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Exploiting lymphatic vessels for immunomodulation: Rationale, opportunities, and challenges[☆]

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Abstract

Lymphatic vessels are the primary route of communication from peripheral tissues to the immune system; as such, they represent an important component of local immunity. In addition to their transport functions, new immunomodulatory roles for lymphatic vessels and lymphatic endothelial cells have come to light in recent years, demonstrating that lymphatic vessels help shape immune responses in a variety of ways: promoting tolerance to self-antigens, archiving antigen for later presentation, dampening effector immune responses, and resolving inflammation, among others. In addition to these new biological insights, the growing field of immunoengineering has begun to explore therapeutic approaches to utilize or exploit the lymphatic system for immunotherapy.

Keywords

Immunotherapy; Nanotechnology; Cancer; Immune regulation; Lymphangiogenesis; Lymph node targeting

1. Lymphatic vessels and their transport functions in immunology

Lymphatics drain extracellular fluid and its solutes, including antigens (Ag), as well as cells and particulates like exosomes from peripheral tissue to draining lymph nodes (LNs) and eventually into systemic circulation (Fig. 1). While traditionally considered as passive, exciting new insights in recent years have revealed multiple ways that the various transport functions of lymphatics may help shape immune responses. In this section we will give an overview of lymphatic transport from an immunology perspective and highlight new findings, including (i) how lymphatic endothelial cells (LECs) can actively modulate fluid and solute transport as well as store Ag, (ii) how antigen-presenting cells (APCs) and lymphocytes traffic within the lymphatic vasculature and potentially affect fluid drainage, and (iii) how the lymphatic-APC interface can modulate Ag distribution.

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1.1. Lymphatic drainage of fluid and antigens

Drainage of fluid and Ags from interstitial spaces of tissues occurs mainly via the initial lymphatic capillaries. These blind-ended structures are non-muscular, have discontinuous basement membrane, and display overlapping cell-cell junctions anchored to the extracellular matrix by elastic filaments [1,2]. Consequently, lymphatic drainage has been considered passive (i.e. based on physical Starling forces), modulated by small, local pressure differences across the vessel wall. However, recent work from our lab has demonstrated that transcellular transport of fluid and solutes mediated through intracellular vesicles is an important component together with paracellular mechanisms, indicating that lymph formation can be actively regulated by LECs [3,4]. Backflow into the interstitium is prevented by a system of valves in initial lymphatics and contractile collecting lymphatics, the latter of which separate segments called lymphangions [1,2]. The collecting lymphatics transport lymph into the LNs through the subcapsular sinus, capsular sinusoids, then medullary sinusoids as well as reticular fibers; finally, the lymph leaves the LNs through the afferent lymphatics, and is eventually drained into the blood via the thoracic duct [5–7].

The free Ags carried by lymph are taken up by APCs in the LN. Ag uptake and presentation in the LN has distinct roles in activating immune responses compared to Ag picked up by peripheral DCs and brought to the LN. This is both because of differences in time to presentation – free Ag arrives within minutes, while DC-carried Ag can take several hours – and because of different functions of LN-resident vs. peripheral DCs [8–10]. Lymph-borne Ags (foreign or mutated self) that arrive together with inflammatory cytokines, danger signals, or other signals from the peripheral site, promote DC maturation in the LN and drive robust T cell education leading to effector T cell phenotypes [11]. Additionally, lymph-borne Ag can be taken up by subcapsular macrophages and B cells to mount humoral immunity [12,13].

Lymph is also rich in peripheral tissue Ags, which, in the absence of danger signals, can be taken up by immature dendritic cells (DCs) or LN stromal cells (LNSCs) and presented to autoreactive T cells for deletional tolerance [14,15]. Interestingly, work from the Santambrogio lab has demonstrated that lymph carries a large pool of tissue- and cell-derived peptides, including those derived from caspases and matrix metalloproteases, that are distinct from DC-presented self-Ag in terms of epitope repertoire [16–21]. In this way, lymphatic drainage in both steady-state and various inflammatory conditions is likely important for maintaining peripheral tolerance to self-Ags. Furthermore, regulatory T (T_{Reg}) cells reside in LNs, and their activation is thought to take place there, further leading to tolerance against peripheral tissue Ags [22].

That lymphatic drainage is important for both effector immunity and maintenance of peripheral tolerance through distinct mechanisms highlights the complex roles lymphatic vessels can have in regulating immunity. Using K14-VEGFR-3-Ig mice that lack dermal lymphatics [23], our lab showed that without lymphatic drainage of an intradermally (i.d.) delivered vaccine, B cell immunity was severely impaired, and T cell immunity was delayed (but could be initiated in the spleen). In contrast, tolerance to a foreign Ag could not be induced with a classical skin hypersensitivity assay, and older mice developed signs of autoimmunity to skin Ag [24]. Much earlier work using skin transplantation in rats

demonstrated that local induction of tolerance required lymphatic drainage from the transplant [25,26]. More recently, using melanomas implanted i.d. into K14-VEGFR-3-Ig mice, we demonstrated that lymphatic vessels are essential for immune recognition and response to these melanomas, even though the resulting tumor immune infiltrate was highly suppressive [27]. Without dermal lymphatic drainage, tumors grew as if in an immune privileged site. Therefore, Ag transport by lymphatics is critical to initiating all types of immune responses.

1.2. Lymphatic transport of leukocytes

Extensive research has demonstrated that while immune cells enter tissue via blood, they use lymphatic vessels to exit tissue, and a number of key factors that help facilitate or control this trafficking have been identified. The most well-studied cell types are DCs and T cells, which we will briefly review here. For more extensive reviews we refer the reader to several excellent review articles [28–31].

Upon scavenging peripheral Ags, DCs home to and enter lymphatic vessels using the chemokine receptor CCR7, which is upregulated upon maturation [22,32–34] and allows chemotaxis towards its ligand CCL21, secreted by LECs (and upregulated upon inflammation) [35, 36]. Blocking CCR7 signaling has been shown to severely compromise peripheral DC migration to LNs [22,30,32,37,38]. Two variants of CCL21 exist: a leucine-containing variant (CCL21-leu) that is constitutively expressed by LECs, and thus important for DC entry into peripheral lymphatics, and a serine-bearing variant (CCL21-ser) secreted by high endothelial venules (HEVs) and fibroblastic reticular cells along with the other known CCR7 ligand CCL19 [11,39–41], and thus primarily found in the LN. In mice lacking functional CCL19 and CCL21-ser, DCs migrated into peripheral lymphatic vessels but showed impaired trafficking into and within the LN stroma [39–41]. There are a vast number of receptors that aid in DC migration by facilitating transendothelial migration and promoting diapedesis that have been described in various review articles [11,30]. Interestingly, some of these molecules differentially modulate lymphatic trafficking of different DC subsets: for instance, Galectin-1 decreases mature DC migration while promoting tolerogenic (immature or regulatory) DC migration by modulating tyrosine kinase phosphorylation [42,43].

In contrast, T cells generally enter LNs from the blood circulation through HEVs, which are specialized to facilitate T cell trafficking. T cells leave the LN via either HEVs or lymphatic vessels (e.g. from an upstream LN). T cell egress from the LNs is mediated by Sphingosine-1-phosphate receptor 1 (S1P1) signaling [44,45], and S1P1 blockade prevents T cells from exiting the LN. CCR7 is also important in T cell entry and egress from the LN, since naïve T cells express CCR7 (and are thus attracted to the LN), but downregulate CCR7 expression as they differentiate towards effector types [29,32,46]. Importantly, T_{Reg} cells as well as central memory T cells, but not effector memory or effector T cells, also maintain high levels of CCR7 expression and reside in the LN. When comparing cell populations in afferent vs. efferent lymph, the efferent was found to be mostly composed of T cells, with more CD4⁺ vs. CD8⁺ T cells [47,48], while afferent lymph consisted mostly of αβ T cells, DCs, monocytes, B cells, and some granulocytes [49]. CD4⁺ T cells seem to be more

efficient than CD8⁺ T cells at exiting peripheral tissue via afferent lymphatics [49–52], and of the T cells found in the afferent lymph, most are either memory or T_{Reg} cells [50,53–56]. However, some research has suggested that naïve T cells may use afferent lymphatics when migrating from peripheral tissues [51,57–60]. In a mouse model of Crohn's disease, the lack of T cell-specific CCR7 prolonged inflammation [46,61], and in a model of skin inflammation, T cell overexpression of CCR7 resulted in faster resolution [61].

