., 2015; Hagerling et al., 2018; Yang et al., 2019). Taken together, these results suggest that VE-cadherin, in addition to mediating cell-cell adhesion, acts also as a membrane signaling hub to transfer intracellular signals in LECs.

Tight Junction Proteins

Tight junctions join endothelial cells of all blood vessels and are most abundant in cerebrovascular endothelial cells. In brain microvessels, multiple interconnected strands of tight junction structures are present at the apical borders of joining endothelial cells, thereby limiting permeability of the blood-brain barrier (Stamatovic et al., 2008; Dejana et al., 2009b; Greene et al., 2019). As mentioned previously, in LECs both buttons and zippers express the endothelial tight junction constituents claudin-5, occludin and ZO-1, as well as the associated proteins JAM-A and ESAM. Although the distribution of those proteins overlaps with VE-cadherin (Baluk et al., 2007; Dejana et al., 2009a; Yao et al., 2012), VE-cadherin deletion or functional inactivation does not alter the distribution of tight junction proteins in buttons and zippers, indicating that tight junction organization is independent of adherens junctions in lymphatic vessels (Baluk et al., 2007; Hagerling et al., 2018). As previously shown in epithelial cells and BECs, tight junctions usually occupy the apical end of the junctional clefts, while adherens junctions are more basal (Niessen, 2007; Dejana et al., 2009a). It is possible that this organization is also maintained at buttons and zippers in LECs. The expression of tight junction proteins such as claudin-5, ZO-1 and JAM-A is upregulated in VE-cadherin-deleted LECs, which may represent a compensatory response following the loss of VE-cadherin (Hagerling et al., 2018). Claudin-5 is a key constituent of endothelial tight junctions. *Claudin-5*$^{-/-}$ mice die within 10 h of birth, and their blood-brain barrier function is impaired against small molecules (Nitta et al., 2003). Importantly, *Claudin-5*$^{+/-}$ mice show dilated and leaky lymphatics after ultraviolet B irradiation (Matsumoto-Okazaki et al., 2012). While the exact roles of claudin-5 in different lymphatics have yet to be elucidated, these results indicate that a claudin-5-dependent barrier may be required for the maintenance of collective lymphatic vessel integrity. Finally, the expression of the tight junction-associated protein JAM-A in LECs does not play a role in lymphatic regulation of leukocyte trafficking (Cera et al., 2004), despite the fact that JAM-A expressed in BECs and certain leukocyte populations, such as dendritic cells, neutrophils and monocytes, has been shown to facilitate leukocyte transmigration (Cera et al., 2004; Woodfin et al., 2007; Cera et al., 2009; Schmitt et al., 2014). Taken together, our understanding of lymphatic tight junctions is still limited, and the dynamics of tight junction proteins in lymphatic development and function have yet to be elucidated.

PECAM-1

The endothelial adhesion molecule PECAM-1 is expressed at a high density at the cell borders of all endothelial cells in the blood and lymphatic vasculature (Sauter et al., 1998; Torzicky et al., 2012). In blood vessels, PECAM-1 acts as a regulator of endothelial cell junction integrity and mechanotransduction and plays an important role in leukocyte transendothelial migration (Conway et al., 2013; Privratsky and Newman, 2014; Chistiakov et al., 2016). PECAM-1 distribution in LECs shows partial overlap with VE-cadherin in buttons and zippers, but it does not appear to regulate VE-cadherin localization, or the junctional integrity of buttons (Baluk et al., 2007). In lymphatic capillaries, PECAM-1 lines mostly the tips of flaps that constitute channels of leukocyte entry (Baluk et al., 2007). To this end, LECs express the chemokine CCL21, which is sensed by the leukocyte receptor CCR7 and provides migration cues for skin dendritic cells toward initial lymphatics (Forster et al., 1999; Saeki et al., 1999). *In vitro*, a PECAM-1-blocking antibody inhibits dendritic cell migration across TNFα-inflamed LEC monolayers and skin explants, suggesting that PECAM-1 may regulate leukocyte migration into lymphatics (Torzicky et al., 2012). However, such a role is not supported by *in vivo* evidence, as *Pecam1-null* mice have normal leukocyte trafficking into afferent lymphatics (Baluk et al., 2007). Finally it has been suggested that PECAM-1 may act as a regulator of lymphatic valve formation and a flow sensor in LECs (Wang et al., 2016).

LYVE-1

Lymphatic vessel endothelial receptor 1 is the major receptor for the ubiquitous glycosaminoglycan hyaluronan in the lymphatic vasculature (Banerji et al., 1999) with a preferential distribution in initial lymphatics over large collective vessels (Baluk et al., 2007), although it is also expressed in some macrophage and BEC populations (Mouta Carreira et al., 2001; Cho et al., 2007; Xu et al., 2007; Gordon et al., 2008; Lim et al., 2018).

Lyve-1-null mice exhibit normal lymphatic development and tissue fluid drainage, suggesting that LYVE-1 is not required for these lymphatic functions (Gale et al., 2007; Luong et al., 2009). However a role for LYVE-1 as a lymphatic-specific receptor for leukocyte trafficking has been recently demonstrated (Lawrance et al., 2016; Johnson et al., 2017; Vieira et al., 2018; Jackson, 2019a). Dendritic cells express hyaluronan on their surface, which during *trans*-lymphatic migration binds to LYVE-1-lined ring-like openings at LEC borders (Johnson et al., 2017). This interaction enables dendritic cell docking to the basolateral surface of initial lymphatics, and the openings allow for dendritic cell entry (Johnson et al., 2017; Jackson, 2019a). In addition to dendritic cells, macrophages may also utilize the same LYVE-1-hyaluronan axis for transport through inflamed lymphatic endothelium (Lawrance et al., 2016; Vieira et al., 2018). LYVE-1 internalization (Johnson et al., 2007) or shedding (Wong et al., 2016) from the lymphatic endothelium allows for the subsequent release of leukocytes into the lymphatic vessel lumen. These findings illustrate a role for the LYVE-1-hyaluronan complex in leukocyte trafficking through initial lymphatic openings defined by button junctions. Notably, previous studies have revealed that in inflamed tissues, dendritic cell trafficking into lymphatics requires β1- and β2-integrins on dendritic cells to engage with their counter receptors ICAM-1 and VCAM-2 on LECs (Johnson et al., 2006; Teijeira et al., 2013; Jackson, 2019a). It has been hypothesized that the LYVE-1-hyaluronan and integrin-ICAM-1/VCAM-2 pathways may coordinate the stepwise entry of leukocytes into afferent lymphatics (Jackson, 2019a). However, this stands in contrast to the observations that lymphatic transmigration and many other inflammatory responses remain unaffected in *LYVE-1-null* mice (Gale et al., 2007; Luong et al., 2009). One possibility is that other lymphatic receptors could compensate for the loss of LYVE-1 function during leukocyte transmigration. Further studies to identify such factors and the possible underlying mechanisms are warranted.

Connexins

Connexins are a family of transmembrane proteins consisting of 21 members in human and 20 members in mouse that assemble into pore-forming hexamers called connexons. Connexons can function as hemichannels in the plasma membrane for cytosol-extracellular diffusion or dock together across adjacent cells to form complete intercellular gap junctions that mediate direct cell-cell communication (Goodenough and Paul, 2009). The role of connexin-mediated homocellular or heterocellular coupling of BECs and vascular smooth muscle cells has been well studied in blood vessels (Figueroa and Duling, 2009; Okamoto and Suzuki, 2017; Okamoto et al., 2019). In contrast, much less is known about connexins in lymphatic vessels; their *in vivo* expression pattern and function in LECs and lymphatic smooth muscle cells have only begun to be unveiled in recent years.

Kanady et al. (2011) showed that Connexin37, Connexin43, and Connexin47 are variably expressed in the endothelium of developing and mature collecting lymphatics. Both Connexin37 and Connexin43 are crucial for normal lymphatic development and function, as evident by the various abnormalities in lymphatic vessel patterning and valve formation, and the presence of lymphedema in *Connexin37*-, *Connexin43*- or *Connexin37*;*Connexin43-null* mice (Kanady et al., 2011; Sabine et al., 2012, 2015; Munger et al., 2016, 2017; **Table 1**). During embryogenesis, Connexin37 is required for maintaining the proper size of the jugular lymph sac, and both Connexin37 and Connexin43 critically regulate thoracic duct development. Intriguingly, Connexin43 and Connexin37 are enriched in a subset of LECs in mature lymphatic valves and have a differential distribution at the upstream and downstream sides of the valves, possibly due to unequal flow shear forces exerted on either side of the valve (Kanady et al., 2011). High expression of Connexin37 in valve LECs depends on PROX1, FOXC2 and flow shear stress (Sabine et al., 2012, 2015). Consistent with their abundant expression in valve LECs, deletion of *Connexin37*, *Connexin43* or both, results in partial or complete loss of lymphatic valve formation (Kanady et al., 2011; Sabine et al., 2012, 2015; Munger et al., 2016, 2017). On the other hand, connexin47 expression is highly restricted to a very small portion of valve LECs on the upstream side of the valve, but the ablation of *Connexin47* alone does not result in lymphatic valve defects or lymphedema in mice (Munger et al., 2016; Meens et al., 2017). Interestingly, mutations in *GJA1* (Connexin43) and *GJC2* (Connexin47) have been linked to human primary and secondary lymphedema (Ferrell et al., 2010; Ostergaard et al., 2011; Finegold et al., 2012; Brice et al., 2013). However, Connexin43 or Connexin47 single mutant mice exhibit no lymphedema, and the lymphedema phenotype can only been seen in *Connexin37*$^{-/-}$;*Connexin43*$^{-/-}$ mice and some *Connexin43*$^{-/-}$;*Connexin47*$^{-/-}$ mutant mouse embryos (Kanady et al., 2011; Sabine et al., 2012). Furthermore, human patients with connexin mutations show no signs of the developmental lymphatic valve abnormalities observed in *Connexin47*-, *Connexin43*- or *Connexin37-null* mice (Ferrell et al., 2010; Ostergaard et al., 2011; Finegold et al., 2012; Brice et al., 2013). These discrepancies raise the question of how the mechanism underlying the defective lymphatic vessel and valve development in connexin mutant mice could relate to the pathogenesis of lymphedema in human patients carrying connexin mutations.

The smooth muscle cell layer of collecting lymphatics generates intrinsic spontaneous contractions that are required for propulsion of lymph. Therefore, loss of lymphatic contractile capacity due to smooth muscle cell dysfunction may be a component of lymphedema and other diseases that are characterized by deficient lymphatic transport. Connexin45, the predominant connexin isoform in lymphatic smooth muscle cells, is essential to the entrainment of lymphatic contraction waves. Connexin45 deficiency disrupts the electrical communication in the smooth muscle layer of lymphatics and largely impairs lymphatic contraction and pumping capability (Castorena-Gonzalez et al., 2018). However, these defects do not result in inhibition of lymph transport *in vivo*, unless a chronic gravitational load is imposed. This is consistent with evidence that lymph transport relies not only on smooth muscle contraction, but also on other driving forces (e.g., interstitial

pressure). A partial-to-severe loss of lymphatic pumping function alone is insufficient to abolish forward movement of lymph. Nevertheless, *GJC1* (Connexin45) mutations have not been identified in human lymphedema patients (Srinivas et al., 2018). Thus, whether a Connexin45 deficiency in lymphatic smooth muscle cells is involved in the pathogenesis of this disease remains yet unclear.

Despite the important advances in understanding the roles of connexins in the lymphatic vasculature, many fundamental questions about lymphatic connexins remain unanswered. First and most importantly, are connexins assembled into gap junction structures in adjacent LECs and smooth muscle cells in the lymphatic wall? If yes, are they integrated with other junctional proteins? In which signaling pathways are connexins involved in those cells? Finally, elucidating the interplay between LEC and smooth muscle cell connexins in mediating cytosol-to-extracellular and cell-to-cell communications will be of particular importance in order to understand key lymphatic functions, such as lymphatic vessel growth, contraction and flow responses.

