Angiopoietins and TIE Receptors in Lymphangiogenesis and Tumor Metastasis

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Contents

Abstract

In contrast to the normal lymphatic network comprised of initial and collecting vessels, intratumor lymphatics are disorganized and lack vessel hierarchy due to the continuous lymphangiogenesis. Lymphatic vessels originate from veins during mammalian development, while tumor-associated lymphatics are largely formed by vessel cooption or sprouting from the preexisting lymphatics of adjacent tissues. Among the known lymphangiogenic regulators, angiopoietins and TIE receptors are crucial for the process of lymphatic remodeling to form a mature network. Accumulating evidence from animal and clinical studies has laid a solid foundation that tumor lymphangiogenesis contributes to tumor dissemination. It has been shown in animal tumor models that targeting the key

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D. Marmé (ed.), *Tumor Angiogenesis*,
https://doi.org/10.1007/978-3-319-31215-6_43-1

lymphangiogenic signaling pathways, including ANGPT-TIE mediated signals, could efficiently block lymphatic tumor metastasis. Meanwhile, ANGPT-TIE pathway is also actively involved in modulating tumor immune microenvironment. Therefore, strategies to fine-tune the interaction of lymphatic EC-immune cells could be employed in the prevention of tumor progression.

Keywords
Angiopoietin · TIE receptors · Lymphatic development · Tumor lymphangiogenesis · Lymphatic metastasis · Tumor-immune microenvironment

Introduction

Lymphatic vessels contribute to tissue homeostasis by draining excess tissue fluid together with large substances and immune cells (Tammela and Alitalo 2010; Petrova and Koh 2018). The lymphatic route can also be employed by tumor cells during their metastatic dissemination to distant organs after evasion from immune surveillance (Alitalo 2011; Karaman and Detmar 2014; Stacker et al. 2014). Mechanisms underlying lymphatic formation, including cellular events and molecular players, are largely shared in development and in tumor (Li et al. 2012). However, due to the distinct tissue microenvironment in embryos and tumors, the finally formed lymphatic networks are quite different, including the lymphatic vessel hierarchy, structural integrity, and functionality.

Comparison of Developmental and Tumor Lymphangiogenesis

Origin of Lymphatic Endothelial Cells in Development Versus Tumor

The initiation of lymphangiogenesis differs in development and in tumor (Fig. 1a, b). Following the arterial-vein specification in mammalian development, venous endothelial cells (ECs) are the major source of lymphatic ECs with PROX1 as the key regulator (Wigle and Oliver 1999; Adams and Alitalo 2007; Yang and Oliver 2014; Potente and Makinen 2017). Non-venous origin of lymphatic ECs has been found to participate in mesentery, heart, and superficial dermal lymphatic vessel formation in mice (Klotz et al. 2015; Martinez-Corral et al. 2015, Stanczuk et al. 2015). Venous EC-independent route of LEC initiation was also demonstrated in other species including chicken embryos (Wilting et al. 2003; Mahadevan et al. 2014), Xenopus tadpoles (Ny et al. 2005), and zebrafish (Nicenboim et al. 2015). In comparison with this, tumor-associated lymphatic endothelial cells mainly originate from the preexisting lymphatic network in the surrounding tissues (He et al. 2004). It is uncertain whether there is any differentiation of lymphatic endothelial cells from venous ECs in tumor. One interesting observation is that intratumor lymphangiogenesis mainly occurs in regions undergoing necrosis (Fig. 1b), suggesting that tumor-associated macrophages may be able to trans-differentiate into lymphatic ECs in tumors as demonstrated in inflamed tissues (Maruyama et al. 2005).

Functional Comparison of Lymphatic Network in Embryos and Tumor

The formation of a mature lymphatic system involves the remodeling of primitive lymphatic plexus into structurally specialized network containing initial and collecting lymphatics in development. Although a functionally competent lymphatic system is crucial for maintaining tissue fluid homeostasis in the postnatal life, the primary lymphatic network without collecting vessels is functional for lymph draining during embryonic development. This has been demonstrated in several genetically modified mouse models. For example, there was no lymphedema observed in *Angpt2* deficient embryos or the downstream *Akt1* null mice although there was no collecting vessel formation (Zhou et al. 2010; Shen et al. 2014). However, severe tissue lymphedema occurred in mice without lymph sac formation or with abnormal formation of the primitive lymphatic network in mutants targeting *Vegfc*, *Vegfr3* or *Tie1*