1.3. Lymphatic-DC interactions

While numerous studies have demonstrated that DCs migrate into lymphatics in a CCR7-dependent manner, more recent work has shown that there exists more complex crosstalk. For example, DCs can alter lymphatic drainage functions, and LECs can alter DC maturation and functions (discussed below in the last section). Furthermore, lymph leaks out of collecting lymphatic vessels into the surrounding adipose tissue [62], and lymph-borne solute can be transferred to DCs that surround the vessels [62,63]. The DCs surrounding these vessels adhere in a CCR7-dependent manner and seem to limit lymph leakage, since in CCR7-null mice, collecting lymphatic vessels lacked DC attachment and were hyperpermeable [64]. This was associated with lymphatic vessel fibrosis; permeability and vessel wall thickness were restored to normal only when CCR7 expression was restored in DCs. Similarly, ablating IFN regulatory factor 4 (IRF4) expression in DCs rendered collecting lymphatic vessels hyperpermeable and drove vessel wall thickening. Thus, CCR7⁺ IRF4⁺ DCs appear to have a key role in regulating collecting vessel permeability and fibrosis.

Both lymphatic vessels and draining LNs are usually surrounded by fat tissue, and it appears that lymphatics allow leakage of some lymph into these fat regions. In the fat regions sit CD11c⁺ cells (DCs and macrophages) that are able to rapidly uptake molecules drained via the lymphatic route, indicating that this uptake occurred through drainage and not migration of other Ag-presenting cells [62,63]. Kuan et al. also showed that fat-associated DCs and macrophages sample the lymph and induce local inflammation and T cell recruitment during recall responses. Furthermore, the fat-associated DCs are also recruited to the inflamed LN in a CCR7-dependent manner, similar to recruitment of other tissue resident DCs. Finally, work by our lab and others has illustrated that LECs themselves take up Ag [4,19,65,66]. In addition, Tamburini et al. showed that Ag persists over long periods of time in LECs with purposes of transferring Ag back to DCs and other APCs during memory responses [66]; further discussion can be found in the final section of this review.

1.4. Lymphatic drainage in the brain

The brain was long considered to be devoid of lymphatics and lymphatic drainage, even though decades-old research had hinted otherwise [67,68]. Evidence in the past decade, however, has indicated that in the brain, Ag drainage also occurs via lymphatic vessels. Studies from the 1980s demonstrated that only 10–15% of interstitial fluid passes into the cerebrospinal fluid, suggesting there is a perivascular route for interstitial fluid drainage from the brain [68]. It has since been shown that tracer injected into the parenchyma of the grey matter rapidly diffuses through the extracellular spaces, enters the basement membranes of cerebral capillaries, and then drains into cervical LNs via arterioles and

arteries out of the brain [67–70]. Further support comes from Weller et al. who demonstrated that while cerebrospinal fluid drains to cervical LNs via cribriform plate and nasal mucosa, interstitial fluid and solutes from the brain parenchyma drain via the lymphatics [71]. Interestingly, emerging evidence shows that cerebrospinal fluid drains via nasal and dural lymphatics in addition to the lymphatic vessels associated with cranial/spinal nerve roots [67, 72–74]. It appears that in some mammals up to 50% of cerebrospinal fluid drains via lymphatics [67]. One group suggests that tolerance in the brain may be mediated through cerebral spinal fluid drainage, since tolerance is induced to proteins injected into cerebral spinal fluid [75]. Finally, APCs and T cells have been shown to traffic out of the brain and to the cervical LNs through an unknown route, which is postulated may be the lymphatics [76–80].

2. Lymphangiogenesis in inflammation and immunopathology

Recent evidence has shown that lymphatic growth and expansion, or lymphangiogenesis, occurs in several inflammatory conditions including psoriasis, rheumatoid arthritis, inflammatory bowel disease (IBD), atherosclerosis, chronic airway inflammation, transplant rejection, and lymphedema [81–86]. Lymphangiogenesis is thought to modulate fluid drainage, inflammatory mediator clearance, and leukocyte migration, and thus may significantly impact inflammation and immune responses [81–86]. Lymphangiogenesis can be induced by a variety of factors including Vascular Endothelial Growth Factor (VEGF)-C, -D, and -A, lipopolysaccharide and several inflammatory cytokines such as IL-17, IL-8, CXCL12 [87]. Despite this extensive list, the most notable pro-lymphangiogenic factor is VEGF-C, which acts through its receptor VEGFR-3. VEGF-C and several of the other pro-lymphangiogenic factors have been shown to induce production of cytokines/chemokines by LECs including CCL21, CXCL12, as well as adhesion molecules like ICAM-1 and VCAM-1 that have a major role in leukocyte migration through lymphatic vessels to the LNs as discussed above [87]. The effects of lymphangiogenesis and pro-lymphangiogenic signaling have been studied in a variety of diseases and tissues. In this section we will focus on lymphangiogenesis, particularly that induced by VEGF-C signaling, and its role in inflammation and immunopathology in various pathologies, namely chronic respiratory inflammation, IBD, allergy, autoimmunity, transplantation, and cancer.

2.1. Chronic respiratory inflammation

Lymphangiogenesis has been observed in several chronic respiratory inflammatory conditions. McDonald and colleagues demonstrated that chronic infection with *mycoplasma pulmonis* caused significant VEGF-C-mediated lymphangiogenesis. When lymphangiogenesis was blocked with a VEGFR-3 blocking antibody, lymphatic drainage from the lung was substantially impaired [81,88,89]. In addition, they observed that VEGFR-3 blockade resulted in smaller LN size, which may indicate that there is less inflammation present. In other studies, virally transduced VEGF-C in the lung has been used as a model of obliterative bronchiolitis, a leading cause of morbidity in transplant rejection; in this model, blocking VEGF-C actually reduced infiltration of CD4⁺ T cells and disease development during transplantation [90–92], although the mechanisms underlying these observations are not entirely clear.

There are several other chronic inflammatory diseases in the lung associated with either lymphangiogenesis or lymphatic hyperplasia. In idiopathic pulmonary fibrosis (IPF) and nonspecific interstitial pneumonia, lymphatic hyperplasia (i.e., increased vessel size but not density) has been correlated with severity of fibrosis [93–95], although this seems to occur in the alveolar spaces and not in the fibroblastic foci [96]. Interestingly, this seemed to be driven not by VEGF-C, which was surprisingly lower in the bronchoalveolar lavage fluid from patients with IPF compared to healthy controls, but by increased levels of short-fragment hyaluronan, which could drive proliferation in LECs [96]. The mentioned changes in lymphatic vessels are hypothesized to impair alveolar clearance and thus worsen prognosis in IPF [94,97]. Lymphangiogenesis has also been observed in chronic obstructive pulmonary disease [97,98], where lymphangiogenesis is thought to be involved in the trafficking of distal lung immune cells. In smokers, soluble VEGFR-3, which inhibits lymphangiogenesis, was found to be decreased compared to non-smokers [99].

Finally, quite extreme lymphangiogenesis is seen in the rare lung disease lymphangioleiomyomatosis (LAM), driven by VEGF-D. In LAM, loss of the TSC gene induces cancer-like lesion formation in the lungs. It is thought that LAM cells grow into lymphatic vessels, thus dilating and obstructing the drainage from these vessels, since lymphatics are found throughout and surrounding LAM lesions [96]. LAM cells have also been shown to produce VEGF-D, and the extent of lymphangiogenesis is correlated with disease severity, making VEGF-D a suitable biomarker for diagnosis of LAM that also indicates disease stage.