LEC JUNCTIONS IN DEVELOPMENT

In mice, the development of the lymphatic vasculature is initiated at approximately E9.5, when a population of endothelial cells in the anterior cardinal vein start expressing PROX1 and become lymphatic endothelial progenitor cells (Wigle and Oliver, 1999; Srinivasan et al., 2007; Escobedo and Oliver, 2016). The VEGF-C/VEGFR3 signaling axis subsequently drives budding of PROX1-positive cells from the cardinal vein to form primitive lymphatic structures called lymph sacs (Karkkainen et al., 2004; Hagerling et al., 2013). As developmental lymphangiogenesis proceeds, new lymphatic vessels grow out centrifugally of the lymph sacs and eventually form the majority of the lymphatic vascular tree (Vaahtomeri et al., 2017). Of note, recent lineage-tracing studies (Klotz et al., 2015; Martinez-Corral et al., 2015; Stanczuk et al., 2015) have identified additional LEC progenitors from non-venous origins (e.g., hemogenic endothelium) in multiple organs including skin dermis, heart and mesentery that also give rise to new lymphatics. The precise cell identity of these precursors deserves further studies.

Interestingly, developing lymphatics have zippers, but not buttons. Yao et al. (2012) showed that LECs in the E12.5 jugular lymph sacs and E16.5 trachea lymphatics are joined exclusively by zipper junctions. Importantly, LEC progenitors budding from the cardinal vein at E10.5−E12.5 are also connected to each other by zippers (Yang et al., 2012; Yao et al., 2012). Similar junctions are present at the tips of growing lymphatic sprouts regardless of age (Baluk et al., 2007; Yang et al., 2012; Yao et al., 2012). Hence, when lymphatic budding or sprouting takes place, zipper junctions are developed to maintain close cell-cell contacts between LECs in such a manner that these cells can migrate as a cohesive unit. This is in agreement with the general requirement for tight cell-cell junctions during collective cell movement, in order to maintain tissue integrity, control cell polarity and ensure cell-cell communication (Ilina and Friedl, 2009; Friedl and Mayor, 2017). Indeed, targeted disruption of VE-cadherin in LECs starting at E10.5 prevents primitive lymphatic structures from remodeling and maturing, supporting the essential role of stable zipper junctions during lymphatic development (Hagerling et al., 2018). On the other hand, excessive stabilization of endothelial cell junctions in mice due to covalent fusion between VE-cadherin and α-catenin strongly suppresses embryonic lymphangiogenesis (Dartsch et al., 2014), suggesting that lymphatic development critically depends on the balanced adhesive strength of zipper junctions. PROX1 (Johnson et al., 2008), RASIP1 (Liu et al., 2018), GATA2 (Mahamud et al., 2019) and miR-126 (Mahamud et al., 2019) have been identified as important regulators of VE-cadherin as well as other junction proteins in developing lymphatics. In conclusion, several lines of evidence support that VE-cadherin dynamics at adherens junctions of zippers are very important for lymphatic vessel development (Baluk et al., 2007; Yang et al., 2012, 2019; Yao et al., 2012; Hagerling et al., 2018). Whether tight junction and gap junction proteins contribute to the formation of zipper junctions and collective cell migration in developing lymphatics is currently unknown.

Buttons start to replace zippers in initial lymphatics at E16.5−E17.5 (Yao et al., 2012; Zheng et al., 2014). As lymphatic capillary networks develop, buttons become more abundant from E17.5 to birth and into adulthood and eventually occupy almost 100% of junctional points in the lymphatic capillaries at postnatal day 70. Intermediates of the two junction types appear during the process of button formation (Yao et al., 2012). At the same time, junctions in the endothelium of collecting lymphatic vessels remain zippers (Yao et al., 2012; Zheng et al., 2014). Taken together, the facts that (1) the appearance and disappearance of intermediate junctions coincide with a decrease in zippers and increase in buttons, and (2) all of these junction types have the same protein composition strongly suggest that zippers in developing initial lymphatics transform into buttons via intermediate forms. This process, referred to as "zipper-to-button" transformation, may involve redistribution of existing junctional structures at cell borders rather than assembly of new ones. The precise molecular mechanisms responsible for button formation during development remain largely unknown. It has been postulated that birth may induce rapid button formation in order to guarantee efficient clearance of amniotic fluid by lung lymphatics after the onset of breathing and immediate absorption of dietary fat by lacteals after initiation of feeding (Kulkarni et al., 2011; Yao et al., 2012; Zhang et al., 2018). The emergence of buttons is coincident with the establishment of a fully functional immune system, raising the question of whether developing immune cells are involved in the regulation of button formation and maturation. Previous studies have suggested that dexamethasone and the growth factor angiopoietin-2 (ANGPT2) promote button formation during development (Yao et al., 2012; Zheng et al., 2014), whereas inflammation or inhibition of a few endothelial factors leads to a reversal process, "button-to-zipper" transformation (Yao et al., 2012; Bernier-Latmani et al., 2015; Zhang et al., 2018). These findings will be discussed subsequently.

LEC JUNCTIONS IN LIPID UPTAKE AND OBESITY

One of the main functions of lymphatic vessels of intestinal villi (lacteals) is to absorb dietary lipids, or chylomicrons, from the gut and transport them to the blood. The intestinal lymphatic system consists of lacteals, which reside at the center of intestinal villi and take up chylomicrons, and mesenteric collectors that drain chylomicrons into the thoracic duct and eventually into the venous circulation (Randolph and Miller, 2014; Bernier-Latmani and Petrova, 2017; Petrova and Koh, 2018; Cifarelli and Eichmann, 2019). Despite the recognized importance of lacteals as mediators of lipid uptake, the cellular mechanisms controlling chylomicron entry into lacteals have remained elusive for years. Some reports have suggested that chylomicrons are preferentially taken up by a specialized subset of cells located at the lacteal tip (Lee, 1986; Randolph and Miller, 2014), while studies based on electron microscopy have suggested that chylomicrons enter through LEC junctions in lacteals or pass through lacteals by transcytosis (Dixon, 2010a,b). We and others have shown that under baseline conditions, lacteals display predominately buttons (Bernier-Latmani et al., 2015; Zhang et al., 2018), thus demonstrating features of initial lymphatics. Ultrastructural visualization of wild-type mouse intestinal villi has clearly shown that chylomicrons enter the lacteal lumen through junctional openings on the lacteal wall (Palay and Karlin, 1959; Casley-Smith, 1962; Zhang et al., 2018). Indeed, lipid absorption by lacteals requires the presence of button junctions. Certain endothelial signaling pathways, such as VEGF-A/VEGFR2, DLL4/Notch and ROCK signaling, critically control lipid uptake by regulating lacteal junction morphology (Bernier-Latmani et al., 2015; Zhang et al., 2018, see below). Most importantly, inducing button-to-zipper transformation by targeting the aforementioned signaling pathways, prevents lipid uptake into the bloodstream, thereby rendering mice resistant to diet-induced obesity and associated metabolic syndromes. Similarly, microbiota depletion in the small intestine has been shown to compromise lacteal maturation and maintenance and induce lacteal junction zippering, leading to impaired lipid absorption (Suh et al., 2019). Thus, gut microbiota is also essential for maintaining lacteal button junctions, and plays a crucial role in lipid uptake in the small intestine (Pascale et al., 2019). As such, gut microbiota might be a suitable therapeutic target against obesity and other metabolic disorders. Collectively, these findings suggest that chylomicron uptake by lymphatics relies on lacteal button junctions, and that zippering (closing) of the lacteal junctions represents an attractive novel strategy for preventing plasma lipid uptake and obesity.

Prox1$^{+/-}$ mice, which present dilated and leaky mesenteric lymphatics possibly due to lymphatic cell junction defects, exhibit excessive accumulation of perimesenteric fat and adult-onset obesity (Harvey et al., 2005; Johnson et al., 2008; Escobedo et al., 2016). In line with this, *Apelin-null* mice fed with a high fat diet for 5 weeks develop a more obese phenotype, which is attributed to impaired lymphatic and blood vessel integrity and increased leakiness during lymph transport (Sawane et al., 2013). Taken together, it appears that loss of junction integrity on collecting lymphatics leads to chronic lymph leakage and can trigger late-onset obesity. Thus, tightening junctions in lymphatic collectors may also be useful in order to prevent or treat adult obesity.

LEC JUNCTIONS IN INFLAMMATION-ASSOCIATED CONDITIONS

A number of inflammatory diseases, such as respiratory tract inflammation and inflammatory bowel disease, are accompanied by lymphangiogenesis (Kim et al., 2014). Detailed characterization of trachea lymphatics after *Mycoplasma pulmonis* infection suggests that such newly formed inflammation-induced lymphatics have predominantly zippers (Baluk et al., 2007; Yao et al., 2012). This is in agreement with the idea that zippers are a general feature of all growing lymphatics under physiological and pathological conditions. Notably, buttons in existing lymphatics also transform into zippers during sustained inflammation and then gradually revert to buttons upon inflammation resolution (Yao et al., 2012), but the molecular basis of inflammation-induced LEC junction remodeling is still largely unknown. Functionally, junction zippering would make the inflamed lymphatics less permeable, thereby reducing fluid entry and clearance and aggravating tissue edema. In support of this, lymph flow is decreased during chronic inflammation (Huggenberger et al., 2010; Zhou et al., 2010; Cromer et al., 2015), although increased lymph flow may take place at the onset of acute inflammation (Benoit et al., 1989; Zhou et al., 2010). As described previously, dendritic cells also utilize lymphatic button junctions; thus junction zippering may be detrimental for dendritic cell trafficking into lymphatics. Yet, enhancement of lymphatic trafficking of dendritic cells has been frequently described in the context of inflammation (MartIn-Fontecha et al., 2003; Johnson et al., 2006; Vigl et al., 2011; Russo et al., 2013). This raises the possibility that leukocytes can migrate across zipper junctions in lymphatic vessels under inflammatory conditions, similarly to what has been described for blood vessels (Muller, 2015). Clearly, more research in this area is required to understand how inflammation-induced alterations in lymphatic junctions affect immune cell trafficking and resolution of tissue inflammation.

Notably, inflammation and infections have been implicated in the pathogenesis of secondary lymphedema (Yuan et al., 2019). Although how exactly inflammation alters lymphatics structurally and functionally in the context of lymphedema remains to be determined, it is possible that junction zippering, along with other potential lymphatic dysfunctions such as retrograde lymph flow, underlies or contributes to localized fluid retention and tissue swelling in patients with inflammation-associated lymphedema. If this hypothesis is true, promoting button formation may improve lymphatic drainage function and reduce edema in these patients, hereby representing a novel therapeutic approach against this, notoriously difficult to treat, disease.

MOLECULAR REGULATION OF LEC JUNCTIONS

ANGPT2

Angiopoietin-2 is a multifaceted growth factor that regulates blood and lymphatic vascular functions. In the blood vasculature, it can function as an agonist or an antagonist of ANGPT1/TIE2 pathway in different contexts (Kim et al., 2016; Korhonen et al., 2016). In the lymphatic endothelium, ANGPT2 promotes embryonic and postnatal lymphangiogenesis by acting primarily as an agonist of the TIE2 receptor (Gale et al., 2002; Dellinger et al., 2008; Souma et al., 2018). LECs express low levels of the orphan receptor TIE1 and no VE-PTP, which catalyzes the dephosphorylation of TIE2. This expression pattern is thought to contribute to the preferential role of ANGPT2 as a TIE2 agonist in lymphatics, given that ANGPT2-mediated TIE2 antagonism is largely dependent on TIE1/TIE2 interaction and VE-PTP activity (Song et al., 2012; Souma et al., 2018). Additional information on the context-dependent roles of ANGPT2 in angiogenesis and lymphangiogenesis is reviewed in detail by Akwii et al. (2019).

Beyond these activities, ANGPT2 has also been identified as an essential regulator of LEC junctions in developing lymphatic vessels. Inhibition of ANGPT2 prevents transformation of junctions from zippers to buttons in initial lymphatics during late gestation (Zheng et al., 2014), suggesting a key role of ANGPT2 in promoting junction maturation. Consistent with this, loss of ANGPT2 function leads to compromised drainage capacity and edema (Dellinger et al., 2008; Zheng et al., 2014). Interestingly, ANGPT2 inhibition also concomitantly reduces VE-cadherin phosphorylation at tyrosine residue 685 in developing initial lymphatics (Zheng et al., 2014), raising the possibility that ANGPT2 promotes button formation by controlling the phosphorylation state of VE-cadherin. In addition, ANGPT2 blockage or deletion also largely impairs junction integrity and valve formation in collecting lymphatics before and after birth, thereby resulting in lymph leakage and chylous ascites (Dellinger et al., 2008; Zheng et al., 2014). In summary, ANGPT2 is required for proper patterning of both button and zipper junctions in lymphatics during development. However, the involvement of ANGPT1 and TIE2 receptor in this process has yet to be determined.