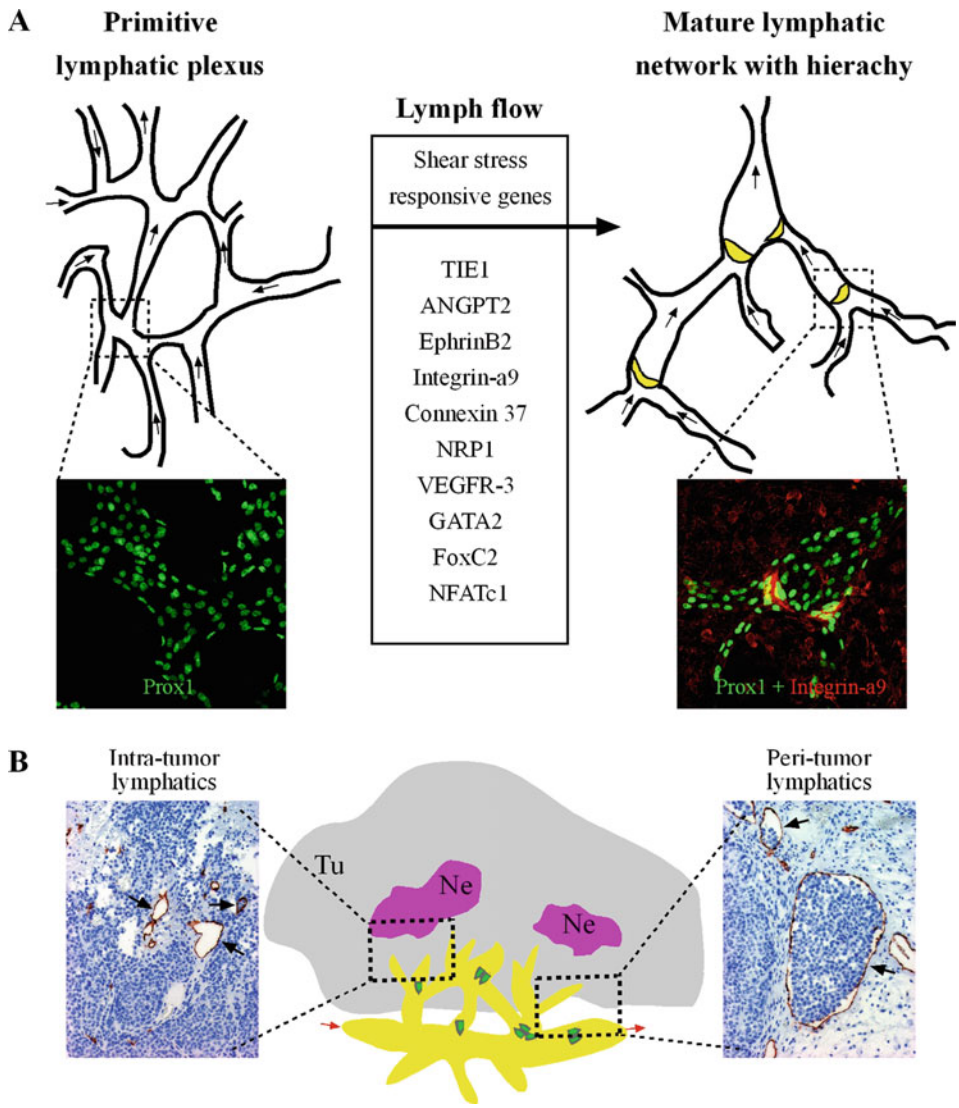

Fig. 1 Comparison of lymphatic network formation in development and tumor. (**a**). Lymphatic development involves the first formation of primitive lymphatic plexus followed by the process of lymphatic remodeling to form collecting vessels with intraluminal valves (green for PROX1 to indicate lymphatic ECs, and red for Integrin-α 9 to indicate lymphatic valves; and images are modified from Supplemental Figure II and Figure 3 in Arterioscler Thromb Vasc Biol. 2014;34:1221–1230, by permission of Wolters Kluwer Health Inc., through Copyright Clearance Center's RightsLink® service). This process is likely to be driven by lymph flow generated shear stress, which could induce a number of key lymphatic regulators as listed in the illustration. (**b**). Tumor-associated lymphatic vessels are formed by vessel cooption or sprouting from the pre-existing lymphatics of adjacent tissues. Intratumoral lymphatic vessel growth is often detected in necrotic areas, which is connected to the dilated peritumoral lymphatic network for tumor cell dissemination (red for LYVE1, and images are modified from Figure 5 in Cancer Res. 2005;65:4739–46, by permission from American Association for Cancer Research). Arrows point to the intra- and peritumoral lymphatics and some are already invaded by tumor cells (Tu, tumor, and NE, necrosis)

(Karkkainen et al. 2004; Zhang et al. 2010; Shen et al. 2014). Fluid flow generated shear stress has been shown to regulate the expression of various genes in endothelial cells including the key lymphatic regulators such as TIE1 and ANGPT2 as listed in Fig. 1a (Porat et al. 2004; Tressel et al.

2007; Sabine et al. 2012; Li et al. 2014; Baeyens et al. 2015; Kazenwadel et al. 2015; Sweet et al. 2015). Therefore, it is likely that lymph flow in the primitive lymphatic network plays a critical role in the process of remodeling to form a mature network.

In contrast, the formation and function of tumor-associated lymphatic network may largely be compromised by the specific tumor microenvironment. Tumor-associated lymphatic network is usually lack of vessel hierarchy due to the continuous lymphangiogenesis, which may to some extent resemble the primitive lymphatic plexus observed in development. Factors contributing to the lymphatic abnormality also include the hypoxic and acidic tumor microenvironment, mechanical stress generated by uncontrolled tumor cell proliferation, and high interstitial pressure resulting from the defective vascular wall integrity (Hanahan and Weinberg 2011; Li et al. 2012). The non-homogeneous distribution of lymphatic vessels in tumor tissues (Beasley et al. 2002; He et al. 2005) may partly account for the failure to detect functional lymphatics in the draining assay (Padera et al. 2002). However, lymph node metastasis occurs frequently in solid tumors (Alitalo et al. 2005; Achen and Stacker 2008; Karaman and Detmar 2014). Therefore, at least a proportion of tumor lymphatics are functional after connecting with collecting vessels mainly located at peritumoral regions (Karpanen et al. 2001; He et al. 2005).

Angiopoietins and TIE Receptors in Developmental Lymphatic Remodeling and Maturation

A range of factors have been identified to coordinate the complex processes of lymphatic development, including transcription factors, lymphangiogenic growth factors and membrane-bound receptors, intracellular signal mediators, extracellular matrix proteins, and cell junction molecules (Bertozzi et al. 2010; Schulte-Merker et al. 2011; Li et al. 2012; Bazigou and Makinen 2013; Yang and Oliver 2014; Zheng et al. 2014a; Aspelund et al. 2016; Vaahtomeri et al. 2017). Among the molecular regulators, ANGPTs and TIE receptors are crucial in the regulation of lymphatic cell-cell junction, cell survival, collecting lymphatic vessel formation, and valve morphogenesis (Fig. 2a, b) (Gale et al. 2002; Shimoda et al. 2007; Dellinger et al. 2008; D'Amico et al. 2010; Qu et al. 2010; Shen et al. 2014; Saharinen et al. 2017).

Angiopoietins in Developmental Lymphangiogenesis

ANGPT2 is a ligand for TIE2 and has important roles in both angiogenesis and lymphangiogenesis. In blood vessels, ANGPT2 was reported to antagonize ANGPT1 to destabilize the integrity of formed vasculature and to keep the sprouting ECs free from mural cell coverage. This allows vascular growth and remodeling in response to angiogenic factors such as vascular endothelial growth factor-A (VEGFA) (Maisonpierre et al. 1997; Gale et al. 2002). In *Angpt2* knockout mice, although blood vascular development during embryogenesis was normal, postnatal angiogenesis in retina was retarded and there was also the failure of hyaloid vessel regression (Gale et al. 2002). Furthermore, deletion of *Angpt2* did not affect the formation of lymph sacs and the capillary lymphatic network during embryonic development (Dellinger et al. 2008; Shen et al. 2014). However, ANGPT2 deficiency disrupted the formation of collecting lymphatic vessels with defective valve formation and abnormal recruitment of smooth muscle cells (SMCs) associated with lymphatic capillaries (Fig. 2b) (Gale et al. 2002; Dellinger et al. 2008; Shen et al. 2014). Mice null for *Angpt2* also displayed thinner lymphatic diameter and decreased LEC number in lymphatic vessels in comparison with that of control littermates (Shen et al. 2014). Consistently, transgenic overexpression of ANGPT2 in endothelial cells under the control of tetracycline was shown to increase the caliber of lymphatic vessels and also LEC number (Zheng et al. 2014b). Interestingly, the lymphatic phenotype of *Angpt2* null mice is similar to that of *Akt1* knockout mice (Zhou et al. 2010). In *Akt1* deficient mice, but not in *Akt2* or *Akt3* knockouts, a significant decrease of the diameter and endothelial cell number of lymphatic capillaries

