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Immunomodulatory Roles of Lymphatic Vessels in Cancer Progression

Melody A. Swartz^{1,2}

Abstract

Lymphatic vessels in the tumor microenvironment are known to foster tumor metastasis in many cancers, and they can undergo activation, hyperplasia, and lymphangiogenesis in the tumor microenvironment and in the tumor-draining lymph node. The mechanism underlying this correlation was originally considered as lymphatic vessels providing a physical route for tumor cell dissemination, but recent studies have highlighted new roles of the lymphatic endothelium in regulating host immunity. These include indirectly suppressing T-cell function by secreting immunosuppressive factors and inhibiting dendritic cell (DC) maturation, as well as directly driving T-cell tolerance by antigen presentation in the presence of inhibitory ligands. Furthermore, lymphatic endothelium scavenges and regulates transendothelial transport actively, controlling the sustained delivery of lymph-borne antigens from chronically inflamed tissues to draining lymph nodes where immature DCs, in the absence of danger signals, along with lymph node stromal cells present these antigens to T cells for maintenance of peripheral tolerance to self-antigens, a mechanism that may be hijacked by some tumors. This Masters of Immunology primer aims to present an overview of research in this area and highlight emerging evidence that suggests lymphatic vessels, and lymphangiogenesis, play important immunomodulatory roles in the tumor microenvironment. *Cancer Immunol Res*; 2(8); 701–7. ©2014 AACR.

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Learning Objectives

Many cancers disseminate first through the lymphatic system. Lymphatic vessels were considered originally as only providing a physical route for tumor cell dissemination, but recent studies have highlighted new roles of the lymphatic endothelium in regulating host immunity. Upon completion of this activity, the participant should gain a basic knowledge of the emerging concepts linking transport and immunity by lymphatic vessels and lymphatic endothelium, and the concept of lymphangiogenesis and its various potential roles in modulating antitumor immunity.

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Lymphatic Vessels and Cancer Progression

Many cancers disseminate first through the lymphatic system, and the presence of tumor cells in the sentinel or draining lymph node (DLN) is a major prognostic indicator in many common

cancer types, including breast cancer and melanoma. Since the discovery of the lymphatic-specific growth factor, vascular-endothelial growth factor receptor-3 (VEGFR-3), and its ligands VEGF-C and VEGF-D, scores of studies have demonstrated correlations between these factors and tumor metastasis experimentally and poor prognosis clinically. In patients with cancer, for example, elevated serum levels of VEGF-C have been correlated positively with incidence of metastasis. The earliest, and most straightforward, explanation for this correlation was the notion that tumor lymphangiogenesis increases access of the tumor cells to the lymphatic vessels and thus the downstream lymph nodes, providing an "escape route" for tumor dissemination. Indeed, experimental tumors modified to express VEGF-C or VEGF-D (i.e., lymphangiogenic tumors) were shown consistently to be more metastatic than their unmodified,

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parental counterparts, and blocking VEGFR-3 signaling in such tumor models could prevent or slow metastasis (summarized in refs. 1, 2). However, it is now apparent that in addition to simply transporting tumor cells to distant sites, lymphatic vessels play many other roles in tumor progression.

Soon after the first reports that lymphangiogenic tumor xenografts in mice were more metastatic, other studies questioned the necessity of tumor lymphangiogenesis for metastasis (3), and suggested that intratumoral lymphatics in VEGF-C-expressing tumors may be mostly collapsed or nonfunctional, whereas peripheral (i.e., peritumoral) lymphatic vessels were the primary sites of egress for metastasis (4). In human malignant melanoma, for example, increased lymphatic density in the tumor periphery, but not inside the tumors, was strongly correlated with metastasis together with local tumor invasion into these lymphatic vessels (5). So, although intratumoral lymphatic vessels have not been found in many highly metastatic human tumors such as mammary carcinomas, lymphatic vessel hyperplasia in the tumor periphery and lymphangiogenesis in the DLN, are frequently reported. Local lymphangiogenesis has also been reported in multiple different types of chronic inflammatory diseases (1, 6), and is probably driven primarily by inflammatory macrophages and other leukocyte subsets recruited to the inflamed site (7), although tumor cells can also secrete lymphangiogenic growth factors.

