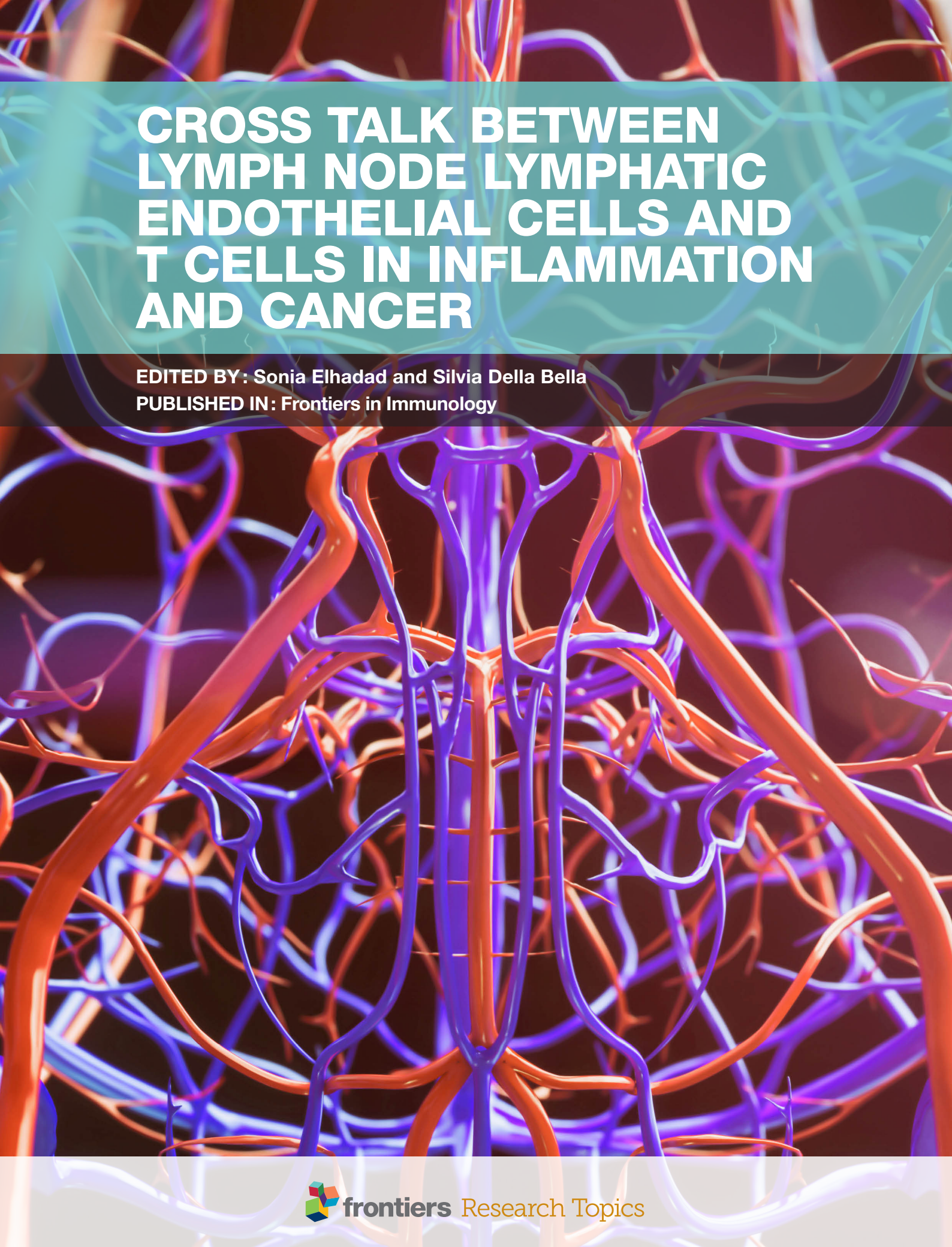

CROSS TALK BETWEEN LYMPH NODE LYMPHATIC ENDOTHELIAL CELLS AND T CELLS IN INFLAMMATION AND CANCER

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CROSS TALK BETWEEN LYMPH NODE LYMPHATIC ENDOTHELIAL CELLS AND T CELLS IN INFLAMMATION AND CANCER

