Chapter 7
Lymph and Lymphatic Capillaries in Cancer

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Abstract Lymph forms from the fluid that is forced out of capillaries and postcapillary venules by hydrostatic pressures, into the interstitium around the vessel. This protein-rich fluid flows through the extracellular matrix and between cells bathing them in nutrients and oxygen and carrying away cellular metabolites and waste products where it is collected by lymphatic capillaries and on to lymph nodes. As with the physiological situation, interstitial fluid and lymph also form within and around tumors, which are collected from cancer-associated tissues. What does change in this situation, however, is the surroundings in which lymph is generated and the tissues exposed to the resulting fluid. The environment in which lymph is formed and transported via can modify its composition and have drastic effects on cells and tissues downstream. This chapter explores the roles of lymphatic function, lymph transport, and their far-reaching implications in cancer development and progression. We pay particular attention to the mechanisms of lymph formation and composition, lymph clearance and resulting cellular effects, the impact on potential antitumour immune responses, methods to identify and measure lymphatic function, and new approaches to exploit or target lymphatics for therapy.

7.1 Introduction

Lymph forms from blood capillary exudates—the fluid that is forced out of capillaries and postcapillary venules by hydrostatic pressures, into the interstitium around the vessel. This protein-rich fluid flows through the extracellular matrix and between cells, bathing them in nutrients and oxygen, and carrying away cellular metabolites and waste products. Responding to pressure gradients within the interstitial space, openings at the junctions between adjacent lymphatic endothelial cells (LECs) drive

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entry of interstitial fluid, which then passes through at least one lymph node (LN). Interstitial fluid and pre-nodal lymph are essentially identical, since very little filtration occurs as a result of crossing the endothelial barrier prior to reaching the lymph nodes. Lymph that exits the efferent vessels following filtration is now a different composition. Lymph nodes filter the lymph to remove waste products, some proteins, fluid, and potential pathogens and/or pathogen components prior to its eventual return to the blood. Many afferent lymphatics from defined catchment areas drain to into the subcapsular sinus of each lymph node. Here, prevented from entering the cortex, particulates and high molecular weight molecules circulate via subcapsular and medullary sinuses before entering the efferent lymphatic and exiting the node. In contrast, lymph carrying sub-80 kDa proteins are able to pass via size exclusion pores (0.1–1 mm diameter) and percolate through narrow conduits within reticular networks (1–200 nm) (Gretz et al. 2000; Roozendaal et al. 2008; Sixt et al. 2005) before entering the medullary sinus. Both routes rely on antigen-presenting cells to screen lymph before its exit.

As with the physiological situation, interstitial fluid and lymph also form within tumors, and are collected from cancer-associated tissues. What does changes, however, is the surroundings in which lymph is generated and the tissues exposed to the resulting fluid. The environment in which lymph is formed and transported via can modify its composition having drastic effects on cells and tissues downstream. This chapter aims to address the roles of lymphatic function, lymph transport, and their far-reaching implications in cancer progression.

7.2 Lymph and the Tumor Microenvironment

Abnormal blood vessels contribute to generation of tumor interstitial fluid: Tumors require the growth of new blood vessels to cope with the increasing oxygen and metabolic demands of the rapidly increasing tumor cell mass. In contrast to the characteristic well-organized branching structures in normal microvasculature, the rapid and uncontrolled manner in which tumor-associated blood microvessels develop renders them tortuous, with abnormal branching patterns. Tumor blood vessels do not undergo normal pruning and stabilization steps such as pericyte recruitment (reviewed by McDonald and Baluk 2002). Tumor vessel endothelial cells have also been noted to be irregularly shaped and present with larger numbers of fenestrations and vesicles, loose cell–cell junctions (Hashizume et al. 2000) as well as reduced connections between endothelium and the normally tightly associated basement membrane (which is also altered in the tumor) (McDonald and Baluk 2002). The combination of these factors means that microvessels are unable to maintain their normal barrier function, and hence a feature of tumor vasculature is abnormally high vessel leakiness. Consequently, fluid and solutes are able to exit vessels much more readily than in normal cases; the accumulation of which contributes to high interstitial fluid pressures (IFP) found within tumors (Boucher et al. 1990; Heldin et al. 2004; Lunt et al. 2008; Wiig et al. 1982; Gutmann et al. 1992).

In comparison to normal tissues, where IFP is typically 0 mmHg or slightly lower (Boucher et al. 1990; Chary and Jain 1989), pressures associated with mouse mammary cancers have been measured at 2.4 mmHg in the superficial layers and rising to 23 mmHg in the center of tumors (Boucher et al. 1990; Wiig et al. 1982). In humans, this range is typically 10–40 mmHg but can rise to 60 mmHg in some tumors (Heldin et al. 2004; Lunt et al. 2008). These pressure gradients drive fluid out of the tumor into the lower pressure environment of peritumoral tissues. The movement of fluid through the tumor and associated tissues forms tumor interstitial fluid (TIF) flow. At the same time, extracellular matrix deposition and remodeling within and around the tumor generate further physical stresses, which compound the elevation seen in IFP (Fig. 7.1).

From vessel exudates to tumor interstitial fluid and lymph formation: In normal situations, the compositions of plasma exudates (immediately after leaving the capillary) and lymph have some similarities. Analysis of the proteome via mass spectrometry and 2D page techniques has begun to shed light onto the composition of lymph compared with plasma. Both lymph and plasma contain typical plasma proteins from albumin and immunoglobulin families (Leak et al. 2004). Lymph is also highly enriched with fibrinogen fragments, enzymes, catabolic products, complement components, extracellular matrix fragments, and cellular constituents (intracellular and membrane) such as histones, mitochondria, and cytoplasmic proteins (Mittal et al. 2009). The abundance of peptide fragments in pre-nodal lymph is also thought to be important for the maintenance of immune homeostasis (Clement et al. 2010, 2011).