Dexamethasone

Dexamethasone is widely used to treat inflammation by acting as an agonist of glucocorticoid receptor. Treatment with dexamethasone has been found not only to reduce VEGF-C-induced lymphangiogenesis, but also to induce the transformation of zippers into buttons in inflamed initial lymphatics (Yao et al., 2010, 2012). This supports the idea that button formation is beneficial to inflammation resolution. Dexamethasone also promotes button formation during neonatal stages in the absence of inflammation (Yao et al., 2012; Zheng et al., 2014), suggesting a direct effect of the steroid on lymphatic junction morphology. The signaling components that mediate the effects of dexamethasone on LEC junctions are not known. As glucocorticoid receptor activation in LECs occurred concomitantly with the formation of button junctions after treatment with dexamethasone (Yao et al., 2012), the junction remodeling is possibly mediated by glucocorticoid receptor signaling. Intriguingly, a number of studies have shown that dexamethasone enhances junctional integrity of BECs by stabilizing the anchorage of adherens junction and tight junction proteins to actin filaments and maintaining the levels of junctional components, thereby reducing vascular permeability under pro-inflammatory conditions (Romero et al., 2003; Blecharz et al., 2008; Salvador et al., 2014). Taken together, these results reveal an interesting context-dependent action of dexamethasone in the regulation of LEC and BEC junctions.

VEGF-A/VEGFR2 Signaling

Vascular endothelial growth factor-A is a major angiogenic growth factor. It signals by binding to its primary receptor VEGFR2 expressed on the surface of BECs and LECs. VEGF-A binding induces tyrosine phosphorylation of VEGFR2 at specific sites and triggers downstream signaling events that are critical for BEC survival, proliferation and migration during angiogenesis (Eichmann and Simons, 2012; Koch and Claesson-Welsh, 2012). VEGF-A signaling through VEGFR2 in LECs is also known to promote lymphatic growth before birth, but does not appear to have important impact on postnatal lymphangiogenesis (Dellinger and Brekken, 2011; Dellinger et al., 2013; Zarkada et al., 2015). In blood endothelium, VEGF-A acts also to transiently increase vascular leak by opening cell-cell junctions (Simons et al., 2016; Dorland and Huveneers, 2017). One well studied pathway that leads to VEGF-A induced BEC junction opening involves the phosphorylation of VEGFR2 tyrosine residue, Y951 (Y949 in the mouse) and the subsequent recruitment of the adaptor protein TSAD, activation of SRC and FAK, VE-cadherin phosphorylation and internalization, and ultimate adherens junction disassembly (Matsumoto et al., 2005; Ruan and Kazlauskas, 2012; Sun et al., 2012; Li et al., 2016). Another pathway involves activation of the small GTPase RhoA and ROCK downstream of VEGFR2, which drives rearrangement of cortical actin into perpendicular stress fibers that bind to the VE-cadherin cytoplasmic tail and pull BEC junctions to open (van Nieuw Amerongen et al., 2003; Bryan et al., 2010; Di Lorenzo et al., 2013).

In addition to VEGFR2, VEGF-A also binds to VEGFR1 (or FLT1) and NRP1 (Hiratsuka et al., 1998; Gelfand et al., 2014). In the intestine, these two receptors are not expressed by intestinal LECs, but only BECs (Jurisic et al., 2012). Previous studies suggested that FLT1 has limited intrinsic tyrosine kinase activity in endothelial cells and rather acts as a decoy that prevents VEGF-A signaling through VEGFR2 (Hiratsuka et al., 2005; Shibuya, 2006). On the other hand, NRP1 is a single-pass non-tyrosine kinase transmembrane receptor that stimulates angiogenesis and tip cell formation via VEGFR2 dependent and independent mechanisms (Fantin et al., 2013, 2015; Gelfand et al., 2014; Aspalter et al., 2015). Interestingly, NRP1 is expressed in lymphatic valves and promotes valve formation together with PlexinA1, via interactions with semaphorin3A (Bouvree et al., 2012). We have recently reported (Zhang et al., 2018) that mice with endothelial cell-specific deletions of both *Flt1* and *Nrp1*

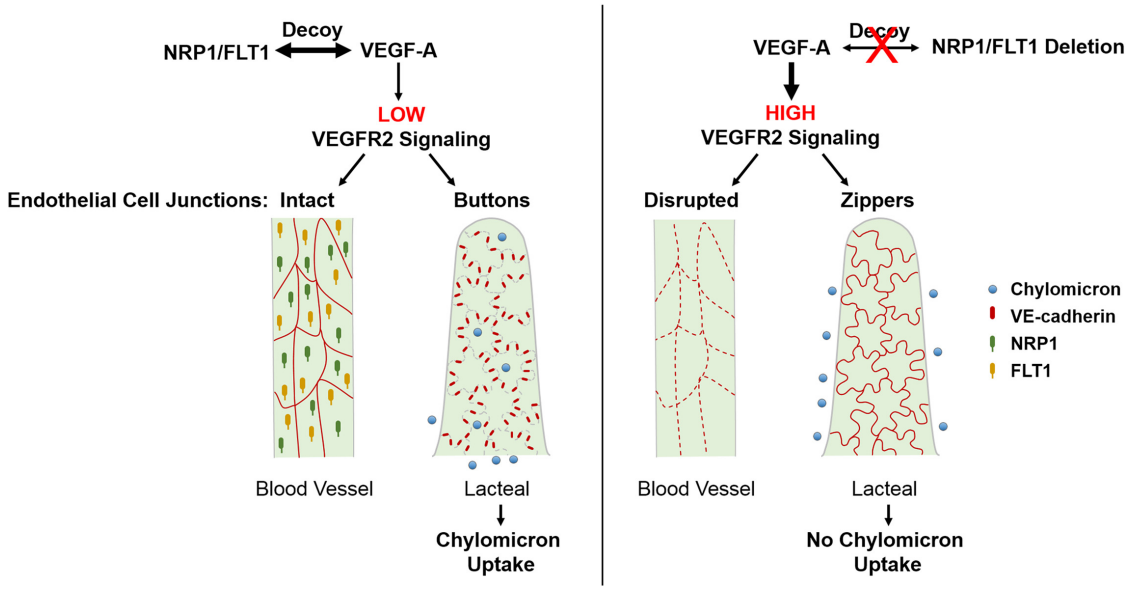

FIGURE 2 | Regulation of endothelial cell-cell junctions by VEGF-A/VEGFR2 signaling in intestinal villi. VEGF-A bioavailability for VEGFR2 is limited due to VEGF-A binding to FLT1 and NRP1 which are only highly expressed in BECs. This results in continuous junctions in BECs and discontinuous buttons in LECs that allow for lacteal chylomicron uptake. Increased levels of VEGF-A or deletion of *Nrp1 and Flt1* in BECs leads to upregulated signaling through VEGFR2, which disrupts BEC junctions and, opposingly, leads to lacteal junction transformation from buttons to zippers. As a result, chylomicron uptake by lacteals is inhibited, rendering mice resistant to diet-induced obesity. BEC, blood endothelial cell; LEC, lymphatic endothelial cell; NRP1, neuropilin1; FLT1, Fms-related tyrosine kinase 1 (or VEGFR1).

had reduced lipid absorption into plasma and were resistant to high fat diet-induced obesity. Mechanistically, endothelial deletion of *Flt1* and *Nrp1* led to enhanced VEGFR2 signaling and induced formation of zipper junctions in intestinal lacteals that inhibited chylomicron uptake. Similarly, increasing VEGFR2 signaling via VEGF-A stimulation promoted zippering of lacteal junctions. This stands in contrast to blood capillaries, where increased VEGFR2 signaling opens BEC junctions. Conversely, transient inhibition of VEGFR2 signaling in the double mutant mice could rescue button junctions and chylomicron uptake. As expected, LEC-specific deletion of *Nrp1* and *Flt1* receptors did not affect lacteal junctions or weight gain in mice on a high fat diet. Taken together, our results point to a cell non-autonomous effect on LEC junctions, mediated by NRP1 and FLT1 expressed by villus BECs. These data show that elevating VEGF-A/VEGFR2 signaling induces lacteal junction zippering, and that FLT1 and NRP1 in BECs function as VEGF-A decoys to antagonize this effect (**Figure 2**). Developmentally, VEGF-A/VEGFR2 signaling together with VEGF-C/VEGFR3 provides prenatal LEC growth signals (Nagy et al., 2002; Wirzenius et al., 2007; Dellinger et al., 2013). However, postnatal lacteal LECs must switch from a growth state with zipper junctions to a functional state with button junctions to allow chylomicron uptake and efficient nutrition of the newborn mouse. This may entail VEGF-A antagonization by NRP1 and FLT1, thereby allowing lacteal junction maturation and chylomicron uptake after birth. This process is likely to be initiated by activation of the chylomicron processing proteins MTP and ApoB at birth (Zhang et al., 2018), which leads to upregulation of FLT1 expression on BECs via ApoB (Avraham-Davidi et al., 2012).

Collectively, these findings identify a novel role for VEGF-A/VEGFR2 signaling in controlling LEC junction remodeling and uptake in intestinal lacteals, although the molecular basis of this effect is still unclear. Interestingly, treatment of wildtype mice with the ROCK inhibitor Y27632 promoted LEC junction zippering and inhibited chylomicron uptake into lacteals (Zhang et al., 2018). This raises the possibility that VEGFR2 signaling in LECs induces junction remodeling by inhibiting RhoA-ROCK signaling-mediated cytoskeletal rearrangement. Signaling through the VEGFR2 tyrosine residue Y949 is another pathway that may mediate VEGF-A-induced lacteal junction zippering. Evidently, further studies are necessary to elucidate the cell autonomous roles of VEGFR2 and downstream signaling pathways that regulate LEC junction remodeling. Finally it would be of interest to understand the opposing effects of VEGF-A on blood and lymphatic vasculature and to identify effectors that regulate the morphological status of lacteal junctions.

DLL4

Delta-like 4 is a major canonical Notch ligand expressed in vascular endothelial cells. DLL4/Notch signaling interplays with many other signaling pathways, such as VEGF-A/VEGFR2 and VEGF-C/VEGFR3 pathways, and has been shown to be essential for sprouting angiogenesis and vascular differentiation under various physiological and pathological conditions (Hellstrom et al., 2007; Lobov et al., 2007; Siekmann and Lawson, 2007; Suchting et al., 2007; Benedito et al., 2012). DLL4 is also highly expressed in the lymphatic endothelium. Genetic inactivation of *Dll4* in LECs leads to lacteal regression accompanied by "button-to-zipper" transformation of lacteal junctions and

reduced lipid uptake and transport capacity (Bernier-Latmani et al., 2015). This underlies, at least in part, the decreased fat accumulation and improved metabolic parameters observed in HFD-fed mice after DLL4 blockage (Fukuda et al., 2012). The cardioprotective peptide adrenomedullin and its signaling partners Calcrl and RAMP2 critically regulate lymphangiogenesis during development or after myocardial infarction (Fritz-Six et al., 2008; Jin et al., 2008; Klein and Caron, 2015; Trincot et al., 2019), while Calcrl has been identified as an important upstream regulator of DLL4-Notch signaling (Davis et al., 2017). Through controlling DLL4 expression in LECs, Calcrl-adrenomedullin signaling is essential for maintenance of proper junction organization in lacteals, thereby contributing to lipid uptake and inflammation resolution (Davis et al., 2017). As *Dll4* deletion can enhance VEGFR2 signaling (Williams et al., 2006; Suchting et al., 2007), it is possible that the "button-to-zipper" conversion phenotype in *Dll4-null* lacteals is a result of increased LEC VEGFR2 signaling, as discussed above. The exact crosstalk between Notch and VEGFR2 and its implications in LEC junction remodeling remain to be further addressed.

