Lymphatic Tissue and Organ Engineering for In Vitro Modeling and In Vivo Regeneration

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The lymphatic system has an important role in maintaining fluid homeostasis and transporting immune cells and biomolecules, such as dietary fat, metabolic products, and antigens in different organs and tissues. Therefore, impaired lymphatic vessel function and/or lymphatic vessel deficiency can lead to numerous human diseases. The discovery of lymphatic endothelial markers and prolymphangiogenic growth factors, along with a growing number of in vitro and in vivo models and technologies has expedited research in lymphatic tissue and organ engineering, advancing therapeutic strategies. In this article, we describe lymphatic tissue and organ engineering in two- and three-dimensional culture systems and recently developed microfluidics and organ-on-a-chip systems in vitro. Next, we discuss advances in lymphatic tissue and organ engineering in vivo, focusing on biomaterial and scaffold engineering and their applications for lymphatic vessels and lymphoid organ regeneration. Last, we provide expert perspective and prospects in the field of lymphatic tissue engineering.

The human body is primarily composed of water, comprising 70% of total body weight. The cardiovascular system of blood vessels and the lymphatic system of lymphatic vessels (LVs) work together to maintain healthy fluid levels. The cardiovascular system is considered a "closed circulatory system" because fluid stays in the blood vessels throughout its circulation (Carmeliet 2003). However, fluids can leak from these blood vessels in physiological conditions due to the pressure difference between the arterial and venous capillaries, triggering the lymphatic system to clear the fluid in tissues (called interstitial fluid) (Wiig and Swartz 2012). The lymphatic system is an "open circulatory system" in which "sponge-like" LVs collect interstitial fluid and drain it into the lymphatic system as lymph (Oliver et al. 2020). The lymph travels to lymph nodes (LNs) and then reenters the cardiovascular system through the subclavian veins.

As mentioned above, LVs maintain fluid homeostasis by draining interstitial fluid (Choi et al. 2012). Since the interstitial fluid contains immune cells, LVs modulate host immunity by draining antigen-presenting cells (e.g., dendritic cells [DCs]) and lymphocytes (e.g., T cells) and

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transporting them to LNs (Rockson 2013; Permanyer et al. 2018; Martens et al. 2020; Lee et al. 2021). Organ-specific lymphatics play roles in different organs (Petrova and Koh 2018). For example, the intestinal LVs absorb dietary fat in the GI tract (Zhang et al. 2018a; Suh et al. 2019); the meningeal LVs transport metabolic products and immune cells in the brain (Louveau et al. 2017, 2018; Da Mesquita et al. 2018, 2021); the ocular Schlemm's canal, expressing LV-specific markers, plays a role in clearance of aqueous humor in the eyes (Aspelund et al. 2014; Kizhatil et al. 2014; Park et al. 2014; Jung et al. 2017; Lu et al. 2020); and the heart LVs regulate cholesterol transport, heart regeneration, and repair (Zhang et al. 2018b; Liu et al. 2020). Therefore, impaired lymphatic function in these organs contributes to numerous human diseases, such as lymphedema, immune disease, glaucoma, metabolic disease, heart disease, and neurodegenerative disease (Noon et al. 2006; Ridner 2013; Teerachaisakul et al. 2013; Da Mesquita et al. 2018; Chakraborty et al. 2019; Kataru et al. 2019a; Lu et al. 2020). In cancer, tumor cells exploit LVs as a route for tumor metastasis (Skobe et al. 2001; Lee et al. 2014, 2015); tumor LVs are also involved in tumor immunity (Fankhauser et al. 2017; Garnier et al. 2019; Kataru et al. 2019b; Vaahtomeri and Alitalo 2020).

Animal models have been widely used to study lymphatic biology for decades. Despite enormous contributions of the animal models, it has been often difficult to isolate the relative contributions of biological and biophysical factors in the animal models. To permit better controlled experiments with decoupled biological and biophysical factors, there have been developments of in vitro model systems on two-dimensional (2D) or three-dimensional (3D) substrates or in microfluidics platforms (called organ-on-a-chip systems). Furthermore, the in vitro tissue-engineered models further expanded toward in vivo applications by providing biomaterial scaffolds for implantation of tissue constructs to promote lymphatic tissue and organ regeneration.

In this article, we overview progress of in vitro tissue-engineered models of lymphatics, focusing on LV differentiation, morphogenesis, and function and lymphoid organ formation. Next, we discuss LV and lymphoid organ engineering in vivo. Last, we provide our perspective and future directions in the field of lymphatic tissue engineering and regeneration. The overview of lymphatic tissue engineering approaches is shown in Figure 1.

LYMPHATIC TISSUE ENGINEERING IN VITRO

Lymphatic Tissue Engineering 2D in Vitro

Early in vitro studies on lymphatic vascular tissue engineering have employed 2D cell culture systems, such as plastic dish and transwell. Especially, the transwell system is featured with upper and lower chambers, which provide 2D monolayers of lymphatic endothelium, chemotactic gradient, and transmural (interstitial) flow (Flores-Pérez et al. 2018). These features allow researchers to study cell and mass transport through the lymphatic endothelium under the interstitial flow condition. For example, Miteva et al. demonstrated that transmural (interstitial) flow serves as a physical cue allowing LVs to change their gene-expression profiles to facilitate immune cell intravasation (Miteva et al. 2010). This model included human lymphatic endothelial cells (LECs) seeded on the porous insert membrane and human DCs in matrigel and collagen matrix on top of the insert. The study has demonstrated that gravity-mediated transmural flow can trigger DC transmigration through the lymphatics due to the increased expression of CC motif chemokine ligand 21 (CCL21) and decreased expression of intercellular adhesion molecule 1 (ICAM-1) and E-selectin in LECs (Miteva et al. 2010). In other studies, transwell models have been used to understand lymphatic mass transport. The widely accepted notion in lymphatic mass transport is that the mass primarily passes through the paracellular space via the lymphatic-specific, button-like cell–cell junctions (Baluk et al. 2007; Murfee et al. 2007; Hu et al. 2008; Dejana et al. 2009a). Triacca et al. (2017) expanded the idea by showing that LECs used both transcellular (endocytosis) and paracellular (cell junctions) mechanisms for solutes uptake in response to transmural flow.