A puzzling element in lymphatic-mediated tumor dissemination is that lymph nodes are normally inhospitable sites for tumor cell growth. This was shown, for example, by implanting B16 melanoma cells into naïve lymph nodes of syngeneic mice, whereupon the tumor cells were readily rejected by cytotoxic T lymphocytes (CTL). In contrast, these same tumor cells thrived when implanted into lymph nodes draining a primary tumor (8). This study and others suggest that DLNs are altered by the primary tumor to allow immune escape (either lack of immune recognition, or recognition and tolerance), and that the correlation between tumor-associated lymphatic vessels and lymph nodes metastasis is more complex than previously realized.

So how and why are tumor-DLNs different from the non-DLNs? For one, they appear to have a more immunosuppressive cytokine environment (6, 9), even though they are tumor antigen-experienced and can mount strong antitumor CTL responses when activated, for example, with a vaccine against a tumor-specific antigen (10, 11). Second, they are lymphangiogenic, and lymph node lymphangiogenesis has been shown to precede metastasis (1, 2). It is important to note that lymph nodes draining *any* chronically inflamed site—be it a tumor, a chronic infection, or even a site of vaccination—undergo expansion in both leukocyte populations and stromal cell populations, the latter of which include lymphatic endothelial cells (LEC), blood endothelial cells, and fibroblastic reticular cells (FRC). Thus, even when the growing tumor does not induce intratumoral lymphangiogenesis, the inflamed microenvironment can drive lymphatic hyperplasia in the periphery and lymphangiogenesis in the DLN.

Given that lymphatic expansion occurs in such a wide range of pathologic situations, including autoimmune diseases like Crohn's disease, it may seem surprising that the function of

lymphangiogenesis, in the lymph node or in any inflamed site, remains controversial and poorly understood. On the other hand, lymphatic vessel function has never been well understood from an integrative perspective, particularly considering that the lymphatic system could be categorized potentially as part of several different organ systems, including circulation, metabolism, immunity, and solute regulation. Indeed, such different research perspectives of the lymphatic system have evolved largely independently from each other and are only recently beginning to be integrated (Fig. 1). Lymphatic vessels were first discovered in the gut as they turned milky white after a meal; we now appreciate this "chyle" as dietary lipids that are taken up specifically by the lymphatics, and some laboratories are focused on targeting this component for oral drug delivery to avoid first-pass metabolism in the liver (12). Vascular and microcirculatory physiologists have traditionally focused on the fluid and solute transport functions of the lymphatic system and its relation to blood capillaries and the interstitial space (13), as well as the regulation of pump function by the collecting lymphatic vessels (14). Tumor biologists have pieced together the molecular regulators of lymphangiogenesis by tumor cells and inflammatory leukocytes in the tumor microenvironment (1, 2), whereas immunologists have traditionally considered mostly the anatomic aspects of the lymphatic system, whereby lymphatic vessels serve to transport leukocytes and antigens to the lymph node, where cell-cell interactions regulate adaptive immune responses (15, 16). Only within the last few years has each of these growing research areas begun to overlap at their boundaries, and at these interfaces entirely new functions of lymphatic vessels have begun to emerge, as the complex, multifaceted roles are now coming to light.

This *Masters of Immunology* primer presents an overview of research in the lymphatic system and highlights new ideas of how lymphatic vessels promote immunity, particularly in the context of tumor progression. Three concepts are discussed below and summarized in Fig. 2. They include the lymph flow of antigens, the lymphatic transport of cells, and the modulation of adaptive immunity by lymphatic endothelial cells. The molecular regulation of lymphangiogenesis in development, inflammation, and cancer is well described in excellent recent reviews (1, 2), and therefore will not be discussed here.