VEGF-C/VEGFR3 Signaling

Vascular endothelial growth factor-C signaling through VEGFR3 is essential for lymphangiogenesis. After the first PROX1-positive LEC progenitors egress from the anterior cardinal vein in mouse embryos at ∼E10.5, VEGF-C/VEGFR3 signaling critically regulates a variety of LEC functions, such as proliferation, migration, differentiation and apoptosis, thereby contributing to prenatal and postnatal expansion and maintenance of the lymphatic vasculature (Secker and Harvey, 2015; Petrova and Koh, 2018). The VEGF-C coreceptor NRP2 modulates lymphatic vessel sprouting together with VEGFR3 (Xu et al., 2010). VEGFR3 signaling also contributes to blood vessel growth (Siekmann and Lawson, 2007; Tammela et al., 2008, 2011; Zarkada et al., 2015) and restrains vascular permeability by limiting VEGF-A/VEGFR2 signaling (Heinolainen et al., 2017). Yet little is known about the role of VEGFR3 signaling in junction dynamics in LECs. *In vitro* studies suggested that stimulation with VEGF-C or the VEGFR3 selective activator VEGF-C156S, slightly decreases transendothelial electrical resistance in cultured LEC monolayers (Breslin et al., 2007). Moreover, systemic delivery of VEGF-C via adenovirus promotes colorectal tumor-associated lymphangiogenesis, while reducing VE-cadherin expression and lymphatic endothelial barrier integrity (Tacconi et al., 2015). However, recent studies have argued that VEGF-C signaling through VEGFR3 does not affect LEC junction organization either *in vivo* or *in vitro* (Zhang et al., 2018). In addition, analysis of newly formed lymphatics following airway infection also suggests that inflammation-associated LEC junction remodeling does not require VEGFR3 signaling (Yao et al., 2012). Further studies will be required for a clear understanding of the roles of VEGFR3 signaling in LEC junction organization in developmental and disease conditions. Additionally, VEGFR3 and VEGFR2 form heterodimers in both BECs and LECs upon VEGF-A or VEGF-C stimulation (Dixelius et al., 2003; Nilsson et al., 2010), and previous studies have underlined the contribution of VEGFR2/VEGFR3 heterodimers to VEGF-C-driven tumor and corneal lymphangiogenesis and pulmonary lymphangiectasia (Yao et al., 2014; Durre et al., 2018). So far no data are available regarding the role of VEGFR2/VEGFR3 dimerization in the regulation of LEC junctions.

CONCLUSION

Since functionally specialized junctions in the lymphatic endothelium were described about a decade ago, their organization and critical contribution to the uptake and transport function of lymphatic vessels have been widely recognized. However, the understanding of LEC junctions is still very limited, and many questions remain unanswered. For example, the molecular dynamics of button formation and maturation during development are poorly understood. This process is most likely to involve cytoskeletal rearrangements driven by Rho small GTPases and other effectors. In fact, the exact molecular architecture of adherens junctions and tight junctions in LEC junctions is virtually unexplored. Whether gap junctions are present at buttons and zippers and how cells communicate with each other in initial and collecting lymphatic vessels are also unknown. LECs undoubtedly share similarities with BECs in the assembly and dynamics of zipper junctions; therefore many of the intracellular partners and associated signaling components of junctional proteins identified in BECs may play similar roles in LECs. However, it is also very likely that certain LEC specific factors contribute to the unique organization and regulation of buttons, given the distinct roles of VEGF-A in opening BEC junctions and zippering LEC junctions. The involvement of these regulatory mechanisms and factors in lymphatic function and pathophysiology of inflammation, obesity and lymphedema remains to be clarified. Increasing our ability to modulate LEC junctions independently of the blood-endothelial barrier will allow to develop novel strategies against diseases characterized by dysregulation of lipid metabolism, lymph circulation and immune reactions.

AUTHOR CONTRIBUTIONS

FZ, GZ, and AE wrote the manuscript. SY contributed to figure preparation.

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Conflict of Interest: AE and FZ are inventors on a patent application (United States Provisional Application No. 62/873,288) submitted by the Yale University that covers compositions and methods for inhibiting dietary lipid uptake.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Systems Biology Will Direct Vascular-Targeted Therapy for Obesity

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Healthy adipose tissue expansion and metabolism during weight gain require coordinated angiogenesis and lymphangiogenesis. These vascular growth processes rely on the vascular endothelial growth factor (VEGF) family of ligands and receptors (VEGFRs). Several studies have shown that controlling vascular growth by regulating VEGF:VEGFR signaling can be beneficial for treating obesity; however, dysregulated angiogenesis and lymphangiogenesis are associated with several chronic tissue inflammation symptoms, including hypoxia, immune cell accumulation, and fibrosis, leading to obesity-related metabolic disorders. An ideal obesity treatment should minimize adipose tissue expansion and the advent of adverse metabolic consequences, which could be achieved by normalizing VEGF:VEGFR signaling. Toward this goal, a systematic investigation of the interdependency of vascular and metabolic systems in obesity and tools to predict personalized treatment ranges are necessary to improve patient outcomes through vascular-targeted therapies. Systems biology can identify the critical VEGF:VEGFR signaling mechanisms that can be targeted to regress adipose tissue expansion and can predict the metabolic consequences of different vascular-targeted approaches. Establishing a predictive, biologically faithful platform requires appropriate computational models and quantitative tissue-specific data. Here, we discuss the involvement of VEGF:VEGFR signaling in angiogenesis, lymphangiogenesis, adipogenesis, and macrophage specification – key mechanisms that regulate adipose tissue expansion and metabolism. We then provide useful computational approaches for simulating these mechanisms, and detail quantitative techniques for acquiring tissue-specific parameters. Systems biology, through computational models and quantitative data, will enable an accurate representation of obese adipose tissue that can be used to direct the development of vascular-targeted therapies for obesity and associated metabolic disorders.

Keywords: systems biology, obesity, angiogenesis, lymphangiogenesis, adipose tissue vasculature, quantitative flow cytometry, computational modeling, VEGFR

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INTRODUCTION

The prevalence of obesity has tripled since 1975, affecting over 650 million adults worldwide (World Health Organization, 2018). Obesity treatment includes diet and lifestyle changes, physical exercise, and/or surgical procedures (Cao, 2014). However, obesity treatment outcomes are often complicated by accompanying metabolic disorders (Muñoz-Garach et al., 2016;

Acharya and Shukla, 2018; Neeland et al., 2018). For instance, metabolically unhealthy obesity is associated with complications such as insulin resistance and type 2 diabetes. Patients with metabolically unhealthy obesity are at a higher risk for chronic disease and death compared to metabolically healthy obese individuals (Durward et al., 2012; Neeland et al., 2018). Alarmingly, the metabolically unhealthy obese phenotype comprises at least 70% of the obese population. Therefore, treating obesity while improving metabolic health is critical for protecting these obese individuals from future health problems (Blüher, 2010; Lemoine et al., 2013; Cao, 2013, 2018; Fukumura et al., 2016; Incio et al., 2018).

The dependency of adipose tissue growth and metabolism on adipose vasculature indicates that vascular-targeted therapies could be used to treat obesity and obesity-associated metabolic disorders (Cao, 2014; Escobedo and Oliver, 2017). Blood and lymphatic vessels formed via angiogenesis and lymphangiogenesis, respectively, are critical for maintaining tissue oxygenation, removing waste products, and regulating adipose tissue expansion and inflammatory responses (Chakraborty et al., 2019). Impaired vasculature can cause tissue hypoxia, leakage of lipids, and chronic inflammation, which are key factors that drive pathological transitions from metabolically healthy to metabolically unhealthy obesity (Corvera and Gealekman, 2014). Restoring vascular health requires a careful balance because upregulation of pro-angiogenic signaling has the downside of accelerating adipose tissue growth during obesity and overexpression of pro-lymphangiogenic factors can increase dietary lipid absorption in the intestinal lymphatics. Therefore, designing a vascular-targeted therapeutic strategy that both reduces adipose tissue mass and also prevents developing adverse metabolic outcomes would require precise control of angiogenic/lymphangiogenic signaling (Nijhawans et al., 2020).

VEGF:VEGFR SIGNALING REGULATE ANGIOGENESIS, LYMPHANGIOGENESIS, ADIPOGENESIS, AND MACROPHAGE SPECIFICATION

Vascular development is primarily regulated by vascular endothelial growth factors (VEGFs) and VEGF receptors (Cao, 2014). VEGFs were initially known as vascular permeability factors that promote tumor vessel permeability (Senger et al., 1983) and, later, they were recognized as vascular growth factors that regulate angiogenesis and lymphangiogenesis. The VEGF family ligands (VEGF-A, -B, -C, -D, and placental growth factor PlGF) selectively bind to three membrane-bound tyrosine kinase receptors, VEGFR1, VEGFR2, and VEGFR3 (**Figure 1A**). Dimerization of VEGFRs forms either homo- or hetero-dimers, enabling downstream signaling that initiates hallmark angiogenic and lymphangiogenic responses, including cell migration, proliferation, survival, and matrix reorganization (Karkkainen and Petrova, 2000; Ferrara et al., 2003; Tammela et al., 2008; Koch and Claesson-welsh, 2012; Karaman et al., 2018). In this section, we discuss how the VEGF:VEGFR system is involved in obesity, with regards to its role in angiogenesis, lymphangiogenesis, adipogenesis, and macrophage specification, and the current state of development of VEGF/VEGFR-targeted therapies for treating obesity.

VEGF/VEGFR: Angiogenesis and Lymphangiogenesis

VEGFR1 and VEGFR2 are the primary angiogenic receptors, with different ligand-induced signaling responses (Rahimi, 2006) which makes knowing the distribution of VEGFR1/VEGFR2 on endothelial cells important for predicting angiogenic outcomes. Here, we highlight six functional differences between VEGFR1 and VEGFR2 that underscore this importance: (1) VEGF-A binds to VEGFR1 with ∼10 times stronger affinity than to VEGFR2 (Cunningham et al., 1999; Von Tiedemann and Bilitewski, 2002; Mamer et al., 2017). (2) However, VEGFR1 initiates lower VEGF-A induced proliferation as measured by 3H-thymidine assays, (3) lower migratory activity as seen in Boyden chamber assays, and (4) less actin reorganization as determined by imaging membrane ruffling, when comparing VEGFR1-transfected to VEGFR2-transfected endothelial cells (Waltenberger et al., 1994). (5) Furthermore, VEGFR1 and VEGFR2 regulate endothelial specification in sprouting angiogenesis: high VEGFR2 is preferentially found on tip cells, the leading cells of an angiogenic sprout, while (6) high VEGFR1 is preferentially found on stalk cells, the trailing cells of an angiogenic sprout (Gerhardt et al., 2003; Bentley et al., 2008; Blanco and Gerhardt, 2013). Hence, VEGFR2 has been established as the major pro-angiogenic VEGFR (Apte et al., 2019) and VEGFR1 is believed to modulate angiogenic signaling and under certain conditions competitively inhibit VEGFR2 signaling. Indeed, the VEGFR1 modulatory versus inhibitory duality lies in its ability to prevent excessive vascular growth in embryos while promoting angiogenesis in cancer, ischemic, and adipose tissues (Carmeliet et al., 2001; Ho and Fong, 2015; Robciuc et al., 2016; Lacal and Graziani, 2018). The dependence of angiogenic outcomes on VEGFR competition makes quantifying the VEGFR distribution a crucial step for predicting angiogenic behaviors in adipose tissue.

It is also prudent to consider VEGFR heterodimers in adipose tissue angiogenesis, as both VEGFR1 and VEGFR3 can heterodimerize with VEGFR2 on vascular endothelial cells, affecting the angiogenic response. Whether VEGFRs preferentially form homo- or hetero-dimeric complexes is an unresolved matter; however, simulations estimate that 10–50% of VEGFRs are heterodimeric. Furthermore, the VEGFR density is predicted to regulate homo- vs. hetero-dimerization: a ratio of 10:1 VEGFR1:R2 or 10:1 VEGFR2:R1 inhibits homodimerization of the less-abundant receptor (R2/R2 or R1/R1, respectively) (Mac Gabhann and Popel, 2007). Functionally, VEGFR1/R2 heterodimerization is significant because it increases VEGFR1 phosphorylation and leads to significantly stronger ligand-induced migration than either VEGFR2/R2 or VEGFR1/R1 homodimerization (Huang et al., 2001; Cudmore et al., 2012). VEGFR2/R3 heterodimers on vascular endothelial cells also result in stronger ligand-induced