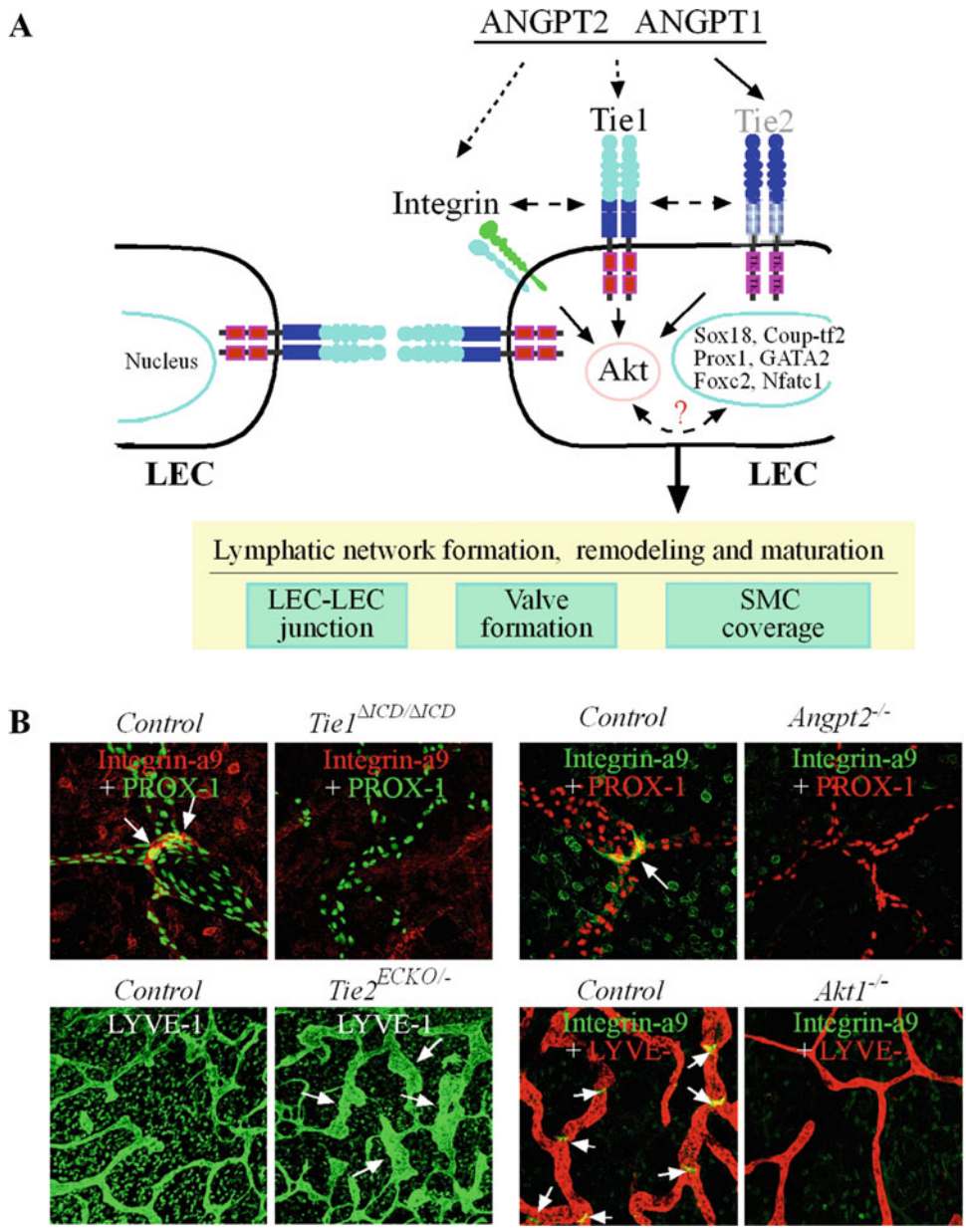

Fig. 2 Regulation of lymphatic development by ANGPT-TIE-AKT pathway. (**a**) Schematic illustration of angiopoietin and TIE receptors, together with other key lymphatic regulators, in lymphatic formation, remodeling, and maturation. AKT1 is a critical signal mediator downstream of TIE pathway and the detailed molecular circuits are yet to be elucidated. (**b**) Lack of collecting lymphatic vessels and valves was observed in the skin of *Tie1*$^{\Delta ICD/\Delta ICD}$ embryos (E18.5, green for PROX1 and red for Integrin-α9). In *Angpt2*$^{-/-}$ mice (E18.5), the diameter of lymphatic capillaries was less than that of control mice (red for PROX1 and green for Integrin-α9), and there were no collecting lymphatic vessels and valves detected in the skin of *Angpt2* mutants. A significant decrease of the diameter of lymphatic capillaries compared with that of control mice was also observed in *Akt1*$^{-/-}$ mice (red for LYVE1, and green for Integrin-α9). In contrast, lymphatic dilation was observed in the skin of *Tie2*$^{ECKO/-}$ mutant mice (green for LYVE1). (Panel B was modified with permission from Figure 3 and 7 in Arterioscler Thromb Vasc Biol. 2014;34:1221–1230 by Wolters Kluwer Health Inc., from Figure 3 in Am J Pathol 2010, 177:2124–2133 by Elsevier, and from Figure 1-figure supplement 3 in Elife. 2016 Dec 22;5. pii: e21032). Arrows point to dilated lymphatics in *Tie2*$^{ECKO/-}$ mice and lymphatic valves in other panels

was also observed, in addition to the abnormal collecting vessel formation as well as valve morphogenesis (Zhou et al. 2010). It is likely that AKT1 acts downstream of ANGPT2-mediated signals for LEC survival, lymphatic remodeling, and maturation during lymphatic development (Fig. 2a).

In contrast, the known biological function of endogenous ANGPT1 in lymphatic formation is still limited. Although local administration of recombinant ANGPT1 to mouse cornea or overexpression of ANGPT1 delivered via adenoviral vectors in ear skin was shown to stimulate lymphatic vessel growth (Morisada et al. 2005; Tammela et al. 2005), systemic treatment with ANGPT1 or other angiopoietins did not produce such an effect with cutaneous lymphatic vessels (Kim et al. 2007). Induction of lymphatic sprouting and filopodia formation by angiopoietins was observed at margins of healing wounds in ear skin at the initial period and also in mouse trachea (Kim et al. 2007). Genetic evidence to support a role of ANGPT1 in lymphatic formation is from this study where lymphatic defects in *Angpt2* deficient mice could be rescued when a cDNA encoding ANGPT1 was placed in the *Angpt2* locus (Gale et al. 2002). However, induced deletion of *Angpt1* during embryogenesis (E16.5) did not affect lymphatic growth in the corneal limbus. While simultaneous deletion of both *Angpt1* and *Angpt2* disrupted lymphatic formation in the corneal limbus, lymphatic vessels could still be detected in nonocular tissues such as ear skin (Thomson et al. 2014). The abnormal lymphatic patterning in *Angpt1*/*Angpt2* double knockout mice could be mainly due to the loss of ANGPT2 as demonstrated by other studies (Dellinger et al. 2008; Shen et al. 2014). It was previously thought that angiopoietins might function via their receptor TIE2 in lymphatic ECs. As to be detailed in the next section, the induced deletion of *Tie2* gene at postnatal stages did not affect the lymphatic network formation and maturation (Shen et al. 2014). Furthermore, Schlemm's canal (SC), formed postnatally, is a type of vessel with venous and lymphatic features. ANGPT1 and TIE2 were shown to be indispensable for SC development, while Angpt2 deficiency alone did not affect SC formation (Thomson et al. 2014; Kim et al. 2017). It is possible that ANGPT1 may exert a tissue-specific role in lymphatic system (Petrova and Koh 2018). At the molecular level, it was proposed that the biological consequences of TIE1/TIE2 interaction complex on cell surface depended on the presence of angiopoietin ligands, which may explain the context dependent function of ANGPT2 as an agonist or antagonist in vascular ECs (Seegar et al. 2010). However, as TIE2 is lowly expressed by lymphatic ECs, it is not known whether such TIE1/TIE2 complexes exist on LEC surface and have a role in lymphatic growth and maintenance.