So, what do tumor-associated lymphatic vessels collect? How does this differ physiological states? The fluid that exits abnormal vessels into the surrounding tumor is referred to as TIF. TIF is a component of the tumor microenvironment that is relatively overlooked compared to angiogenesis and lymphangiogenesis for example, yet is likely to play an equally important role in successful establishment of the tumor niche. Unlike the harvest of normal lymph, which requires vessel cannulation, collection of TIF is technically challenging. Progress in the field has been hampered due to difficulties in (a) accessing the tumor-associated tissues or vessels, given the disorganized tissue architecture, and (b) harvesting TIF without intercellular fluid contamination or directly altering the tissues that are under investigation (methods reviewed by Wiig et al. 2010). Early measurements (Gullino et al. 1964) demonstrated that as with normal lymph, albumin and immunoglobulins form a major fraction of TIF, whereas TIF has high H^+, CO_2, and lactic acid, but low glucose, oxygen, and pH compared with plasma and normal subcutaneous fluid. These differences most likely reflect the differing metabolic needs, and hypoxic environment seen in tumor vs. normal tissue. Representative of the changes in endothelial barrier function, an accumulation of low molecular weight proteins (<25 kDa) has been recorded in TIF compared with plasma, whereas those of >25 kDa are not significantly different (Stohrer et al. 2000). Following the acceptance of the tumor microenvironment as a major influencing factor in defining tumor fate (Hanahan and Weinberg 2011), there has been a renewed interest in the TIF and pre-nodal lymph. Given that these fluids envelop highly bioactive tumor cells, many

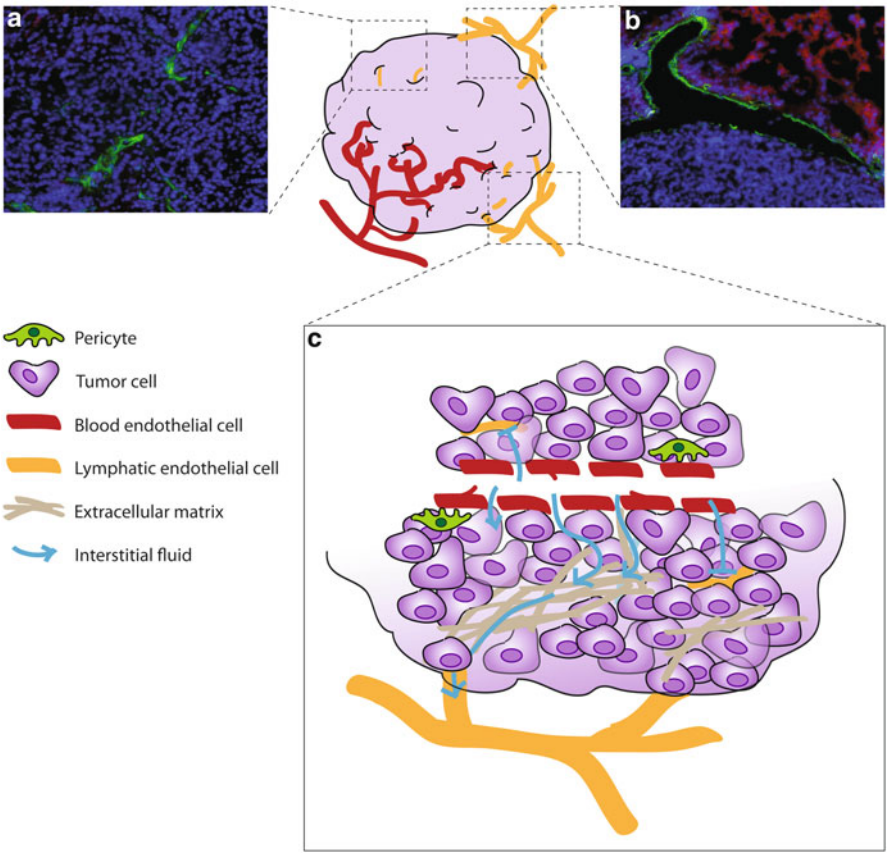

Fig. 7.1 High interstitial fluid pressure (IFP) exists within tumors. (**a**) High IFP and mechanical stresses created by high cell numbers mean that lymphatic vessels (LYVE-1 positive, *green*) within the tumor mass are collapsed and nonfunctional. (**b**) In contrast, vessels at the tumor periphery are hyperplastic and dilated. Nuclei are counterstained with DAPI. (**c**) High IFP results from exudates leaving abnormally leaky vessels within a tumor. Vessels are tortuous and associations with mural cells such as pericytes are looser than in normal tissues. As lymphatics within a tumor are not functional, fluid follows a pressure gradient via non-endothelial channels into the surrounding tissues where resistance is lower. From here, dilated peritumoral lymphatics collect the lymph and any proteins contained within

components of TIF are actively secreted or produced in metabolic exchanges, and thus represent an accurate readout of cellular events in the tumor. Breast (Celis et al. 2004, 2005; Mannello et al. 2009; Wiig et al. 2003), ovarian (Haslene-Hox et al. 2011), and renal cell (Teng et al. 2010) carcinomas are among the first to undergo proteomic profiling of TIF composition. These studies have re-highlighted the abundance of proteins contained within TIF, and identified enriched compounds with potential therapeutic significance. Of those examined, particular enrichment of plasma membrane-associated or predicted extracellular matrix constituents was

recorded when compared with normal adjacent tissue (Teng et al. 2010). Given that TIF is a direct reflection of metabolic state and cellular activity and that a major hallmark of cancer is growth factor overexpression and independence (Hanahan and Weinberg 2011), we can expect that TIF is enriched with numerous other factors in addition to those described above. TIF that passes over tumor cells and surrounding tissues before entering vessels as lymph is also rich in all the growth factors, cytokines, and chemokines secreted from within the tumor microenvironment—irrespective of whether this is from tumor cells, fibroblasts, endothelial cells, or infiltrating immune cells. TIF therefore constitutes a potentially rich hunting ground for tumor-specific biomarker discovery, in both shed (e.g., components released during cell death) and soluble secreted forms (Leak et al. 2004; Clement et al. 2010, 2011; Celis et al. 2004; Haslene-Hox et al. 2011).

7.3 Physical Effects Created as a Result of Tumor-Associated Lymphatic Drainage and Lymph Flow

We have seen that TIF and lymph compositions differ from each other in physiology and disease, but what impact does this have on tumor cells themselves, their immediate surrounding tissues, and those further downstream? This section of the chapter will discuss their role in the context of lymph-mediated effects, as well as the resultant biophysical factors that contribute to the tumor microenvironment.