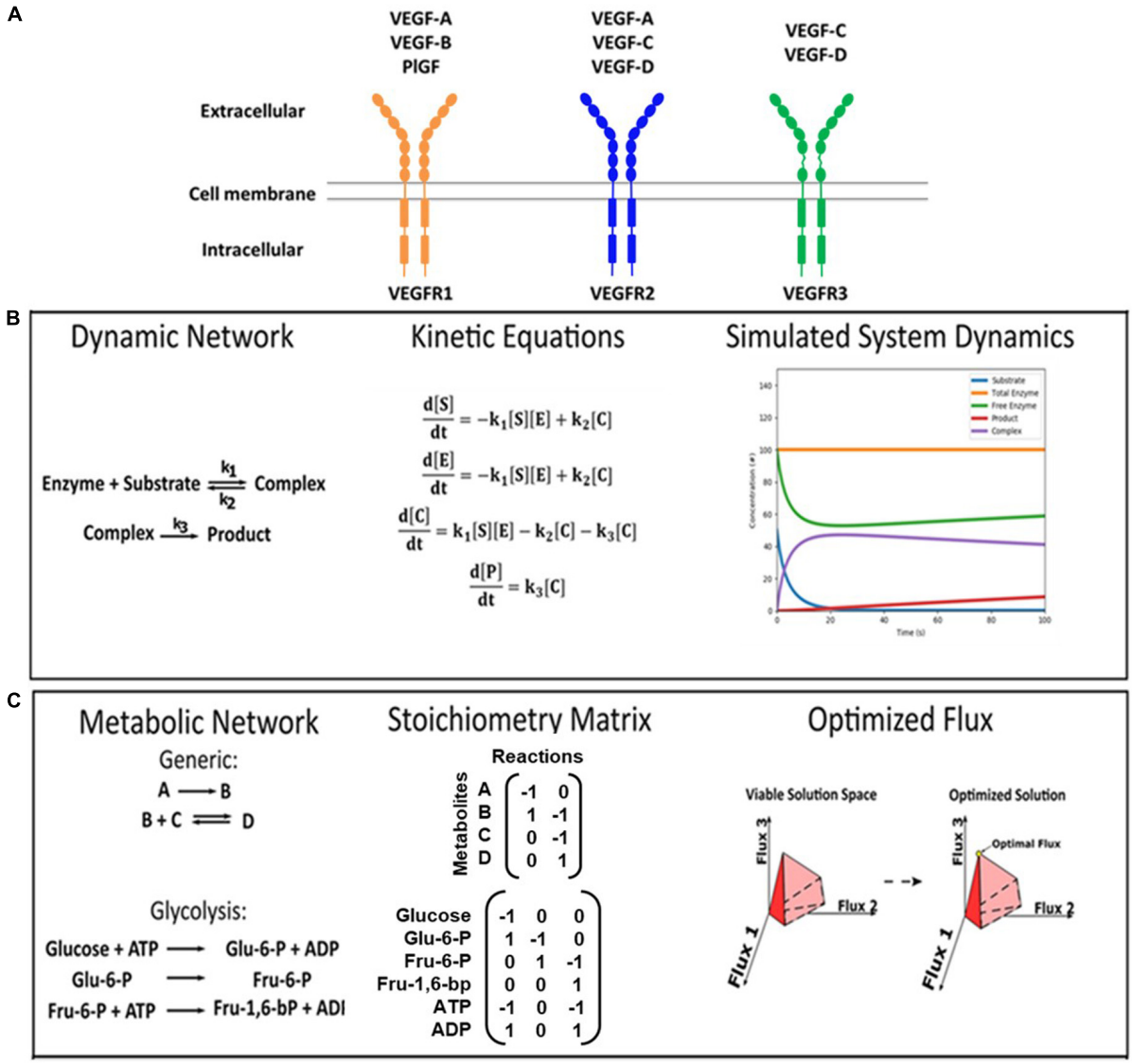

FIGURE 1 | VEGF ligand/receptor interactions and modeling approaches. **(A)** This schematic summarizes the canonical VEGF:VEGFR interactions. VEGF-A binds to both VEGFR1 and VEGFR2. VEGF-B and PlGF exclusively bind to VEGFR1. VEGF-C and VEGF-D bind to both VEGFR2 and VEGFR3. VEGFR2 on vascular endothelial cells primarily induces pro-angiogenic functions, and VEGFR3 on lymphatic endothelial cells primarily induces pro-lymphangiogenic functions. **(B,C)** Formulations of kinetic deterministic and constraint-based models. **(B)** An example of a dynamic enzymatic network was made into kinetic equations, and the output was simulated. **(C)** Examples of inputs for a generic network and glucose metabolism are shown, as well as a visual representation of the output.

angiogenic sprouting than when the heterodimerization was prevented by VEGFR3 antibodies (Nilsson et al., 2010). Overall, VEGFR heterodimerization is a prevalent phenomenon that can upregulate endothelial cell migration and angiogenic sprouting relative to homodimers; thus, accounting for heterodimerization can lead to improved predictions of VEGF-induced cell responses.

Another key vascular process is lymphangiogenesis, which is primarily driven by VEGFR3 activation on lymphatic endothelial cells. VEGFR3 is necessary for lymphangiogenesis as it activates PROX1, the master transcription factor regulating both differentiation of lymphatic endothelial cells from their progenitors and subsequent maintenance of their lymphatic identity (Jha et al., 2018). VEGFR2 is also indispensable for lymphangiogenesis, as inhibition of VEGFR2/R3 heterodimers significantly reduces ligand-induced VEGFR3 phosphorylation (Alam et al., 2004) and completely blocks VEGF-C-induced lymphatic sprouting (Nilsson et al., 2010). Additionally, the VEGFR2 antibody (DC101) prevents the onset of lymphatic vessel growth in adult mice (Goldman et al., 2007). As we have discussed, the relative amounts of VEGFR2 and VEGFR3 will affect the formation of VEGFR2/R3 heterodimers on lymphatic endothelial cells; therefore, quantitative characterization of the distributions of VEGFRs on lymphatic endothelial cells is

similarly required to accurately predict the lymphangiogenic responses of adipose tissue.

VEGF/VEGFR: Isoforms and Signaling Crosstalk

Many VEGF isoforms initiate differential angiogenic and lymphangiogenic responses, which increases the complexity of VEGF:VEGFR signaling. VEGF-C and -D are matured by proteolytic processing, which is necessary for their interaction with VEGFR2 (Joukov et al., 1997; Achen et al., 1998; Shin et al., 2008). Unlike VEGF-C (-D) isoforms, the isoforms of VEGF-A, -B, and PlGF are derived from alternative mRNA splicing; these isoforms have been reviewed in greater detail elsewhere (Weddell and Imoukhuede, 2018; Bowler and Oltean, 2019). For brevity, we discuss only the splice variants of VEGF-A. VEGF-A_{165} is the most abundant isoform; other known VEGF-A isoforms include VEGF-A_{111}, VEGF-A_{121}, VEGF-A_{145}, VEGF-A_{148}, VEGF-A_{183}, VEGF-A_{189}, and VEGF-A_{206} (Bowler and Oltean, 2019). These VEGF-A isoforms differ in their binding affinities with the VEGFR co-receptors and the extracellular matrix (ECM) components, differentially regulating VEGFR binding and activation (Park et al., 1993; Robinson and Stringer, 2001; Jakobsson et al., 2006; Sarrazin et al., 2011; Nieminen et al., 2014; Sarabipour and Mac Gabhann, 2018; King and Hristova, 2019). Therefore, anti-VEGF studies conducted using pan-VEGF antibodies without distinguishing the various isoforms could be potentially less accurate.

There are also VEGF-A isoforms termed VEGF-$A_{(xxx)a}$ and VEGF-$A_{(xxx)b}$ which are generated via exon 8 splicing and designated as pro-angiogenic and anti-angiogenic, respectively (Kopp et al., 2006). These VEGF-$A_{(xxx)a/b}$ splice variants are discussed by several reviews (Ladomery et al., 2007; Biselli-Chicote et al., 2012; Hilmi et al., 2012). Here, we highlight three VEGF-A_{165b} properties that are implicated in obesity: (1) VEGFR binding: VEGF-A_{165b} competes with VEGF-A_{165a} for VEGFR binding in pathological conditions (Woolard et al., 2004; Mamer and Wittenkeller, 2016a,b; Clegg and Mac Gabhann, 2017). Thus, adipose tissue angiogenesis can be inhibited by increasing the VEGF-A_{165b}:VEGFR2 binding. (2) VEGFR activation: VEGF-A_{165b} induces only weak VEGFR phosphorylation and downstream signaling in ischemia (Clegg et al., 2017; Ganta et al., 2017); indeed, markedly lowered angiogenic activity has been observed in VEGF-A_{165b}-high visceral adipose tissue compared to VEGF-A_{165b}-low subcutaneous adipose tissue (Ngo et al., 2014). (3) Systemic VEGF-A_{165b} upregulation: obese patients show systemic upregulation of VEGF-A_{165b} compared to lean patients, which is reduced after significant weight loss following bariatric surgery (Ngo et al., 2014). Thus, systemic upregulation of VEGF-A_{165b} might be a prognostic marker for weight loss outcomes. However, it remains unclear whether this VEGF-A_{165b} upregulation in the systemic circulation and visceral adipose tissue is a compensatory mechanism to reduce the excessive growth of adipose tissue or a pathological mechanism leading to vascular disease.

A recent study of VEGF-$A_{(xxx)b}$ mRNA *in vivo* further complicates this story: VEGF-$A_{(xxx)b}$ mRNA was undetectable in RNA-seq data from approximately 7,000 samples from 50 tissues (Bridgett et al., 2017). They suggested that studies of VEGF-$A_{(xxx)b}$ may be confounded by variants that contain both "a" and "b" C-terminal sequences, termed VEGF-A_x. Such VEGF-A_x variants would be detected by VEGF-$A_{(xxx)b}$ antibody (Eswarappa et al., 2014) which was used in the previous study on VEGF-$A_{(xxx)b}$ in obesity (Ngo et al., 2014). VEGF-A_x also has putative anti-angiogenic effects. Thus, additional studies are needed to determine whether the systemic VEGF-A_{165b} upregulation in obese patients is, in fact, VEGF-A_{165x} upregulation and to determine the functional consequences of these anti-angiogenic isoforms in adipose tissue.

VEGFRs also exist in soluble forms that competitively bind and sequester VEGFs from the membrane-bound VEGFRs. For instance, soluble VEGFR1 is a high-affinity receptor for VEGF-A and thereby downregulates the pro-angiogenic VEGFR2 signaling (Shibuya, 2011). Soluble VEGFR3 can similarly inhibit lymphangiogenesis by preventing VEGF-C/D from binding membrane-bound VEGFR3 (Mäkinen et al., 2001). The anti-VEGF effect of soluble VEGFRs could be a critical factor that alters the outcomes of a VEGF:VEGFR signaling system.

VEGF:VEGFR signaling is further complicated by non-canonical signaling involving the platelet-derived growth factor family (PDGFs). The angiogenic signaling canon describes uni-family interactions: VEGF:VEGFR signaling promoting healthy vascular formation (Tammela et al., 2008; Sina et al., 2011; Simons, 2012; Dellinger et al., 2013; Simons et al., 2016) and PDGF:PDGFR signaling on perivascular cells supporting blood vessel function (Kazlauskas, 2017). Recent discoveries of PDGF binding to VEGFR2 (Mamer et al., 2017; Kazlauskas, 2018), VEGF-A:PDGFR signaling (Ball et al., 2007; Pennock and Kazlauskas, 2012; Pennock et al., 2014), and VEGF regulation of PDGFRs (Chen et al., 2015) reveal that the canonical, uni-family paradigm insufficiently describes vascular development. It would be worthwhile to study whether PDGF contributes to VEGFR signaling in obesity, as (1) PDGF is secreted by macrophages, pre-adipocytes, and adipocytes; (2) the concentration of PDGF ligands in serum and the expression of PDGF mRNA in adipose tissue cells were both shown to increase by $\sim$1.5$\times$ in obese mice; and (3) anti-PDGF antibody reduced endothelial tube formation compared to untreated control (Pang et al., 2008). As such, non-canonical signaling should also be considered when elucidating angiogenic mechanisms in obesity.

VEGF/VEGFRs in Adipogenesis and Obesity

Adipose tissue angiogenesis is driven by either local hypoxia or endocrine signaling (Silvia, 2014). Under hypoxia, rapid adipose tissue expansion activates hypoxia inducible factor (HIF)-regulated genes, inducing the transcription, translation, and secretion of pro-angiogenic factors like VEGFs and PDGFs in both adipocytes and endothelial cells. In the endocrine mechanism, circulating insulin, growth factors (e.g., VEGFs and FGFs), and nutrients (e.g., amino acids and glucose) activate adipocyte transcriptional pathways (Um et al., 2006), among which mTOR activation is the pivotal switch that triggers local

adipose HIF-1 and VEGF production (Jia et al., 2015). Indeed, multiple signaling pathways help coordinate these adipose tissue angiogenesis mechanisms, as detailed in several reviews (Cao, 2013; Escudero et al., 2017; Korybalska, 2018) despite the many angiogenic regulators, VEGF:VEGFR signaling is accepted as the primary adipose angiogenic pathway (Sung et al., 2013). In white adipose tissue, upregulation of VEGF-A:VEGFR2 signaling can promote tissue vascularization, increasing oxygen supply and subsequently enhancing energy consumption (di Somma et al., 2020). In addition, VEGF-A causes vascular fenestration, one of the structural bases that cause vascular permeability (Kamba et al., 2006). VEGF-A:VEGFR2 signaling promotes adipose vascular permeability, which allows the release of free fatty acids from adipose tissue to the circulation when systemic energy is low during fasting (Asterholm et al., 2012). Taken together, VEGF:VEGFR signaling plays an important role in controlling adipose tissue function and energy metabolism through the modulation of the adipose vasculature.