2.2. Inflammatory bowel disease

Lymphatic vessels in the small intestine transport dietary lipids in the form of chylomicrons to the mesenteric LNs and then into the blood. In addition, lymphatic vessels are important throughout the gastrointestinal tract for draining Ag and cells from the periphery to the LNs. One of the chronic inflammatory conditions of the gut that has been extensively associated with lymphangiogenesis is inflammatory bowel disease (IBD). As early as the 1940s, lymphangiogenesis was observed specifically in Crohn's disease, a subset of IBD [100]. In this disease, fat wrapping (also termed creeping fat) accompanies B cell-rich lymphoid aggregates, areas thought to have impaired lymphatic clearance [101,102]. The alteration of lymphatic drainage in humans has been observed through recently published dye injection studies, where dye was injected into resected regions of the gastrointestinal tract of IBD patients in areas with or without ulcers or inflammation [103]. These studies found that collecting lymphatics associated with creeping fat and that drain to the mesenteric LNs are remodeled by B cells and innate lymphoid cells invading the lymphatic wall, and that the lymphoid aggregates (also called tertiary lymphoid organs) alter Ag and immune cell trafficking to the mesenteric LN and thus contribute to disease progression [104]. In mouse models, there is not a clear consensus on how lymphangiogenesis affects IBD, and to what extent it is protective vs. pathological. In a mouse model of colitis induced by dextran sulfate sodium (DSS), Wang et al. found that VEGF-C overexpression and associated increased lymphatic vessel density worsened the disease [104], which could be interpreted as lymphangiogenesis contributing to the pathogenesis of IBD. On the other hand, inflammatory lymphangiogenesis already occurs in DSS-induced colitis, and thus these

experiments can only demonstrate that super(patho)physiological levels of VEGF-C, in excess of what is produced in the inflamed environment, exacerbates inflammation and tissue damage resulting from DSS.

However, in direct contradiction to this study, D'Alessio et al. found that adenovirally delivered VEGF-C reduced the severity of inflammation in both DSS-induced colitis as well as in a second mouse model using IL-10-null mice, which spontaneously develop IBD [105]. Interestingly, inhibiting VEGF-C signaling with a VEGFR-3 blocking antibody worsened the disease in both models. They further found that the VEGF-C/VEGFR-3 pathway skews macrophages into a hybrid M1–M2 phenotype and that the protective function of VEGFC is mediated by so-called resolving macrophages in a STAT6 dependent manner [105]. This corroborated an earlier study by Jurisic et al., who found that VEGFR-3 blockade in IL-10-null mice further aggravates inflammation, with increased submucosal edema and leukocyte infiltration as well as tortuous lymphatic vessels [106,107]. These studies suggest that the inflammatory lymphangiogenesis resulting from colitis is protective rather than pathological.

Nonetheless, it is important to note that these mouse models lack many key features of human IBD, which is neither chemically induced nor occurs in the absence of IL-10. Lymphangiogenesis likely plays pleiotropic and complex roles in IBD, and thus a much deeper understanding of these roles is needed before we can begin to consider therapeutic manipulation of the VEGF-C/VEGFR-3 signaling pathway for modulating the immune response in IBD [106,107].

2.3. Allergy and hypersensitivity

Many allergic diseases including allergic eye disease, hypersensitivity pneumonitis, and allergic airway inflammation are associated with lymphangiogenesis. In allergic eye disease, corneal lymphangiogenesis is induced by VEGF-C and VEGF-D in the cornea. Lee et al. demonstrated that Ag-loaded DCs use these newly formed lymphatics to egress from this normally immune-privileged site [108], and that preventing corneal lymphangiogenesis with VEGFR-3 blockade led to reduced disease severity as well as the type 2 inflammatory response and serum IgE levels, two important markers of allergic inflammation.

Allergy and inflammation in the lung has also been associated with lymphangiogenesis, although not without controversy and conflicting reports. In hypersensitivity pneumonitis, increased levels of VEGF-D and VEGF-C have been demonstrated in patients, as well as increased LEC expression of CCL21 that drive CCR7⁺ immune cell aggregates around lymphatic vessels [109]. Shin et al. reported that lymphangiogenesis did not occur in an ovalbumin (OVA) model of allergic airway inflammation, but lymphangiogenesis could be induced, along with increased lymphatic clearance from the lungs, by blocking the Th2 cytokines IL-4 or IL-13 [110]. They further examined the direct effects of IL-4 and IL-13 on LECs in vitro, showing that these cytokines prevent tube formation and implying that Th2-type inflammatory cytokines are anti-lymphangiogenic. In contrast, reports by Baluk and colleagues have demonstrated increases in lymphatic sprouting and lymphatic vessel expansion in OVA-alum models of respiratory allergy [81,88,89]. Furthermore, Moldobaeva et al. demonstrated in a house dust mite-mediated rat model of allergic airway inflammation that lymphangiogenesis occurs, and that LECs isolated from inflamed lungs showed

increased proliferation, tube formation, and chemotaxis compared to LECs isolated from naïve rat lungs [111]. Finally, Shinoda et al. recently demonstrated that lymphangiogenesis occurs in a model of induced bronchus-associated lymphoid tissue (iBALT) [112], where LECs secrete the chemokines IL-33, CCL21, and CCL19 to recruit memory and naïve T cells to the iBALT regions. In addition, LECs in iBALT were shown to produce IL-7, which helps maintain T cell memory, suggesting further roles that lymphatics may play in airway allergies.

2.4. Autoimmunity

Lymphangiogenesis has been reported in several autoimmune diseases including chronic skin inflammation, psoriasis, rheumatoid arthritis, atopic dermatitis, and contact hypersensitivity in obesity-related dermatitis. In the case of rheumatoid arthritis, synovial specimen from patients show increased lymphatic density and VEGF-C expression [83,113]. In mice, blocking lymphangiogenesis using VEGFR-3 blockade led to decreases in lymphatic drainage as well as the size of the popliteal LNs and numbers of VEGF-C⁺ CD11b⁺ myeloid cells in the draining LNs [84]. Interestingly, blockade of VEGFR-2 also inhibited lymphangiogenesis and reduced joint inflammation in this model [84].

In atopic dermatitis (eczema), lymphangiogenesis and increased lymphatic drainage have also been reported in a transgenic model of IL-4 overexpression in the skin (K14-IL-4-Tg) [114]. In this model, increased numbers of CD11b⁺ dermal macrophages that produce VEGF-C were the likely source of pro-lymphangiogenic signaling, along with increased macrophage recruitment and activation. However, the disease implications of lymphangiogenesis are unclear, and the effects of blocking lymphangiogenesis in atopic dermatitis have not been addressed.

Interestingly, lymphatic density seems to be decreased in contact hypersensitivity in obesity-related dermatitis. Savestky et al. found that obese mice have decreased lymphatic density in the skin, and their lymphatic capillaries are increasingly leaky, thus reducing lymphatic clearance in obese mice. When lymphangiogenesis was induced using recombinant VEGF-C, lymphatic drainage was improved and contact hypersensitivity was decreased in obese mice, suggesting that lymphatic function may directly mediate contact hypersensitivity [113]. This was also consistent with a report by Thomas et al. showing that transgenic mice lacking dermal lymphatic drainage had exacerbated swelling in response to contact hypersensitivity and, unlike mice with normal lymphatic drainage, could not be tolerized to the sensitizing agent [24].

2.5. Transplantation

Significant work has been done on the role of lymphatic vessels in transplantation and transplant rejection. In corneal allografts, lymphangiogenesis is mediated by CD11b⁺ macrophages [115]. In addition, it has been shown that VEGFR-3 signaling induced corneal alloimmunity, and that blocking VEGFR-3 suppresses the trafficking of corneal APCs to the draining LN, in turn leading to a delay in transplant rejection [117]. VEGF-A also contributes to lymphangiogenesis in the cornea, and trapping VEGF-A was shown to inhibit lymphangiogenesis and angiogenesis, decrease mononuclear phagocyte recruitment into the

graft, and improve long-term graft survival [116,117]. In islet transplantation, blocking VEGFR-3 also improves graft acceptance, specifically by preventing lymphangiogenesis around islets and in the draining LNs, in turn inhibiting T cell accumulation in the islets. In human kidney transplants, lymphangiogenesis and increased microvascular density have been observed, and endothelial activation was found to be significantly correlated to glomerulopathy, interstitial fibrosis, and graft failure [118]. In experimental models of kidney transplant, Palin et al. found that blocking lymphangiogenesis can prevent chronic kidney allograft injury and thus reduce transplant rejection [119]. Finally, Kerjaschki et al. have demonstrated that donor-derived endothelial progenitor cells may contribute to lymphangiogenesis in renal transplantation [120].