Tumor-associated lymphatic vessels: Within tumors, the elevated IFP from fluid and protein accumulation, along with the mechanical stresses imposed by proliferating tumor cells within a confined space results in the compression of intratumoral lymphatic vessels, rendering them nonfunctional (Leu et al. 2000; Jain and Fenton 2002; Padera et al. 2002). The loss of intratumoral lymphatic functionality and reduction in fluid clearance from the tumor interstitium compound the problem of high fluid pressure within the tumor. Instead, fluid is able to move through a tumor via non-endothelial, matrix-rich channels oozing out into surrounding tissues that pose the least resistance (Padera et al. 2002). Here, TIF can be collected by peritumoral lymphatics. In many tumor types, the tumor periphery contains an abundance of lymphatic vessels (Mandriota et al. 2001; Shields et al. 2004; Skobe et al. 2001). These vessels are either co-opted preexisting vasculature or newly formed and remodeled vessels. Peritumoral lymphatics are frequently hyperplastic and functionally abnormal having malformed valves and retrograde flow (Hagendoorn et al. 2006; Isaka et al. 2004). That being said, even with abnormal function, tumor-associated lymphatics collect the protein-rich fluids exiting the tumor mass (Fig. 7.2).

Many human cancers metastasize via the lymphatic system. To maximize the opportunity for dissemination, tumor-derived expression and secretion of vascular endothelial growth factor-C and -D (VEGF-C and VEGF-D, the major lymphangiogenic growth factors) are frequently observed in tumor cells (Mandriota et al. 2001; Shields et al. 2004; Skobe et al. 2001; Stacker et al. 2001; Mattila et al. 2002) and

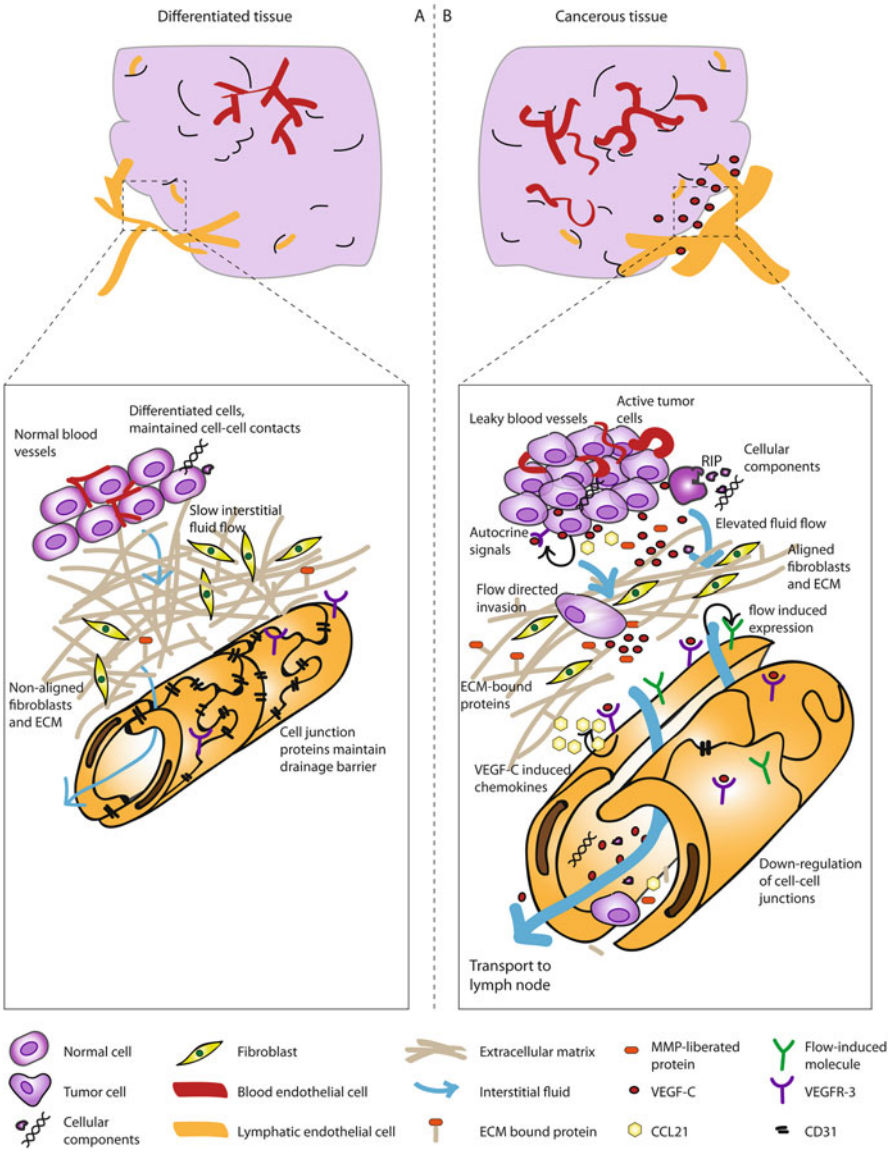

Fig. 7.2 The effects of tumor interstitial flow on tumor-associated tissues. (**a**) To ensure the maintenance of tissue homeostasis in normal differentiated tissues, plasma exudates leaving blood vessels are slowly transported through the interstitial space via pressure gradients before entering lymphatic capillaries. Cell adhesion molecules such as CD31 maintain lymphatic endothelial cell (LEC) junctions. Self-antigens and potential pathogenic components are also transported to help maintain immune homeostasis. (**b**) In the tumor microenvironment, enhanced interstitial flow created by high IFP exits the tumor and drains into peritumoral lymphatics. In the process of passing over metabolically active tumor cells, TIF and later lymph pick up growth factors (e.g., vascular endothelial growth factor-C [VEGF-C]) and tumor cell components, which enter the lymphatic

surrounding tissues (Gallego et al. 2011; Schoppmann et al. 2006). VEGF-C expression in tumor-associated tissues reflects a strong correlation with the incidence of lymph node metastasis and poor prognosis in patients (Emmett et al. 2010; Kurahara et al. 2010; Nakamura et al. 2005). VEGF-C directly acts on lymphatic vessels at the tumor periphery through the ligation of its cognate receptor VEGFR-3, stimulating both de novo vessel formation and remodeling (hyper-proliferation) of those preexisting. The expanded lymphatic network presents a tumor with greater chance of encountering a vessel, and a portal for tumor metastasis that can directly help tumor progression.