Angiogenesis also supports adipose tissue expansion by providing a niche for preadipocyte recruitment and differentiation (Tang et al., 2008; Volz et al., 2016). Preadipocytes recruited to the vascular niche differentiate into mature adipocytes via adipogenesis, assisting existing non-proliferative adipocytes in storing glucose, lipids, and cholesterol. Newly formed adipocytes cluster around active angiogenic sprouts, as observed via live adipose tissue imaging (Nishimura et al., 2007). This intimate spatial association enables paracrine signaling that increases both adipogenesis and angiogenesis. Microvascular endothelial cells enhance adipogenesis by secreting insulin-like growth factors and fibroblast growth factors (FGF) (Volz et al., 2016). FGF-1 was shown to facilitate differentiation of human preadipocytes that was initiated by PPARγ (Hutley et al., 2004), which is the master transcriptional regulator of adipogenesis (Sarjeant and Stephens, 2012; Pellegrinelli et al., 2016). Reciprocally, preadipocytes and mature adipocytes secrete various pro-angiogenic factors, including VEGF-A and FGF-2, which permit further angiogenesis and adipose tissue expansion (Volz et al., 2016; Jiang et al., 2017). This endothelial-adipocyte crosstalk has been reviewed in further detail elsewhere (Volz et al., 2016). Altering either angiogenesis or adipogenesis will inevitably affect this paracrine loop, resulting in changes to both processes.

Impaired lymphangiogenesis is also implicated in the progression of obesity. The peripheral lymphatic vessels are required for lipid transport and immune cell clearance in adipose tissue. Impaired lymphatic vasculature, commonly seen in obesity, can leak lipid-containing lymph into peripheral tissue, inducing adipogenesis, subcutaneous fat deposition, and weight gain in mice (Escobedo et al., 2016; Escobedo and Oliver, 2017). Inactivating mutations in VEGFR3 in Chy mice induce lymphatic vessel leakiness and cause abnormal adipose tissue accumulation adjacent to these impaired vessels. Treating the Chy mice with an adenovirus encoding VEGF-C can stimulate lymphatic growth, suggesting VEGFR3 activation with an excess of its ligand is sufficient to overcome the lymphatic impairment (Karkkainen et al., 2001). Because VEGF-C can also stimulate vascular permeability via binding VEGFR2, this study further suggested that VEGFR3-specific ligand (VEGF-C156S) is a more attractive choice for therapeutically activating VEGFR3 and stimulating lymphangiogenesis. Thus, upregulating VEGFR3 activation could be useful for restoring lymphatic vessel integrity and reducing adipose tissue expansion. In addition, obese mice with leaky lymphatic vessels exhibited blunted immune cell trafficking toward lymph nodes, higher peak inflammatory responses, and delayed clearance of inflammatory responses when compared to lean mice with healthy vessels (Savetsky et al., 2015). The same study showed that VEGF-C (a VEGFR3 ligand) upregulated lymphangiogenesis, improved immune cell clearance, and decreased tissue inflammation, suggesting a therapeutic approach for alleviating adipose tissue inflammation in obesity. Altogether, repairing the impaired lymphatics can help reduce adipose tissue expansion and inflammation in obesity, which can be done by normalizing VEGFR3 activation.

Lacteals, the specialized lymphatic vessels adjacent to the small intestines, are important for absorbing digested dietary lipids and conveying the lipids to the circulation (Harvey, 2008; Jiang et al., 2019). Emerging evidence suggests that reducing lacteal permeability can inhibit lipid absorption during high-fat diet, additional work is needed to develop a therapy targeting the lacteals (Shew et al., 2018; Cifarelli and Eichmann, 2019). Lacteal permeability can be reduced via upregulating VEGF-A:VEGFR2 signaling, which transforms discontinuous button-like cell junctions to continuous zipper-like junctions, limiting lipid entry into the lacteals (Zhang et al., 2018). Thus, administration of VEGF-A or VEGFR1 inhibitors are promising therapeutic options for reducing lacteal permeability and lipid uptake. A further understanding of the lacteal zippering mechanism holds promise for the identification of therapeutic targets for obesity treatment.

Established *in vivo* obesity models should be used in further studies investigating the molecular and cellular mechanisms that link angiogenesis/lymphangiogenesis, adipogenesis, and related metabolic outcomes. High-fat diet (HFD)-fed mice are widely used models for obesity research because they exhibit (1) increased triglyceride storage via adipocyte hypertrophy, (2) increased overall body weight (Jo et al., 2009) and (3) metabolic dysfunction, such as insulin resistance, i.e., impaired control of blood glucose via insulin (Samuel et al., 2010). Future efforts should take advantage of these *in vivo* models to collect quantitative data on the molecular and cellular changes caused by VEGF/VEGFR-targeted therapies; such efforts will help identify key signaling mechanisms that affect obesity progression and response to therapies.

VEGF/VEGFR: Macrophage Function in Adipose Tissue

Pro-inflammatory macrophage accumulation is considered a major factor that perpetuates chronic inflammation in obese adipose tissue. HFD-induced obesity increased the number of pro-inflammatory M1 macrophages by 65-fold while increasing anti-inflammatory M2 macrophages by only six-fold (Fujisaka et al., 2009). M1 macrophages secrete pro-inflammatory molecules, like IL-6 and TNF-alpha, and are associated with

an impaired ability to control blood glucose by insulin, also known as insulin resistance. Conversely, M2 macrophages are more prevalent in lean adipose tissue, and they maintain insulin levels by releasing anti-inflammatory cytokines (Mills et al., 2000; Lumeng et al., 2007; Castoldi et al., 2016). Increasing the number of M2 relative to M1 macrophages may improve metabolic outcomes of obese patients, as an increased M1:M2 ratio is associated with metabolically unhealthy obesity (Fujisaka et al., 2009).

Macrophages are VEGFR-expressing cells and the ratio of M1/M2 macrophages in obese adipose tissue can be normalized by modulating the VEGF/VEGFR expression. For instance, VEGF-A antibody can reduce the number of M2 macrophages in the hypoxic adipose tissue tip region, which is the region of active adipose outgrowth characterized by a dense vascular network; these M2 macrophages are important for tissue outgrowth as they secrete matrix metalloproteinases (MMPs) (Cho et al., 2007). The evidence that VEGF-A regulates M2 abundance is further substantiated by a later study, which showed that overexpression of VEGF-A can drive the composition of macrophages in adipose tissue toward low-M1/high-M2, protecting mice from diet-induced insulin resistance (Elias et al., 2012). Meanwhile, VEGFR1 signaling in macrophages was also found to favor M2 macrophage accumulation, both during wound healing in diabetic mice (Okizaki et al., 2016) and during tumor growth in obese mice (Incio et al., 2016). Evidence from an *in vitro* study suggests that this increase in M2 macrophages could be a result of M1 macrophages differentiating into M2 macrophages, rather than M2 recruitment. This study found that VEGFR1 mRNA was highly expressed in M1 macrophages but only at low levels in M2 macrophages and that VEGF-A:VEGFR1 signaling stimulates M1 recruitment and subsequent differentiation of M1 macrophages into M2 macrophages (Wheeler et al., 2018). Altogether, VEGF-A and VEGFR1 promote the accumulation of anti-inflammatory M2 macrophages, which provides a useful therapeutic strategy to lower inflammation in obese adipose tissue. Future studies should investigate the differential VEGF:VEGFR signaling mechanisms underlying the recruitment and differentiation of M1 and M2 macrophages, such that their responses can be reliably predicted.

Besides the role of M1 and M2 macrophages in inflammation, they also differentially regulate the initiation and continuation of angiogenesis (Corliss et al., 2017). M2 macrophages secrete MMP-9, which degrades the extracellular matrix to initiate vascular sprouting into the adipose tissue tip region (Cho et al., 2007). Both M1 and M2 macrophages secrete pro-angiogenic growth factors, such as VEGFs and PDGFs, and it has been shown that the conditioned medium of either M1 or M2 macrophages promoted endothelial tube formation (Jetten et al., 2014). However, coculture of endothelial cells with M1 macrophages exhibited significantly less tube formation than M2-endothelial cell co-culture (Jetten et al., 2014). Tube formation is necessary for the continuation and stabilization of angiogenic sprouting; the coculture results indicate that the M1-endothelial cell contact is anti-angiogenic, although the M1 paracrine signaling is pro-angiogenic. The mechanisms for this anti-angiogenic behavior remain unclear; however, they could be related to macrophage-endothelial interactions seen in tumor metastasis, where macrophages disrupt endothelial tight junctions to facilitate cell extravasation (Kim et al., 2019). It is also possible that the high VEGFR1 on M1 macrophages sequesters VEGF-A from acting on endothelial cells in coculture. Future studies are needed to investigate how VEGFR signaling is regulated among M1, M2, and endothelial cells present in the same microenvironment.

Although macrophages are conventionally characterized by M1 and M2 subtypes, other classification schemes based on macrophage functions are useful for identifying the roles of macrophages in specific pathologies (Mosser and Edwards, 2008). As we have discussed, VEGF:VEGFR signaling in macrophages affects their recruitment and differentiation into different subtypes, but it is not clear whether these subtypes are strictly M1 or M2 macrophages. Moreover, it is notable that VEGFR-expressing cell subpopulations can express drastically different amounts of VEGFR proteins and generate heterogenous VEGF-induced cell responses, even though they are identified as the same cell type (Weddell and Imoukhuede, 2014). Thus, a VEGFR-based macrophage classification may help identify the subpopulations and functions of macrophages involved in adipose tissue inflammation, angiogenesis, and lymphangiogenesis. Future data on VEGFR distributions in adipose tissue macrophages are necessary.

Vascular-Targeted Therapies for Obesity

Emerging evidence has shown that adipose vasculature is a promising anti-obesity target (Daquinag et al., 2011; Cao, 2010, 2016). Indeed, since angiogenesis is required for adipose tissue expansion, researchers have already begun testing anti-angiogenic treatments to reduce adipose tissue mass and prevent obesity-associated metabolic disorders. A promising vascular-targeted drug, adipotide (a prohibitin-targeting peptide), has previously shown rapid weight loss in mice by specifically inducing apoptosis of adipose endothelial cells (Kolonin et al., 2004), however, its first human trial was discontinued in 2019 due to unspecified reasons.

Anti-VEGF and anti-VEGFR treatments have also largely been shown to be beneficial in treating obesity in pre-clinical obesity models (Cao, 2014; Leong-Poi, 2014). However, as we have discussed, angiogenesis and adipogenesis are concomitant processes, so there is a delicate balance that needs to be maintained. It is a reasonable concern that anti-angiogenic treatment could reduce adipogenesis to below what is necessary to relieve the metabolic stress of excessively hypertrophic adipocytes (Cao, 2007; Rutkowski et al., 2009; Lemoine et al., 2012; Haczeyni et al., 2018). Indeed, inhibiting angiogenesis can block necessary adipogenesis and two studies have independently shown that: (1) administration of VEGF-A inhibitors not only decreases angiogenic sprouting but also reduces adipogenesis in obese mice (Nishimura et al., 2007); (2) VEGFR2 antibody inhibits both vascular growth and adipogenesis in murine preadipocyte implants, similar to the effect of gene suppression of PPARγ (Fukumura et al., 2003). Thus, anti-angiogenic treatments might worsen metabolic dysfunction in metabolically unhealthy obese patients, who already show impaired angiogenesis and adipogenesis (McLaughlin et al., 2007; Kusminski et al., 2016;

Longo et al., 2019). Next, we focus on the consistent and discrepant outcomes from two anti-VEGFR and two anti-VEGF pre-clinical obesity studies; along with other relevant evidence, we discuss the considerations for future anti-VEGFR/VEGF therapies for obesity treatment.