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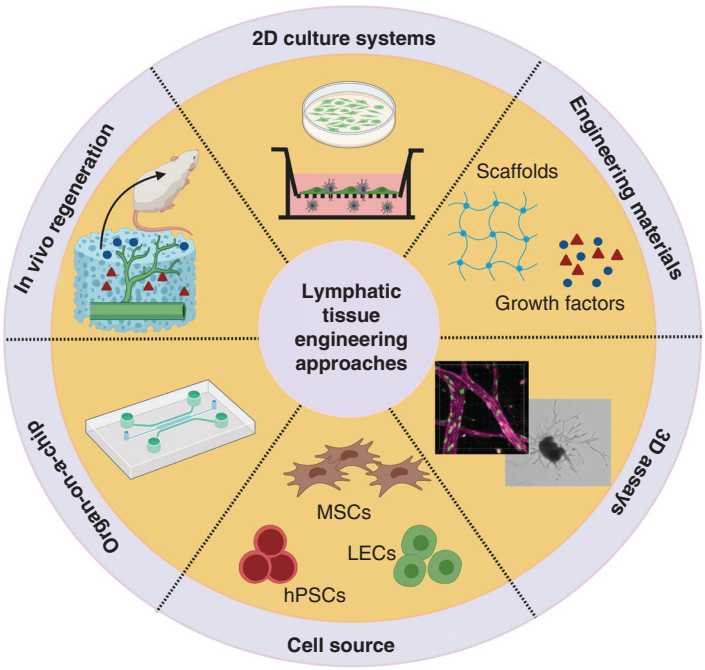

Figure 1. Schematic drawing of strategies in lymphatic tissue engineering and regeneration. Early studies on lymphatic tissue engineering launched with 2D culture systems. Researchers observed lymphatic vessel morphogenesis and homeostasis under interstitial flow and different biochemical stimuli. As an alternative to 2D systems, 3D in vitro assays were developed to study lymphatic tissue development. For most studies, primary lymphatic endothelial cells (LECs) isolated from human or murine lymph nodes were used. Organ-on-a-chip systems are advantageous at investigating cell–cell interactions, cell–extracellular matrix (ECM) interactions, biomechanical, and biochemical cues with a spatial and temporal distribution of those factors. A 3D porous construct for in vivo lymphatic tissue and organ regeneration is often cross-linked with prolymphangiogenic growth factors that can be released in a controlled manner to facilitate functional tissue formation. (The figure was generated using BioRender.) (Schematic drawing has combined figures adapted with permission from Marino et al. 2014 and with permission from The American Association for the Advancement of Science © 2014; and Detry et al. 2011 under the terms of the Creative Commons CC BY license.)

Lymphatic culture in 2D has also been used to understand lymphatic endothelial cell differentiation and morphogenesis. In a study involving mouse embryonic stem cells (ESCs), LECs were differentiated from vascular endothelial growth factor receptor 2 (VEGFR2)-positive, E-cadherin-negative mesodermal cells, cocultured with OP9 cells (a mouse bone marrow–derived mesenchymal stromal cell) (Kono et al. 2006). In another study, transfection of VEGFR3 in VEGFR2-positive endothelial cells increased LV endothelial hyaluronan receptor 1 (LYVE-1) expression and promoted lymphatic endothelial differentiation (Suzuki et al. 2005). Lymphatic morphogenesis was studied in 2D wound-healing assays. In this assay, CD34/VEGFR3-positive progenitor cells could differentiate into lymphatic capillary-like cells, making lymphatic invasion and sprouting to fill the wound gap, showing the importance of VEGF-C/VEGFR3 pathway (Tan et al. 2014). A Boyden chamber assay, another type of transwell, proposed VEGF-C and VEGFR3 signaling as a crucial component of LV growth, migration, and survival (Mäkinen et al. 2001). Alderfer at al. studied the role of biophysical factors in lymphangiogenesis with tube formation assay. They showed that LECs cultured on softer substrates formed intracellular vacuoles and up-regulated crucial LEC markers such as LYVE-1, prospero homeobox protein 1 (Prox-1),

and VEGFR3. Intracellular vacuoles can further merge into coalescence vacuoles, which are precursors of hollow vessel structures. Up-regulation of VEGFR3 receptor enabled effective stimulation with VEGFC leading to higher expression of matrix metalloproteinases (MMPs), which facilitates LEC invasion and migration in lymphatic tube formation (Alderfer et al. 2021).

Lymphatic Tissue Engineering 3D in Vitro

LEC cultures on 2D substrates have a limitation since they do not recapitulate the 3D in vivo organization of the lymphatic vasculatures (Baker and Chen 2012; Rogic et al. 2018; Gong et al. 2019). To study lymphatic development and differentiation in vitro 3D, the Detmar group differentiated LECs from mouse ESCs using embryo-like structures called "embryoid bodies" (EBs). In the normoxic E27-day-old EBs, lymphatic development was induced by VEGF-C and VEGF-A, but not by fibroblast growth factor-basic (bFGF) (Liersch et al. 2006). Similarly, another group used the EB assays and showed that combined treatment with VEGF-C and VEGF-A promoted LV sprouting from EBs; however, other growth factor combinations or hypoxic environments without VEGF-C and VEGF-A had little or no effect (Fig. 2A,B; Kreuger et al. 2006). Cueni et al. (2010) created a fusion protein consisting of podoplanin and an Fc region of immunoglobulin, showing that interfering with podoplanin function negatively impacts lymphangiogenesis in EB models in the presence of VEGF-C/A. Foskett et al. performed microarray gene analysis to compare EBs cultured in various conditions: normoxia, hypoxia, presence or absence of VEGF-A/C, different ECMs—collagen I versus laminin—in blood, and lymphatic endothelial EBs. They showed that hypoxia combined with growth factors (VEGF-A/C) promoted angiogenesis in general, whereas hypoxia combined with VEGF-A/C stimulation on specific ECM (collagen I rather than laminin) favored lymphangiogenesis (Foskett et al. 2011).