Lymph Flow of Antigens

The term "lymph" refers to the fluid inside lymphatic vessels, which largely represents the local interstitial fluid of the tissue it drains. This fluid thus contains not only interstitial solutes and serum proteins that have leaked out of the blood capillaries, but it also carries cytokines, growth factors, and cell debris from this space. During any inflammatory event, blood capillaries rapidly become hyperpermeable and an influx of immune cells induce local extracellular matrix proteolysis; thus, lymph from inflamed or irritated tissues is rich in peptides derived from the extracellular matrix (due to proteolysis by matrix metalloproteinases) or from apoptotic cells and other factors present in the inflamed tissue (17). Therefore, lymph is enriched with local antigens from the tissue it drains, and constantly bathes cells in the lymph node with these antigens.

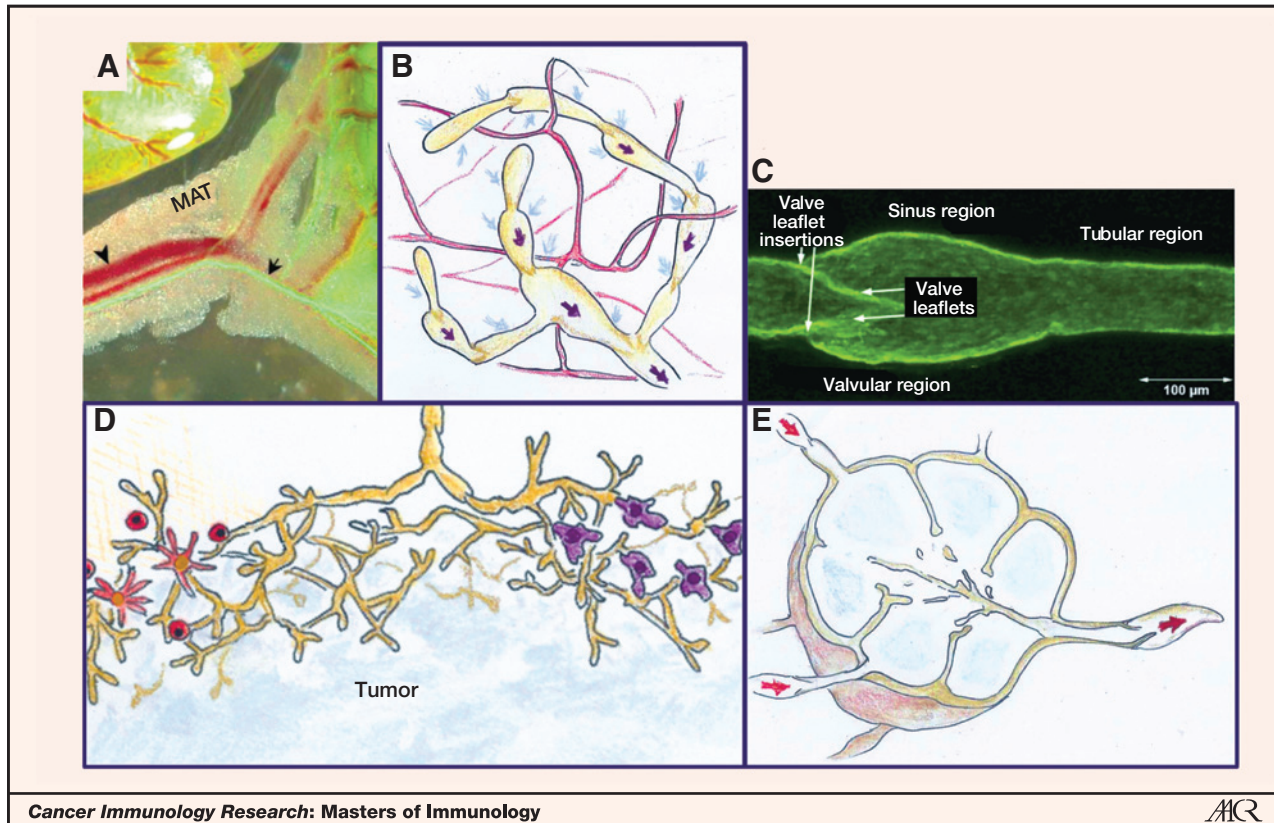


Figure 1. Several domains of lymphatic research that evolved for the most part independently have recently begun to overlap and merge. A, lymph was first discovered in the mesenteric vessels draining the small intestine after a lipid-rich meal, as they absorb and transport lipids in the form of chylomicrons. Shown is an overlaid bright-field image of the mesentery with a fluorescence image of a lymphatic collecting vessel (arrow) and surrounding mesenteric adipose tissue (MAT) in the small intestine after feeding a fluorescently labeled lipid to a mouse; image courtesy of Ryan Oliver (EPFL, Lausanne, Switzerland.) B, interstitial fluid balance, whereby osmotic and hydrostatic pressure forces are considered to drive plasma out of blood capillaries and into lymphatic vessels with small net driving forces, has been the subject of a great deal of quantitative investigation over the past 50 years. C, a valve separating two lymphangions in a collecting lymphatic vessel. The pump function and valve system of collecting lymphatics have received substantial research attention over the past few decades, and genes critical for valve development have been found to be mutated in congenital lymphedema. Image adapted from Figure 5A of ref. (43). D, the molecular regulation of tumor lymphangiogenesis has greatly improved our understanding of molecular cross-talk and signaling involved in lymphatic proliferation. E, until very recently, lymphatic research in immunology was mostly limited to its roles in antigen and cell transport and cell-cell communication necessary for adaptive immune responses, i.e., from peripheral tissues to the lymph node, and within various compartments of the lymph node. Drawings in B, D, and E are courtesy of Katie Hubbell, University College London, UK.