In lung transplantation, the role of lymphangiogenesis in transplant rejection is less clear. Obliterative airway disease remains the leading cause of chronic rejection after lung transplantation, and a study by Krebs et al. showed that overexpression of VEGF-C in the lungs resulted in lymphangiogenesis and obliterative bronchiolitis with increased epithelial activation, neutrophil chemotaxis, and a shift towards a Th17 adaptive immune response [90]. In this mouse model, blocking VEGFR-3 signaling inhibited both lymphangiogenesis and angiogenesis, and reduced the CD4⁺ T cell infiltration into the lungs. In addition, blocking VEGFR-3 led to DC entrapment in the allografts, which could lead to decreased T cell priming and activation. In contrast, studies by Cui et al. in a murine model of acute lung allograft rejection suggest that a lack of lymphangiogenesis promotes allograft rejection [121]. In this model, lymphangiogenesis did not occur within seven days of transplantation, the time during which acute lung allograft rejection occurs. It was hypothesized that lack of lymphatic drainage contributes to rejection in part due to an increased accumulation of hyaluronan in the lungs, a molecule often associated with lung injury. When VEGF-C was delivered, there was a reduction in T cell and macrophage infiltration into the allografts, indicating that lymphangiogenesis may ameliorate established acute lung allograft rejection. Thus, lymphangiogenesis may play multiple and complex roles in transplant rejection, and the therapeutic targeting of lymphangiogenesis to prevent transplant rejection may need to be evaluated on an organ-specific basis.

2.6. Cancer

A large fraction of the existing literature on lymphangiogenesis and VEGF-C expression stems from studies in cancer. There is a plethora of evidence from clinical and experimental studies indicating that VEGF-C and/or tumor lymphangiogenesis correlates strongly with metastasis in most solid tumors [122–126], ever since Skobe et al. showed two decades ago that VEGF-C overexpression and lymphangiogenesis in breast cancer xenografts caused LN and lung metastasis [124]. More recently, Tacconi et al. have shown that VEGF-C disrupts the lymphatic barrier by increasing lymphatic permeability and promoting transendothelial migration by colon cancer cells [125]. Similarly, several groups have shown in mice that blocking VEGFR-3 signaling during tumor progression prevents metastasis [122,123].

Our laboratory has previously demonstrated that tumor VEGF-C strongly upregulates LEC expression of CCL21 [127], which in addition to chemoattracting DCs and other immune cells into the lymphatics, can also promote the chemotaxis of many breast cancer types,

since CCR7 expression on breast cancer cells is positively correlated to their metastatic potential. In this way, VEGF-C-activated LECs may promote metastasis even before extensive proliferation occurs. Cancer cells can also secrete CCL19 and CCL21, which form skewed gradients in the presence of interstitial flow, thus facilitating ‘autologous chemotaxis’ towards the nearest draining lymphatic vessel [126]. Finally, our group also showed that chronic expression of CCL21 in the tumor microenvironment, at physiologic levels similar to those seen in lymph nodes, can induce lymphoid-like stromal changes that promote both T cell infiltration and immune suppression and tolerance [128]. CCL21 can attract naïve and T_{Reg} cells to the tumor environment, which in turn can suppress the antitumor response due to the suppressive microenvironment. Additionally, lymphatics are known to produce immunosuppressive factors indoleamine-2,3-dioxygenase (IDO) and nitric oxide (NO) [129,130] and are capable of suppressing DC-mediated T cell activation [131], thus potentially aiding in cancer-mediated immunosuppression.

While tumor-associated lymphangiogenesis or activation is correlated with the hallmarks of immune suppression in the tumor microenvironment, we have also showed that lymphatic drainage is necessary for initiating an immune response [27], a notion that may seem paradoxical at first but unsurprising when considering that immune suppressive mechanisms only develop in response to an immune attack. In wildtype C57BL/6 mice, B16-F10 melanomas evolve with substantial immune infiltrates and a suppressive microenvironment; however, when implanted into mice lacking dermal lymphatics, they grew robustly without significant immune infiltrates or immune suppressive features. In human melanoma, a lymphangiogenic gene signature was strongly correlated with those of immune infiltrates [27]. These findings may have significant implications for immunotherapy.

3. Targeting lymphatic vessels for delivery of immunomodulatory agents to LNs

Because of their role in lymph filtration and their particular architecture, LNs are specialized sites for the initiation of adaptive immune reactions. By concentrating lymph-borne soluble Ags, activated APCs trafficking from peripheral tissues, and the pool of circulating naïve and memory lymphocytes, LNs promote activation and clonal expansion of Ag-specific T and B cells. This physiologic function can be exploited in the context of vaccination and tolerance induction, by designing specific LN-targeting strategies. Currently explored LN-targeting approaches include the direct injection of immunomodulatory agents into cutaneous LNs or lymphatic vessels, and the indirect targeting of LNs by the use of drug delivery systems with optimal lymphatic uptake and retention properties.

Because lymphatic capillaries drain local tissue, interstitial fluid flow is always directed towards them. These convective forces become increasingly important compared to diffusion for interstitial transport of molecules and particulates as size increases. On the other hand, the extracellular matrix is dense and complex, which can hinder convective transport of very large particles towards lymphatics. Based on these size exclusion limits, macromolecular constructs and particulate agents can be designed to be taken up into lymphatics to target the draining LN from peripheral tissue. We have previously shown that an optimal size range for

targeting lymphatics after i.d. injection, at least in the mouse, is between 5 and 50 nm when delivered as a low-pressure infusion that did not locally increase interstitial fluid pressure (i.e., with a catheter injection pressure set at the minimum required for flow) [132]. It is important to note that most i.d. injections into mice are done as a bolus injection at a very large volume relative to the tissue volume, which induces extremely high pressure initially and can damage the tissue. Injection pressure and relative volume can drastically affect lymphatic delivery upon i.d. administration, and LECs themselves can actively take up even large (~1 μm) particles by macropinocytosis.

“Lymphotropic” particulate contrast agents have been used for decades to image lymphatic vessels and draining LNs with the aim to assess lymphatic dysfunction or to identify the tumor-draining LN (tdLN) [133–135]. More recently, a series of studies have thoroughly defined the size range and chemical properties that result in preferential lymphatic absorption over blood absorption, as discussed below. This knowledge has led to the design of refined lymphatic-targeting drug delivery systems, which are finding increasing application in the field of cancer immunotherapy, tolerance induction, LN-targeting for treatment of metastasis and lymphatic imaging.

3.1. Design of delivery systems for lymphatic targeting

Our laboratory previously investigated the impact of particle size on lymphatic uptake and retention by using poly(ethylene glycol) (PEG)-stabilized poly(propylene sulfide) (PPS) nanoparticles (NPs) [132, 136]. Following injection into the mouse tail, fluorescent 20 nm PPS-NPs rapidly allowed the visualization of the lymphatic network, while 45 nm and 100 nm PPS-NPs resulted in a faint or absent lymphatic staining, respectively, indicating progressively reduced lymphatic uptake with increased NP size [132]. 20 and 45 nm PPS-NPs (but not 100 nm NPs) were also recovered in high amount in the dLNs, where they co-localized with MHCII⁺ APCs [132]. PPS-NPs were shown to drain more rapidly and efficiently to local LNs and reach higher blood concentrations when injected i.d. rather than intramuscularly (i.m.) [137], which is consistent with the higher lymph flow from the skin [138].

Manolova et al. reported that polystyrene NPs up to 200 nm could freely travel to dLNs via lymphatics and be internalized by LN resident myeloid cells [139]. Of note, NPs from 500 to 2000 nm were still transported to the LNs, although to a lesser extent and with delayed kinetics. This delayed transport occurred via in situ NP uptake by skin-resident DCs and subsequent DC migration to the LN [139]. Differences in the lymphatic-targeting properties of NPs may be directly reflected in the efficacy as Ag delivery systems for vaccination. Varypataki et al. compared the in vivo activity of two NP-based vaccine formulations with different size and composition: poly-(lacticoglycolic-acid) (PLGA) NPs and cationic liposomes [140]. They found vaccine formulations based on cationic liposomes were more potent than PLGA-NP formulations, at least in part to due to the enhanced lymphatic transport of the smaller size liposomes (150–200 nm) compared to PLGA NPs (250–350 nm).