To recapitulate complexity of the native LVs, one hurdle is recreating endothelial cell organization in a hierarchical manner from larger vessels into smaller branches and capillaries. To address this, ring assays ex vivo, previously used for studying angiogenesis (Masson et al. 2002; Maquoi et al. 2004), bridged the gap between in vivo and in vitro experiments in lymphatic biology by allowing more realistic lymphangiogenesis studies in 3D. A major advantage of the ring assay was the use of thoracic ducts obtained from wild-type or genetically modified animals to identify possible regulators of lymphangiogenesis. The ring assay was used to examine lymphatic sprouts and the interconnected network formation. To study the potential role of MMPs in 3D growth of LVs, researchers isolated thoracic ducts fragment from wild-type mice or matrix metallopeptidase 2 (MMP2) knockout mice and cultured the isolated thoracic ducts ex vivo in 3D collagen. This model mimicked LEC sprouting from existing lymph vessels, migration and assembly into lumen-containing lymphatic capillaries, and suggested a role of MMP2 in lymphangiogenesis (Bruyère et al. 2008). Using the method, Detry and colleagues (2011) observed (1) elongation and alignment of cord-like lymphatic structures, (2) coalescence of vesicles to create a lumen and formation of vacuoles through vesicle invagination, and (3) remodeling matrix enabling the conditions for LV formation (Fig. 2C). The ring assay was also used to observe and assess LV sprouting in hypoxia (Moog et al. 2020). Hypoxia preconditioned plasma (HPP) is a blood-derived autologous growth factor composition that is being used clinically for promoting tissue regeneration (Hadjipanayi and Schilling 2014). Moog et al. (2020) tested the HPP in their ring assays of thoracic ducts and showed the versatility of blood-derived secretomes to induce lymphangiogenesis.

More complex, coculture methods have been used to provide a functional blood and LV network in 3D in vitro. A study showed a bioengineered dermal vascular plexus for skin grafts composed of both lymphatic and blood vessels by coculturing human dermal microvascular endothelial cells (HDMECs) isolated from human foreskin with human fibroblasts on 3D fibrin matrix (Marino et al. 2014). HDMECs contained both blood and LEC populations, so that the coculture gave rise to lumenized lymphatics and blood capillaries, and the lymphatic and blood vessels did not intermix during vessel develop-

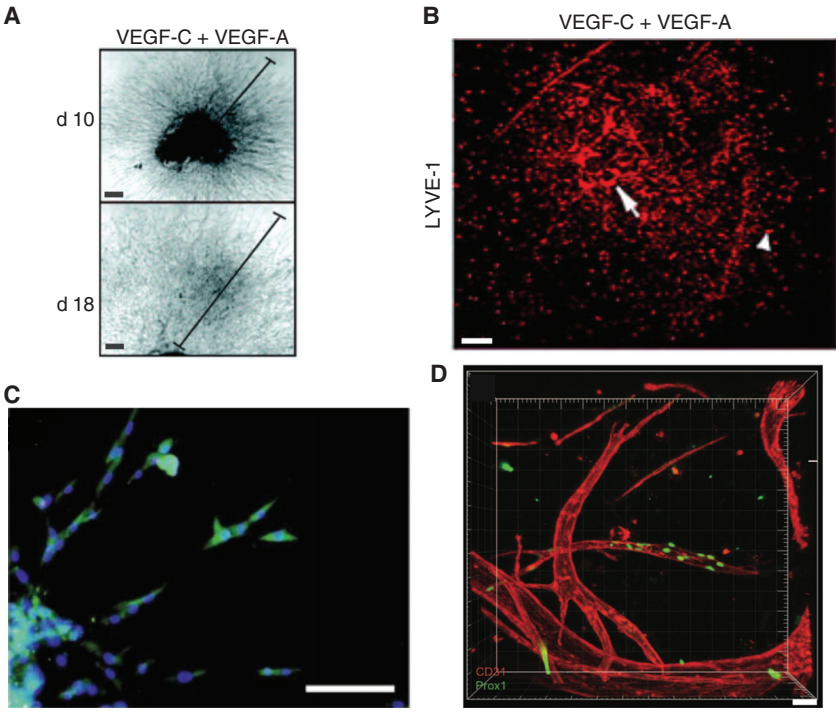

Figure 2. 3D assays in vitro for study of lymphangiogenesis. (*A*) Embryoid bodies (EBs) embedded in 3D collagen matrix, treated with vascular endothelial growth factor C and A (VEGF-C and VEGF-A), imaged at 10 d and 18 d of culturing. Scale bar, 300 µm. (*B*) Characterization of EBs, treated with VEGF-C and VEGF-A with immunostaining of lymphatic vessel endothelial hyaluronan receptor 1 (LYVE-1) (red). Also shown are networks of LYVE-1-positive structures (arrow) and single cells (arrowhead). Scale bar, 300 µm. (*C*) Lymphatic ring assays. Once embedded into 3D type I collagen and cultured, lymphatic rings demonstrate an outgrowth of lymphatic endothelial cells (LECs) into capillary-like structures. Characterization of LEC outgrowth from the ring assay with immunostaining of LYVE-1 (green) and nuclear staining with DAPI (blue). Scale bar, 100 µm. (*D*) A bioengineered skin graft composed of both lymphatic and blood vessels by coculturing human dermal lymphatic endothelial cells (HLECs) and human dermal microvascular endothelial cells (HDMECs) isolated from human foreskin with human fibroblasts on a 3D fibrin matrix. The system showed lumenized lymphatics (identified with red CD31 and green Prox1-positive nuclei) and blood capillaries ($CD31^+/Prox1^-$) that did not intermix during development. Scale bar, 50 µm. (Panels *A* and *B* adapted from Kreuger et al. 2006, with permission, from Wolters Kluwer Health © 2006; *C* adapted from Detry et al. 2011 under the terms of the Creative Commons CC BY license; and *D* adapted from Marino et al. 2014, with permission, from The American Association for the Advancement of Science © 2014.)

ment (Fig. 2D). After transplanting the human skin grafts into an immunocompromised rat wound–healing model, both lymphatic and blood capillaries underwent anastomosis with the recipient's plexus and the engineered lymphatics supported fluid drainage (Marino et al. 2014). In a similar study, lymphatic and blood endothelial cells (BECs) formed a distinguishable microvascular network from HDMECs, having both lymphatic and BEC populations, in the coculture with adipose-derived stromal cells (ASCs) in 3D fibrin substrate (Knezevic et al. 2017). Matsusaki et al. (2015) generated vascularized human skin tissues using a cell-coating technique where BECs and LECs formed a dense capillary network when stacked between ECM sheets (gelatin and fibronectin) and epidermal keratinocyte layers. A lymphatic-like capillary network has been also engineered without using hydrogels or exogenous growth factors. Isolated LECs from human fore-

skin in coculture with fibroblasts formed capillaries driven by endogenous bFGF and VEGF-C secreted by stromal cells (Gibot et al. 2016, 2017).

