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Chapter 4
Plasticity of Airway Lymphatics in Development and Disease

Li-Chin Yao and Donald M. McDonald

Abstract The dynamic nature of lymphatic vessels is reflected by structural and functional modifications that coincide with changes in their environment. Lymphatics in the respiratory tract undergo rapid changes around birth, during adaptation to air breathing, when lymphatic endothelial cells develop button-like intercellular junctions specialized for efficient fluid uptake and transport. In inflammatory conditions, lymphatic vessels proliferate and undergo remodeling to accommodate greater plasma leakage and immune cell trafficking. However, the newly formed lymphatics are abnormal, and resolution of inflammation is not accompanied by complete reversal of the lymphatic vessel changes back to the baseline. As the understanding of lymphatic plasticity advances, approaches for eliminating the abnormal vessels and improving the functionality of those that remain move closer to reality. This chapter provides an overview of what is known about lymphatic vessel growth, remodeling, and other forms of plasticity that occur during development or inflammation, with an emphasis on the respiratory tract. Also addressed is the limited reversibility of changes in lymphatics during the resolution of inflammation.

4.1 Introduction

Plasma leakage, edema, and remodeling of the airway wall are hallmarks of inflammatory airway diseases (Dunnill 1960; Ebina 2008; Wilson and Hii 2006). Lymphangiogenesis and lymphatic remodeling are among the features of sustained respiratory inflammation (El-Chemaly et al. 2008). Lymphatics proliferate in pneumonia (Mandal et al. 2008; Parra et al. 2012), regress in asthma (Ebina et al. 2010), and undergo remodeling and growth in idiopathic pulmonary fibrosis (Yamashita

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et al. 2009; El-Chemaly et al. 2009). Understanding the contribution of lymphatic changes to disease pathophysiology and the clinical implications is still at an early stage. Elucidation of the causes, consequences, and reversibility of changes in airway lymphatics will offer new therapeutic targets and treatment strategies.

In a mouse model of sustained inflammation associated with infection by the respiratory pathogen *Mycoplasma pulmonis*, lymphatics in the airways and lung undergo rapid proliferation and remodeling (Baluk et al. 2005; McDonald 2008). Lymphatic growth and remodeling typically occur together but represent different vascular responses, and the driving factors and consequences are likely to be different. As diseases worsen, the lymphatic microvasculature undergoes progressive changes in structure and function. By comparison, lymphatic growth and remodeling during development are a natural process.

The pulmonary lymphatic network arises by sprouting lymphangiogenesis from lymphatic precursor channels and sacs that initially arise from the cardinal vein. The segmental pattern of tracheal lymphatics is established before birth, but maturation of lymphatics evidenced by specialized button-like intercellular junctions occurs postnatally. Studies of changes in lymphatic junctions around birth have provided a better understanding of lymphatic dynamics during development. Lymphatic changes found in inflammation recapitulate aspects of physiological growth and remodeling in perinatal mice (Yao et al. 2012). Understanding the plasticity of lymphatics under normal conditions and in disease, along with the underlying mechanisms, is necessary for identifying new therapeutic strategies and developing new diagnostic procedures.

Most studies of lymphangiogenesis have focused on preventing rather than reversing changes in lymphatics. In prevention studies, treatment with growth factor inhibitors starts with or before the lymphangiogenic stimulus. This approach can test the involvement of factors that promote lymphangiogenesis and elucidate the contribution of lymphatic changes to disease. In reversal studies, lymphatic changes are established before the onset of treatment. This design can be used to detect factors that are responsible for the maintenance of newly formed lymphatics, develop strategies for eliminating dysfunctional lymphatics, and elucidate the consequences of lymphatic reversal in relation to disease severity. Reversal of lymphatic changes to the baseline state could be clinically important, but the understanding of this reversibility is still at an early stage.