TIE Receptors in Lymphatic Network Formation

TIE1 as a Critical Regulator of Collecting Lymphatic Vessels

TIE1 has high homology to TIE2, and lymphatic endothelial cells co-express TIE1 with PROX1 (Qu et al. 2010). High expression of TIE1 was detected in valve lymphatic ECs (Iljin et al. 2002; Shen et al. 2014). Mice null for *Tie1* exhibited edema and hemorrhage due to abnormal blood and lymphatic vascular development (Puri et al. 1995; Sato et al. 1995; Qu et al. 2010; Shen et al. 2014). Specifically, TIE1 deficiency was shown to result in abnormal lymphangiogenesis during embryogenesis (D'Amico et al. 2010; Qu et al. 2010). The primary lymphatic network became disorganized with a significant increase in the number of abnormal lymphatic connections (Shen et al. 2014). Furthermore, TIE1 deficiency led to the failure of lymphatic remodeling to form collecting vessels during embryogenesis (Fig. 2b) (Shen et al. 2014; Qu et al. 2015). The postnatal deletion of *Tie1* also disrupted lymphatic network formation with a significant decrease of intraluminal valves, suggesting an important role of TIE1 in lymphatic maturation and maintenance (Shen et al. 2014). It is worth pointing out that *Tie1* mutant model ($Tie1^{\triangle ICD/\triangle ICD}$) (Shen et al. 2014) is different from those by D'Amico et al. (2010) and Qu et al. (2010). The specific difference in genetic targeting between the models was detailed in the original articles. It was originally aimed to generate a mutant mouse model

expressing the truncated TIE1 lacking the intracellular domain (TIE1$^{\Delta ICD}$) for the characterization of TIE1 tyrosine kinase in vascular development. Unfortunately, the expression level of TIE1$^{\Delta ICD}$ was low in *Tie1*$^{\Delta ICD/\Delta ICD}$ mice compared with that of wildtype *Tie1* allele, which may be due to the nonsense-mediated mRNA decay (Amrani et al. 2006). However, it is possible that TIE1$^{\Delta ICD}$, in spite of its low expression, retains some functions of TIE1. This may account for the discrepancy, such as lymph sac formation, between the *Tie1*$^{\Delta ICD/\Delta ICD}$ mutants (Shen et al. 2014) and other genetic models targeting *Tie1* gene (D'Amico et al. 2010; Qu et al. 2010).

TIE2 in Lymphatic Versus Blood Vessel Formation

TIE2 (also named TEK) is expressed by endothelial cells and several other cell types and mediates a crucial pathway in vascular formation and maturation (De Palma et al. 2005; Augustin et al. 2009; Shen et al. 2014; Teichert et al. 2017). Angiopoietins are the ligands of TIE receptors, with ANGPT1 expressed by vascular mural cells and platelets while ANGPT2 mainly from endothelial cells (Davis et al. 1996; Li et al. 2001; Fiedler et al. 2004). TIE2 is activated by ANGPT1 with a tetrameric or higher order of multimeric structure (Cho et al. 2004). ANGPT1-TIE2 pathway-mediated signals are required for blood vascular endothelial cell (BEC) survival, migration, and the establishment of vascular wall integrity. Although mice deficient of TIE2 showed embryonic lethality with defective cardiovascular development (Dumont et al. 1994; Sato et al. 1995), the underlying mechanism was not defined. It has been shown recently that *Tie2* deletion induced by gene targeting leads to defective vein formation and maintenance during embryogenesis and the postnatal development. Further biochemical analysis revealed that TIE2 participated in the specification of venous EC identity via AKT-mediated regulation of COUP-TFII protein stability (Chu et al. 2016). Consistently, *Angpt1* deficiency produced similar vascular defects as observed in *Tie2* null mice (Suri et al. 1996). It was revealed that myocardial-specific *Angpt1* deletion disrupted the coronary vein formation and atrial chamber morphogenesis (Arita et al. 2014; Kim et al. 2018). The requirement of ANGPT1 in vascular development is time-dependent as *Angpt1* deletion at E13.5 or later did not produce any obvious vascular defects (Jeansson et al. 2011).

In the lymphatic system, TIE2 expression in lymphatic ECs was much lower compared with that in blood vascular ECs (Shen et al. 2014). This was also confirmed by *Tie2-GFP* transgenic mice, where no GFP positive lymphatic vessels were detected in ear skin examined (Dellinger et al. 2008). The expression of TIE2 in lymphatic vessels was suppressed in lymphatic ECs with high expression of PROX1 (Petrova et al. 2002; Kim et al. 2010). As *Tie2* null or *Angpt1* deficient mice died before the emergence of lymphatic vessels during embryogenesis, conditional gene knockout models targeting TIE pathway were employed for further studies. It was found that induced deletion of *Tie2* in neonate mice did not affect lymphatic growth (Shen et al. 2014). However, abnormal dilation of lymphatic vessels was observed when *Tie2* deletion was induced at earlier stages of embryogenesis (Fig. 2b) (Chu et al. 2016; Souma et al. 2018). As mutant mice with Tie2 insufficiency had abnormal blood vascular development with hemorrhage and edema (Chu et al. 2016), it is possible that the lymphatic defects may be secondary to the increase of blood vascular leakage. Further studies are needed to characterize the role and underlying mechanism of TIE2 in lymphatic development. In addition, lymphatic defects resulting from inactivating mutations have been reported with several factors including VEGFR3, GATA2, and FOXC2 (Fang et al. 2000; Karkkainen et al. 2000; Petrova et al. 2004; Kazenwadel et al. 2012; Brouillard et al. 2014). However, there is still no evidence linking *Tie2* gene mutation to any lymphatic malformation, although a number of activating mutations have been identified with *Tie2* gene in human patients with blood vascular abnormalities including cutaneomucosal venous malformations and ventricular septal defects (Vikkula et al. 1996; Wouters et al. 2010).