For NPs and macromolecular constructs bigger than ~10 nm (or ~16 kDa) lymphatic vessels are the main route of absorption from interstitial tissues [141,142]. Indeed, K14-VEGFR-3-

Ig mice that lack dermal lymphatics fail to efficiently absorb NPs upon i.d. injection [137]. In contrast, smaller particles and macromolecules are more likely to diffuse out of lymphatic capillaries and be directly absorbed into the blood, since the blood flow rate is 100–500 times greater than lymph flow [141]. Indeed, gadolinium-labeled dendrimers smaller than ~9 nm were shown to be inefficiently retained in lymphatics and resulted only in a faint and short-lived LN visualization by magnetic resonance [143].

Aside from size requirements, the surface charge of particulates is known to affect lymphatic uptake. Neutral or negatively charged NPs and liposomes are absorbed faster into the lymphatic circulation, while positively charged materials tend to interact with the negatively charged extracellular matrix and are retained in the interstitial tissue [144,145]. Nevertheless, a cationic particle surface may promote cell internalization and result in better DC targeting and LN retention once particles reach the LNs [146].

Lastly, LN-retention of NPs can be promoted by exploiting specific receptor-ligand interactions. Lee et al. screened protein NPs of different compositions and found that NPs made of human ferritin heavy chain are persistently retained in LNs as consequence of interaction with the ferritin receptor expressed on T and B lymphocytes [147]. In addition, a number of works investigated the use of mannose surface coating to promote NP binding to LN-resident APCs expressing the mannose receptor, including DCs and macrophages [148–150]. Mannose-functionalized NPs of different size and composition were repeatedly shown to result in a better LN and DC-targeting than their non-functionalized counterparts [148–151]. Prolonged NP retention in LNs and efficient uptake by APCs both favor the presentation of NP-coupled Ag to LN-resident T and B cells, and are thus particularly useful in vaccination strategies (discussed below).

3.2. Lymphatic-targeting vaccines for cancer immunotherapy

Cancer immunotherapy encompasses all the approaches aimed to promote the detection and killing of neoplastic cells by the immune system. The potential of immunotherapy for cancer treatment has been proven in the last decade by the striking clinical success of antibodies that target inhibitory receptors on T cells, such as anti-CTLA-4 and anti-PD-1 (also referred to as checkpoint blockade therapy) [152]. Although these therapies have achieved remarkably high percentages of long-term clinical responses, the efficacy appears to be limited to specific subsets of patients within certain cancer types [152]. One of the major determinants of checkpoint blockade efficacy is the pre-existence of a tumor-specific CD8⁺ T cell response than can be reactivated by blocking antibodies, which is patient- and cancer type-specific [153, 154]. Tumor-specific T cells can be expanded by anti-cancer vaccination, which is expected to work in synergy with checkpoint blockade therapies and conventional cancer therapy. Anti-cancer vaccination has been intensively investigated since the early 90s and can aid in expanding the pool of tumor-reactive T cells [155]. Nevertheless, the clinical efficacy of anti-cancer vaccines has been very limited so far, likely due to suboptimal choice of targeted tumor Ag, Ag delivery systems, treated patient populations, and combination with other therapies [156]. Due to the multiple challenges faced in the development of effective anti-cancer vaccines and the potential of immune-based cancer treatments, a vast number of studies in the last decade have focused on the development of new technologies

and approaches for anti-cancer vaccination. For this reason, although lymphatic-targeting vaccines for the treatment of infectious disease have also been reported [157, 158], this review specifically focuses on vaccines for cancer immunotherapy.

NP-based vaccines have been extensively investigated not only for their LN-targeting properties (as discussed above), but also due to their ability to promote Ag uptake by APCs. They allow optimal Ag and adjuvant co-delivery, and induce DC maturation (even in absence of classical immunological adjuvants). DCs exposed in vitro to Ag-loaded NPs display increased Ag uptake, cytokine secretion and upregulation of MHC molecules and costimulatory receptors, compared to free Ag and adjuvants mixtures [159–161]. Consistently, co-delivery of Ag and adjuvants by NPs was repeatedly shown to result in superior vaccine efficacy in vivo compared to control vaccine formulations [150,161–166]. Efficient Ag and adjuvant co-delivery can be achieved even when these two components are loaded into distinct particles, as long as the particles have identical size, biodistribution properties, and allow the simultaneous uptake of Ag and adjuvant by LN-resident DCs [162].

Many NP-based lymphatic-targeting vaccines have been initially evaluated in melanoma mouse models, due to the better understanding of melanoma immunology compared to other cancer types and its relatively high immunogenicity [167]. Xu et al. and Zhuang et al. developed two different NP formulations, mannose-decorated lipid/calcium/phosphate NPs [150] and lipid-coated zinc phosphate hybrid NPs [166], for co-encapsulation of peptides from melanoma Ags (Trp2 and/or gp100) with Th1-inducing adjuvants (CpG or monophosphoryl lipid, MPLA). Both proposed systems had suitable size for optimal lymphatic targeting (30–40 nm) and showed accumulation in local LNs following subcutaneous (s.c.) or i.d. injection. NP-based vaccines were shown to delay the growth of B16-F10 mouse melanomas with enhanced efficacy compared to control free Ag and adjuvant mixtures [150,166]. Guo et al. used ~150 nm erythrocyte membrane-enveloped PLGA NPs to simultaneously deliver the human gp100_{25–33} melanoma peptide and MPLA [148]. The NPs were found to both persist at the skin injection site and be drained to local LNs, indicating a mixed pattern of NP uptake by skin and LN-resident APCs, which is consistent with the >100 nm particle size [148]. This vaccination system induced strong Ag-specific T cell immunity and control of B16-F10 tumor growth in both prophylactic and therapeutic schedules. Muraoka et al. showed that 40–60 nm nanogels loaded with long peptide Ag preferentially target medullary macrophages in the LNs and are able to delay the growth of established mouse fibrosarcomas and colon carcinomas [168].

Besides using molecularly defined tumor Ags, anti-cancer vaccination can be achieved using whole tumor Ags in the form of lethally irradiated tumor cells, tumor-derived exosomes or mRNA, or tumor protein lysates (TL) [169]. These approaches potentially allow induction of an immune response against multiple Ags at the same time, including patient-specific neoantigens [169]. Mannose-coated chitosan NPs loaded with TL were recently reported to induce DC maturation and T cell activation in the B16 melanoma model, and confer a moderate advantage in controlling tumor growth compared to vaccination with TL only [151]. Although poorly explored so far, delivery of whole TL through NPs currently represents a promising strategy for anti-cancer vaccination.

An alternative way to maximize cancer vaccine delivery to LNs, which does not require the use of NPs, is the direct intralymphatic administration by injection into a LN under ultrasound guidance [170–174] or by lymphatic cannulation [175,176]. Studies in mouse models demonstrated that the intranodal (i.n.) injection of peptide vaccines [170] and DNA vaccines [172], compared to i.d. or s.c. administration, enhances vaccine immunogenicity and protection against viral or tumor challenges.

A series of clinical trials on different late stage cancers (including co-lorectal cancer, prostate cancer, renal cell carcinoma and melanoma) have investigated the direct intralymphatic administration of DC vaccines [171,173–176]. DC vaccines require the ex vivo generation of mature DCs from autologous peripheral blood mononuclear cells, which are subsequently loaded with tumor Ags and reinjected into the patient [155]. These vaccines are most commonly administered intravenously (i.v.) or s.c./i.d. Compared to these injection routes, i.n. injection increases the percentage of injected cells that reach the LN, although this does not necessarily translate into a stronger induction of antitumor immunity [171,174]. In addition, although potentially useful, intralymphatic vaccine administration is more technically challenging than other injection methods and requires highly trained medical personnel, practically limiting its feasibility.