Microfluidic Devices or Organs-on-a-Chip in Lymphatic Tissue Engineering

Microfluidic devices add unique value to the current cell culture systems, improving the classical 2D/3D approaches to be more in vivo–like. In the past, a vast majority of research with microfluidic devices has mostly focused on blood vessels (Ryu et al. 2015). However, recently, scientists have also begun to develop microfluidic systems that can mimic the native structure and function of LVs. Microfluidic devices have multiple compartments and channels ranging from tens to hundreds of micrometers in diameter enabling for controllable experiments with a small volume of fluid under controlled hydraulic pressure, shear stress, and chemical gradient (Whitesides 2006). Microfluidic systems have the advantage to investigate cell–cell interactions, cell–ECM interactions as well as biomechanical (e.g., interstitial pressure, luminal flow, stretching motion) and biochemical (e.g., growth factor gradient, hypoxia) cues with spatial and temporal distributions of those factors in a controlled manner (Kim et al. 2010; Boussommier-Calleja et al. 2016).

A multichamber radial flow device that allowed 3D experiments with real-time imaging was performed to study LV network formation (Bonvin et al. 2010). In the study, interstitial flow and biomechanical cues were crucial to recapitulate lymphangiogenesis-like lymphatic sprouting. Another microfluidics approach was used to understand the formation of lymphatic capillaries and lymphatic invasion in response of multiple growth factors, such as VEGF-A, bFGF, interleukin 8, sphingosine 1-phosphate (S1P), hepatocyte growth factor (HGF), and/or multiple ECMs, such as fibronectin, collagens, and laminins, which can interact with various integrins expressed in LECs. They demonstrated the important roles of VEGF-C, S1P, bFGF, HGF, fibronectin, and integrin β1 in the formation of lymphatic capillaries and lymphatic invasion using their microfluidic device (Kumaravel et al. 2020). Microchannel-based LV-on-a-chip was generated to understand laminar flow mediated lymphatic spouting and the underlying Notch-dependent mechanism (Choi et al. 2017). In the study, polydimethylsiloxane (PDMS) casting mold was fabricated using soft lithography (Henderson et al. 2020), inlets and outlets were punched in the polymerized PDMS device to create media reservoirs and ECM ports, and the whole structure was bonded to a glass coverslip. In the hollow channel, LECs were seeded, luminal flow (~2 dyne/cm^2) was introduced, then lymphatic sprouting was measured. Interestingly, luminal flow promoted LV sprouting, but reduced blood vessel sprouting. This distinctive response was possibly because the applied laminar flow induced shear stress (~2 dyne/cm^2) was much lower than the physiological levels of shear stress in blood vessels (>10 dyne/cm^2). In that experimental setting, they proposed a mechanism that laminar flow activated calmodulin to facilitate a physical interaction between Krüppel-like factor 2 (KLF2), the major regulator of shear responses, and Prox1, the master regulator of lymphatic development. The KLF2/Prox1 complex up-regulated the expression of Notch E3 ligase, down-regulating Notch1 activity and enhancing lymphatic sprouting. In another study, a microlymph (μLYMPH) system composed of a single lymphatic channel embedded in the collagen matrix was developed (Fig. 3A; Gong et al. 2019). LECs seeded in microchannel of the μLYMPH system formed a monolayer of cells expressing VE-cadherin, zonula occludens 1 (ZO-1), and CD31 with the vessel lumen in the range of 200–250 μm. The μLYMPH system enabled the investigation of secretory profiles of LECs and BECs in steady-state and during coculture with breast cancer–associated fibroblasts (Gong et al. 2019). Henderson et al. presented a bioengineered microfluidic 3D in vitro LV model to investigate the role of integrin α5 in lymphatic barrier function. Within the microfluidic device, they altered integrin α5 activity in LECs by pretreating LECs with anti-integrin α5 antibodies (clone: SNAKA51), fibronectin, or a vehicle while monitoring vessel permeability and junction tightening. They discovered that anti-integrin α5 antibodies (clone: SNAKA51) or