Regulation of Lymphatic Remodeling and Maturation

Lymphatic Endothelial Cell Junctions in Initial and Collecting Vessels

During lymphatic development in mammals, a primitive lymphatic plexus is first formed with a homogeneous tubular structure. Subsequent remodeling leads to the formation of a functionally specialized vascular network containing initial and collecting lymphatic vessels. Both types of lymphatic vessels are lined by a single layer of lymphatic ECs. The major structural differences lie in the lymphatic endothelial cell-cell junctions between them, in addition to the differential investment with basement membrane, mural cell coverage, as well as the existence of intraluminal valves (Tammela and Alitalo 2010; Schulte-Merker et al. 2011; Yang and Oliver 2014). By immunostaining for an adherens junction molecule, VE-Cadherin, it was found that endothelial cells of mature initial lymphatic vessels were joined by discontinuous button-like junctions while collecting lymphatic vessels contained continuous zipper-like junctions (Baluk et al. 2007). Interestingly, initial lymphatic ECs of primitive lymphatic plexus were first joined by continuous zipper-like junctions, which were transformed into button-like junctions at later stages of embryonic development and postnatally (Yao et al. 2012). Although genetic studies have revealed the essential requirement of several genes in the process of lymphatic remodeling and maturation, mechanisms underlying the establishment of distinct lymphatic vessel identity are still incompletely understood.

It has been shown that *Angpt2* gene deletion or ANGPT2 blockage by neutralizing antibody disrupted the button-like junction formation in initial lymphatic vessels due to the suppression of VE-Cadherin phosphorylation at the tyrosine residue 685 (Zheng et al. 2014b). Disorganization of primary lymphatic network was also observed in *Tie1* mutant mice at both embryonic and postnatal stages (Shen et al. 2014). In blood vascular endothelial cells, TIE1 has been shown to associate with trans-endothelial complexes including TIE2 and VE-PTP, which support endothelial junction integrity by associating with VE-cadherin, a key component in adherens junctions (AJs) (Saharinen et al. 2008; Frye et al. 2015). In addition, several integrins have been shown to interact with both TIE receptors and angiopoietins (Cascone et al. 2005; Felcht et al. 2012; Lee et al. 2013), which may coordinate their effects in lymphatic network formation and remodeling. It has also been shown recently that CELSR1, a planar cell polarity protein, suppressed the stabilization of lymphatic endothelial AJs by delaying VE-Cadherin recruitment during the rearrangement of valve forming lymphatic endothelial cells (Tatin et al. 2013). Furthermore, it has been reported recently that the increased VEGFA-VEGFR2 signaling, in the absence of NRP1 and VEGFR1, induced lacteal junction zippering and disrupted chylomicron absorption (Zhang et al. 2018). Further studies are required for elucidating whether there is any effect secondary to the increased blood vascular permeability resulting from excess VEGFA bioavailability after VEGFR1 deficiency. This could be answered by employing the genetic mouse models with specific *Vegfr2* gene knockout in lymphatic endothelial cells, in combination with *Vegfr1* gene deletion. So far, the available information on this topic is still fragmented, and a system approach is required to explore how the above-mentioned factors interact with each other in this process.

Lymphatic Valve Morphogenesis

Valve morphogenesis occurs in collecting lymphatic vessels, veins, and heart, which ensures the unidirectional fluid flow (Bazigou and Makinen 2013). Interestingly, some key factors identified in lymphatic valves are also expressed by venous valve endothelial cells (Bazigou et al. 2011), suggesting a similar regulatory mechanism underlying vascular valvulogenesis. Lymphatic valves are semilunar structures with its leaflet composed of a connective tissue core invested by lymphatic ECs on both sides and are positioned close to vessel bifurcations (Zhou et al. 2010). The process of valve morphogenesis involves extracellular matrix organization including fibronectin fibril assembly mediated via the interaction of

integrin-α9 (ITGA9) and Fibronectin-EIIIA (FN-EIIIA) (Bazigou et al. 2009). Valve-associated endothelial cells are from vessel wall via the process of cell rearrangement including lymphatic EC elongation, reorientation, and migration (Tatin et al. 2013). Valve lymphatic ECs express higher levels of PROX1, FOXC2, ITGA9, TIE1, and cell junction molecules such as connexins (Petrova et al. 2004; Kanady et al. 2011; Sabine et al. 2012; Shen et al. 2014). Genetic studies have revealed that valve morphogenesis is disrupted in mutant mice targeting the following genes, including *Tie1* or *Angpt2* (Dellinger et al. 2008; Shen et al. 2014; Qu et al. 2015), *Foxc2* (Petrova et al. 2004), *Efnb2* (Makinen et al. 2005), *Cx37* (Kanady et al. 2011; Sabine et al. 2012), *Itga9* and *Fn-EIIIA* (Bazigou et al. 2009), and *Akt1* (Zhou et al. 2010). It remains to be clarified whether the defects with valvulogenesis are primary or secondary to the failure of lymphatic remodeling to form collecting vessels. Conditional knockout models in combination with valve LEC expressing Cre transgenic mice, such as *Nfatc1-Cre* (Qu et al. 2015), are needed to better elucidate their specific roles in valve development and maintenance. In addition, it is still incompletely understood how these factors coordinate to control the process of lymphatic valve morphogenesis. It has been found recently that GATA2, a zinc finger transcription factor, was shown to regulate the expression of factors involved in lymphatic maturation, including PROX1, FOXC2 and NFATC1, ITGA9, and ANGPT2 (Kazenwadel et al. 2012, 2015). BMP9, acting via ALK-1, could also induce several genes involved in valve formation including FOXC2, CX37, Ephrin-B2 (EFNB2), and NRP1, but suppresses LYVE-1 expression (Levet et al. 2013). The findings suggest a synergistic effect of the above-mentioned factors in different aspects during lymphatic development.

SMC Coverage with Collecting Lymphatics

Besides the valve morphogenesis during the process of lymphatic remodeling and maturation, another important event is the formation of a continuous basement membrane and SMC coverage with the collecting vessel wall. However, valve regions of collecting lymphatics are free of mural cells so that intraluminal valves could open and close freely during the SMC-mediated contraction to move lymph forward. There is also no mural cell investment with initial lymphatic vessels lined by a single layer of lymphatic ECs, where overlapping endothelial flaps function as primary valves for fluid draining.

