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Immunology of the Lymphatic System

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Contents

Chapter 1
Introduction

Stanley G. Rockson and Laura Santambrogio

The disciplines of lymphatic biology and medicine are in the midst of much-needed and much-anticipated renaissance. New and expanded insights into the normal and abnormal function of the lymphatic system can be predicted to have a transformative impact upon our grasp of human physiology in health and in disease.

While it is difficult to fully comprehend the antecedents, it is unfortunately true that the lymphatic system has been the recipient of centuries of passive neglect. However, awareness of the importance of the lymphatic function to human health and disease is experiencing unprecedented growth. The lymphatic health continuum can readily be defined to encompass inflammation, autoimmune diseases, transplant rejection, cancer, cardiovascular and metabolic disorders, obesity, and many other expressions of human functional disorders.

This book delves into several concepts that have emerged in the last few years. These concepts provide a deeper understanding of lymph formation, its cellular and proteomic composition, circulation, filtration, as well as establishing a context in which to understand the defining attributes that govern the development and function of the lymphatic system in contrast to the blood circulatory system.

The last 20 years have eclipsed the notion that lymphatic capillaries are a mere footprint of the vascular system. There has been delineation of the specific growth factors and transcription factors that drive lymphatic biogenesis from endothelial cells expressing a unique lymphatic signature. Novel concepts have also emerged from the anatomy and ultrastructural morphology of the lymphatic system: these acknowledge several similarities to, but also important differences with, the blood vascular system. The concept of the lymphangion, as a functional unit defined by

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the lymphatic valve and characterized by the lymphatic wall capable of active spontaneous contractions, has also been further developed and elucidated. This active driving force is essential to transport lymph against the opposing net hydrostatic gradient. Important progress has been achieved in the attempts to define the cellular and matrix composition of the lymphangions and their changes under physiological or pathological conditions.

Novel insights into lymph formation have also been derived. The classical Starling principle, which states that fluid filtration rates between plasma and interstitial fluid is determined by differences in the hydrostatic and colloid osmotic pressures across the arterial and venous microvascular ends, has been revisited. Indeed, it has been shown that reabsorption at the venous end of the capillary bed is actually negligible; in fact, the greater part of tissue fluid drainage occurs through lymphatic means. This has created an even greater emphasis on the process of lymph formation and the role of plasma ultrafiltration in determining lymph composition.

A major development in the analysis of lymph composition has been made possible through the highly sophisticated and sensitive proteomic approaches developed in the last decade. Recently performed proteomic analyses have allowed a deeper insight into the "omic" composition (proteomic, metabolomic, and lipidomic) of the prenodal lymph and their relationships to those of plasma. Published analyses of lymph, under physiological and pathological conditions, reveal the rich tissue antigen composition of the lymph that supersedes the mere process of plasma ultrafiltration, reflecting the overall signature of the tissues of origin. Such analyses hold promise for the discovery of tissue-specific biomarkers under physiological and pathological conditions.

Novel insights into the cellular composition and circulation of the lymph have also been derived during the last decade. Emerging concepts regarding lymph-bound regulatory T cells and regulatory dendritic cells, and the inability to maintain immunological tolerance in mouse strains that have abrogated lymphatic transit, raise important questions regarding the role of lymph in the maintenance of immunological tolerance.

Finally, the role of lymphatic function, lymph transport, and their far-reaching implications in cancer progression and metastasis formation has begun to be elucidated.

Each chapter provides an historical prospective and a summary of current understanding of lymphatic angiogenesis, lymph formation, transport, circulation, and composition, thereby providing a comprehensive knowledge of "what is known" and "what is new" in the field of lymphatic biology.

Chapter 2
Lymphangiogenesis

Andrea M. Foskett, Sanjukta Chakraborty, and Mariappan Muthuchamy

Abstract Lymphatic vessels are intimately involved in the maintenance of tissue homeostasis, immune cell trafficking, and transport of dietary lipids. During embryonic development, growth of new lymphatic vessels or lymphangiogenesis occurs from preexisting blood vessels in a tightly regulated manner, which then undergoes remodeling and maturation to form the extensive lymphatic network. However, aberrant lymphangiogenesis is also associated with a number of pathological conditions, such as inflammatory diseases, allograft rejection, and cancer metastasis, while insufficient lymphangiogenesis underlies the debilitating condition of lymphedema. This chapter aims to provide an overview of the different cellular mechanisms and key molecular players involved in the regulation and progression of normal lymphatic vascular development (or physiological lymphangiogenesis) and pathological lymphangiogenesis. Understanding the mechanisms of lymphatic vascular development or its role in these pathological processes is a prerequisite for the efficient development of key therapeutic interventions for lymphatic-associated diseases.