Increased lymphatic drainage: The stimulation of extra lymphatic vessels also confers indirect effects that help support a growing tumor. TIF that oozes out of a tumor is collected as lymph by peritumoral lymphatics. VEGF-C (and other growth factors such as FGF) present in the vicinity of the tumor is sufficient to induce high peritumoral vessel density, so although hyperplastic and functionally abnormal, the increase in number is translated to increased functional output and enhanced capacity for fluid clearance (Hoshida et al. 2006). Moreover, VEGF-D has been shown to act further downstream in the vessel hierarchy via a mechanism distinct from VEGF-C. Rather than stimulating endothelial proliferation, VEGF-D induces collecting lymphatic vessel dilation in a prostaglandin-dependent manner (Karnezis et al. 2012). This would imply that the synergies between tumor-derived VEGF-C and -D extend beyond the induction of new lymphatic vessels, towards modulating their functionality in the tumors' favor.

Fluid flux effects on cell behavior: The physical movement of fluid through tumor tissues and the tissues immediately surrounding it is a tumor-promoting stimulus in its own right. Firstly, it is likely that tumor cells in the process of detaching from the main tumor bulk are assisted as a consequence of increasing lymph flow. Shedding cells may be physically carried along fluid channels towards draining lymphatics and further downstream, augmenting delivery of "flushed" cells to the draining lymph node (Hoshida et al. 2006). In lymphatic-rich tumors, there is generally more cellular movement between the tumor and lymph nodes (Shields et al. 2010; Hoshida et al. 2006; Lund et al. 2012; Hoshida et al. 2006). Fluorescently labeled microbeads have enabled researchers to define specific migrating cell populations and trace cell movements. Not surprisingly, the major cell types identified as trafficking via the tumor lymphatics are immune cells (immune context discussed later) (Shields et al. 2010; Lund et al. 2012). In addition to physically

Fig. 7.2 (continued) system towards the draining lymph node where further changes occur in preparation for metastasis. Tumor-derived growth factors can synergize with flow to act in an autocrine fashion increasing cell invasiveness, or paracrine on cancer-associated fibroblasts and LECs. VEGF-C stimulates lymphatic growth (enhancing drainage capacity), and also the secretion of lymph homing chemokines such as CCL21. Proteases, skewed by flow, further liberate proteins within the matrix amplifying gradient effects. Flow itself is sufficient to induce environmental changes, stimulating remodeling of the interstitium and modification of vessel functionality (via down-regulation of junction proteins and changes in surface expression)

assisting tumor cell detachment and metastasis, fluid convection created by lymphatic drainage can act as a morphogenetic cue, synergizing with the local chemical and physical environment. Together, these forces can significantly influence the environment and cellular behaviors. Oncogenic stresses and physical pressures associated with tumor development stimulate tumor cells to secrete growth factors, chemokines, etc.; for example, high pressures within a tumor have been shown to stimulate tumor proliferation (Hofmann et al. 2006) and the release of VEGF-C (Nathan et al. 2009). These factors exert their effects on cells within their immediate proximity in (a) autocrine, (b) paracrine fashion, or are (c) transported to downstream tissues to exert their effects remotely (Gretz et al. 2000; Helm et al. 2005; Miteva et al. 2010; Ng et al. 2004). *Autocrine effects on tumor cells*: On exposure to subtle fluid flows, transcellular chemokine gradients are generated that are biased towards functional, draining lymphatic vessels. In this sense, a tumor cell can follow an autologously generated cue that directs it to the nearest functioning vessel—and escape route (Fleury et al. 2006; Haessler et al. 2012; Shields et al. 2007a; Polacheck et al. 2011). This phenomenon, referred to as autologous chemotaxis, was shown in vitro in tumor cells that utilize a CCL21–CCR7 signaling loop, but has the potential to exist for any number of tumor-derived factors. Similarly, VEGF-C is able to ligate its receptor VEGFR-3 in an autocrine manner leading to increased tumor cell motility and proteolytic capacity (Issa et al. 2009). *Paracrine effects on cells within the stroma*: Proteins carried within the fluid that is collected from a tumor maintain the capacity to influence neighboring cells encountered en route to lymphatic vessels, including cancer-associated fibroblasts and the LECs themselves. Fibroblasts proliferate and become activated upon exposure to flow. Flow-dependent responses measured include the up-regulation of smooth muscle actin for contractility, reorientation to align themselves and collagen fibers perpendicular to flow, the induction of factors such as TGF-β_1, and enhanced degradation of surrounding matrix (Ng et al. 2005; Ng and Swartz 2003, 2006; Shieh et al. 2011). In this way, flow through tissue spaces can "prime" the tumors surroundings in preparation for metastasis. Indeed, it has been shown that activated cancer-associated fibroblasts guide escaping tumor cells away from the primary mass towards vessels (Shieh et al. 2011; Gaggioli et al. 2007). We also know that the extracellular matrix which fibroblasts deposit modulates the physical environment via the mechanical stresses they exert, but matrix components can also amplify the paracrine (and some degree autocrine) effects generated by interstitial fluid flow. Many proteins bind to matrix components leading to sequestered pools of locally high concentrations of e.g. latent TGF-β_1, VEGF, or CCL21 (Shieh et al. 2011; Patel et al. 2001; Zilberberg et al. 2012). Proteases transported within the interstitial fluid are able to rapidly liberate any bound factors. Directional fluid flow and protein release can then synergize to form directed and intensified chemical gradients that cancer-associated fibroblasts or egressing tumor and immune cells can respond to (Ng et al. 2004, 2005; Haessler et al. 2012; Shields et al. 2007a, b; Polacheck et al. 2011). Movement of lymph as it enters lymphatics (referred to as transmural flow) and lymph constituents modulates LEC properties too. The lymph node homing chemokine CCL21 is produced and secreted in