Several factors have been found to participate in the regulation of SMC investment with lymphatic vessels, including ANGPT2, TIE1, FOXC2, EFNB2, or SEMA3a. ANGPT1 is known to regulate EC-mural cell interaction in the process of blood vessel maturation while ANGPT2 blocks this event to allow vessel sprouting during angiogenesis (Zhang et al. 2003; Hammes et al. 2004; Feng et al. 2007). Deletion of *Angpt2* leads to the abnormal SMC coverage of lymphatic capillaries (Gale et al. 2002; Shimoda et al. 2007; Dellinger et al. 2008; Shen et al. 2014), suggesting that ANGPT2 plays a similar role in lymphatic development to create a mural-cell free lymphatic vessels. *Tie1* deficient mice also showed similar defects with mural cell coverage with lymphatic capillaries (Qu et al. 2015). There was an increased expression of endoglin in capillary lymphatic vessels of *Tie1* null mice, which may account for the abnormal recruitment SMCs (Li et al. 1999; Qu et al. 2015). Increase of SMC coverage with lymphatics was detected in *Foxc2* deficient mice (Petrova et al. 2004), and in *Efnb2* mutant mice lacking its C-terminal PDZ interaction site (Makinen et al. 2005). SMC coverage at lymphatic valve region was reported in *Sema3a* null mice or mice treated with neutralizing antibodies blocking SEMA3A binding to NRP1 (Bouvree et al. 2012; Jurisic et al. 2012). It seems that lymphatic ECs in valve regions are able to generate signals to exert an inhibitory role in mural cell recruitment. FOXC2 and NFATC1 could cooperate in the transcriptional control of several genes involved in vascular development such as downregulation of PDGF-B. This may account for the lack of mural cell recruitment in certain lymphatic regions (Petrova et al. 2004; Norrmen et al. 2009).

Interestingly, FOXC2 has been shown to regulate *Angpt2* expression by direct activation of its promoter (Xue et al. 2008). On the other hand, Reelin, an ECM glycoprotein secreted by lymphatic ECs, might mediate SMC-LEC interaction during lymphatic maturation. It was reported that reelin deficiency led to the reduction of SMC recruitment with dermal collecting lymphatic vessels (Lutter et al. 2012).

ANGPT-TIE Pathway in the Modulation of Tumor-Associated Lymphangiogenic Microenvironment

Angiopoietins in Tumor Lymphangiogenesis and Lymphatic Metastasis

Consistent with the observation made in developmental lymphangiogenesis, intratumor lymphatic vessel growth occurs after tumor angiogenesis (He et al. 2005). Tumor also actively remodel the preexisting lymphatic network, including lymphatic sprouting and vessel dilation, in adjacent tissues to facilitate its dissemination and the establishment of metastatic foci in lymph nodes and other organs (Fig. 3a, b). The molecular regulators identified in development are also essentially required for tumor-associated lymphangiogenesis, including VEGFR3, angiopoietins, and TIE receptors (Alitalo et al. 2005; Augustin et al. 2009; Saharinen et al. 2017). In animal tumor studies, lymphatic metastasis could be efficiently suppressed by blocking VEGFR3 and TIE signaling pathways. This has been demonstrated by using soluble receptors or peptide-Fc fusion protein for ligand-trapping (Karpanen et al. 2001; He et al. 2002; Krishnan et al. 2003; Karlan et al. 2012; Atkins et al. 2015), receptor activating and/or blocking antibodies (Roberts et al. 2006; Caunt et al. 2008; Tammela et al. 2008; Park et al. 2016), and small molecules tyrosine kinase inhibitors (Demetri et al. 2013; Garcia-Manero et al. 2015; Smith et al. 2015; Saharinen et al. 2017). Therapeutic targeting on angiopoietins and their receptors has been nicely reviewed by Dr. Kiss and Dr. Saharinen in this series.

Angiopoietins are expressed by tumor and tumor-associated stromal cells. In addition to its secretion from vascular mural cells and platelets, ANGPT1 expression was detected in tumor cells (Stratmann et al. 1998; Augustin et al. 2009; Holopainen et al. 2009). ANGPT1 could compensate for the loss of ANGPT2 in lymphatic development (Gale et al. 2002), suggesting that its function in lymphatic ECs is comparable to that of ANGPT2 when expressed in the proper environment. Transgenic expression of both ANGPT1 and ANGPT2 in pancreatic β cells of Rip1Tag2 mice showed an increase of peritumoral lymphangiogenesis (Fagiani et al. 2011). Consistently, ANGPT1 delivered via an adenoviral vector was shown to increase the rate of lymph node metastasis (Holopainen et al. 2009). The metastasis enhancing effect of ANGPT1 was abolished by the administration of soluble TIE2. Surprisingly, tumor-associated lymphangiogenesis was not inhibited by the soluble TIE2 (Holopainen et al. 2009). This is consistent with the observation that TIE2 is lowly expressed by lymphatic ECs and the postnatal deletion of *Tie2* did not affect the lymphatic vessel formation and maintenance (Shen et al. 2014). It is likely that the soluble TIE2Ig trapped ANGPT2 and ANGPT1, which were required for the lymphatic remodeling to form a functional network for tumor cell dissemination to the sentinel lymph nodes. Furthermore, TIE1 expression is increased in tumor vasculature and endothelial-specific deletion of *Tie1* led to the suppression of tumor angiogenesis and growth. Tie1 deletion in combination with soluble TIE2 treatment produced an additive inhibition of tumor progression (D'Amico et al. 2014). It is worth noting that although the restoration of tumor vascular perfusion is essential for therapeutic drugs targeting tumor cells, vascular normalization by ANGPT1 treatment could also promote both hematogenous and lympahtic tumor metastasis as described (Holopainen et al. 2009). There is an elegant review article on tumor vessel normalization by Dr. Koh and colleagues in this series.

In contrast, ANGPT2 is expressed in activated endothelial cells in tumors and plays a crucial role together with VEGFA in tumor-associated