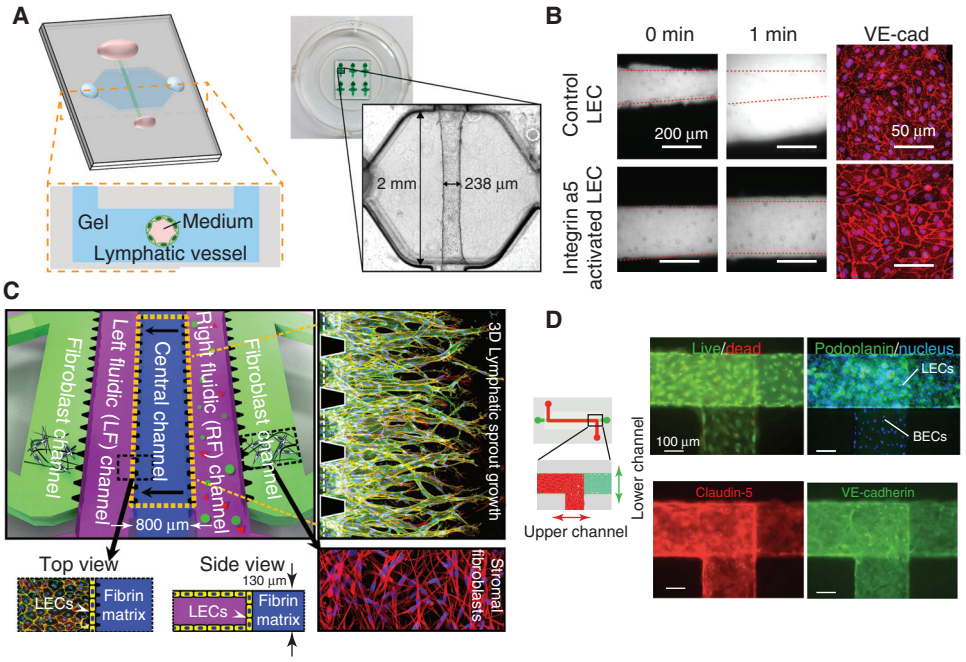

Figure 3. Microfluidic approaches to study lymphatic biology. (*A*) (*Left*) A single-channel microfluidic device (microlymph [µLYMPH]) seeded with lymphatic endothelial cells. (*Right*) A bright field image of the cell-seeded channel in the device. (*B*) (*Left*) Lymphatic endothelial cells (LECs) in collagen 1 were pretreated with anti-integrin α5 antibodies (clone: SNAKA51) to activate integrin α5 in LECs. Control LECs or LECs with activated integrin α5 were cultured in lymphatics-on-a-chip for 3 days. 70 kDa dextran was introduced to the lymphatic lumens and the dextran diffusion was observed under microscopy. Superimposed red dashed lines represent the edges of the vessel lumens. (*Right*) The fixed samples were stained with anti-vascular endothelial (VE)-cadherin antibodies (red) to visualize adherens junctions. (*C*) A multichannel microfluidic system with coculture of LECs with stromal fibroblasts. Endothelial cells are spatially separated with fluidic (*left* and *right*) channels allowing for paracrine signaling communication. This setup reconstituted lymphangiogenesis in vitro. LECs exhibited sprouting behavior in response to biochemical factors and flow-mediated stimulation. (*D*) (*Left*) A microfluidic device with cocultured blood endothelial cells (BECs) (red cells) and LECs (green cells). Two different types of endothelial cells are seeded in two aligned channels interconnected with a porous membrane. (*Right*) Analysis of lymphatic endothelial cells by immunostaining for lymphatic markers: podoplanin (green), junctional protein claudin-5 (red), and adhesion molecule VE-cadherin (green). (Panel *A* adapted from Gong et al. 2019, with permission, from Elsevier © 2019; *B* adapted from Henderson et al. 2021, with permission, from John Wiley and Sons © 2021; *C* adapted from Kim et al. 2016, with permission, from Elsevier © 2016; and *D* reprinted from Sato et al. 2015 under the terms of the Creative Commons Attribution License.)

fibronectin-mediated integrin α5 activation is responsible for the decreased permeability and increased junction tightening in lymphatic endothelium (Fig. 3B; Henderson et al. 2021). Kim et al. developed a biomimetic microfluidic device that included human LEC–fibroblast interactions, biochemical cues, and interstitial flow (Kim et al. 2016), which was based on their previous platform containing BECs (Kim et al. 2013). This research demonstrated that lymphatic sprouting is increased by interstitial flow directed toward the tip of the sprouts accompanied by addition of prolymphangiogenic growth factors (Fig. 3C). In the following study of the same group, cancer cells were cocultured with primary fibroblasts, BECs and LECs, to mimic angiogenesis and lymphangiogenesis induced by tumor–stroma interactions (Chung et al. 2017). Another group featured both blood and LV barrier integrity on one device with upper and lower channels

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that are partially interconnected. In overlapping channel sections, LECs and BECs were separated by a porous membrane, aligned to each other back-to-back to show mass transport between the blood and LVs (Fig. 3D; Sato et al. 2015).

Recently, 3D microfluidic devices have been used for lymphoid organ formation to understand immunological response in vitro. In many cases, researchers used ex vivo LN tissues or cells and cultured them in vitro to study T-cell and B-cell responses. The Pompano group excised LNs from animals and cultured the sliced LN tissues in vitro using a microfluidics platform, demonstrating drug deliveries to a specific region of the LN slices, such as T-cell or B-cell zone (Ross et al. 2017). The same group investigated spatial organization of cultured LNs, reflecting the 3D spatial complexity of the organ (Belanger et al. 2021). Another group used mouse LN tissues to generate B-cell and T-cell organoids for studying lymphocyte development, activation, and antibody production (Purwada and Singh 2017; Purwada et al. 2019). Bovay and coworkers studied LN formation by focusing on recruitment of lymphoid tissue inducer cells (LTi) to the LN region. They primarily used animal models, but in part used a microfluidic device to study how interstitial flow affects LTi recruitment and revealed CXC motif chemokine ligand 13 (CXCL13) as one of the key drivers for the LTi recruitment and LN formation (Bovay et al. 2018).

Currently, many blood or LVs-on-a-chip systems are being developed in conjunction with cancer research. Especially, the blood or LVs-on-a-chip systems are evolving from physiological perspectives of those vessels toward more complex mechanisms of tumor metastasis and immunotherapy through the blood and LVs (Nguyen et al. 2019; Kwak and Lee 2020b,c; Liu et al. 2021); interactions with stromal cells and immune cells (Chung et al. 2017; Parlato et al. 2017; Lugo-Cintrón et al. 2020, 2021; Kolarzyk and Lee 2021); and drug screening, delivery systems, and discoveries (Bourland et al. 2018; Cao et al. 2019). However, given this area of research is beyond the scope of this article, we cite the following review articles for more information (Lee et al. 2016; Kumar and Varghese 2019; Sontheimer-Phelps et al. 2019; Greenlee and King 2020).

LYMPHATIC TISSUE AND ORGAN ENGINEERING FOR REGENERATION IN VIVO

Lymphatic Structure in Vivo

LV networks develop by distinct molecular mechanisms from those in the blood vessel networks, having lymphatic-specific vascular architectures of the initial (capillary) and collecting LVs (Huethorst et al. 2016; Alderfer et al. 2018). Thin-walled initial LVs are composed of unique oak leaf–shaped endothelial cells connected through discontinuous button-like junctions with "flap valves" (or primary valves), facilitating fluid and leukocyte entry (Baluk et al. 2007; Pfeiffer et al. 2008; Dejana et al. 2009a; Tammela and Alitalo 2010; Alitalo 2011). Lymphatic capillaries attach to the ECM via anchoring filaments that are enabling movements of overlapping cell layers in response to the change in interstitial pressure. When there is a higher interstitial fluid pressure, anchoring filaments are pulled from the ECM that leads to wider opening of LV lumen and increased uptake of interstitial fluid, which prevent vessels from collapsing (Leak and Burke 1968; Tammela and Alitalo 2010; Schulte-Merker et al. 2011). Lymphatic capillaries are not covered with smooth muscle cells (SMCs) or pericytes and have sparse basement membrane containing portal-like structures facilitating DC entry (Pflicke and Sixt 2009; Norrmén et al. 2011). Lymphatic capillaries merge into collecting LVs, which have continuous zipper-like junctions, bi-leaflet valves (or secondary valves) to prevent backflow, a basement membrane, and covered by an SMC layer (Dejana et al. 2009b). Subsequently, the collecting vessels drain lymph and leukocytes to LNs. Given the specialized anatomy, lymphatic tissue–engineering approaches need to consider bona fide lymphatic vascular structure to better recapitulate lymphatic function in vivo (Schaupper et al. 2016; Knezevic et al. 2017). Especially, bioengineered models for initial lymphatics require button-like cell–cell junctions and their primary valve function with anchoring filaments. Also, tissue-

engineered models for modeling collecting lymphatics need to reconstitute contractile motion of lymphangion with lymphatic muscle cells and luminal valve formation.