response to transmural flow, acting as a potential homing signal for CCR7-expressing tumor cells in the early stages of metastasis. CCL21 can in turn simulate VEGF-C up-regulation, which as we saw earlier may further contribute to lymphatic mediated events. Furthermore, even at low flow rates of 0.1 μm s^{-1} (representative of measured interstitial fluid velocity), entry of lymph into vessels results in delocalization and down-regulation of cell junction proteins such as VE-cadherin and CD31 (Miteva et al. 2010), but up-regulation of cell adhesion molecules such as ICAM and E-selectin; a preparatory step prior to cellular transmigration and entry into lymphatic circulation. *Downstream effects within vessels and lymph node*: Once inside a vessel, tumor cell behavior continues to be influenced by lymph flow and lymph-borne factors. When compared with the harsh environment of the blood system, lymph flows seen in tumor-draining lymphatics provide a setting more conducive with cell survival—with velocities typically an order of magnitude lower than blood (1–10 vs. 100–1,000 μm s^{-1}, respectively; Berk et al. 1996; Leu et al. 1994a, b; Swartz et al. 1996). Tumor emboli within vessels have been shown to utilize the low shear stresses found in lymphatics to their advantage. Under low flow conditions ($\tau = 2.5$ dyn cm^{-2}; Byers et al. 1995), highly invasive E-cadherin negative or defective tumor cells are much more likely to detach from their counterparts in response to lymph flow and lodge in local lymph nodes than E-cadherin positive epithelial cells (Byers et al. 1995).

Lymph drainage from the tumor site clears the excess fluid from leaky blood vessels, which as we discussed can have autocrine and paracrine effects. As lymph flows along a vessel hierarchy, its components also pass this same route and therefore imply that tumors can utilize this path to remotely access distant tissues. One of the primary reasons a tumor might want to communicate with distant tissues is in order to prepare the new environment for metastasizing cells, otherwise known as the pre-metastatic niche (reviewed by Peinado et al. 2011; Psaila and Lyden 2009). Changes to lymph nodes immediately downstream of tumor-bearing sites *prior* to arrival of metastasizing cells have frequently been recorded in experimental models (Harrell et al. 2007; Hirakawa et al. 2005, 2007; Mumprecht et al. 2010; Ruddell et al. 2008), and similar changes in clinical samples are showing prognostic potential (Kurahara et al. 2010; Jakob et al. 2011). In mouse models of lymph node metastasis, tumor-draining lymph nodes present with greatly enlarged and supernumerary lymphatic sinuses before metastasis occurs, whereas lymph node metastases are not observed in the absence of LN lymphatic expansion (Hirakawa et al. 2007). In contrast, metastasis is frequently observed where LN lymphangiogenesis had preceded, and further distant lesions, e.g., lung, are more likely to occur (Hirakawa et al. 2007). Whether this directly relates to an enhanced delivery of tumor cells to the node, or some secondary survival advantage provided via environmental adaptation is still not clear. Consistent with earlier data on VEGF-C effects, lymph flow to the nodes is enhanced suggesting that tumor-derived VEGF-C not only impacts tumor cells and LECs at the tumor periphery but that VEGF-C can also be transported in lymph to the LN to stimulate lymphangiogenesis there.

7.4 Lymph Effects on the Tumor Immune Response

Of course, there is a flip side to every story. In the case of lymph and lymphatics from the context of a tumor, we know that tumor-derived factors and proteins contained within lymph can help to increase the probability of a tumor cell finding lymphatic vessels. We have also seen that the lymph flow generated as a consequence can both physically detach cells and act as a guidance cue. However, in providing a route for tumor cell dissemination, lymphatic expansion also reinforces the connection to our immune system. To survive, therefore, a tumor must develop methods to suppress potentially destructive immune responses. Early clues alluding to a cooperative relationship between a tumor, lymphatics, and the host immune system came from studies illustrating that immune cells recruited to a tumor and within the draining LN actively stimulated lymphangiogenesis and tumor progression (Schoppmann et al. 2006; Harrell et al. 2007; Jeon et al. 2008; Moussai et al. 2011). More recently, research advances indicate that tumors are capable of exploiting normal aspects of immunology into their advantage, confirming that lymph and lymphatic vessels are important mediators of this. Maintenance of tissue fluid balance requires an equilibrium between fluid that enters the interstitial space and that which leaves via the lymphatics. This ensures a flow of lymph from the periphery via lymph nodes and towards the thoracic duct before reentering blood circulation. The anatomical sites of lymphatic capillaries in peripheral tissues mean that they are in close proximity to the environment, and therefore represent a major route of immune surveillance and protection. In addition to plasma proteins and cellular waste products lymph also acts as a sampling reservoir, transporting soluble antigens from peripheral tissues towards the LN (Clement et al. 2010; Sixt et al. 2005; Roozendaal et al. 2009). Here, antigen and small molecular weight proteins penetrate the lymph node to the T cell zones via fine conduit structures and lymph node-resident antigen-presenting cells sample the unfiltered fluid, take up, and cross present antigen. These responses are rapid (within minutes), much more so than mobilizing peripheral antigen-presenting cells (8–12 h). Delivery of soluble antigens represents a rapid efficient way to monitor events at the periphery and adapt accordingly, either by inciting effective immune responses or by preventing inappropriate tissue-damaging responses. Many tumors evolve to express mutated antigens and overexpress normal tissue antigens (e.g., EGFR), differentiation antigens specific to tissue types (e.g., Melan-A specific to skin), or cancer/testes antigens, normally only expressed in germline tissues. In normal circumstances such changes would be detected and eliminated, however this is not the case in cancer. Something clearly goes awry. With collection of tumor-derived factors, vesicles, chemokines, inflammatory mediators, and cells by lymphatics it is also likely that shed tumor-derived antigens are carried in the soup. Influenced by this altered milieu, normally reactive immune cells undergo changes to functional phenotype (e.g., induction of T_{reg}), functional inactivation (loss of co-stimulatory molecules in dendritic cells), or die (deletion and exhaustion of tumor-reactive T cells). Indeed in physiological situations, lymph node LECs and stromal cells, which are continually exposed to