2.1 Introduction

2.1.1 Structure and Function of the Lymphatic Vasculature

From a historical perspective, the first descriptions of vessels containing a colorless fluid, referred to as "white blood" or "arteries containing milk," were made as early as 300 BC (Gnepp 1984). However, it was not until 1622, that an Italian anatomist

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and physician Gasparo Asellius observed unique vessels containing white blood in the mesentery of the dog and he called these vessels lacteals. The discovery of the lymphatic system has a longstanding history with contributions from numerous anatomists and physicians over the course of many centuries. However, since these first reports on lymphatic vessels, research on the development and function of the lymphatic system has progressed dramatically only over the last two decades, enabling a better appreciation for this complex system.

The major roles of the lymphatic system include maintenance of blood and tissue volume (Taylor et al. 1973), absorption and transport of dietary lipids from the intestine to the liver (Tso 1994), and immune cell trafficking (Angeli and Randolph 2006; Casley-Smith 1974; Johnson et al. 2006; Randolph et al. 2005). To accomplish these roles, the lymphatic system consists of a network of vessels of varying caliber that are connected through a series of lymph nodes and other lymphoid organs. The lymphatic system is responsible for the absorption of 20–50 % of the plasma volume and 50–100 % of the plasma proteins from the interstitium and drainage back into the systemic circulation daily (Yoffey and Courtice 1970). Furthermore, specialized lymphatics called lacteals present within the villi of the small intestine absorb dietary lipids that are secreted by enterocytes in the form of chylomicrons (Backhed et al. 2007; Tso 1994). In addition, lymphatic vessels function as active conduits for the passage of extravasated leukocytes and immune cells such as antigen-presenting dendritic cells, T lymphocytes, and macrophages thus representing an important step in the regulation of the immune response (Angeli and Randolph 2006; Casley-Smith 1974; Johnson et al. 2006; Randolph et al. 2005).

Lymphatic capillaries, also known as initial lymphatics, act as an entry point for interstitial fluid and macromolecules from the interstitial spaces. Anatomically, lymphatic capillaries are irregularly shaped, blind-ended, and thin-walled vessels. In order to facilitate permeability to large macromolecules and migrating cells, lymphatic capillaries are comprised of a single layer of overlapping oak leaf-shaped endothelial cells that are connected by loose discontinuous button-like junctions or flap-like valves (Baluk et al. 2007; Dejana et al. 2009). These lymphatic capillaries neither have a continuous basement membrane nor are they invested with muscle cells. To prevent the collapse of the lymphatic capillaries, they are physically tethered to the surrounding extracellular matrix (ECM) by bundles called anchoring filaments, which are composed of collagen, fibrillin, and emilin-1 (Danussi et al. 2008; Leak and Burke 1966, 1968).

The lymphatic capillaries coalesce to form a larger network of precollector vessels, leading to muscular collecting lymphatics, lymphatic trunks, and finally the lymphatic ducts (Gnepp 1984). The collecting lymphatic vessels possess a diverse structure that can differ dramatically in various tissues depending on its position in the lymphatic network. The two most distinguishing characteristics of the collecting lymphatics are the presence of numerous unidirectional bicuspid valves and varying amounts of muscle cell layers (Baluk et al. 2007; Schmid-Schonbein 1990). Lymphangions are the functional unit of the muscular collecting lymphatic that are arranged in series along the length of the vessel separated by valves (Gashev 2002). These unique structural features of the collecting lymphatics render its functional