Biomaterial Scaffolds for Lymphatic Tissue and Organ Engineering

A great number of approaches in tissue engineering depend on employing biomaterial scaffolds. Specifically, natural hydrogels have served as 3D microenvironments for vascular network assembly. Hydrogels are characterized with low toxicity, biocompatibility, biodegradability, high water content, and viscoelastic properties (Drury and Mooney 2003; Park and Gerecht 2014). These hydrogels recapitulated natural ECM, which plays a key role in lymphangiogenesis, mediating LEC proliferation, migration, and communications with the environment (Ji 2006; Wiig et al. 2010). Hydrogel scaffolds can be combined with cells and/or prolymphangiogenic growth factors to initialize LV formation in vitro, and even in vivo when they are implanted (Huethorst et al. 2016).

Naturally derived hydrogels can be divided into three groups: protein-based, polysaccharide-based, and decellularized hydrogel. Protein-based hydrogels are used most often due to their abundance in natural ECM. Each type of hydrogel is composed of collagen, fibrin, gelatin, and elastin, which manifest different characteristics making them suitable for intended applications (Catoira et al. 2019). They are naturally degraded by MMPs secreted by cells in the scaffold, allowing them to inherently reshape the growing tissues after implantation (Drury and Mooney 2003). ECM stiffness is one of the physical characteristics to be considered when culturing endothelial cells (Gordon et al. 2020). Studies showed LECs to be influenced by substrate stiffness in vitro (Alderfer et al. 2021) and in vivo (Frye et al. 2018; Choi et al. 2019). Chemokines and cytokines are commonly cross-linked to the hydrogel matrices to induce lymphangiogenesis in vitro and in vivo. The VEGF-C and VEGFR3 axis is the most validated pathway when considering addition of prolymphangiogenic signaling molecules into the scaffolds (Rauniyar et al. 2018). VEGF-D is another ligand that binds to VEGFR3 receptor. VEGF-D was dispensable for LV development during embryogenesis in mice (Baldwin et al. 2005), but VEGF-D could rescue lymphatic hypoplasia in *VEGFC* knockout mice, when it is overexpressed, which means that VEGF-D is functionally redundant with VEGF-C in the stimulation of lymphangiogenesis through the VEGFR3 receptor (Haiko et al. 2008). Collectively, it is important to consider biomaterial properties, such as stiffness, surface topography, structure, origin, composition, incorporation of cells, and prolymphangiogenic growth factors and chemokines when designing an approach to support LV growth in applications for regenerative therapies in vivo (Park and Gerecht 2014; Sestito and Thomas 2019).

Lymphatic Tissue and Organ Regeneration in Vivo

Lymphatic deficiency owing to either hereditary or acquired reasons can cause lymphatic diseases (Rockson 2006; Ridner 2013). It is believed that VEGF-C expression leads to formation of new LVs to improve lymphatic function in the context of lymphatic deficiency (Schindewolffs et al. 2014; Vivien et al. 2019). However, often overexpression of VEGF-C produces hyperplastic lymphatic networks (Jeltsch et al. 1997; Saaristo et al. 2002; Goldman et al. 2005), provoking LV hyperpermeability (Tammela et al. 2007) and dysfunctional intraluminal valves in collecting LVs driving retrograde luminal flow (Isaka et al. 2004). To induce well-tuned, physiologically relevant lymphangiogenesis, Güç et al. engineered fibrin-binding VEGF-C (FB-VEGF-C), which is a controlled release system regulated by proteases produced by the matrix-infiltrating cells (Fig. 4A, B; Güç et al. 2017). They showed that single, low-dose implantation of FB-VEGF-C, but not free VEGF-C, improved diabetic wound healing in vivo by inducing local lymphangiogenesis (Fig. 4C) without affecting downstream collecting lymphatics or prompting excessive angiogenesis. FB-VEGF-C-mediated lymphangiogenesis also attracted immune cells to the newly formed lymphatics (Fig. 4D,E). Another study used biodegradable alginate hydrogels ionically cross-linked