4.2 Structure of Lymphatic Vasculature in Normal Airways

4.2.1 Segmental Arrangement of Initial Lymphatics

The trachea is the largest airway in mice but is similar in size to small bronchioles in humans. When compared to the lung, the trachea is anatomically simpler and easier

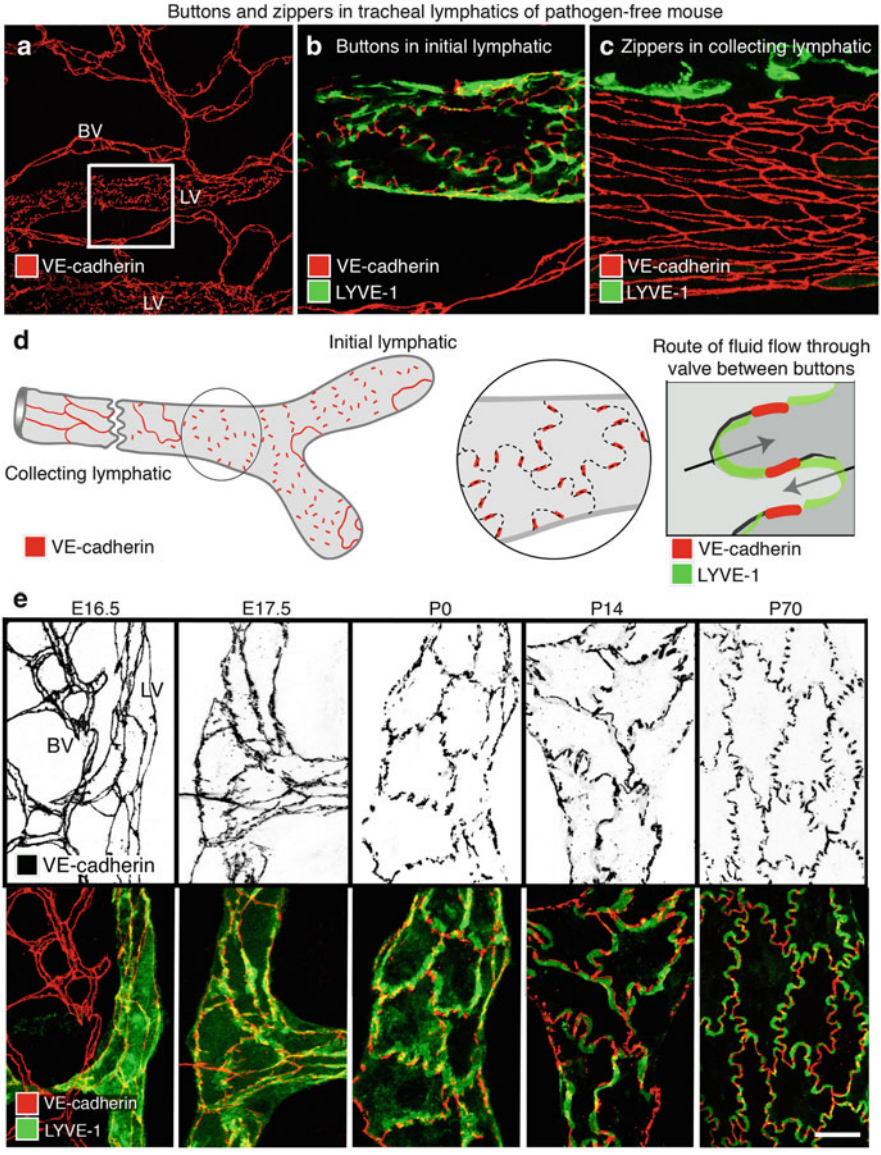

Fig. 4.1 Plasticity of tracheal lymphatics in development. Buttons in initial lymphatics and zippers in collecting lymphatics of a normal mouse trachea. (**a**) Low magnification of VE-cadherin (*red*) stained button-like junctions in initial lymphatics (LV) and zipper-like junctions in blood vessels (BV). (**b**) The *box* in (**a**) is enlarged here to show discontinuous segments of VE-cadherin immunoreactivity (*red*) at buttons and segments of LYVE-1 staining (*green*) between buttons in an initial lymphatic. (**c**) VE-cadherin immunoreactivity (*red*) at zippers in the endothelium of a collecting lymphatic with little or no LYVE-1 staining. LYVE-1-positive leukocytes (*green*) are present outside the lymphatic. (**d**) Schematic diagram showing buttons and zipper in lymphatics revealed by VE-cadherin immunoreactivity. *Middle panel* shows the oak leaf-shaped endothelial cells marked by *dashed lines*. The *right panel* shows the enlarged diagram of buttons (*red*) at the sides of cell border flaps (*green*). Fluid is believed to flow through the junction-free