ability to transport lymph from one lymphangion to the next via active pumping mechanisms while also preventing backflow. The collecting lymphatics consist of a continuous layer of endothelial cells with "zipper-like" intercellular adherens and occludin junctions (Baluk et al. 2007; Dejana et al. 2009). A continuous basement membrane is also present in these vessels, which prevents leakage of lymph. Collagen and elastic fibers are randomly distributed in the spaces between the endothelial cells and the layers of muscle cells. Muscle cell layers are usually associated with the lymphangion segments and wrap around the endothelial cell-lined vessel wall, while usually at the valve site there are fewer muscle cells (Gnepp 1976; Gnepp and Green 1980). There exists a huge variation in the density, orientation, and organization of the muscle cells in different calibers of collecting vessels and among various species. As an example, the thoracic duct in a human has circular muscle cell layers that are oriented in a circumferential fashion (Gnepp 1984; Petrenko and Gashev 2008). However, in the rat diaphragm, muscle cells are arranged circumferentially near valve regions, while they are more longitudinally or spirally arranged between valves (Ohtani and Ohtani 2001). Remarkably, the different structural components of the lymphatic system work in concert to accomplish its principal task—the transport of lymph. The mechanisms of lymph transport have been discussed in another chapter of this book.

Dysfunction of the lymphatic system either due to genetic mutations that cause improper development or surgical procedures that damage lymphatic vessels result in a wide range of pathologies. One of the most debilitating outcomes of impaired lymph transport is lymphedema, a chronic progressive disease with no cure that is characterized by disfiguring swelling and impaired immunity. Other pathological conditions include filariasis, chylous ascites, and cyclothorax, inflammatory and autoimmune diseases, and the involvement of lymphatics as routes for tumor metastasis. This chapter focuses on the lymphangiogenesis processes in normal development and in pathological conditions.

2.2 Physiological Lymphangiogenesis

2.2.1 Embryonic Development of the Lymphatic Vasculature

American anatomist, Florence Sabin is credited with presenting the earliest and most widely accepted model of lymphatic development in 1902 (Sabin 1902, 1904, 1916). Based on elegant dye-injection experiments, she proposed that endothelial cells bud from veins to form primary lymph sacs, which in turn sprout in a centrifugal pattern to form dense lymphatic networks in surrounding tissues and organs (Sabin 1902, 1904, 1916). Several years later in 1910, Huntington and McClure argued an alternative theory suggesting that the initial lymph sacs originated from the mesenchyme, independent of the veins and only subsequently established venous connections (Huntington and McClure 1910). Over the past 100 years or so,

we have moved down a rather slippery slope debating repeatedly over the origin of lymphatics. Only recently has this question been irrevocably answered with evidence from Cre/Lox-P-based lineage tracing studies by Srinivasan et al. (2007), which conclusively corroborates Sabin's model. Srinivasan et al. (2007) demonstrated that lymphatic endothelial cells (LECs) sprouted, proliferated, and migrated from venous-derived lymph sacs, giving rise to the entire lymphatic vasculature, and that hematopoietic cells did not contribute to this process (Srinivasan et al. 2007). The venous origin of LECs has also been documented in other models including *Xenopus laevis* (Ny et al. 2005) and zebrafish (Yaniv et al. 2006).

The key steps outlining the development of the lymphatic vasculature is schematically represented in Fig. 2.1. In mice, this process is initiated around embryonic day 9.0 (E9.0) when some local induction signal, albeit sill unknown, triggers the process of commitment of a few endothelial cells (ECs) lining the anterior cardinal vein, toward a unique LEC identity (Albrecht and Christofori 2011; Oliver 2004; Oliver and Alitalo 2005; Oliver and Srinivasan 2008; Tammela and Alitalo 2010). The early expression of markers such as vascular endothelial growth factor receptor-3 (VEGFR-3), also known as Fms-like tyrosine kinase 4 (Flt4) as well as lymphatic vessel endothelial hyaluronan receptor 1 (LYVE-1) identify this unique population of lymphatic endothelial fate-competent cells. However, the exact developmental, functional, or regulatory relevance of LYVE-1 is further confounded by studies in LYVE-1$^{-/-}$ mice that demonstrate the development of a normal functional network of lymph vessels and lymph nodes (Gale et al. 2007; Luong et al. 2009). These findings implicate a possible compensatory mechanism that can overcome the loss of LYVE-1.