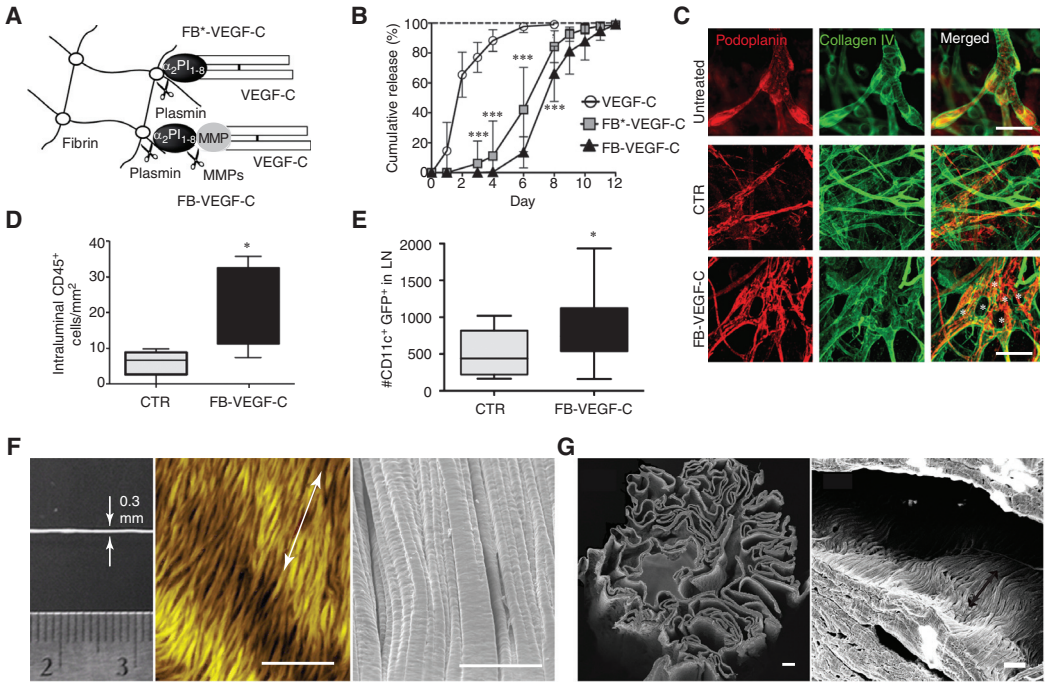

Figure 4. Hydrogel scaffolds for promoting lymphangiogenesis in vivo. (*A*) Engineered fibrin-binding vascular endothelial growth factor C (FB-VEGF-C) construct. VEGF-C is released by matrix infiltrating cells. Cleavage occurs at α_2-$PI_{1\text{-}8}$ or α_2-$PI_{1\text{-}8}$–matrix metalloproteinase (MMP) substrate domain fused to the amino-terminal site of VEGF-C. (*B*) VEGF-C is released from the FB-VEGF-C construct in a controlled manner in contrast to rapid release of free VEGF-C. (*C*) Confocal images of untreated dorsal skin and treated for 21 days with just fibrin (CTR: control) or FB-VEGF-C construct. FB-VEGF-C-treated skin showed extensive lymphangiogenesis (indicated by asterisks on the image) and wider vessel lumen formation (*asterisks represent lumen-like structure). Lymphatic vessels (LVs) were stained with podoplanin (red) and collagen IV (green). Scale bars, 50 µm. (*D*) VEGF-C fibrin construct attracted trafficking of CD 45^+ leukocytes to newly formed LVs (Lyve-1^+). $^*P < 0.05$. (*E*) Enhanced migration of $CD11c^+$ leukocytes to lymph nodes (LNs). $^*P < 0.05$. (*F*) (*Left*) Microscopic view of BioBridge collagen scaffolds prior to implantation into a porcine model of lymphedema. (*Middle*) Atomic force microscopy showing aligned collagen fibrils. Scale bar, 50 µm. (*Right*) Structure of thread-like nanofibrillar collagen scaffolds characterized by scanning electron microscopy (SEM). Scale bar, 20 µm. (*G*) (*Right*) SEM images showing porous structure of the scaffold, in which the pores are aligned in parallel orientation. Scale bar, 1 µm. (*Left*) Lumen of the thread-like scaffolds with parallel aligned fibrils. Scale bar, 1 µm. (Panels *A*, *B*, *C*, *D*, and *E* reprinted from Güç et al. 2017 under the Creative Commons Attribution 4.0 International License; *F* and *G* adapted from Hadamitzky et al. 2016, with permission, from Elsevier © 2016.)

with VEGF-C/D to study LV formation in the chick chorioallantoic membrane (CAM) assay (Campbell et al. 2017). LECs exhibited a distinct response to VEGF-C and VEGF-D, showing strong 3D sprouting at a constant concentration of VEGF-C or at high initial concentration of VEGF-D. Hadamitzky et al. used aligned nanofibrillar collagen scaffolds (also known as "BioBridge") to treat secondary (acquired) lymphedema in porcine models (Fig. 4F,G). This study resulted in two pilot human trials (Hadamitzky et al. 2016; Rochlin et al. 2020) and currently in a phase II clinical trial. Originally, the BioBridge was created to study its ability to modulate cytoskeleton rearrangement of human BECs (Huang et al. 2013). In the earlier study, the authors focused on BEC alignments based on the previous observation of Drs. Shu Chien and Martin Schwartz showing that healthy BECs exhibited an alignment pattern in response to physiological

laminar flow (Tzima et al. 2001; Chien 2008). Huang et al. fabricated collagen membranes by liquid collagen shearing techniques enabling formation of resorbable, porous, aligned nanofibers. Primary human BECs cultured on the aligned nanofibers exhibited elongated nucleus and organized cytoskeleton assembly along the nanofibers in contrast to randomly arranged actin bundles on disorganized collagen nanofibers. The authors further demonstrated that aligned nanofibers modulate inflammatory responses in BECs and boost their survival after implantation into ischemic rodent hindlimbs (Huang et al. 2013). More recently, the same group applied the BioBridge to treat obstructed LVs by guiding lymphangiogenesis in a porcine secondary lymphedema model in vivo (Hadamitzky et al. 2016; Rochlin et al. 2020). The BioBridge implantation into the porcine lymphedema model was performed together with LN transfer. VEGF-C was cross-linked to collagen via heparin to serve as an exogenous prolymphangiogenic source. The BioBridge implantation with VEGF-C combined with LN transfer were biocompatible and well-integrated, whereas control animals without the BioBridge method developed fibrous scar tissues.

Lymphoid organs, such as LNs, thymus, tonsils, and spleens, have complex structures and functions to maintain human immunity (Drayton et al. 2006). Lymphoid-like tissue and organ engineering has evolved to study and control immune responses in vivo as well as to serve as a model to study immunotherapies against tumors and immune diseases (Irvine et al. 2008; Kwak and Lee 2020a). In lymphoid-like tissue and organ engineering, biocompatible hydrogels provide controlled release of chemokines, positioned in geometrically well-defined 3D structures to provide proper chemotactic gradients. These hydrogels allow for incorporation of different cell types to mimic LN formation and function. The 3D porous structure also modulates trafficking of immune cells through newly formed vascular networks (Kobayashi and Watanabe 2010; Hickey et al. 2019; Najibi and Mooney 2020; Jia et al. 2021). Watanabe's group contributed to this field by generating synthetic secondary lymphoid-like organoid in vivo. They embedded thymus-derived stromal cell line (TEL-2-LTα) along with activated bone marrow–derived DCs into porous collagen scaffold, which was implanted at the renal subcapsular space in mice. The transplanted tissue constructs induced high endothelial venule (HEV)-like vessel development as well as the formation of follicular DCs (FDCs) and fibroblastic reticular cells (FRCs) in the B-cell follicles and T-cell zones. The engineered organoid also generated antibodies in naive and severe combined immunodeficiency (SCID) mice following immunization (Suematsu and Watanabe 2004). In their follow-up study, transplantation of artificial LNs like tertiary lymphoid organs (artTLOs) into SCID mice prompted lymphocyte migration from axillary LNs to the spleen and bone marrow (Okamoto et al. 2007). Watanabe's group also developed a strategy to obtain artificial LNs without the necessity of incorporation of stromal cells into the scaffold. This approach was based on entrapment of lymphotoxin $\alpha_1\beta_2$ and a cocktail of multiple chemokines in slow releasing gel beads incorporated into 3D collagen matrix (Fig. 5A). The implanted 3D structures with entrapped chemokines prompted a potent and antigen-specific response with production of memory B and T cells and functional capillaries (Fig. 5B; Kobayashi and Watanabe 2016). Another group engineered secondary lymphoid-like tissues using decellularized scaffolds derived from animals. Cuzzone et al. decellularized murine LNs with detergent sodium dodecyl sulfate. The decellularized scaffolds preserved native tissue architecture and did not trigger severe host immune inflammation and recellularized scaffolds with donor splenocytes survived in vivo after implantation in the muscular pocket (Cuzzone et al. 2015). More recently, Lenti et al. developed functional lympho-organoids by seeding LN stromal progenitor cells in gelatin matrix without using decellularized animal scaffolds (Fig. 5C). The implanted lympho-organoids were able to restore lymphatic function, such as drainage and perfusion, and upon immunization provoked antigen-specific immune responses (Lenti et al. 2019). Collectively, these studies showed that tissue-engineered lymphoid organs composed of hydrogel matrices recreated the native tissue microenvironment with organized areas of B cells and T cells and triggered immune responses.