3.3. Targeting the tumor-draining lymph nodes

So far, most studies on lymphatic-targeting cancer vaccines did not evaluate how the choice of the specific LNs to be targeted affects vaccine efficacy. In mouse models, i.d. or s.c.-injected vaccines have been administered either on the same side of the tumor (potentially targeting the tdLNs), or on the contralateral side (reaching non-tdLNs), while in clinics some vaccines (such as DC-based vaccines) are administered systemically [177]. However, recent works suggest that LNs close to the tumor host a very different microenvironment compared to distant nodes, which should be considered when designing vaccination approaches. Tumor-derived factors transported to the tdLNs through the lymph are known to induce a deep remodeling of LN structure and immune functions, already before the appearance of lymphatic metastases. TdLNs are markedly enlarged respect to their non-draining counterparts, with higher LN cellularity and an increased proportion of LNSC components [178,179]. The expression of homeostatic cytokines that regulate LN-homing and survival of T cells (including CCL21 and IL-7) is downregulated in tdLNs, resulting in reduced LN-resident T cells (especially the naïve subsets) and aberrant immune cell organization [178]. In addition, tdLNs have increased and more active T_{reg} populations and higher concentrations of immunosuppressive cytokines (such as IL-10) that promote a tolerogenic microenvironment [180, 181]. On the other hand, the direct connection of tdLNs with the tumor mass inevitably results in an enhanced exposure of tdLN-resident immune cells to tumor-associated Ags (TAAs). Moreover, the structural alterations of tdLNs and the expansion of peritumoral lymphatic vessels under the influence of tumor-derived VEGF-C can both contribute to enhanced transport of solutes (including TAAs) to the tdLNs [178,182]. Thus, although more immunosuppressed, the tdLNs also represent a precious reservoir of TAAs, which could be exploited to boost the antitumor immunity through immunotherapy. Based on these premises, we hypothesized that delivering immunomodulatory agents to the tdLN by lymphatic-targeting NPs could revert tumor-

induced immunosuppression, switch the LN microenvironment from tolerogenic to immunogenic, and produce efficient TAAs presentation and T cell activation (Fig. 2). We loaded small (~30 nm) pyridyl disulfide-NPs with the TLR9 agonist CpG or low dose paclitaxel and injected them i.d. into either the ipsilateral or contralateral side with respect to B16-F10 tumors (i.e., either targeting the tdLN or non-draining LN, respectively) [165]. We found that when injected ipsilaterally, the NPs exerted more robust immunomodulatory effects than when injected contralaterally, delaying tumor growth and increasing the cytotoxic CD8⁺ T cell response [165]. This effect correlated with higher frequencies of TAA-specific, IFN γ -secreting CD8⁺ T cells in the tdLN compared to non-tdLNs prior to vaccination [164].

The interest in targeting the tdLNs is further supported by studies showing that the intratumoral administration of TLR agonists is sufficient to boost antitumor immunity and can mediate tumor regression when combined with other therapies [183–185]. Intratumorally injected vaccines, especially if formulated as NPs, are supposed to efficiently reach the tdLNs, following the same lymphatic pathway of tumor-secreted factors and disseminating tumor cells. Although in these studies it is difficult to discriminate to what extent T cell activation is initiated within the tdLNs rather than directly within the tumor site, we can hypothesize that (due to their professional role in orchestrating lymphocyte priming and expansion) the tdLNs are critical players in this process.

Targeting the tdLNs potentially allows the activation of cells with multiple Ag specificities by taking advantage of the high TAA concentration in these sites (Fig. 2). TdLNs are in certain circumstances surgically removed together with the tumor mass in order to treat or prevent lymphatic metastasis, depending on the histologic type, stage and metastatic status of the tumor [186–188]. In cases in which surgical lymphadenectomy is not associated with significant survival benefits, we propose that the specific targeting of the tdLNs with lymphatic-targeting vaccines should be investigated as candidate approach for cancer immunotherapy.

3.4. Tolerance induction

Besides vaccination, lymphatic-targeting approaches have been explored to induce Ag-specific tolerance for the treatment of allergies, autoimmunity and prevention of anti-drug immune reactions. Optimal delivery of immunosuppressive drugs and Ags to LNs can be achieved by using NP-based formulations administered i.d. or s.c., or by i.n. injection of free Ags.

Dane et al. developed amphiphilic PEG-bI-PPS micelles able to encapsulate hydrophobic anti-inflammatory and immunosuppressive drugs [189]. The small micelle size (50 nm) led to lymphatic drainage and uptake by LN-resident immune cells following injection into the mouse tail [189]. Once loaded with the glucocorticoid anti-inflammatory agent mometasone, PEG-bI-PPS micelles prevented CpG-induced DC activation into the dLNs [189]. This system was also used to deliver other immunosuppressant drugs, such as rapamycin and tacrolimus, and prolong skin allograft survival [189]. Maldonado and co-workers also encapsulated the immunosuppressive drug rapamycin into PLGA NPs [190]. NP-encapsulation promoted drug uptake by DCs and accumulation into dLNs upon s.c.

injection, and in the spleen and liver following i.v injection [190]. This approach was able to inhibit the generation of both T cell and B cell immunity against specific Ags co-administered with rapamycin, even in presence of strong immune adjuvants [190]. Treatment with rapamycin-NPs protected mice from hypersensitivity reactions, experimental autoimmune encephalomyelitis and generation of anti FVIII antibodies in a mouse hemophilia model [190]. In a different study, liposomes delivering NF- κ B inhibitors and model Ags were reported to accumulate preferentially in the spleen and mesenteric LNs following i.v. injection and in the dLNs following s.c. injection, and be taken up by MHCII⁺ APCs in target organs [191]. NF- κ B inhibition resulted in impaired APC maturation, tolerogenic Ag presentation and attenuated inflammation in an experimental model of Ag-induced arthritis [191].

We previously showed that ultra-small PPS-NPs target skin-draining LNs upon i.d. injections (discussed above). The same particles can be used to target lung-draining LNs upon intranasal administration, since they are specifically taken up by pulmonary DCs and transported to local LNs [192,193]. CpG-loaded PPS-NPs, administered intranasally, were employed to modulate house dust mite-induced allergic airway inflammation by inducing a Th2 to Th1 switch of the immune response. Induction of a Th1 immune response to the house dust mite allergens resulted in a reduction of Th2 cytokine secretion, eosinophil infiltration and mucous production in the lung mucosa, indicating an attenuation of the allergic response [192].

Another interesting possibility for targeting immunosuppressant drugs to the lymphatic system is exploiting the lymphatic absorption of dietary lipids following oral administration. Han et al. developed lipophilic prodrugs of the immunosuppressive drug mycophenolic acid (MPA, an inhibitor of lymphocyte proliferation) including highly lipophilic alkyl derivatives of MPA and a triglyceridemimetic MPA (2-MPA-TG) [194]. 2-MPA-TG was found to be absorbed much more efficiently in the intestinal lymph following intraduodenal administration in rats than native MPA [194]. Consistently, higher concentrations of 2-TG-MPA were found in lymphocytes collected from the intestinal lymph and in mesenteric LNs [194]. The proposed mechanism of 2-TG-MPA absorption involved hydrolysis into the intestinal lumen, absorption by enterocytes and incorporation into lymph-transported lipoproteins, following an analogous pathway to dietary triglycerides [194].

Lastly, enhanced tolerance induction can be achieved by direct intralymphatic administration of Ag, which does not require the use of NPs for delivery. Different clinical studies compared the efficacy of standard s.c. immunotherapy for the induction of allergen tolerance to intralymphatic allergen administration by injection into inguinal LNs [195,196]. Although both administration routes induced long lasting tolerance and amelioration of allergy clinical symptoms, intralymphatic administration consistently reduced the treatment period (8 weeks vs. 3 years), caused less adverse events, and improved patient compliance [195,196].

3.5. Lymph node-targeting beyond immunomodulation

Lymphatic-targeting particulate systems have been widely exploited to deliver cytotoxic drugs to LNs for the treatment of lymphatic cancer metastasis. In addition, imaging agents with enhanced lymphatic uptake and retention are employed in sentinel LN mapping for

cancer staging and surgical treatment. These latter applications are not the main focus of this review and have been extensively described elsewhere [197,198]. However, it is worth noting that the same rules of particle design described for particulate vaccines largely apply also to the design of lymphatic-targeting systems carrying cytotoxic drugs or imaging agents. Thus, many NP-based systems originally developed to deliver chemotherapeutic drugs or imaging agents may have the potential to be converted to immunomodulatory systems, and vice versa, or be employed to simultaneously deliver multipurpose agents.