In the lymph nodes, many cell types can take up lymph-borne antigen, but perhaps most well studied are lymph node-resident immature dendritic cells (DC). In the absence of stimulatory cytokines or danger signals, antigen uptake by these cells helps to maintain peripheral tolerance to self-antigens because they present antigen to cognate T cells with low levels of costimulatory molecules. Obviously, soluble pathogenic antigens flow to the lymph nodes upon infection or vaccination, but these pathogenic antigens are also taken up by patrolling DCs in the periphery in the presence of danger signals and inflammatory cytokines, which cause DC maturation as they travel to the lymph nodes. Because soluble antigen flows to the lymph node within minutes, and because lymph node-resident DCs are already in the vicinity of T cells as they begin to mature and respond to the danger signals, they activate T cells more rapidly but less potently than the second wave of peripheral DCs that were activated in the presence of higher local concentrations of cytokines and danger signals

(e.g., the site of infection) and had sufficient time to upregulate costimulatory receptors (18, 19). This further rationalizes why lymph flow may be critical for fine-tuning immune responses and to avoid autoimmune reactions in the process of activating effective immunity against pathogens.

In addition to immature DCs, other cells in the lymph nodes can also directly access lymph-borne antigens, depending mostly on antigen size (20). Large or opsonized antigens are taken up by subcapsular macrophages, whereas smaller antigens flow into small conduits lined with follicular DCs in which they can be taken up directly by antigen-specific B cells (21). On the other hand, LECs have access to all lymph-borne antigens, and it was recently shown that LECs can actively scavenge these antigens, process them intracellularly, and cross-present them to T cells for deletional tolerance (22, 23), which is discussed in more detail later. Furthermore, LECs can retain antigen for long periods of time and may play a role in recalling memory T cells (24).