lymph-borne antigens, are able to process and present endogenous antigen, resulting in the deletion of self-reactive T cells and maintenance of immune homeostasis and tolerance (Lee et al. 2007; Cohen et al. 2010; Fletcher et al. 2010). More recently, this scenario was translated to the pathological setting of the tumor. Tumors that express the model antigen ovalbumin together with VEGF-C developed extensive peritumoral lymphatic vessels networks, significant immune infiltrates which concentrated in close proximity to the vessels, and enhanced lymphatic drainage (Lund et al. 2012). This study demonstrated that tumor-specific antigen could be scavenged by LECs of the tumor and draining LN and presented in Class I MHC complexes. This leads to impaired activation, function, and deletion of ovalbumin-specific, tumor-reactive T cells that infiltrate the tumor (Lund et al. 2012). Consistent with this, lymph node LECs are capable of iNOS production upon inflammatory stimulation, which attenuate T cell proliferation (Lukacs-Kornek et al. 2011) and may promote regulatory T cell development (Niedbala et al. 2007). In areas of chronic inflammation such as the tumor microenvironment, the associated immune cells can further contribute to immune suppressive effects by signaling to collecting lymphatics in their vicinity. iNOS secreting immune cells such as myeloid-derived suppressor cells (MDSC) cause collecting vessel relaxation and diminished vessel contraction strength (Liao et al. 2011). While the interpretations of these and other studies differ, they all support the hypothesis that tumors employ complex strategies to manipulate our immune system, but thanks to the major role that lymphatics and their cargo play in immune homeostasis, modifications to lymphatic function are a key weapon in a tumors arsenal.

7.5 Lymph Measurement in Tumors

The steps leading to hematogenous metastasis have been extensively studied (Butler and Gullino 1975; Condeelis and Segall 2003; Liotta et al. 1974) and until recently, lymphatics had not received the same attention. However, genetically modified mouse models are now proving invaluable tools to aid our understanding of how lymphatic vessels function in both normal and diseased tissues.

Detection of lymphatic vessels: Coincident improvements to intravital imaging techniques and development of transgenic lines with fluorescent proteins such as GFP, mOrange, tomato red, and luciferase under the transcriptional control of lymphatic-specific genes *Prox1* or *Vegfr3* have made the accurate identification of lymphatic vessels possible and allowed real-time, minimally invasive tracking of the cells that contribute to the changes (Choi et al. 2011; Hagerling et al. 2011; Martinez-Corral et al. 2012; Truman et al. 2012). This is particularly useful for studying the onset and progression of lymphangiogenesis in cancer both at primary and metastatic sites (Harrell et al. 2007; Mumprecht et al. 2010; Martinez-Corral et al. 2012).

Measuring functionality in tumors: As we have seen, vessel functionality is essential for the establishment and propagation of tumors. Using murine models of

cancer, lymph exit from tumors and transit to lymph nodes have been measured by magnetic resonance imaging (MRI) technology (Ruddell et al. 2008; Dafni et al. 2002) and fluorescence microlymphangiography using fluorescently labeled tracers (dextrans and quantum dots) (Hoshida et al. 2006; Harrell et al. 2007; and reviewed by Cohen et al. 2011). Both methods have advantages and disadvantages. MRI allows three-dimensional imaging, which can be achieved for both superficial and deeper structures (Pathak et al. 2005). Contrast agents such as low molecular weight gadolinium-based agents (e.g., dimeglumine gadopentate, Gd-DTPA), however, can rapidly diffuse out of lymphatic vessels so are not suitable for long-term imaging in small animal models. Gd-DTPA has proven useful for short-term kinetic studies of tumor-associated lymph flow in mice, where rapid uptake into lymphatics enabled measurement of the increased lymph flow from tumors to lymph nodes and back into the circulation of tumor-bearing mice (Ruddell et al. 2008). In contrast, quantification of lymph flow velocity is achieved using fluorescence lymphangiography, which tracks the convective movement of photobleached spots within fluorescently loaded vessels (Hoshida et al. 2006). Alternative methods to quantify lymphatic function involve the measurement of depot clearance of labeled albumins or dextrans (Emmett et al. 2011; Karlsen et al. 2012). Fluorescent and near-infrared optical imaging are useful for imaging of superficial lymphatic vessel drainage, however, they do have the drawbacks that images are of relatively low resolution and deeper vessels cannot be detected (Harrell et al. 2007; Kwon and Sevick-Muraca 2007). Recently, optical frequency domain imaging, a second generation of optical coherence tomography, has been described as an exciting new technique for intravital imaging of tumors, utilizing elastic light scattering properties to yield high resolution images in 3D. Signals are generated based upon the intrinsic movement of erythrocytes so no contrast agents are required. Without the need for tracers, both angiography and lymphangiography can be employed at the same time and distinguished based on contrast. This method is being advocated as a high volumetric imaging technique with sufficiently high resolution and offering enhanced tissue penetration up to depths in the order of millimeters (Vakoc et al. 2009).

Lymph flow measurements in cancer patients: In patients, tracers that rely on the functional properties of lymphatics for selective uptake and transport of either radiolabeled/radio-opaque colloids or inert dyes are most commonly used to locate sentinel lymph nodes (Lai and Rockall 2010; Mouli et al. 2010). There are few studies that have measured lymph flow in cancer patients. Indocyanine green, a near-infrared fluorophore, has recently been used to measure lymph velocities, and lymphatic functionality in breast cancer patients undergoing sentinel lymph node mapping (Rasmussen et al. 2009; Sevick-Muraca et al. 2008). This noninvasive technique measured lymph velocities in patients ranging from 0.08 to 0.32 cm s^{-1} (4.8–19.2 cm min^{-1}) (Sevick-Muraca et al. 2008) in contrast to normal arms where lymph velocities have been measured at 8.9 cm min^{-1} (Modi et al. 2007a, b). Near-infrared fluorescence and other imaging techniques are also being utilized to map vessel functionality in patients suffering from complications associated with

surgical resection of lymph nodes. These modalities improve the efficiency of manual lymph drainage therapy (Maus et al. 2012; Sevick-Muraca 2012), and can also be applied to help detect early changes or deficits in vessel function that could account for lymphatic dysfunction preceding postmastectomy lymphedema (Modi et al. 2007a; de Rezende et al. 2011; Stanton et al. 2006; Szuba et al. 2007).