Ruiz-de-Angulo and co-workers developed oxide-filled micelles that allow both gallium loading for tissue imaging by single photon emission computed tomography, and coating with CpG and OVA for anti-cancer vaccination [161]. Consistently with the small micelle size (40 nm), tracking revealed rapid distribution to dLNs (3 h) following s.c. injection, and LN retention for at least 24 h [161]. When used as vaccine system, these particles enhanced expansion of OVA-specific CD8⁺ T cells and Ag-specific IFN γ secretion over vaccination with free OVA + CpG, and conferred superior protection against challenge with B16-F10-OVA melanoma cells [161]. Our lab previously developed NPs with optimal lymphatic-targeting properties that can be loaded both with CpG (by chemical crosslinking with the NP surface) and with paclitaxel (by encapsulation into the hydrophobic core) [164,165]. Low doses of paclitaxel-loaded NPs were found to exert immunomodulatory effects and shape the immune infiltrate into the tumor site and tDLNs [165]. Although the effects of higher doses of paclitaxel-loaded NPs on LN metastases were not investigated, NPs represent a promising system for the simultaneous delivery of immunomodulatory agents and chemotherapeutic drugs in order to achieve both direct tumor cell killing and boosting of antitumor immunity.

Other interesting lymphatic-targeting approaches have been developed exclusively for imaging purposes or for the delivery of cytotoxic drugs, which could be modulated for administration of immunomodulatory drugs. Huang et al. used drug-loaded NPs chemically cross-linked to the cell membrane of autologous T cells to increase drug delivery to secondary lymphoid organs [199]. In this approach, T cells were expanded in vitro under conditions that maintain the expression of CD62L and CCR7 molecules, necessary for T cell homing to lymphoid organs, and conjugated with NPs carrying the topoisomerase I inhibitor SN-38 [199]. When reinfused in vivo in a mouse Burkitt lymphoma model, NP-carrying T cells trafficked to lymphoma-bearing LNs, resulting in higher drug concentrations in those sites compared to just drug-loaded NPs and free drug [199]. Bhang and coworkers developed colloiddally stable hyaluronic acid-quantum dot conjugates that have tunable sizes between 50 and 120 nm [200]. Here, the surface-exposed hyaluronic acid specifically bound to the Lyve-1 receptor expressed on LECs and allowed long term retention of fluorescent quantum dots into lymphatic vessels and high resolution optical imaging of mouse dermal lymphatics [200].

To date, an extremely high number of lymphatic-targeting formulations have been developed and showed to be effective for various applications in preclinical animal models. However, the clinical translation of these systems, as well as of most nanotechnology-based systems, is still limited, mainly because of the high costs and long times associated with clinical evaluation and approval of new drugs for human use [201]. We expect an increasing number

of lymphatic-targeting systems to reach the stage of clinical testing and prove their actual efficacy for the treatment of human diseases.

4. Emerging research in LEC immunobiology

Aside from transport and trafficking functions, the lymphatic endothelium can have direct interactions with immune cells. These interactions are made possible through anatomical location of LECs, chemoattraction and expression of adhesion molecules, as previously mentioned. A summary of the immunomodulatory functions of LECs are presented in Fig. 3, and details will be discussed in this section.

4.1. LEC modulation of DC function

In addition to the attraction of DCs through the CCL21-CCR7 signaling pathway, LECs have also been shown to interact directly with DCs. Indeed, inflamed LECs were shown to impair DC maturation in vitro through ICAM-1/Mac-1 contact-dependent interactions [131]. More recently, LECs were shown to suppress DC maturation through anti-inflammatory prostacyclin synthesis and TGF- β secretion [202]. These mechanisms underline the ability of LECs to act as a negative regulator of the immune response via DC modulation. Furthermore, two studies suggest that LECs can transfer Ag to professional APCs for subsequent education. First, exogenous Ag archiving by LECs required the presence of hematopoietic cells for priming of Ag-specific CD8⁺ T cells [66]. Second, Ags endogenously expressed specifically by LECs at steady-state could not directly prime CD4⁺ T cells, but required hematopoietic cells for education, suggesting Ag transfer [203]. Mechanisms of Ag transfer are still unclear, as their effects on the immune response involve multiple cell types.

4.2. LECs help maintain T cell homeostasis

As mentioned earlier, LECs have an important architectural role in LNs, where T cells reside at steady-state. The maintenance of this T cell pool, which includes naïve and memory T cells, critically relies on IL-7 signaling [204]. Using IL-7 GFP knock-in mice, LECs were shown to be a major source of IL-7 [205,206]. Quite interestingly, this expression seems to be modulated depending on the environment. For instance, LEC production of IL-7 was upregulated following viral infection [207]. Upon inflammation induced by lipopolysaccharide, LECs also express IL-15, suggesting an additional role in the maintenance of memory T cells, natural killer, and natural killer T cells [208]. Very recently, a study also reported the contribution of the chemoattractant sphingosine 1-phosphate, secreted by LECs, to promoting naïve T cell survival [209].

Furthermore, LEC-secreted IL-7 seems to play functional roles during inflammatory responses. In a model of allograft tolerance, T_{Reg} cells had enhanced sensitivity to IL-2 in response to LNSC-derived IL-7, thereby increasing T_{Reg} cell immuno-inhibitory effects [210]. Finally, in a model of chronic allergic inflammation, Thy1⁺IL-7-expressing LECs in the lung provided a survival niche for memory Th2 cells [112]. Taken together, these observations suggest LECs are important players in the maintenance of T cell survival and homeostasis.

4.3. Modulation of T cell activation

Recently, LECs were shown to serve as a negative feedback loop of the immune system to counteract T cell activation. Specifically, inflamed LNSCs inhibit proliferation of T cells through secretion of NO [130], thereby keeping in check the pool of activated T cells in the LN. Quite similarly, inflamed LECs can inhibit division of T cells through expression of IDO [129]. More recently, another report suggested the ability of LNSCs to suppress activated CD4⁺ T cells in the tumor microenvironment in a contact-independent manner [211]. In this study, the specific contribution of lymphatics was not investigated. Interestingly though, the reduction of tumor stroma development through lymphotoxin- β receptor inhibition resulted in delayed tumor growth and enhanced CD4⁺ T cell activation and infiltration. These observations suggest inhibitory properties of LECs under inflammatory conditions.

4.4. Antigen presentation to CD8⁺ T cells

Ag presentation by LNSCs has first emerged by the study of peripheral immune tolerance. About ten years ago, it was first shown that a mechanism of peripheral tolerance induction of CD8⁺ T cells involved radio-resistant LN-resident cells [212]. Similarly, in the mesenteric LN, radio-resistant LNSCs were shown to promote tolerance in the intestine [213]. These studies led investigations on whether LECs can act as APCs. To this effect, Magnusson et al. reported that LNSCs can tolerize autoreactive CD8⁺ T cells in the intestine [214]. Furthermore, evidence has been accumulating regarding the ability of LECs to present peripheral tissue Ags (PTAs) to promote tolerance. Cohen et al. reported the expression of melanocyte-specific protein tyrosinase in an Aire-independent mechanism, leading to deletion of tyrosinase-specific CD8⁺ T cells [215]. In addition, the pattern of PTA expression seems to depend on both the LNSC subset and the inflammatory stimulus [215, 216]. Subsequent studies looked at the mechanisms of LEC-mediated tolerance induction. Our lab and others have shown that dysfunctional activation of CD8⁺ T cells by LECs results from low expression of costimulatory molecules CD80 and CD86 combined with high expression of PD-L1 [217]. PD-L1 expression is confined to LN LECs and controlled by lymphotoxin- β receptor [218], and is upregulated upon IFN γ stimulation [219].

In a murine model of lymphatic-enriched melanoma, we have found that VEGF-C-stimulated LECs in the tdLNs tolerize antitumor CD8⁺ T cells through cross-presentation of tumor Ags [182]. Very recently, PD-L1 expression by LECs was shown to have a crucial role in the inhibition of CD8⁺ T cell response in the context of tumor [220]. These studies underline the ability of LECs to extend their Ag presentation abilities beyond PTAs, therefore broadening their role as an active player in immune responses.

Interestingly, it has been shown that LECs can retain viral Ag for extended periods of time, a phenomenon termed Ag archiving [66]. Tamburini et al. propose a model with Ag transfer to hematopoietic cells: during viral infection clearance, LECs undergoing apoptosis during LN retraction are engulfed by DCs for further Ag presentation and induction of memory T cells. More recently, our group has found that the non-apoptotic fraction of LEC-primed CD8⁺ T cells exhibited phenotype and functions similar to those of memory T cells (Vokali et al.,

unpublished). These two studies suggest an interesting role of the lymphatic endothelium in the generation and maintenance of memory T cells reactive to exogenous Ag.