to access experimentally. Moreover, the stereotypic architecture of tracheal lymphatics has proven advantageous for studying growth and remodeling of lymphatics in mouse models. The distinctive segmented arrangement of blood vessels and lymphatics is evident in flattened tracheal whole mounts stained by immunohistochemistry (Baluk et al. 2005; Yao et al. 2010) (Fig. 4.1a). Blind-ended initial lymphatics and pre-collecting lymphatics with valves are largely restricted to regions of mucosa between cartilage rings. Few lymphatics are present over cartilage rings. Large collecting lymphatics with valves and weaker LYVE-1 immunoreactivity are located downstream on the adventitial surface of the trachea. Smooth muscle cells are absent in the initial lymphatics and pre-collecting lymphatics and are sparse on collecting lymphatics. Lymphatics from the trachea join lymphatics from the esophagus and drain into mediastinal lymph nodes (Van den Broeck et al. 2006).

4.2.2 Button-Like Endothelial Junctions in Initial Lymphatics

Lymphatic endothelial cells have specialized intercellular junctions (Fig. 4.1b–d). The organization of junctional proteins is specialized to meet functional requirements, similar to those in blood vessels (Baluk et al. 2007; Dejana et al. 2009a, b). Endothelial cells of initial lymphatics are connected by discontinuous junctions called "buttons" (Fig. 4.1b). The scalloped flaps between buttons overlap each other and are thought to open and close in response to elevated interstitial fluid pressure. Live cell imaging has shown that valve-like gaps located between buttons are preferential sites of cell entry (Pflicke and Sixt 2009; Tal et al. 2011). Endothelial cells of collecting lymphatics are joined by continuous intercellular junctions called "zippers" (Baluk et al. 2007).

Understanding the nature of junctions in the initial lymphatics has evolved in multiple stages. In the 1960s, electron micrographs of thin cross sections of lymphatic vessels suggested that lymphatic endothelial cells had partially or completely open intercellular junctions (Leak and Burke 1966, 1968; Casley-Smith 1972). About ten years ago, a concept for the initial lymphatics serving as an entry or primary valve to regulate cell and fluid entry was introduced (Trzewik et al. 2001; Schmid-Schonbein 2003). Subsequent studies by immunohistochemistry with VE-cadherin and other junctional proteins in tracheal initial lymphatics demonstrated the morphological basis of primary valves (Baluk et al. 2007).

Fig. 4.1 (continued) flaps. (**e**) Development of button-like junctions from E16.5–P70 is shown as inverted gray scale images (*upper panel*, VE-cadherin) and in color (*lower panel*, VE-cadherin, *red*; LYVE-1, *green*). Scale bar: 100 μm (**a**); 20 μm (**b**, **c**); 10 μm (**e**). ((**b**, **c**, and **e**) reproduced from (Yao et al. 2012); (**d**) reproduced from (Baluk et al. 2007))

Endothelial cells of lymphatics and blood vessels are joined by two types of intercellular junctions, adherens junctions and tight junctions (Dejana et al. 2009b; Leak and Burke 1966; Komarova and Malik 2010). Buttons and zippers are composed of the same junctional proteins that include VE-cadherin at adherens junctions and occludin, claudin-5, ZO-1, JAM-A, and ESAM at tight junctions (Baluk et al. 2007).

VE-cadherin is a well-characterized marker of endothelial cell adherens junctions. VE-cadherin immunoreactivity is similarly strong in blood vessels and lymphatics (Dejana et al. 2009a, b), but the distribution of the junctional protein is different. Unlike the continuous junctions of blood vessels, VE-cadherin in initial lymphatics is distributed in the form of button-like junctions that are 3.2 μm in length and 2.9 μm apart (Baluk et al. 2007). In the trachea, buttons are most abundant in the first 750 μm from the tip of initial lymphatics and are replaced by zippers after about 1,500 μm, which is the region where pre-collecting lymphatics with valves begin.