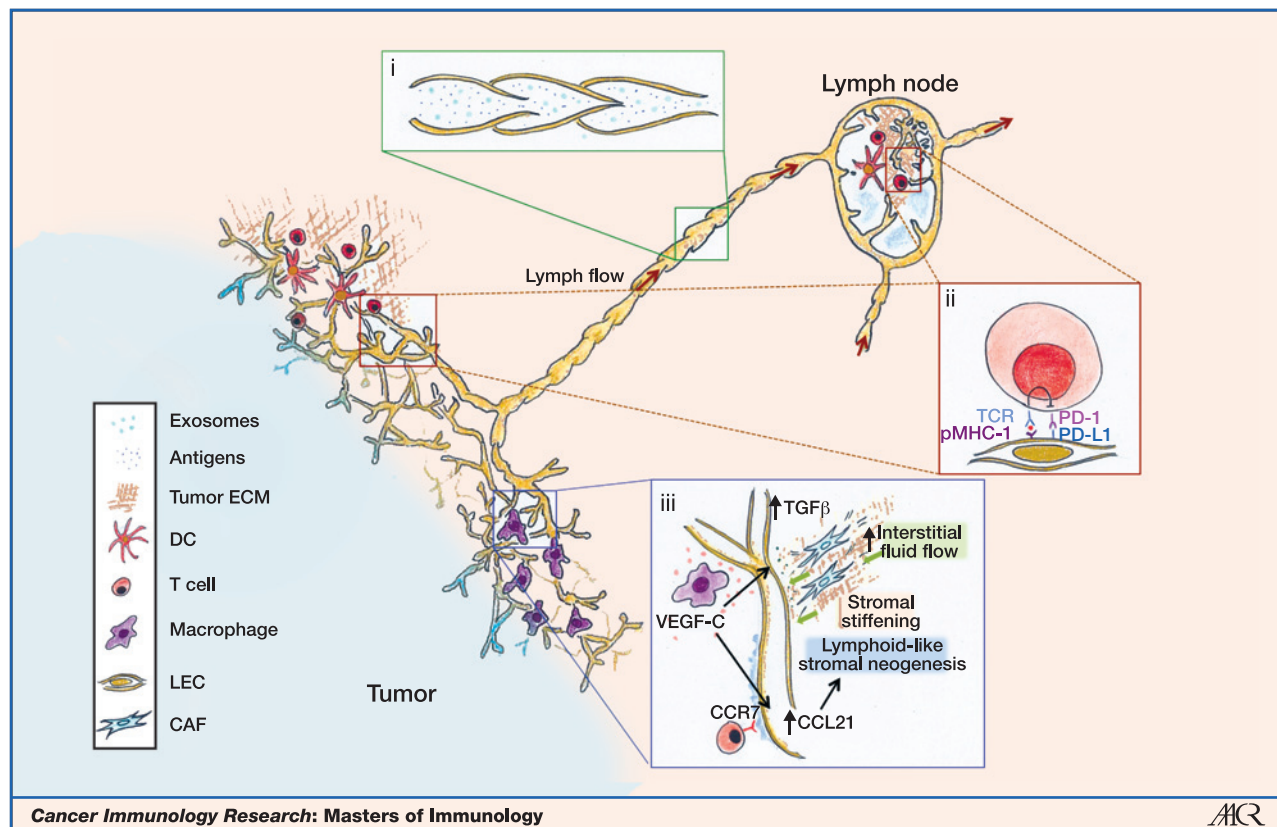


Figure 2. This is a schematic diagram incorporating results from recent work that identified multiple complementary mechanisms by which tumor-associated lymphatic vessels may promote immune escape. (i) Many solid tumors induce increased lymphatic drainage, delivering tumor antigens and tumor-secreted exosomes to the DLN. Because lymphatic drainage plays important roles in adaptive tolerance to newly introduced antigens, increased tumor drainage may exploit the natural mechanisms of self-tolerance in the lymph node. (ii) Tumor-associated LECs can directly scavenge and cross-present tumor antigen in the absence of costimulatory molecules and in the presence of inhibitory ligands such as PD-L1 to drive CD8⁺ T-cell apoptosis. (iii) VEGF-C, secreted by tumor-associated macrophages, upregulates LEC expression of the lymphoid chemokine CCL21, which attracts naive and regulatory T cells and drives the transformation of lymphoid-like stroma that facilitates T-cell education. VEGF-C also induces TGFβ secretion by LECs (J. Munson and M. Swartz, unpublished data), which can promote regulatory T-cell education and drives fibroblast-to-myofibroblast transformation and stromal stiffening. Finally, increased interstitial flow in the tumor stroma due to high pressure gradients at the tumor margin also promotes TGFβ activation, myofibroblast transformation, and stromal stiffening (44), and promote fibroblast-led tumor-cell invasion (45). Drawings are courtesy of Katie Hubbell, University College London, UK.