7.6 Lymph and Lymphatics as Therapeutic Targets

With increasing insight into the role of lymphatics, their function, and the compounds they carry, comes an increasing aspiration to exploit or manipulate these features as therapeutic targets for the treatment of cancer (Fig. 7.3).

Manipulation of VEGF-C/VEGFR signaling axis: Numerous studies have been conducted with a goal to assess the therapeutic potential of antibodies raised against lymphatic-specific epitopes. In experimental cancer models, neutralizing antibodies and antibody fragments against VEGF-C and its receptors inhibit the growth of peritumoral lymphatics and ultimately the incidence of lymph node metastasis but have no effect on growth of the primary tumor (Rinderknecht et al. 2010; Roberts et al. 2006; Tvorogov et al. 2010; Yang et al. 2011). Major effects on the primary tumor and metastasis were only observed when combinations of both anti-VEGFR-2 and -VEGFR-3 were used. Moreover, antibodies designed to block shared markers of blood and lymphatic endothelium such as ephrins and angiopoietins impact both systems but the mechanisms of actions are yet unclear, whether they exert their beneficial effects on lymphatics due to direct inhibition, or whether the observed lymphatic inhibition is a downstream consequence of the forced reduction in blood vessel density within the tumor (Abengozar et al. 2012; Holopainen et al. 2012; Hwang-Bo et al. 2012). Small molecule inhibitors such as Sunitinib, Sorafineb, PTK787/ZK222584, and E7080, and mTOR inhibitors such as Rapamycin have also been shown to impact tumor lymphangiogenesis and lymphatic dissemination (Kodera et al. 2011; Matsui et al. 2008; Patel et al. 2011; Schomber et al. 2009). While effective in preclinical models, these compounds, e.g., broad-spectrum tyrosine kinase inhibitors, are not lymphatic-specific, therefore off-target secondary effects need to be carefully evaluated. These data would suggest that with the complex survival strategies put in place by a growing tumor, targeting either lymphatics or blood vessels alone is not the best way to attack tumors.

Particulates that exploit the lymphatics: As we learn more about how lymphatics function and the proficient manner in which lymphatics are able to rapidly but selectively take up pathogens such as viruses and virus-like particles up to 150 nm in diameter, there is an increasing thrust to harness and exploit these properties. With this in mind, researchers are aiming to improve the efficacy of drug delivery, and develop novel "vaccines" that target both the tumor and the downstream immune checkpoints bridged by the lymphatics. Compounds loaded within particulates that are drained and carried via

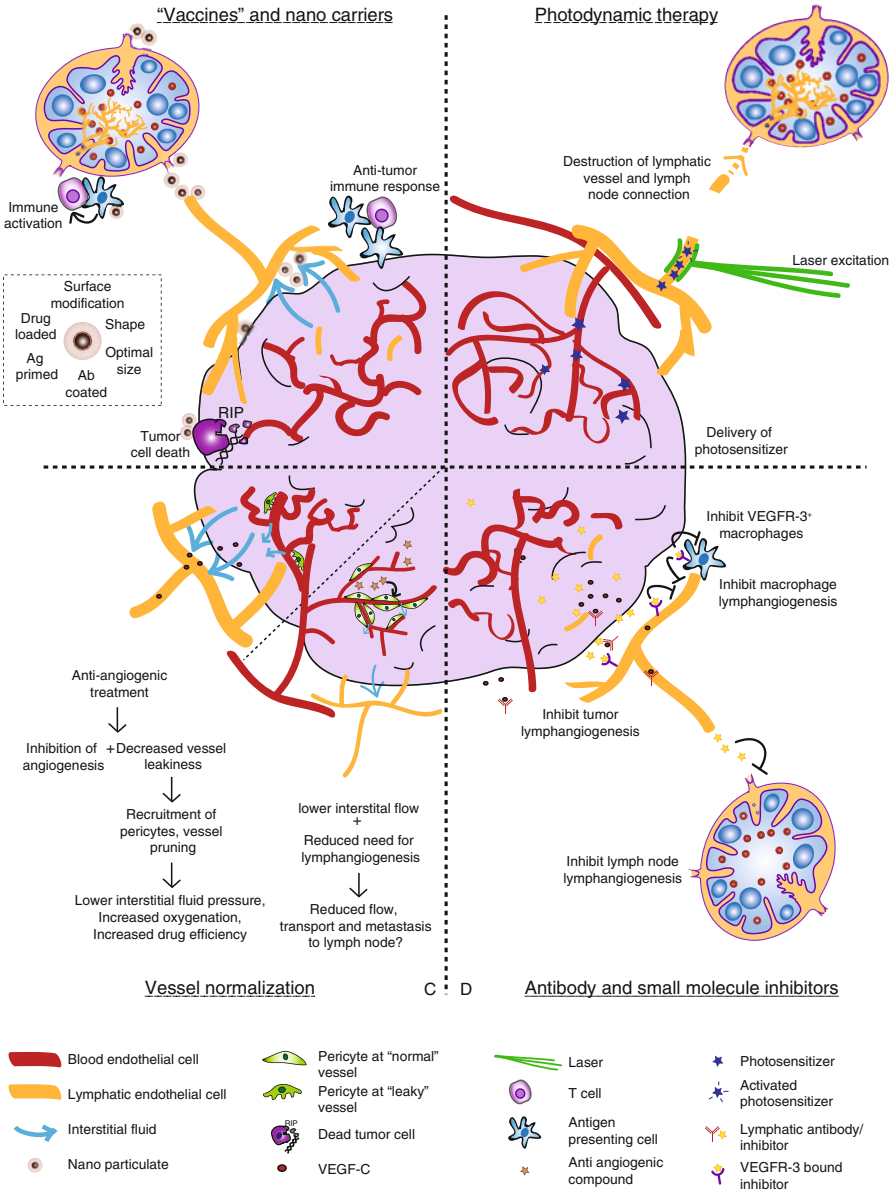

Fig. 7.3 Lymph-associated therapeutic strategies. (**a**) The implementation of "vaccines" and nanocarriers aim to improve efficiency of therapies by exploiting the properties of lymph and their transport via lymphatics. These particulates can come in many guises due to their pliability. Researchers hope to use this strategy as an immunotherapy, directly targeting the draining lymph node and immune system, to more effectively deliver cytotoxic payloads or kill tumor cells as a direct consequence of particle properties. (**b**) Photodynamic therapy (PDT) relies on the accumulation of photosensitizers within tissues, but cytotoxic activation only occurs once the specific tissue is exposed to light of defined wavelengths. Preclinical models demonstrated this method could