Subsequent to the initiating step, at around E10, expression of the homeobox transcription factor prospero-related homeobox 1 (Prox1) commences in VEGFR-3^{+}/LYVE-1^{+} cells and becomes restricted in a polarized manner to a subpopulation of ECs located on one side of the vein (Oliver 2004; Oliver and Alitalo 2005; Oliver and Srinivasan 2008). Unlike LYVE-1, the functional significance of Prox-1 in lymphatic development is well understood and remains the most valuable regulator of LEC specification and maintenance (Hong et al. 2002; Wigle et al. 2002; Wigle and Oliver 1999). Prox1^{+} LECs bud out from the vein, migrate, and aggregate to form primary lymph sacs, eventually giving rise to a primitive lymphatic plexus. Consequently, Prox1$^{-/-}$ mouse embryos have been shown to completely lack a lymphatic vasculature, not due to an arrest in LEC budding, but rather as a result of a failure in lymphatic cell specification (Wigle et al. 2002; Wigle and Oliver 1999). On closer examination, it was apparent that the ECs that budded from the anterior cardinal vein still expressed blood vascular markers but in the absence of Prox1 failed to commit to a lymphatic identity. Prox1^{+} budding LECs also provides feedback regulation allowing Prox1 to be continuously upregulated in the veins, which is believed to provide the spatial and temporal cues that influence the location and timing of the newly forming lymphatic primordia (Wigle et al. 2002). Also, overexpression of Prox1 was determined to be sufficient to reprogram blood endothelial cells (BECs) into LECs, by suppressing blood vascular-specific genes and up regulating LEC-specific genes (Hong et al. 2002, 2004; Petrova et al. 2002). In addition