The notion that lymph flow of local soluble antigen to the DLNs might be important in maintaining immune tolerance was first suggested more than 40 years ago, when a contact hypersensitivity tolerance assay on transplanted mouse skin islands failed unless the skin was functionally connected to lymphatic drainage (25). More recently, this notion was revived using genetically modified mice lacking dermal lymphatics; the studies showed that without drainage from the skin to the lymph node, these mice could not generate tolerance in similar skin tests but could still elicit a robust, but delayed, T-cell response to intradermal vaccination (26). The importance of the delivery of soluble antigens to the lymph nodes for modulating adaptive immunity, particularly in the context of local tolerance, requires further investigation and is a growing area of research.

In the context of solid tumors, there is mounting evidence that lymph flow from tumors is elevated, particularly from lymphangiogenic tumors, correlating with increased interstitial fluid pressure (IFP) at the margin. IFP is increased because intratumoral blood vessels, which are rapidly and

haphazardly formed as the tumor grows and therefore are less mature and more leaky than normal vessels (27). Thus, the sharp gradient in fluid pressure at the tumor margin drives heightened interstitial flow in the tumor stroma and increases lymphatic drainage from the tumor to the DLN (6, 13). In turn, the increased flow of lymph from the tumor—i.e., the tumor interstitial fluid—to the DLN implies a greater rate of antigen flow, and thus antigen uptake and presentation, in the tumor-DLN. Also, tumor-secreted exosomes, which are vesicles secreted by tumor cells that contain a variety of microRNAs relevant to metastasis, are largely taken up by lymphatic vessels; because of their size (50–100 nm), exosomes do not diffuse readily in tissue but rather flow with the interstitial fluid into tumor-draining lymphatic vessels. In turn, the lymph delivers these exosomes to the DLN and eventually to the blood, where they can help "seed the soil" of these distant sites for future metastatic colonization (28, 29). Coupled with a suppressive cytokine milieu and the absence of danger signals, it is thus conceivable that the increased lymphatic drainage from a tumor could

increase the rate or strength of tolerogenic pressure to tumor antigens in the DLN. In this way, tumor-driven increases in lymph flow might promote host tolerance to tumor antigens and exosomes by increasing their abundance in the lymph nodes.

Lymphatic Transport of Cells

In addition to fluid, antigens, exosomes, and cytokines, lymph provides a conduit for cell transport between peripheral tissues, the lymph node, and the systemic blood circulation. Cell transport is thus another mode by which lymphatics may modulate adaptive immunity. DC transport in lymphatics has been the most studied; DCs upregulate the chemokine receptor CCR7 as they mature to sense the lymphoid chemokine CCL21 in the periphery that is secreted by LECs and accumulates in the basement membrane, and later to detect CCL21 and CCL19 in the lymph nodes, secreted by FRCs. Without CCR7, DCs are unable to home to the lymph node after activation (30). LECs also express adhesion molecules such as ICAM-1 and e-selectin to facilitate DC transmigration, and LECs can modulate their expression of CCL21, ICAM-1, and e-selectin, among other cell-surface molecules, according to inflammatory cues (31). One such inflammatory cue is interstitial flow, which is rapidly upregulated upon cellular insult or injury (i.e., within seconds, due to rapid onset of capillary leakiness). In fact, increasing interstitial flow not only upregulates these molecules and in turn increases DC transmigration, but LECs rapidly down-regulate these molecules in the absence of mechanical flow signals (32).

Because CCR7 expression by tumor cells has been particularly well correlated with lymph node metastasis in many tumor types, including mammary, colorectal, lung, prostate, and others, it is likely that leukocyte-like, CCR7-driven homing toward lymphatics and lymph nodes plays an important role in cancer metastasis. Recent studies have shown that LEC secretion of CCL21 is strongly upregulated upon VEGFR-3 ligation, such that VEGF-C-secreting, CCR7⁺ tumor cells are far more chemotactic toward LECs than their non-VEGF-C-secreting counterparts (33). In this way, tumor VEGF-C drives increased tumor invasion into existing peripheral lymphatic vessels by increasing lymphatic chemoattraction of these cells.