4.2.3 Comparison of Initial Lymphatics to Collecting Lymphatics

The continuous zipper-like junctions between endothelial cells of collecting lymphatics stain for VE-cadherin at the cell border, similar to the junctions of blood vessels (Baluk et al. 2007) (Fig. 4.1c). The exclusive presence of zippers in collecting lymphatics is consistent with their importance for transport of lymph.

4.3 Plasticity of Lymphatics During Pre- and Postnatal Development

4.3.1 Dependence of Lymphatic Growth and Survival on VEGF-C/VEGFR-3 Signaling

Activation of VEGF-C/VEGFR-3 signaling is necessary for the growth and development of the lymphatic system (Lohela et al. 2009). The requirement of VEGF-C starts from the initial steps of lymphatic development when lymphatic endothelial cells sprout from venous endothelium in the early embryo (Karkkainen et al. 2004). VEGF-D is also a ligand for VEGFR-3, but appears to be dispensable in the embryo because lymphatic development proceeds normally in the absence of VEGF-D (Baldwin et al. 2005).

After proteolytic cleavage to the mature 20 kDa protein, VEGF-C can bind and activate both VEGFR-3 and VEGFR-2 (Joukov et al. 1997). Activation of VEGFR-2 can promote lymphangiogenesis (Nagy et al. 2002; Hong et al. 2004; Wirzenius

et al. 2007). Activation of VEGF-C/VEGFR-3 signaling alone is usually sufficient to induce lymphangiogenesis, but VEGFR-2 and VEGFR-3 form heterodimers, and signaling through VEGFR-2/VEGFR-3 heterodimers could be involved under some conditions (Joukov et al. 1997; Nilsson et al. 2010).

Continuous signaling by VEGFR-3 is required for the survival of lymphatic endothelial cells during development, but this requirement is lost after birth (Makinen et al. 2001; Karpanen et al. 2006). Inhibition of VEGFR-3 activation can lead to regression of lymphatics until 2 weeks of postnatal age, but prolonged VEGFR-3 inhibition in the adult has no apparent effects on established lymphatics despite strong VEGFR-3 expression in lymphatic endothelial cells in the adult (Pytowski et al. 2005). The function of VEGFR-3 in maintaining the integrity of mature lymphatics deserves further investigation.

4.3.2 Transformation of Zipper- to Button-Like Junctions During the Perinatal Period

Intercellular junctions of endothelial cells can change under physiological conditions (Dejana et al. 2009a). Studies of changes in lymphatic cell junctions around the time of birth provide a better understanding of the dynamic features of lymphatics in the airways of neonatal mice (Yao et al. 2012). At E16.5, the segmental pattern of airway lymphatics is largely established, but the lymphatic endothelial cells are joined by zipper-like junctions and lack button-like junctions typical of the adult. Zippers begin to be replaced by buttons at E17.5. This transformation is particularly rapid at birth and largely complete by P28 (Fig. 4.1e). Similar perinatal transformation of zippers to buttons also occurs in the initial lymphatics of the diaphragm (Yao et al. 2012). During the transformation, the junctional proteins stay the same.

The rapid increase in number of buttons around birth is consistent with the need for efficient clearance of fluid from the lungs during the transition from an intrauterine environment of water to an external environment of air. The importance of lung lymphatics in this process is evident in transgenic mice that overexpress the extracellular domain of VEGFR-3 that traps VEGF-C and VEGF-D and thereby blocks VEGFR-3 signaling in the lungs (Kulkarni et al. 2011). These newborn mice have pulmonary lymphatic hypoplasia, increased lung weight, and high mortality.