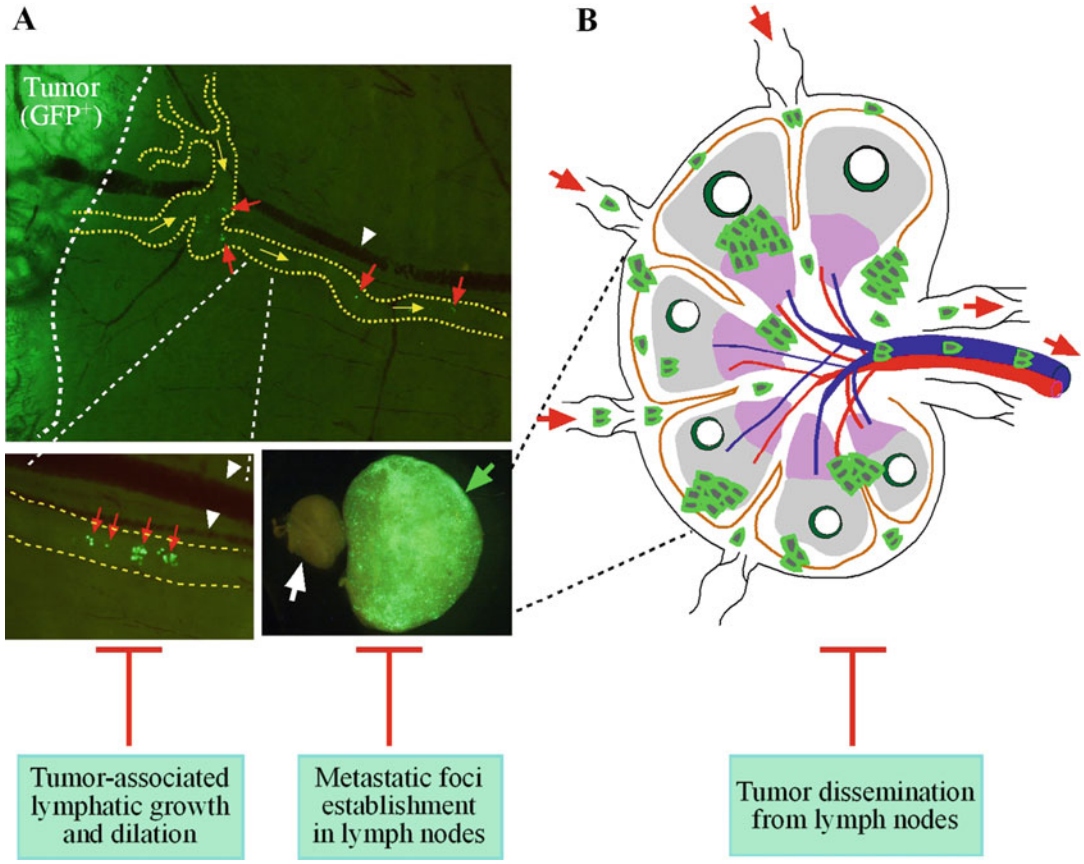

Fig. 3 Lymphatic regulators as targets for blocking lymphatic tumor metastasis. (**a**) Tumor cells (GFP^+) invaded into the lymphatic system are transported via the dilated collecting lymphatic vessels (dotted yellow lines) of adjacent normal tissues to the draining lymph nodes (yellow arrows indicate the flow direction; red arrow indicate GFP^+ tumor cells; and white arrowheads indicate blood vessels). Single tumor cell or tumor emboli (green, GFP^+) were detected in collecting vessels. (Images are modified with permission from Figure 2 and 4 in Cancer Res. 2005;65:4739–46). (**b**). Establishment of metastatic tumor foci in lymph nodes and schematic illustration of further tumor cell dissemination via efferent lymphatic vessels and blood vessels to distant organs. Candidate drugs targeting the key signaling pathways including ANGPTs and TIE receptors are in clinical development, including peptide-Fc fusion protein for ligand-trapping, blocking antibodies and small-molecule tyrosine kinase inhibitors. Green arrow points to the axillary lymph node with GFP^+ tumor cells, and white arrow to the contralateral axillary lymph node without tumor metastasis

vascular growth and metastasis (Holash et al. 1999; Oliner et al. 2004; Augustin et al. 2009). VEGFA was also shown to increase the endothelial ANGPT2 expression via the calcineurin and nuclear factor of activated T cells (NFAT) pathway. ANGPT2 upregulation was implicated in the preparation of premetastatic niche to facilitate the establishment of tumor metastasis (Minami et al. 2013). Circulating ANGPT2 levels was shown to increase in patients with pancreatic cancer, which correlated with lymph node metastasis (Schulz et al. 2011). ANGPT2 overexpression promoted tumor lymphangiogenesis and lymph node metastasis in mice with the subcutaneous pancreatic and lung tumor xenografts (Schulz et al. 2011; Holopainen et al. 2012). *Angpt2* deficiency was shown to suppress tumor angiogenesis at early stages of tumor progression and increased mural cell coverage with blood vessels in mouse models (Nasarre et al. 2009). Consistently, ANGPT2-blocking antibodies suppressed tumor-associated lymphangiogenesis and enhanced the integrity of

endothelial cell-cell junction (Holopainen et al. 2012). Furthermore, ANGPT2 was shown to promote glioma cell invasion (Hu et al. 2003, 2006) and breast cancer metastasis by upregulation and activation of matrix metalloprotease 2 (MMP-2) (Imanishi et al. 2007, 2011). The effect is mediated via α5β1 integrin pathway but independent of TIE-2 signaling (Imanishi et al. 2007). Similar mechanism may also account for the role of ANGPT2 in lymphatic formation as TIE2 expression is low in lymphatic ECs.

Lymphatic Regulator-Mediated Modulation of Tumor Immune Response

There is an active interaction between lymphatic ECs and immune cells during tumor progression. On one hand, tumor-infiltrating leukocytes modulate the tumor vascular network by stimulating angiogenesis and lymphangiogenesis, and create a protumor inflammatory microenvironment (Mantovani et al. 2008). In addition to neutrophils and tumor-specific T cells, mononuclear phagocytotic lineage, comprising of tumor-associated macrophages, dendritic cells, and monocytes, constitutes the major component of infiltrating leukocytes (Pollard 2004). Macrophages are the major source of lymphangiogenic factors such as VEGF-C (Kerjaschki 2005; Condeelis and Pollard 2006; Kataru et al. 2009), and VEGF-C expression was induced by TNFα via NF-κB pathway (Ristimaki et al. 1998; Baluk et al. 2009). Blockage of the macrophage recruitment reduced lymph node metastasis by suppressing VEGF-C expression in tumor (Fischer et al. 2007; Iwata et al. 2007). In addition to the intratumoral lymphangiogenesis, active lymphangiogenesis was detected in tumor draining lymph nodes before the arrival of metastatic tumor cells (Hirakawa et al. 2005; Van den Eynden et al. 2007; Rinderknecht and Detmar 2008; Ruddell et al. 2008). Besides the lymphangiogenic factors transported with lymph from tumor, immune cells in lymph nodes also actively participate in the regulation of lymph node-associated lymphatic vessel growth. Follicular B cells could produce lymphangiogenic factors such as VEGF-A to stimulate lymphangiogenesis in lymph nodes (Angeli et al. 2006; Shrestha et al. 2010), while T cells have been found to modulate lymphatic growth in a negative manner via secreting IFN-γ (Kataru et al. 2011).