4.5. Antigen presentation to CD4⁺ T cells

Low expression of MHCII at steady-state on endothelial cells has been described many years ago [221]. MHCII expression is driven by the class II transactivator domain (CIITA), whose expression is controlled by different promoters [222–224]. While the pI promoter is constitutively active in DCs, pIV is IFN γ -inducible and turned on during type I inflammation. Moreover, LNSCs were shown to have another mechanism to display MHCII molecules: scavenging peptide-MHCII complexes from DCs. LNSCs loaded with scavenged Ag induced apoptosis in Ag-specific CD4⁺ T cells after T cell co-culture [19]. Therefore, at steady-state, most MHCII displayed on LECs is derived from DCs. However, during inflammation, MHCII is driven by IFN γ stimulation [19].

Studies have reported the role of Ag presentation in LNSCs during inflammatory reactions, including negative regulation of the CD4⁺ T cell response in a model of viral infection [225], and maintenance of T_{Reg} cells in a model of LN transplantation [226]. At steady-state, LECs do not seem to present Ag on MHCII since they do not translate their own MHCII and do not express H2M, a molecule involved in the loading of Ag on MHCII [203]. Instead, endogenously expressed MHCII seems to be transferred to DCs to promote tolerance. The specific contribution of LEC Ag presentation during inflammation remains to be understood except for the described studies [225,226] and the fact that both MHCII and H2M are controlled by CIITA and are upregulated during inflammation [219,224,226] suggest that LECs may actively modulate the immune response through Ag presentation to CD4⁺ T cells.

4.6. Targeting the lymphatic endothelium to manipulate immune responses

The described studies underline the role of active immune regulator that the lymphatic endothelium plays and therefore open opportunities for therapeutic targeting. Ciré et al. used an elegant lentiviral approach to target MHCII-expressing cells such that the transgene was not expressed in hematopoietic cells, therefore targeting primarily LNSCs [227]. The delivery of MHCI and MHCII-restricted Ags resulted in dysfunctional activation of CD8⁺ and CD4⁺ T cell responses, respectively. The specific contribution of LEC Ag presentation, however, was not investigated.

Other approaches to target the lymphatic endothelium are possible, most likely by taking advantage of lymphatic-specific receptors such as VEGFR-3 or Lyve-1. In such cases, the specificity of targeting is an issue since some macrophages express Lyve-1 and VEGFR-3 [35]. Guo and colleagues targeted LECs via Lyve-1 in vitro, by using Lyve-1 binding PEG ultrasmall superparamagnetic iron oxide NPs [228]. Similarly, Tiantian and colleagues used low molecular weight hyaluronic acid-modified liposomes, which proved to be a promising drug carrier to specifically target lymphatics in the mouse [229]. Alternatively, in the context of tumor, the cyclic peptide LyP-1 specifically targeted tumor lymphatics and tumor cells [230]. This approach was used to specifically deliver doxorubicin to tumor cells and tumor LECs by using LyP-1-conjugated liposomes [231]. Still, these promising approaches need to be validated for efficient delivery of Ag in vivo.

5. Conclusions

LECs have a diverse portfolio of immune functions that on the one hand initiate and facilitate adaptive immune responses, while on the other hand resolve inflammation, suppress immunity, or even promote tolerance. Manipulating lymphatics (e.g., by ablation, or by inducing or blocking lymphangiogenesis) can affect all of these functions at the same time, yielding uncertainty on the therapeutic outcomes or even lacking a clear therapeutic rationale in the first place. For example, in murine cancer models where lymphangiogenesis promotes metastasis, blocking VEGFR-3 signaling in a prevention setting could reduce metastatic dissemination [123,232,233], but had no benefit when delivered therapeutically [234]. Furthermore, while our lab has demonstrated that tumor VEGF-C promotes an immune suppressive microenvironment [27,183], we have also found that blocking VEGFR-3 signaling can, paradoxically, decrease efficacy of immunotherapy (Fankhauser et al., under revision).

The research field of lymphatic immunobiology is nascent but growing, and we believe it has enormous potential for future therapeutic strategies in a wide range of disease targets. Despite huge advances in the last decade, however, we still have much to do, both on increasing our basic mechanistic understanding as well as on developing ways to better manipulate or exploit lymphatics. Both are evolving symbiotically, since tools to manipulate lymphatics locally or in more controlled or specific ways will facilitate the basic research, while increasing our understanding of how lymphatics affect immunity is necessary to provide the rationale for, and instruct the development of, new immunotherapeutic strategies.

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Abbreviations

Ag	Antigen
APC	antigen-presenting cell
DC	dendritic cell
DSS	dextran sodium sulfate
HEV	high endothelial venule
iBALT	induced bronchus-associated lymphoid tissue
i.d.	Intradermal
IDO	indoleamine-2,3-dioxygenase
i.m.	Intramuscular

i.n.	Intranodal
IPF	idiopathic pulmonary fibrosis
i.v.	Intravenous
LAM	Lymphangioliomyomatosis
LEC	lymphatic endothelial cell
LN	lymph node
LNSC	lymph node stromal cell
MPA	mycophenolic acid
MPLA	monophosphoryl lipid
NO	nitric oxide
NP	Nanoparticle
OVA	Ovalbumin
PEG	poly(ethylene glycol)
PLGA	poly-(lacticoglycolic-acid)
PPS	poly(propylene sulfide)
PTA	peripheral tissue antigen
S1P1	Sphingosine-1-phosphate receptor 1
s.c.	Subcutaneous
TAA	tumor-associated antigen
tdLN	tumor-draining LN
T_{Reg} cell	regulatory T cell
VEGF	vascular endothelial growth factor.

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Fig. 1.

Antigen and cell trafficking through lymphatics. Antigens and leukocytes travel from peripheral tissue sites to the draining lymph nodes and finally back into systemic circulation via the lymphatics. In particular, antigen presenting cells like dendritic cells and lymphocytes migrate through the lymphatics via CCL19/21-mediated chemotaxis, as these cell types express the receptor CCR7. Dendritic cells also sit between lymphatic endothelial cells and appear to mediate vessel integrity in a CCR7-dependent manner. Lymphocytes can enter the LN via lymphatics, if drained from the periphery, or via high endothelial venules from the systemic circulation. This also occurs in a CCR7-dependent manner. In the LN, dendritic cells present antigens (taken up locally or peripherally) for lymphocyte education. Finally, educated lymphocytes egress from LN via efferent lymphatics and either home to the peripheral tissues or remain in the systemic circulation.

Fig. 2.

Targeting the tumor-draining lymph nodes (tdLNs) with NP vaccines. TdLNs collect lymphborne tumor-associated-antigens (TAAs). Lymphatic-targeting NP vaccines can be injected into a healthy tissue (e.g. the skin) directly connected to the tdLNs via lymphatic vessels. Particulate vaccines in the 10–200 nm-range are directly uptake into the lymphatics, most likely mainly by transcellular transport through LECs, while particles above 200 nm mostly use internalization and transport by DCs to enter the lymphatics. In the LNs, resident DCs take up NP vaccines and become activated by delivered immune adjuvants. Activated DCs can present both TAAs contained in the vaccine and TAAs already present in the tdLNs, resulting in the stimulation of T cells with multiple antigen specificities.

Fig. 3.

Summary of immunomodulatory roles of lymphatic endothelial cells (LECs) during steady state and inflammation. LECs modulate immune responses both via direct interactions with immune cells, including dendritic cells and T cells, as well as through secretion of chemokines and cytokines. In the steady state, direct LEC-T cell interactions and antigen transfer to DCs can lead to increased CD4⁺ T cell apoptosis or anergy and dysfunctional CD8⁺ T cell activation. During inflammation, LECs can produce factors such as IDO and NO that inhibit T cell proliferation. LECs also upregulate adhesion molecules, MHC molecules, and other factors that aid in the maintenance of regulatory and memory T cell populations, and inhibit dendritic cell (DC) maturation. Following resolution of inflammation, LECs can retain antigens for prolonged time periods (a phenomenon called antigen archiving) and transfer them to DCs, thus contributing to the maintenance of immunological memory.