Finally, cell transport toward and into lymphatic vessels can be directly influenced by interstitial flow, especially in the heightened flow environment of the tumor margin. Because CCL21 is strongly matrix-binding, predominantly to proteoglycans in the basement membrane, its broadcast distance is very short particularly when considering that interstitial flow into the lymphatic vessel opposes diffusion away from the vessel. Interestingly, some CCR7⁺ tumor cells, as well as DCs, secrete CCR7 ligands. On the one hand, autologous expression of chemokine should weaken the ability of the cell to sense a downstream paracrine source of chemokine due to local dilution of the signal; on the other hand, interstitial flow should skew the distribution of autocrine chemokine and form a gradient that increases in the flow direction. Even if the resulting gradient is small, chemokine sensing is known to be extremely sensitive such that even a 1% to 2% concentration difference across a cell can drive chemotaxis. Indeed, recent

studies have shown that interstitial fluid flow alone can drive CCR7-dependent chemotaxis in CCL21⁺ tumor cells, and that this autocrine gradient synergizes with the steep local paracrine gradient from a CCL21⁺ lymphatic vessel (34). This "autologous chemotaxis" can also occur with other chemokine-receptor autocrine loops, for example as shown in CXCR4-dependent flow-driven invasion of glioma cells, which also express its ligand CXCL12 (35). This is yet another way in which lymphatic vessels in the tumor microenvironment can facilitate tumor cell metastasis, i.e., by virtue of the interstitial flow that they create by their drainage function, always directed toward and into the vessel.

Lymphatic Endothelial Cells Modulate Adaptive Immunity

Several recent studies have highlighted new roles of LECs on T-cell fate and function, through both direct and indirect mechanisms. First, it has been shown that LECs can directly dampen DC maturation (36), in turn reducing their ability to activate effector T cells. Second, LECs (as well as FRCs) constitutively express and present self-antigen on MHC class I molecules to delete autoreactive T cells and thus help maintain peripheral tolerance to these self-antigens (37, 38). Third, as mentioned above, it is now known that LECs can actively scavenge exogenous lymph-borne antigens and process them for cross-presentation on MHC class I molecules (22, 23). Because LECs lack costimulatory molecules necessary to activate effector CD8 T cells, and instead they express high levels of the inhibitory ligand PD-L1 (39), LEC presentation of the peptide-MHC class I complex can drive deletional tolerance of naïve CD8 T cells (22, 23). Even though the relative importance of exogenous antigen presentation by LECs in the context of DCs and other antigen-presenting cells is not known, it is likely to have interesting new implications in our understanding of tumor immunity, and may constitute a major reason why lymphangiogenesis in the tumor margin or DLN is positively correlated with metastasis.

For example, tumor-driven lymphangiogenesis or lymphatic hyperplasia in the tumor margin or DLN substantially increases the local surface area of LECs, providing both more cells that can scavenge and present antigens as well as more surface area for interacting with T cells. Also, by presenting both scavenged and endogenous antigens, LECs could potentially help maintain tolerogenic pressure even in the midst of frequent antigenic mutations as occurs in tumors.

Implications for Cancer Immunotherapy

These emerging concepts in lymphatic immunobiology suggest LECs as interesting potential therapeutic targets to combine with tumor immunotherapy. If indeed tumor-associated LECs can dampen effector T-cell responses, deliver tumor antigens along with immunosuppressive cytokines to immature DCs in the lymph nodes, and even directly maintain selective tolerogenic pressure by scavenging and presenting tumor antigens to T cells, then their inhibition, destruction, or alteration could help promote more effective antitumor immune responses. Currently, our ability to alter lymphatic

vessels specifically is very limited; VEGFR-3-blocking antibodies have been shown to inhibit lymphatic proliferation without affecting preexisting lymphatic vessels, but it may affect angiogenic blood vessels and tumor-associated macrophages. It might also be possible to ablate lymphatic vessels locally using photodynamic therapy (40, 41). Such lymphatic modulation might be effective when combined with antitumor immunotherapy strategies, including vaccination with tumor lysate or peptides, or adoptive DC therapy, or adoptive transfer of engineered T cells (42). Research on such topics is only in their beginning stages, and clearly much more work is needed to clarify the immunologic roles of tumor-associated LECs and

how they alter the tumor immune microenvironment, but further research into how the immune functions of LECs are coupled to lymphatic transport functions will likely grow in the coming years and could have considerable potential for therapeutic impact.

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