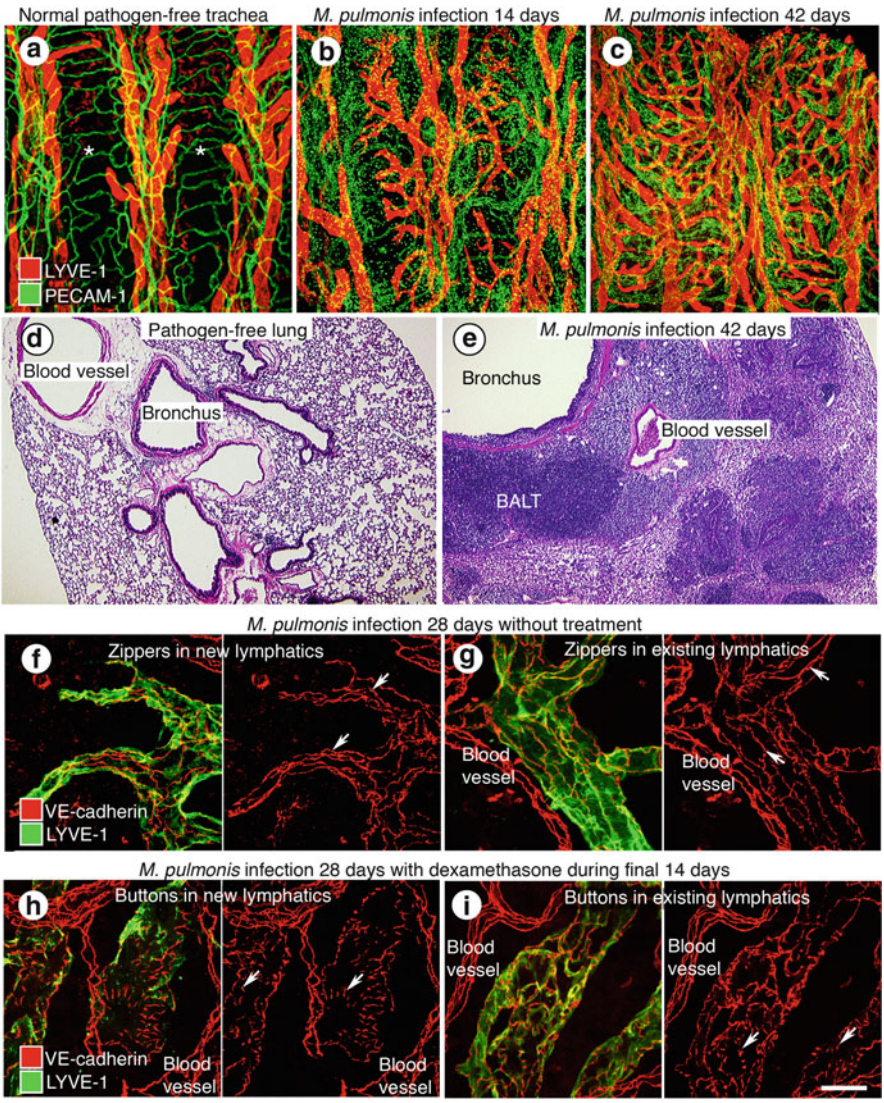

Fig. 4.2 Plasticity of lymphatics in airway inflammation. Changes in tracheal lymphatics after *M. pulmonis* infection. Confocal micrographs of mouse tracheal whole mounts stained for lymphatics (*red*, LYVE-1) and blood vessels (*green*, PECAM-1). (**a**) Few or no lymphatics are located over the cartilage rings (*asterisks*) in the pathogen-free mouse. (**b**) Tracheal lymphatics are present over cartilage rings in a mouse infected for 14 days. (**c**) Tracheal lymphatics are abundant and disorganized in a mouse infected for 42 days. H&E stained sections of mouse left lung. (**d**) No BALT is present in the pathogen-free lung. (**e**) BALT is abundant around the large bronchus and blood vessel in the lung of a mouse infected for 42 days. (**f**, **g**) Zipper-like junctions ("zippers," *arrows*) are present in the endothelium of tracheal lymphatics after infection for 28 days. (**h**, **i**) Button-like junctions ("buttons," *arrows*) are present in the endothelium of tracheal lymphatics when dexamethasone was given during the final 14 days of a 28-day infection. Scale bar: 200 μm (**a**–**c**); 400 μm (**d**, **e**); 20 μm (**f**, **i**). ((**a**–**c**) reproduced from (Yao et al. 2010); (**f**) reproduced from (Yao et al. 2012))