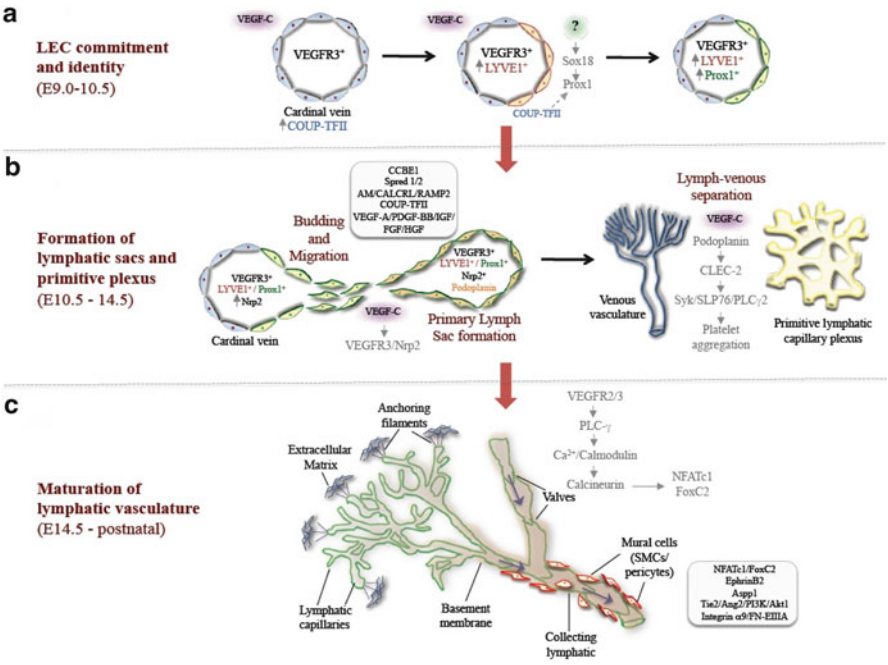

Fig. 2.1 Development of the lymphatic vasculature. (**a**) Lymphatic endothelial cell (LEC) commitment and identity (E9.0–10.5)—LECs are specified in embryonic veins, which initially express high levels of VEGFR-3. LYVE-1, the earliest known lymphatic marker, identifies a unique subpopulation of lymphatic endothelial fate-competent cells in the large veins. Following this initiation step, Sox18 induces the expression of Prox1 in VEGFR-3^{+}/LYVE-1^{+} cells and becomes restricted in a polarized manner to a subpopulation of ECs located on one side of the vein. (**b**) Formation of lymphatic sacs and the primitive plexus (E10.5–14.5)—LEC biased cells also begin to express Nrp2. VEGFR-3/VEGF-C provides a guidance mechanism required for the budding and migration of LECs thus forming primary lymph sacs. Other lymphangiogenic factors such as CCBE1, Spred 1/2, and COUP-TFII among others have also been implicated in the sprouting potential of LECs. The LECs now begin to express podoplanin, which further activates the CLEC-2/Syk/SLP76 signaling pathway. This leads to platelet aggregation, which blocks the connections between the blood and lymphatic vasculatures, thus severing the two vasculatures from each other. (**c**) Maturation of the lymphatic vasculature (E14.5-postnatal)—Maturation of the primitive lymphatic plexus encompasses differentiation into lymphatic capillaries and collecting lymphatic vessels. The lymphatic capillaries are irregularly shaped, thin-walled vessels comprised of a single layer of overlapping oak leaf-shaped endothelial cells. The lymphatic capillaries neither have a continuous basement membrane nor are they invested with muscle cells. They are physically tethered to the surrounding extracellular matrix by bundles called anchoring filaments. The process of maturation of the collecting lymphatic vessels involves complex morphological remodeling events necessitating the occurrence of several critical steps, such as valve formation, muscle cell recruitment, and vessel specification. The calcineurin/NFATc1/FoxC2 pathway plays an important role in the formation of valves. The investiture of muscle cell layers is accomplished by several key players, such as, EphrinB2, FoxC2, Aspp1, Tie2/Ang2, and integrin α9

to regulating lymphatic specification, Prox1 is also critical for the maintenance of this lymphatic endothelial phenotype during later stages of development and in adulthood, since heterozygous Prox1$^{+/-}$ mice exhibit impaired lymphatic function and abnormalities in lymphatic network patterning (Harvey et al. 2005). These

findings and those of other groups have confirmed that Prox1 is the crucial hallmark gene that is both required and sufficient to confer a LEC phenotype (Hong et al. 2002, 2004; Oliver and Detmar 2002; Petrova et al. 2002; Wigle et al. 2002; Wigle and Oliver 1999).

As a consequence, this raises the next logical question: what mechanism regulates Prox1, the master regulator of lymphatic development? The exact mechanisms upstream of Prox1 induction remain elusive, although some suggestions have been proposed in the literature. For instance, it is known that IL-3 and IL-7 can induce Prox1 expression in cultured human BECs (Al-Rawi et al. 2005; Groger et al. 2004). However, the involvement of IL-3 and IL-7 in inducing Prox1 expression in vivo is yet to be determined. As another example, Francois et al. (2008) demonstrated the direct induction of Prox1 in cultured BECs by transcription factor Sox18, while Sox18$^{-/-}$ mouse embryos lacked the development of lymphatic vasculature. Intriguingly, Sox18 expression was identified in a subset of cells on the cardinal vein believed to have a lymphatic bias, prior to Prox1 induction leading to lymphatic specification in those very same cells (Francois et al. 2008). Nuclear receptor COUP-TFII that is best known for its role in maintaining venous cell identity has recently been shown to interact with Prox1 (Lee et al. 2009; Srinivasan et al. 2010; Yamazaki et al. 2009) and was identified as a co-regulator of Prox1 function in maintaining LEC specification (Lin et al. 2010). Furthermore, the conditional ablation of COUP-TFII during early embryonic time points compromised the development of the lymphatic vasculature implicating its role in the establishment of LEC identity (Lin et al. 2010).

2.2.2 Lymphangiogenic Growth Factors: Sprouting, Proliferation, and Migration

Subsequent to LEC specification, the polarized budding of lymphatic fate-committed Prox1^{+} cells is driven by a receptor–ligand guidance mechanism. Although several lymphangiogenic growth factors and their receptors have been identified, the best characterized and vital to this directed migration process are vascular endothelial growth factors (VEGF)-C and VEGF-D and their cognate receptor VEGFR-3 (Achen et al. 1998; Joukov et al. 1996; Karkkainen et al. 2004; Lohela et al. 2009). Even before the onset of LEC specification by master regulator Prox1, VEGFR-3 is expressed on some ECs of the cardinal vein that are presumed to be competent to acquire a lymphatic phenotype (Albrecht and Christofori 2011; Oliver 2004; Oliver and Alitalo 2005; Oliver and Srinivasan 2008; Tammela and Alitalo 2010). During early murine development, VEGFR-3 is initially expressed by both the blood and lymphatic endothelium, but becomes mostly restricted to the lymphatic endothelium in late development and adulthood (Kaipainen et al. 1995; Wigle et al. 2002). Findings from studies using VEGFR-3$^{-/-}$ mice highlighted a role for VEGFR-3 in mediating sprouting and remodeling of the primary vascular plexus. Because VEGFR-3$^{-/-}$ mice exhibit a dramatic blood vascular phenotype and are