Anti-VEGFR treatments have shown varied effects on weight loss and metabolic enhancement in HFD-fed and chow-fed models. Systemic anti-VEGFR2 treatment significantly slowed weight gain after 6 weeks of HFD, though no weight suppression was shown in the first 6 weeks (Tam et al., 2009). In comparison, systemic anti-VEGFR1 treatment showed no effect on body weight throughout the HFD treatment (Tam et al., 2009). Although targeting VEGFR1 did not suppress weight gain in obese mice, systemic anti-VEGFR1 treatment resulted in positive metabolic outcomes in chow-fed mice, which exhibited increased adipose vascular density, reduced adipocyte size, and enhanced metabolic energy expenditure (i.e., increased non-shivering thermogenic capacity). These features were observed over a 10-day anti-VEGFR1 treatment but not observed with anti-VEGFR2 treatment (Seki et al., 2018). Moreover, targeted gene deletion of endothelial VEGFR1 also led to a healthier metabolic profile, including lowered blood levels of free fatty acids, glycerol, cholesterol, fasting serum levels of glucose, and serum levels of insulin in HFD-fed mice (Seki et al., 2018). Altogether, both anti-VEGFR1 and anti-VEGFR2 have shown benefit in either weight loss or metabolic improvement; however, it is unclear what variables may affect individual responses to treatments.

Improved metabolic function was also observed in two pre-clinical anti-VEGF studies, although there were discrepancies in the weight loss outcomes (Honek et al., 2014; Wu et al., 2014). One study showed anti-VEGF treatment decreased serum glucose levels in HFD mice, suggesting that anti-VEGF treatment can mitigate HFD-induced insulin resistance (Honek et al., 2014). The other study showed that anti-VEGF treatment prevented HFD-induced hepatic glucose production but did not alter muscle glucose production, suggesting that anti-VEGF treatment can reduce serum glucose by inhibiting its production in the liver (Wu et al., 2014). However, Wu et al. (2014) did not observe a change in adipocyte size or mouse weight while Honek et al. (2014) observed reductions in both obesity measures. Honek et al. (2014) showed that anti-VEGF-induced adipocyte size reduction was greater in older mice, so the different outcomes regarding adipocyte size reduction may be explained by the age differences between the Wu and Honek studies (2-month vs. 7-month old, respectively). Future studies should also precisely control the dosage and duration of anti-VEGF treatment because prolonged and high-potency anti-VEGF is also known to trigger hypoxia and adverse systemic metabolic changes (Cao, 2013; Usui-Ouchi and Friedlander, 2019).

VEGFR3-targeted therapeutics for obesity have not yet been significantly explored, although the VEGFR3 activation must be maintained or upregulated to prevent lymphatic vessel leakiness, excess adipocyte hypertrophy, and insulin resistance (Cifarelli and Eichmann, 2019). A recent mouse model indicated that increasing the VEGFR3 on the lymphatic endothelium can restore impaired lymphangiogenesis in HFD-fed hyperglycemic conditions (Wu et al., 2018). However, VEGF-C/D:VEGFR3 signaling is also chemotactic, inducing macrophage accumulation and metabolic dysfunction. For instance, dermal production of VEGF-C gene induced pro-inflammatory macrophage accumulation and worsened metabolic parameters such as insulin resistance in transgenic obese mice (Karaman et al., 2015, 2016). Interestingly, adipose-specific overexpression of VEGF-D gene increased lymphatic vessel density in obese adipose tissue and prevented insulin resistance under HFD (Chakraborty et al., 2019). Taken together, upregulating VEGFR3 signaling is a potential therapeutic strategy to repair lymphangiogenesis in obesity and adipose-targeted administration of VEGFR3 ligands might be more beneficial than systemic approaches. Systemic and adipose-targeted administration of VEGF-C (-D) or other VEGFR3 agonists remain to be tested in obese models.

While vascular-targeted therapies are promising, their efficacy in treating obesity remains unclear in humans. Many factors should be considered in future studies targeting VEGF/VEGFR, including age, treatment duration, as well as the routes of administration (systemic or adipose-targeted). For example, systemic administration of VEGFR2 antibody could undesirably increase lacteal permeability, leading to higher lipid uptake from the small intestines to the lymphatics (Zhang et al., 2018). Other factors that we have discussed in this review, such as the abundance of membrane VEGFRs, the distributions of VEGFRs in different cell types, and VEGF splice variants, could also confound the therapeutic outcomes. We believe establishing biologically faithful platforms that predict how the VEGF:VEGFR signaling system regulates vascular density and metabolism in humans through systems biology could offer a mechanistic approach to develop effective therapeutics for obesity and obesity-related metabolic disorders.

SYSTEMS BIOLOGY PROVIDES A PLATFORM FOR INVESTIGATING VASCULAR SIGNALING AND METABOLISM IN ADIPOSE TISSUE

Systems biology offers a mechanistic understanding of complex biological networks through mathematical and computational modeling (Mac Gabhann and Popel, 2009; Weddell and Imoukhuede, 2018; Papin and Mac Gabhann, 2019). Systems biology approaches have led to discoveries of therapeutic biomarkers and have helped design effective vascular-targeted therapies (Weddell and Imoukhuede, 2018). We highlight two highly used systems biology modeling approaches that would be useful for designing better vascular-targeted therapies to treat obesity: (1) mass-action kinetic modeling and (2) constraint-based modeling. We also provide an overview of quantitative techniques for acquiring biologically faithful parameters for accurate modeling.

Mass-Action Kinetic Modeling

Simulations of VEGF/VEGFR targeted therapy in human patients can be made at the protein level using mass-action

kinetic modeling. This approach reconstructs a reaction network using ordinary differential equations (ODEs) (**Figure 1B**), which requires quantitative parameters on concentrations of ligands and receptors and kinetic reaction rates to simulate behaviors such as protein binding, trafficking, and phosphorylation over time (Loskot et al., 2019). Mass-action kinetic models of VEGF:VEGFR signaling are well-established in the field of angiogenesis (Weddell and Imoukhuede, 2018). These validated models provide a framework that can be easily adapted to represent obesity with adipose tissue-specific parameters. Indeed, several of these models are available in Systems Biology Markup Language, a file format that can be readily used by several software, including MATLAB's SimBiology and Wolfram's SystemModeler (SBML, 2016; Software Guide/SBML Software Matrix – SBML.caltech.edu). Reactions and parameters can be modified to test new model implementations in these software. There is also a well-developed community that can critique VEGF:VEGFR model results, which improves the quality of the model and leads to greater credibility in its findings (10 Simple Rules with Conformance Rubric | Interagency Modeling and Analysis Group). Therefore, adipose tissue-specific VEGF:VEGFR models would be in line with the field, while offering a platform for predicting the efficacy of VEGF/VEGFR targeted therapies in obese patients.

The model developed by Finley et al. (2015) provides a useful framework to simulate VEGF/VEGFR targeted therapy in obese adipose tissue. It is the most recent in a series of models investigating the pharmacokinetics of anti-VEGF therapy and was validated with published clinical measurements (Finley et al., 2011, 2013, 2015). The base model is an early benchmark model consisting of 40 ODEs that described receptor trafficking, VEGF secretion and transport, and association and dissociation of free VEGF-A_{121} and VEGF-A_{165} isoforms to membrane VEGFRs, NRP1, and the ECM (Stefanini et al., 2008). Finley et al. added drug, molecular, and lymphatic mechanisms that were relevant to breast cancer, expanding the base model to 161 ODEs. For simplicity, we highlight three features that would be relevant to an obesity adaptation: (1) interactions with the anti-VEGF drug Aflibercept, which could be directly used to model anti-VEGF therapy in obesity; (2) VEGF:soluble VEGFR1 binding, which sequesters VEGF from interacting with membrane VEGFRs, could downregulate membrane VEGFR activation and subsequent angiogenesis/adipogenesis in adipose tissue. Additional predictions of soluble VEGFR1 dynamics can be found in earlier computational studies (Wu et al., 2009, 2010); and (3) lymphatic drainage of macromolecules from tissue compartments into the blood compartment, which can model changes in lymphatic vessel integrity seen in obese and diabetic conditions (Savetsky et al., 2014; Scallan et al., 2015) and can be extended to clarify whether normalizing VEGFR3 signaling can restore lymphatic functions. Overall, adapting the Finley et al. (2015) model with adipose tissue-specific parameters (e.g., VEGFR concentrations on adipocytes and adipose stromal-vascular cells) will allow accurate prediction of the pharmacokinetics of anti-VEGF therapies in obese patients.

However, many other signaling mechanisms could affect the efficacy of vascular-targeted therapies, like the competition between pro- and anti-angiogenic VEGF-A isoforms, cross-family PDGF-VEGFR2 signaling, and paracrine signaling between the endothelium and adipocytes/macrophages, as we detailed earlier. To more accurately predict how obese adipose tissue responds to vascular-targeted therapies, future models should integrate the ODEs that have been developed to model: the impact of VEGFR heterogeneity on anti-VEGF efficacy (Weddell and Imoukhuede, 2014) the differential VEGF signal transduction by VEGFR homo- and hetero-dimers (Mac Gabhann and Popel, 2007; Mamer et al., 2019b), the significance of VEGF-A_{165b} in ischemic conditions (Chu et al., 2016; Clegg et al., 2017), the effect of cross-family PDGF-VEGFR binding on VEGFR occupancy (Mamer et al., 2017), the VEGFR mechanisms inducing macrophage migration (Weddell et al., 2018), the recruitment of macrophages to the lymphatic endothelium (Bianchi et al., 2015), and the pro- and anti-inflammatory signaling of adipose tissue macrophages (Díaz et al., 2009). Modeling these molecules, interactions, and cell responses would allow us to determine which mechanisms in adipose tissue could be targeted to normalize VEGF:VEGFR signaling in obesity.

Constraint-Based Modeling

There is also a significant need to understand and improve how vascular-targeted therapies affect metabolism. Mass-action kinetic modeling has only been applied by a few laboratories for modeling metabolic networks, as it is a challenge to acquire the reaction rates of all the interactions in these large and complex systems (Rizzi et al., 1997; Chassagnole et al., 2002; Yugi et al., 2005; Tran et al., 2008; Jamshidi and Palsson, 2010). In this regard, constraint-based modeling (CBM) has been an effective alternative to simulate a metabolic network and identify its regulatory mechanisms and dysfunctional pathways (Mardinoglu et al., 2013b; Bordbar et al., 2014). CBM does this via three steps: (1) Describing each pathway via stoichiometric equations: **Figure 1C** exemplifies one glycolysis reaction in stoichiometric form; (2) Incorporating experimental data on metabolite concentrations: unlike mass-action kinetic modeling, CBM requires only concentration measurements. Metabolite concentrations in pathological, healthy, and treatment conditions can be obtained from primary experiments, mining literature, or databases like the Human Protein Atlas [available from[1] Uhlen et al. (2015)] and NCBI's Gene Expression Omnibus (Edgar, 2002; Barrett et al., 2019). These concentrations are used as the initial conditions of the model; and (3) Simulating and analyzing the pathway: these final steps reveal the rates, also known as "fluxes", through each pathway. The stoichiometric equations built in Step 1 dictate how the model proceeds from the initial conditions set in Step 2, and comparing the flux profiles of different conditions in Step 3 will indicate which pathways are used at a greater or lesser flux (Orth et al., 2010).

[1] http://www.proteinatlas.org

In order to make a CBM for studying metabolic changes in obese adipose tissue in response to vascular-targeted therapies, we highlight a model that is available in SBML format and can be used as a benchmark model for studying pathogenesis and therapeutic strategies in adipocyte metabolism (Mardinoglu et al., 2013a)[2]. All known metabolic reactions of the human adipocyte were curated from prior models, experimental evidence, and database mining. This model ultimately involved 1809 genes which regulated 6160 reactions between 4550 metabolites. This model can be adapted to mechanistically explore the transcriptional pathways that connect VEGF:VEGFR signaling and adipocyte metabolism. For instance, this model can be used to reconstruct the VEGF-activated mitochondrial thermogenic activity and the browning of white adipocytes, and identify the key mitochondrial genes that regulate the VEGF expression in such processes (di Somma et al., 2020). In addition, metabolites were separated by organelle (e.g., cytosol, mitochondria, extracellular space) to obtain a compartment-specific reconstruction. While several other CBM approaches have investigated metabolism in physiological adipogenesis, obesity, and diabetes (Si et al., 2007; Coskun et al., 2010; Bordbar et al., 2011; Väremo et al., 2013; Montastier et al., 2015) we believe this model would provide the best benchmark for researchers to use for investigating the response to vascular-targeted therapies due to its thorough reconstruction of adipocyte metabolism and inclusion of VEGFR-related pathways.