4.4 Plasticity of Lymphatics in Pathological Conditions

4.4.1 Lymphangiogenesis in Chronic Airway Inflammation

M. pulmonis infection has multiple attributes for studying lymphangiogenesis in sustained inflammation in the airways of mice (Lindsey and Cassell 1973). Regions overlying the cartilage rings of airways, which are normally almost free of lymphatics, have increasingly abundant lymphatics after infection (Fig. 4.2a–c). Lymphatics eventually outnumber blood vessels in the inflamed airway mucosa after *M. pulmonis* infection. Allergens have been used to sensitize and challenge the respiratory tract in studies of lung inflammation, but sustained inflammation depends on continued challenge, and few changes have been reported in respiratory lymphatics (Chu et al. 2004; Kretschmer et al. 2013).

Robust immune responses are essential for driving rapid and sustained changes in lymphatics and blood vessels of the airway mucosa after infection (Aurora et al. 2005). The lymphatic growth factors VEGF-C and VEGF-D and other cytokines released from macrophages, neutrophils, and other cells play important roles in lymphangiogenesis associated with inflammation (Baluk et al. 2005, 2009, 2013). Blocking VEGFR-3 signaling inhibits inflammatory lymphangiogenesis and reduces the enlargement of sentinel lymph nodes (Baluk et al. 2005), consistent with the contribution of cytokines, lymphatic fluid, and cell transit from inflamed airways. In this regard, defective lymphangiogenesis during airway inflammation could contribute to bronchial lymphedema and exaggerated airflow obstruction, but further studies of this issue are needed.

Bacteria or viral infection can induce immune responses that are accompanied by the development of bronchus-associated lymphoid tissue (BALT) in the lungs (Yao et al. 2010; Moyron-Quiroz et al. 2004; Rangel-Moreno et al. 2007; Kahnert et al. 2007). This so-called tertiary lymphoid tissue, consisting mainly of B-cell follicles, T cells, dendritic cells, and stromal cells, is commonly found at sites of chronic inflammation in the lung. BALT and its more peripherally located variants are abundant in the lungs of patients with chronic obstructive lung disease and are sites of lymphangiogenesis (Mori et al. 2013). BALT also accumulates in peribronchial and perivascular regions of lungs after *M. pulmonis* infection (Fig. 4.2d, e) (Yao et al. 2010). Lymphangiogenesis is reported to occur preferentially in regions of BALT (Baluk et al., unpublished findings).

4.4.2 Button-to-Zipper Transformation in Chronic Airway Inflammation

The physiological consequences of lymphangiogenesis and remodeling in chronic inflammation are poorly understood. The microvasculature of the chronically

inflamed airway mucosa has abnormalities in endothelial barrier function (McDonald 1994, 2001; Schoefl 1963). The endothelium of normal blood vessels has continuous zipper-like intercellular junctions, but remodeled blood vessels have focal gaps along intercellular junctions. Remodeled blood vessels are also abnormally sensitive to inflammatory mediators that evoke plasma leakage. Mucosal edema is usually present in sustained inflammation despite widespread lymphangiogenesis. The presence of edema indicates that fluid uptake exceeds the capacity for drainage through lymphatics and other routes.

Some clues toward reconciliation of the presence of edema despite more abundant lymphatics could lie in changes in endothelial cell junctions that result in impaired fluid uptake (Baluk et al. 2005; Yao et al. 2012). In inflamed airways, newly formed lymphatics have zippers instead of buttons (Fig. 4.2f) and existing lymphatics undergo button-to-zipper transformation (Fig. 4.2g) which reverses the transformation that occurs in development (Baluk et al. 2005; Yao et al. 2012).

4.5 Reversibility of Lymphatic Growth and Remodeling

4.5.1 Reversal of Inflammation by Dexamethasone

4.5.1.1 Reversal of Lymphangiogenesis

Dexamethasone has broad anti-inflammatory activity including inhibitory effects on angiogenesis and lymphangiogenesis (Folkman and Ingber 1987; Barnes 2005). Treatment of *M. pulmonis*-infected mice with dexamethasone has distinct but different effects on blood vessels and lymphatics. Remodeled blood vessels rapidly return to their baseline state after treatment, but newly formed lymphatics are more resistant. Many new lymphatics persist after the inflammation is resolved.