embryonically lethal by E9.5 before the emergence of the lymphatic vasculature, its exact role in lymphatic development cannot be fully addressed (Dumont et al. 1998; Hamada et al. 2000). However, the identification of missense mutations in VEGFR-3 in patients with hereditary lymphedema (Karkkainen et al. 2000) has provided support for its role in lymphatic development.

In concert with VEGFR-3, there is now overwhelming evidence for the chemotactic role of VEGF-C as a potent inducer of lymphatic sprouting from the cardinal vein (Enholm et al. 2001; Karkkainen et al. 2004; Karpanen and Alitalo 2008; Oh et al. 1997). Findings from in vivo studies demonstrate that over expression of VEGF-C in the mouse skin results in lymphangiogenesis and hyperplasia of cutaneous lymphatics (Enholm et al. 2001; Jeltsch et al. 1997), while VEGF-C is capable of regenerating the cutaneous lymphatic network in skin of mice with lymphedema (Karkkainen et al. 2001). Furthermore, VEGF-C$^{-/-}$ embryos are perinatally lethal around E15.5 and completely lack lymphatic vessels due to defective budding and migration of the Prox1^{+} LECs to form lymph sacs (Karkkainen et al. 2001, 2004). Also in zebrafish, the VEGFR-3/VEGF-C signaling axis has been implicated in the development of the lymphatic vasculature (Kuchler et al. 2006; Yaniv et al. 2006). On the other hand, VEGF-D can bind both VEGFR-2 and VEGFR-3 (Stacker et al. 1999). VEGF-D has been shown to be a potent inducer of lymphangiogenesis and is capable of mediating LEC migration (Byzova et al. 2002; Rissanen et al. 2003; Tammela et al. 2005a). In the mouse skin model, over-expression of VEGF-C or VEGF-D induced lymphangiogenesis of lymphatic capillaries, without affecting angiogenesis (Jeltsch et al. 1997; Veikkola et al. 2001). However, the lymphangiogenic potential of VEGF-D was foreshadowed by evidence in mice with a targeted inactivation of VEGF-D that developed normal lymphatic vasculature (Baldwin et al. 2005). Therefore, VEGF-D may be dispensable during the sprouting of embryonic lymphatic capillaries or perhaps VEGF-C compensates for VEGF-D in its absence.

It has been suggested that the sprouting potential of LECs is not only mediated by VEGFR-3 but also by its transmembrane co-receptor neuropilin-2 (Nrp2), which is also a receptor for class III semaphorins classically involved in neuronal axon guidance (Neufeld et al. 2002). Nrp2 selectively controls the formation of large and small caliber lymph vessels as Nrp2$^{-/-}$ mice exhibit a transient absence or severe reduction of small lymphatic capillaries during development (Yuan et al. 2002). Recent evidence further suggests that Nrp2 interacts with VEGFR-3 to promote dermal lymphatic vessel sprouting in mice in response to VEGF-C (Xu et al. 2010). Several additional growth factors have been implicated either in vitro or in vivo to be involved in various aspects of the lymphangiogenesis process namely proliferation, migration, and formation of the primitive lymphatic plexus. These include hepatocyte growth factor (HGF), fibroblast growth factor-2 (FGF-2), FGF-3, platelet-derived growth factor-BB (PDGF-BB), and insulin growth factors-1 and -2 (Auguste et al. 2003; Bjorndahl et al. 2005a; Cao et al. 2004; Kajiya et al. 2005; Kubo et al. 2002; Shin et al. 2006).

As budding and sprouting progresses to give rise to the primitive lymphatic network, the connections between the blood and lymphatic vasculatures are lost. Lymphatic capillaries begin to separate away from the veins except at the junction