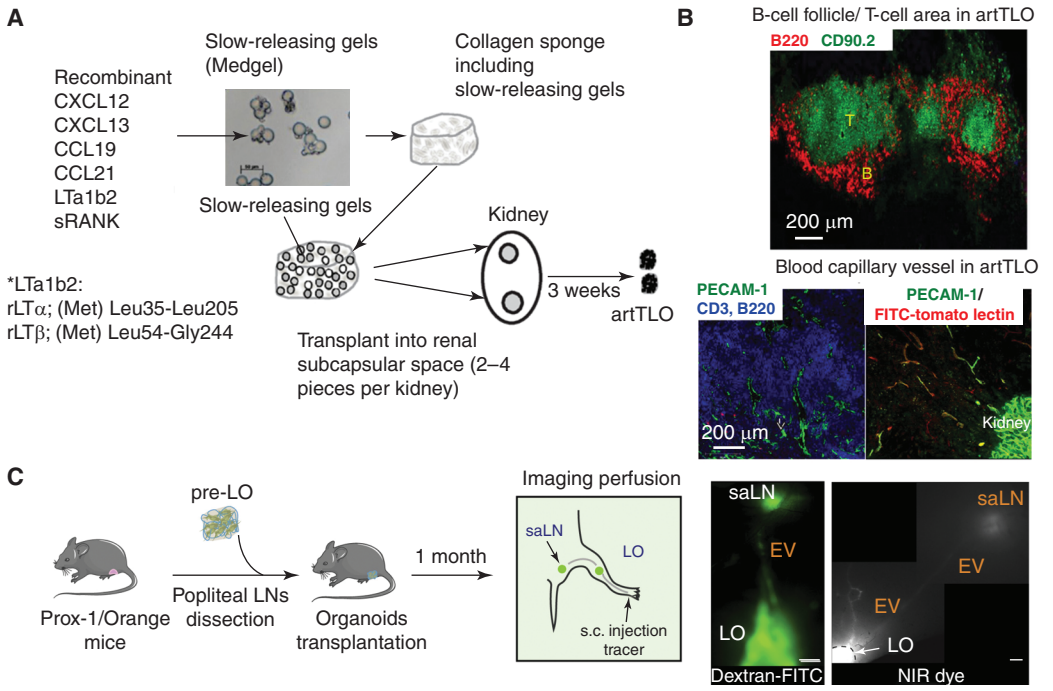

Figure 5. Strategies for engineering lymphoid organs in vivo. (*A*) Workflow showing generation of artificial lymph node (LN)-like tertiary lymphoid organs (artTLOs). Functional artTLOs are developed by transplantation of collagen sponge scaffolds containing trapped cytokines in slow-releasing gel beads. The construct was transplanted into the renal subcapsular space of mice and organized into lymphoid tissues. (*B*) T cells (CD90.2, green), B-cell follicles (B220, red), and blood capillary vessels (PECAM-1 and fluorescein isothiocyanate [FITC]-tomato lectin positive, yellow) were detected in artTLOs. (*C*) (*Left*) Strategy for generation of LOs, which are composed of LN stromal progenitors and decellularized scaffolds. Pre-LOs were implanted at the site of popliteal dissected LNs. (*Right*) FITC and near infrared (NIR) fluorescence images showing integration of LOs with existing lymphatic popliteal efferent vessel (EV) and sacral LN (saLN). Scale bars, 1 mm. (Panels *A* and *B* reprinted from Kobayashi and Watanabe 2016 under the terms of the Creative Commons Attribution License (CC BY); *C* adapted from Lenti et al. 2019, with permission, from Elsevier © 2019.)

CONCLUDING REMARKS

In this article, we discuss in vitro tissue-engineered models that reconstitute lymphatic tissues and organs to permit controlled experiments, investigating lymphatic differentiation and morphogenesis, and lymphoid organ formation. There is an unmet need for an advance over current technology by creating both initial and collecting LVs and combining these vessels with LNs to comprehensively investigate lymphatic function. Models for initial LVs still need to recapitulate button-like cell–cell junctions and their primary valve function, which is important to better understand the interstitial pressure-driven mass transport through the initial lymphatic junctions. Dead-ended vessel geometry, well-defined interstitial fluid pressure, and ECM composition would be critical for creating button-like junctions in the initial lymphatics. Meanwhile, models for collecting LVs need to reconstitute lymphangion-specific contractile motion by adding lymphatic muscle cells to the systems, and to consider luminal valve formation to better recapitulate unidirectional fluid mechanics in the collecting lymphatics. A 3D printing approach would be one of the ideal methods to provide realistic collecting LV structure, dimension, and valves as previously shown in their applications for blood vascular tissue

engineering (Wu et al. 2011; Paulsen and Miller 2015; Gao et al. 2018). These platforms can be applied for a variety of context-dependent topics, such as heart regeneration, lacteal absorption of dietary fat, amyloid-β clearance by the brain LVs, tumor cell entering the lymphatics in metastatic cancers, and antigen-presenting cells entering the lymphatics under infection. Human-induced pluripotent stem cell (iPSC)-derived LECs and lymphatic SMCs would also improve individualized medicine, given currently limited lymphatic cell sources.

We also discuss in vivo applications for lymphatic tissue and organ engineering. Controlled VEGF-C delivery in a well-tuned manner is required for regenerating functionally sound LVs to treat lymphatic deficiency. Given the hierarchical structure of the lymphatics, bioprinting-mediated generation of prepatterned initial and collecting LV scaffolds with valves would be worth investigation. In addition to lymphatic deficiency, structure of LVs are critical in regulating immune responses in many different contexts such as immune rejection after organ transplantation (Maltzman et al. 2015; Mehrabi et al. 2020; Wong 2020), which must be carefully considered, regarding the timing, the degree, and the junctional structure of LVs. Last, organ-specific lymphatic tissue regeneration, defining new lymphangiocrine factors, single-cell genomics approaches, and modulation of preexiting lymphoid organs by using biomaterials and scaffolds would improve current tissue repair and immunomodulation strategies. Obtaining relevant cells and ECM for organ-specific lymphatics would also be important to better recapitulate organ-specific lymphatic function.

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