the lymphatics display reduced nonspecific cytotoxicity and enhanced bioavailability (e.g., protection from enzymatic degradation and first pass clearance from liver metabolism) compared with intravenous delivery. Furthermore, the pliability of such particulates, e.g., particle material, core composition, size, structure, shape, and surface modification adds to their attractiveness as a therapeutic vehicle. For example, the potential to modify their surface chemistry can enhance lymphatic uptake (sub-50 nm diameter), retention (avidin coating), and cell-specific uptake. Ingestion particularly by antigen-presenting cells therefore potentiates these nanoparticles as immune activators. Although the use of patient-specific therapeutic vaccines is still some way off, the prospect of individualized lymph-targeted immune-modulatory therapies is immensely exciting. The number of scientific research groups, biotech start-ups, and pharmaceutical giants buying into this technology indicates that the notion of lymph-borne therapeutics is in fact likely to become a powerful therapeutic platform.

Photodynamic ablation therapy: With the knowledge that systemic delivery of blocking antibodies is less effective and specific than hypothesized, alternative and more targeted ideas are under exploration. Photodynamic therapy (PDT) typically works via the accumulation of compounds with photosensitizing properties at your tissue of choice. Exposure of this area to visible light activates the photosensitizer generating reactive oxygen species and cytotoxicity tissues exposed to the compound (reviewed by Juarranz et al. 2008). The benzoporphyrin derivative verteporfin is clinically applied to patients with diseases such as age related macular degeneration (Zhao et al. 2010). It is delivered to patients in the form of liposomes. Recently, taking advantage of lymphatic functional characteristics, these particulates were applied to experimental models of melanoma. Upon intradermal inoculation, liposomes in the tumor-associated interstitium drained into local lymphatics where they were retained for up to 2 h. Upon subsequent activation by 639 nm laser light, activation of the verteporfin resulted in specific destruction of tumor-associated lymphatics but also cancer cells contained within them in transit to the lymph node (Tammela et al. 2011). Developments of this nature are particularly exciting because of their low toxicity and off-target effects when compared to conventional cytotoxic therapies. Further advantages offered by PDT in the context of tumor-associated lymph formation are that this therapy can also (a) induce vascular shutdown which may help to interrupt the nutrient supply chain but also switch off vascular leakage and high interstitial pressures that drive lymph formation; and (b) activate the immune response

Fig. 7.3 (continued) effectively ablate tumor-draining lymphatics and cancer cells within them. How this would impact tissue fluid balance, however, is not clear. (**c**) Vessel normalization strategies use anti-angiogenic therapies to temporarily restore the balance of pro- and anti-angiogenic signals, thereby reducing vessel leakiness and fluid pressure, improving oxygenation and drug delivery. Reduction of fluid pressure may then decrease lymph flow and the effects it and its cargo impart on lymphatics and downstream tissues. (**d**) Anti-lymphatic antibodies and small molecule inhibitors may prevent VEGFR-3 signaling, and therefore lymphangiogenesis at the tumor and lymph node by blocking the receptor or scavenging ligands on responsive cells (e.g. endothelium and infiltrating immune cells). Secondary advantageous effects may also occur via interactions with blood endothelial cells

which is compromised in tumors, partly as a consequence of the immune modulating components transported within tumor-derived lymph.

Vessel normalization strategies: Rather than directly targeting lymph and lymphatic vessels, the route of *vessel normalization* is also being explored to target abnormal vessels within the tumor. The concept came from the observation that in preclinical models, treatment with anti-angiogenic agents transiently restored vessel function in tumors, improving oxygenation and reducing fluid pressure (Tong et al. 2004; Winkler et al. 2004; Yuan et al. 1996). In this window of opportunity, the efficacy of cytotoxic drugs was also improved (Winkler et al. 2004; Mazzone et al. 2009). The reinstatement of a more normal vessel network by balancing pro- and anti-angiogenic signals within the tumor microenvironment may therefore help to reduce the manifestation of lymph-mediated disease downstream stemming from high TIF pressure, irrespective of whether this occurs through direct effects on vessel wall components or via inhibition of angiogenic myeloid cells (reviewed by Carmeliet and Jain 2011). Direct evidence to the benefits of this concept in human tumors is still missing, however recent studies in colorectal cancer and glioblastoma have reported changes in Bevacizumab-treated patients that are consistent with the notion of normalization (Batchelor et al. 2007, 2010; Willett et al. 2004). Although in theory, vessel normalization may attenuate lymphatic promotion of tumor dissemination by reducing TIF generation and flow thereby reducing need for lymphangiogenesis and decreasing transport of soluble pro-tumor factors, recent work has demonstrated that caution is indeed warranted: compounds with potential to induce vessel normalization (and increase drug delivery) may also promote lymphatic metastasis (Grepin et al. 2012; Liu et al. 2011). Therefore understanding the delicate balances that exist within the tumor environment will be critical when designing and optimizing the doses, combinations, or timings of future therapeutic platforms.

7.7 Concluding Remarks

There is no doubt that lymph via its generation, transport, and the vessels that facilitate this plays an essential role in the pathophysiology of cancer, and carries widespread implications. As a result, lymphatics and their function are rapidly becoming a major therapeutic target. It is also clear to see that the relationship between tumor blood vessels, lymphatics, and their microenvironment is a convoluted one with multiple levels of complexity. But as the field grows and expands into previously unexplored niches, we have begun to embrace new methodologies. The integration of tumor biology with disciplines, such as physics, proteomics, and bioengineering for example, is helping to unravel these relationships layer by layer at continually evolving and accelerating rates. It is through collaborations such as these that the biggest breakthroughs will transpire; an event we await with much anticipation.

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