The findings from the Mardinoglu et al. (2013a) model also provide an example of the information that can be gained through CBM (Mardinoglu et al., 2013a). Using various proteomics data from lean and obese adipose tissue (e.g., uptake/secretion rates of non-esterified fatty acids (NEFAs), triacylglycerol (TAG), and glucose), they simulated the dynamics of lipid droplet formation and acetyl-CoA production, the central metabolite of the mitochondria. They found that lean patients showed larger fluctuations in the size of lipid droplets than obese patients, as determined by comparing the flux through a reaction that synthesized lipid droplets from its constituent components (TAGs, NEFAs, cholesterol, phospholipids, etc.). They also found that obese patients exhibited lower acetyl-CoA production, which indicated potential mitochondrial dysfunction. They then extended the former investigation to include gene regulation using proteomic and genomic data from a clinical study on obese and non-obese individuals (SOS Sib Pair study), which allowed them to study global metabolic changes occurring in obesity. They predicted downregulated flux through all mitochondrial metabolic pathways in the obese patients of this study, further supporting the link between obesity and mitochondrial dysfunction (Bournat and Brown, 2010). Additionally, they identified three upregulated metabolites that could serve as biomarkers for obesity: (1) androsterone, a steroid hormone that may increase adipose tissue deposition (O'Reilly et al., 2014), (2) heparan sulfate proteoglycan (HSPG) degradation products, where HSPGs regulate the transport of lipoprotein lipase across endothelial cells to adipocytes (Saxena et al., 1991), and (3) ganglioside GM2, a molecule associated with insulin signaling in adipocytes (Tanabe et al., 2009). These findings demonstrated that CBM can identify specific changes in metabolic pathways and biomarkers, which can be extended to discover the potential adverse effects of vascular-targeted therapies on metabolic health and biomarkers for predicting patients' metabolic outcomes.

[2]metabolicatlas.org

Techniques for Acquiring Quantitative Parameters for Predictive Models

Quantitative data are necessary to construct and validate computational models that are predictive of vascular signaling in adipose tissue so that they can be used to improve vascular-targeted therapies for obesity. Binding kinetics parameters, such as VEGF:VEGFR association and dissociation rates, are necessary for mass-action kinetic modeling. Binding kinetics can be acquired with affinity assays, commonly using radiolabeling and surface plasmon resonance (SPR) (Mamer et al., 2019a). In addition, protein concentrations, such as serum VEGF concentrations and membrane VEGFR concentrations, are required for both CBM and mass-action kinetic modeling. Soluble protein concentrations (e.g., VEGF-A, soluble VEGFRs) are commonly obtained using quantitative enzyme-linked immunosorbent assays (ELISAs) and radiolabeling assays as well. However, there is a lack of a standardized method for obtaining quantitative data on membrane VEGFR concentrations, which are especially important for predicting VEGF/VEGFR signaling outcomes.

Quantitative flow cytometry (qFlow) is an emerging quantitative standard for measuring proteins on the plasma membrane (Chen et al., 2017; Wu and Finley, 2017). qFlow employs calibration beads to translate the mean fluorescence intensity values to fluorophore molecules per cell (Imoukhuede and Popel, 2011; Chen et al., 2017). VEGFR quantification has applied phycoerythrin (PE)-conjugated VEGFR antibodies to cells (Chen et al., 2018), and PE calibration beads are used to convert the PE signal intensity to the number of PE molecules per cell. The number of PE molecules per cell equals the number of PE-conjugated receptors per cell, due to the 1:1 protein/fluorophore ratio. Thus, applying qFlow with PE-conjugated antibodies allows absolute quantification of membrane VEGFR levels.

Furthermore, cell-by-cell analysis of qFlow data can unmask population heterogeneity when specific subpopulations depart from the "mean" behavior (Altschuler and Wu, 2010; Weddell and Imoukhuede, 2014). To identify and characterize such subpopulations in VEGFR-expressing cells (e.g., endothelial cells and macrophages), the qFlow measurements should be analyzed in 2 steps: (1) fitting the cell-by-cell VEGFR distribution to multiple log-normal distributions using mixture modeling and (2) optimizing the fitting by applying Bayesian information criterion (BIC) to identify the number of subpopulations without overfitting (Chen et al., 2018). BIC is preferred over Akaike information criterion (AIC) because AIC can report more false-positive subpopulations in qFlow data, increasing mixture model complexity with small sample sizes (Vrieze, 2012). Therefore, cell-by-cell qFlow analysis and mixture modeling should be used for identifying cell subpopulations exhibiting distinct receptor expression and VEGFR-mediated cell responses in adipose tissue.

qFlow has successfully measured membrane receptor levels on tumor xenograft-derived cells (Imoukhuede and Popel, 2014; Chen et al., 2018), primary mouse skeletal muscle endothelial cells under normal (Imoukhuede and Popel, 2012), and ischemic (Imoukhuede et al., 2013) conditions, and several *in vitro* cell lines (Chen et al., 2015; Weddell et al., 2018; Chen and Imoukhuede, 2019). To summarize some of the key qFlow findings, human umbilical vein endothelial cells (HUVECs) present 1800 ± 200 VEGFR1/cell, 4900 ± 400 VEGFR2/cell, and 2800 ± 400 VEGFR3/cell. These VEGFR concentrations on the membrane are regulated by VEGFs. A 24-h VEGF-A treatment increases the concentration of membrane VEGFR1 while decreasing membrane VEGFR2 on HUVECs; likewise, a 24-h VEGF-C treatment decreases the concentration of membrane VEGFR3 on in-vitro human lymphatic endothelial cells (Imoukhuede and Popel, 2011). The membrane VEGFR distributions have also been examined in the endothelium from healthy mouse skeletal muscle and tumor xenograft models, where endothelial cells stably present low membrane VEGFR2 (<1700 VEGFR2/cell) across normal and tumor xenografts (Imoukhuede and Popel, 2012, 2014; Chen et al., 2018) despite VEGF-A:VEGFR2 binding being the primary pro-angiogenic signaling pathway (Olsson et al., 2006). On the other hand, endothelial cells from breast tumor xenografts overexpress membrane VEGFR1 relative to HUVECs, presenting an average of 15,000 membrane VEGFR1/cell. Although it is still unclear why VEGFR1 overexpression is associated with pathologically active angiogenesis, qFlow measurements have enabled predictions of the outcomes of VEGFR1 overexpression in pathological angiogenesis and cell migration (Weddell and Imoukhuede, 2014, 2018; Mamer et al., 2017; Weddell et al., 2018). For example, computational modeling has predicted that tumor endothelial cells overexpressing membrane VEGFR1 contribute to the therapeutic resistance to anti-VEGF-A treatment because the VEGF-A ligands captured by membrane VEGFR1 can dissociate to increase free VEGF-A levels in tumors, counteracting the anti-VEGF-A treatment (Weddell and Imoukhuede, 2014). This model also predicted that the subpopulation of tumor endothelial cells expressing >35,000 VEGFR1/cell is a potential marker of therapeutic resistance to the anti-VEGF treatment (Weddell and Imoukhuede, 2014). This prediction was further supported by the clinical observation that VEGFR1 overexpression in tumor tissue was correlated with decreased overall survival in patients treated with the anti-VEGF agent bevacizumab (Weickhardt et al., 2015). This prediction was possible because of data from qFlow measurements of VEGFR levels on breast tumor xenograft endothelial cells. Thus, computational models can yield clinically relevant findings when coupled with qFlow data.

To date, there are minimal proteomic data on VEGFR concentrations on adipose-derived cells, even though adipose tissue is one of the highest VEGFR gene-expressing tissues (**Figure 2**). qFlow can be used to establish the VEGFR distribution in non-obese and obese adipose tissue and characterize adipose tissue cell heterogeneity through cell-by-cell analysis and mixture modeling. qFlow can also address the unmet need for a repository, similar to the GTEx portal (a genomic database), of quantitative protein concentrations in various tissues, particularly adipose tissue. Such a database would enable researchers across labs to contribute and acquire quantitative proteomic data to build systems biology models.

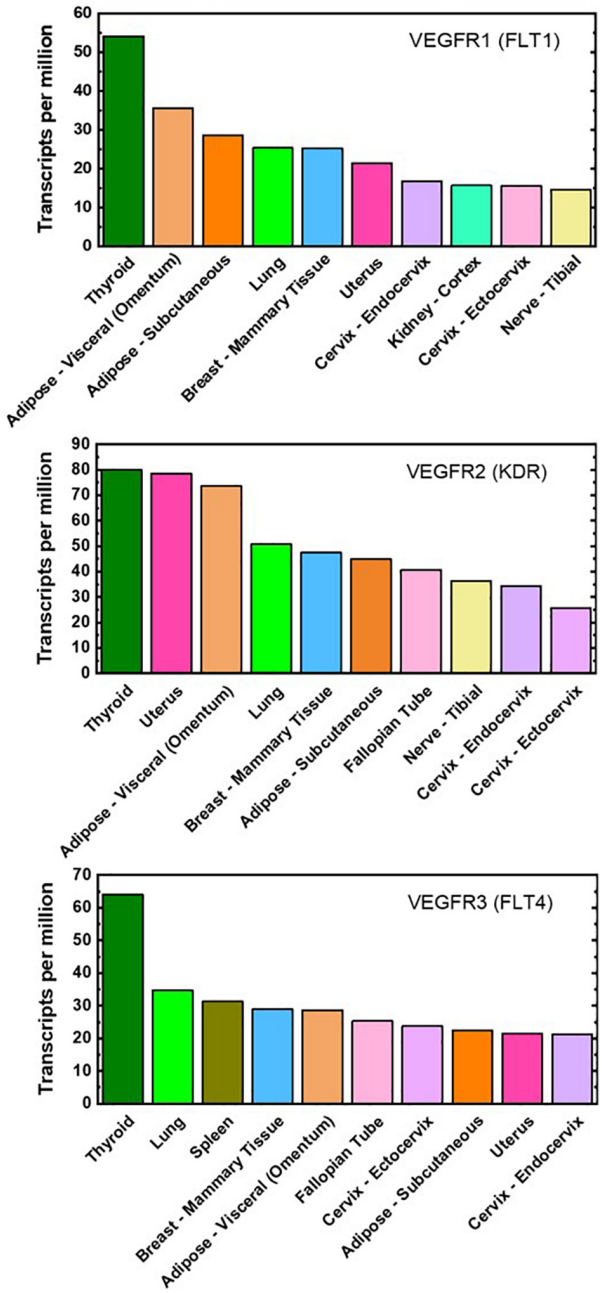

FIGURE 2 | Top ten VEGFR mRNA-expressing tissues. Gene expression of VEGFR1, VEGFR2, and VEGFR3 are ranked highly in both visceral and subcutaneous adipose tissues (light- and dark-orange bar, respectively). These plots are reproduced with median gene-level of VEGFRs and include the top 10 tissues representing the highest VEGFR gene expression. The full database of 53 examined tissues can be accessed at https://gtexportal.org/home/.

SYSTEMS BIOLOGY ENABLES FUTURE VASCULAR-TARGETED THERAPY FOR OBESITY

The predictive power of computational modeling paired with quantitative data should be used to design and direct vascular-targeted therapies for obesity. With the established quantitative techniques (ELISA, SPR, radiolabeling) and literature data, the VEGF:VEGFR kinetics and VEGF concentrations in lean, obese, and treated adipose tissue can be acquired. However, there are minimal proteomic data on VEGFR concentrations on adipose-derived cells, even though adipose tissue is one of the highest VEGFR gene-expressing tissues. qFlow should be used to acquire membrane-bound VEGFR concentrations in adipose tissue; furthermore, cell-by-cell analysis and mixture modeling will be particularly helpful for identifying functionally distinct cell subpopulations within adipose tissue endothelial cells and macrophages.

We have reviewed benchmark models that will provide the basis for future efforts to predict the effects of vascular-targeted therapies on obesity and metabolic responses. These models predict VEGF:VEGFR signaling outcomes and metabolic network regulation via mass-action kinetic modeling and CBM, respectively; mass-action kinetic modeling can be used to design effective vascular-targeted therapeutic strategies, and CBM can identify metabolic pathways that are significantly affected by vascular-targeted therapies. Systems biology, through future quantitative data and computational models, will enable a better understanding of obesity-associated vascular dysregulation and advance vascular-targeted therapies for obesity and its associated metabolic disorders.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

YF led the manuscript preparation. TK contributed to the computational modeling review and contributed critical discussion on this work. YF, TK, and PI prepared the manuscript. All authors contributed to the article and approved the submitted version.

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