On the other hand, the tumor-associated lymphatic system regulates immune responses by delivering antigen presenting cells (APCs) and lymph containing soluble antigens from tumor to the draining lymph nodes. After reaching the subcapsular sinus of lymph nodes, small lymph-borne antigens are delivered directly to B cell follicles and paracortical T cell zones via the reticular conduit system while large antigens were taken up and transported by macrophages (Roozendaal et al. 2009). Interestingly, the sinus lymphatic endothelium acts as a physical sieve depending on diaphragms formed by PLVAP (plasmalemma vesicle-associated protein) fibrils in transendothelial channels (Rantakari et al. 2015). Lymphatic ECs also actively participate in the regulation of immune cell entry and emigration from lymphatic vessels via the expression of chemokines and adhesion molecules (Forster et al. 2008; Card et al. 2014). VEGF-C was shown to upregulate chemokine expression in lymphatic ECs (e.g., CCL21), which are immobilized by glycosaminoglycans (e.g., podoplanin) on the luminal surface of lymphatic ECs to guide the migration of immune cells expressing CCR7 (Forster et al. 2008; Alitalo 2011). Lymphatic semaphorin-3A was shown to promote actomyosin contraction during the DC entry into lymphatic vessels (Takamatsu et al. 2010), and lymphatic ECs lining the ceiling of subcapsular sinus also expressed CCRL1, a scavenger receptor for CCL21/CCL19, to create a chemokine gradient for DC trafficking into the parenchyma (Ulvmar et al. 2014). Furthermore, it is known that tumor-associated macrophages have poor antigen-presenting capability and express immunoinhibitory factors to suppress T cell proliferation in comparison with macrophages derived from normal tissues (Forster et al. 2008). However, there exist distinct populations of dendritic cells (DCs) including the resident and migratory DCs in lymph nodes and the periphery tissues. It has been shown that a subset of dendritic cells

($CD103^+/CD141^+$) expressing CCR7 in melanoma were critical for trafficking tumor antigens via afferent lymphatics to prime $CD8^+$ T cells in the draining lymph nodes. Increase of T cell infiltration in tumor showed survival benefits for patients (Roberts et al. 2016). Consistently, lymphatic absence or dysfunction was shown to impair antitumor immune responses (Kimura et al. 2015; Lund et al. 2016). Specifically, xenograft melanoma implanted intradermally displayed a markedly reduced leukocyte infiltration and failed to mount an antitumor immunity in response to dermal vaccine delivery in a transgenic mouse model lacking skin lymphatics. The finding was further verified in metastatic human cutaneous melanoma samples where tumor immune cell infiltrates correlated well with the expression level of lymphatic markers (Lund et al. 2016).

In addition to the involvement of lymphatic system in immune defense, it also promotes self-tolerance (Card et al. 2014). DCs constantly migrate via afferent lymphatic vessels to the draining lymph nodes, carrying self and foreign antigens from the periphery tissues (Forster et al. 2008). This is important for tolerance induction towards environmental antigens and may also be employed by tumor to evade the immune surveillance. VEGF-C was shown to promote immune tolerance in murine melanoma, and lymphatic ECs are involved in maintaining peripheral immune tolerance by inducing CD8 T-cell deletion (Cohen et al. 2010; Lund et al. 2012). As innate immune cells including macrophages and DCs express VEGFR-3, it is also likely that VEGF-C may have a direct role in the restriction of their inflammatory activation (D'Alessio et al. 2014; Zhang et al. 2014). Interestingly, in spite of the immunosuppressive tumor microenvironment, it was also reported that VEGF-C induced lymphangiogenesis could enhance the antitumor immunotherapy resulting from the increased naïve T cell infiltration dependent on CCL21 in the antigen-expressing melanoma (Fankhauser et al. 2017). Furthermore, lymphatic ECs in lymph nodes were found to function as tolerogenic APCs by expressing major histocompatibility complex (MHC) class I and II molecules as well as immunoregulatory factors (Card et al. 2014). Lymphatic ECs rely on DCs to present peripheral tissue antigens to CD4 T cells to induce anergy (Rouhani et al. 2015). Expression of programmed death-ligand 1 (PD-L1) by lymphatic ECs transmitted an inhibitory signal to suppress the proliferation of antigen-specific T cells via its receptor PD-1 (Tewalt et al. 2012).

Interestingly, ANGPT-TIE pathway plays an important role in the regulation of tumor immune microenvironment. There is a subset of TIE2-expressing macrophages (TEMs) identified in tumor, which interact with vascular ECs to promote tumor progression dependent on ANGPT2-TIE2 pathway (Mazzieri et al. 2011; Matsubara et al. 2013). Overexpression of ANGPT2 promoted tumor-infiltrating macrophages and neutrophils while ANGPT1 suppressed this event (Fagiani et al. 2011). Consistently, myeloid cell-specific deletion of *Tie2* or *Angpt2* deficiency, or the administration of ANGPT2 blocking antibodies, led to the suppression of tumor growth and relapse after chemotherapy or antiangiogenic therapy in animal tumor studies (Nasarre et al. 2009; Brown et al. 2010; Mazzieri et al. 2011; Chen et al. 2016). Endothelial-derived ANGPT2 was elevated in mice with the bevacizumab-resistant murine glioblastoma model. The combined inhibition of VEGF and ANGPT2 was shown to extend survival of tumor-bearing mice, accompanied by the favorably altered immune microenvironment, including the suppression of M2-polarized macrophages as well as an increase of intratumoral T cell infiltration (Scholz et al. 2016). ANGPT2 also stimulated IL-10 release by TEMs from tumor to suppress T cell proliferation while promoting regulatory T cell (T_{reg}) expansion (Coffelt et al. 2011). Inhibition of ANGPT2 with simultaneous TIE2 activation was shown to reduce T_{reg} cells in tumor (Park et al. 2016). Modulation of T_{reg} cell-mediated immune suppression by lymphatic EC-derived cytokines such as angiopoietins could be another important mechanism contributing to the immune tolerance to tumor-derived antigens.

Summary

Tumor cells disseminate to sentinel lymph nodes via intratumoral lymphatic vessels connecting to the lymphatic network in the adjacent normal tissues. It was frequently observed that there was a dramatic increase of lymphatic vessel diameter at peritumoral areas to facilitate tumor dissemination as single cell or emboli. Lymph node metastasis is an early event in solid tumors and analysis of sentinel lymph node biopsy from cancer patients is routinely practiced for prognostic evaluation in clinic. One long-lasting question is that whether lymph node metastasis contributes to systemic tumor spread to other organs. Two recent articles provided evidence that metastatic tumor cells could spread further via blood vessels from lymph nodes (Brown et al. 2018; Pereira et al. 2018). As anti-lymphangiogenesis treatment had limited effect on tumor progression after dissemination, it is necessary to make early detection of lymphangiogenic event in tumor and/or the draining lymph nodes before tumor cells metastasize. On the other hand, insufficient lymphatic drainage may account for a low level of immune cell infiltration in primary tumors and poor response to immunotherapy. It seems contradictory to enhance the efficacy of immunotherapy by improving the vascular perfusion including lymphatic draining function and to simultaneously suppress the metastatic tumor spread via the tumor-associated vascular network. Further studies are needed to develop combined therapies to fine-tune the interaction of vascular EC-immune cells to block tumor progression.

Cross-References

- Anti-angiogenic Targets: Angiopoietin and Angiopoietin-Receptors
- Benefits and Pitfalls of Tumor Vessel Normalization

Acknowledgments This work was supported by grants from the National Natural Science Foundation of China (81770489, 91539101, 91739304), the Ministry of Science and Technology of China (2012CB947600), and the Priority Academic Program Development of Jiangsu Higher Education Institutions.

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