After dexamethasone, most lymphatics acquire a normal, smooth contour without sprouts. Although some newly formed lymphatics undergo regression—appearing as disconnected lymphatic "islands" with little or no LYVE-1 immunoreactivity—the majority persist. Differences in the reversibility of remodeling of blood vessels and lymphatics in inflammation are also found after treatment of *M. pulmonis* infection with an antibiotic (Baluk et al. 2005).

4.5.1.2 Reversal of Button-to-Zipper Transformation

Reversal of button-to-zipper transformation is another feature of normalized lymphatics (Yao et al. 2012). Dexamethasone treatment restores the oak leaf-shaped cell phenotype typical of initial lymphatics and redistributes LYVE-1 immunoreactivity to the cell borders at sites between the segments of VE-cadherin in buttons

(Fig. 4.2h, i). This action of dexamethasone is restricted in initial lymphatics and does not change zippers in the endothelium of collecting lymphatics and thoracic duct.

The actions of dexamethasone are not limited to anti-inflammatory effects. Glucocorticoid receptor signaling has beneficial effects on perinatal lung maturation (Whitsett and Matsuzaki 2006). Glucocorticoid receptor-deficient mice develop respiratory distress and die after birth (Cole et al. 1995). The observation that dexamethasone activates phosphorylation of glucocorticoid receptors in lymphatic endothelial cells and promotes button formation in neonatal mice could represent direct actions of the steroid that complement the more generalized anti-inflammatory effects (Yao et al. 2012).

4.5.2 Reversal of Lymphangiogenesis in Other Experimental Models

The reversibility of lymphangiogenesis has not been studied as much as the growth of lymphatics, but the results are mixed. As found after *M. pulmonis* infection, newly formed lymphatics persist in skin for many months after withdrawal of overexpression of VEGF-C in transgenic mice (Lohela et al. 2008). Similarly, persistence of radiotherapy-induced lymphangiogenesis has been reported in the skin of patients with breast cancer (Jackowski et al. 2007). New lymphatics that grow in inflamed ear skin are reported to slowly regress after a wound heals (Pullinger and Florey 1937). Lymphangiogenesis in lymph nodes is similarly reported to be reversible after lymph node hypertrophy resolves (Mumprecht et al. 2012). In a suture-induced corneal inflammation model, lymphangiogenesis is described as reversible and undergoing regression more quickly than blood vessels (Cursiefen et al. 2006).

4.6 Summary and Outlook

The extraordinary plasticity of lymphatics in disease fits with the dynamic nature of airway lymphatics during perinatal development (McDonald et al. 2011). Lymphatic vessel proliferation and remodeling are also features of pulmonary lymphangiectasia, lymphangiomatosis, lymphangioleiomyomatosis (LAM), and idiopathic pulmonary fibrosis (El-Chemaly et al. 2008; Henske and McCormack 2012). Because of this plasticity, airway lymphatics serve as indicators of changing tissue requirements during normal development and in pathological conditions. Development of new animal models that recapitulate the changes would provide valuable tools for elucidating underlying mechanisms. Mechanistic insights into the driving factors and consequences of lymphatic plasticity and into the resistance of lymphatics to regression should lead to a better understanding of disease

pathophysiology and new therapeutic approaches. Promotion of lymphatic growth by overexpression of VEGF-C has shown therapeutic potential by relieving the severity of skin inflammation (Huggenberger et al. 2010), improving drainage, and reducing edema in lymphedema models (Szuba et al. 2002). The same approaches could promote lymphatic maturation in other inflammation conditions. In addition, the delineation of factors that influence maturation of lymphatic endothelial cell junctions should advance the understanding of edema formation and resolution. Although it is unclear to what extent tissue edema in these conditions results from impaired lymphatic function, the use of new imaging techniques and other approaches for assessing efficiency of lymphatic fluid and cell transport should provide insights into the implications of lymphatic plasticity in disease.

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