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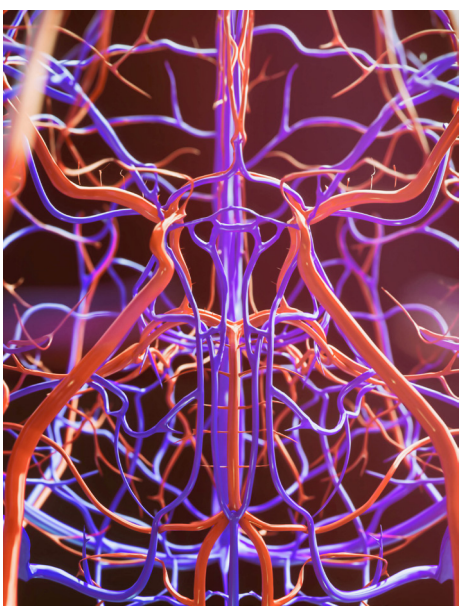

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Lymphocytes constantly survey the lymph nodes in search for potential infection by a pathogen. They enter the afferent lymphatic vessel that serves as a conduit to transport the motile lymphocytes to the draining lymph node. Lymphatic vessels (LVs) are present in most vascularized tissues. They are traditionally regarded as passive conduits for soluble antigens and leukocytes. Afferent LVs begin as blind ended capillaries, which give rise to collecting vessels that merge and connect with draining lymph nodes (dLNs). Initial lymphatic capillaries are composed of Lymphatic Endothelial Cells (LECs) connected by discontinuous cell junctions, which join to form larger collecting lymphatic vessels, and ultimately feed into the LN subcapsular sinus. Within the LN, LECs are localized to the subcapsular, cortical, and medullary sinuses, where they interact with incoming and exiting leukocytes. LECs, and in general LN stromal cells, have emerged in the recent years as active players in the immune response. In support to this,studies have shown that the immune response generated during inflammation and under pathologic conditions is accompanied by modeling of the LVs and generation of new lymphatics, a process known as lymphangiogenesis. These facts strongly suggest that LECs and stromal LN cells in general, are not inert players but rather are part of the immune response by organizing immune cells movement, exchanging information and supplying survival factors.

The purpose of this research topic is to review the role of the LECs during immune homeostasis and cancer. Considering the critical role of lymphangiogenesis in many pathologies like chronic and acute inflammation, autoimmunity, wound healing, graft rejection, and tumor metastasis, it is important to understand the molecular mechanisms that govern the cross talks between the LECs and immune cells during homeostasis and inflammation.

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EDITORIAL
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Editorial: Cross Talk between Lymph Node Lymphatic Endothelial Cells and T-Cells during Inflammation and Cancer

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Keywords: T cells, lymphatic vessels, lymphatic endothelial cells, inflammation, cancer, antigen presenting cells, atypical chemokine receptors, microRNAs

Editorial on the Research Topic

Cross Talk between Lymph Node Lymphatic Endothelial Cells and T-Cells during Inflammation and Cancer

A successful adaptive T cell immune response depends on the encounter of T cells and antigen-presenting cells (APCs). Therefore, T cells constantly patrol the body, recirculating between the blood and the lymph nodes, looking for antigens drained from peripheral tissues. In the lymph node, lymphocytes recognize antigens upon contact with APCs, proliferate to expand few clonally relevant lymphocytes, differentiate into effector T cells, then exit the LNs and migrate to peripheral tissues to ensure immune protection. All these processes involve cellular interactions and migration. Lymph nodes are strategically distributed throughout the body, at the junction of the blood vascular and lymphatic systems. T cells spend several hours in a lymph node sampling the microenvironment, leaving then the lymph node *via* efferent lymphatic vessels (LVs). Traditionally considered as passive conduits, LVs appeared to be active players in the modulation of the immune response. The lymphatic system serves as the primary route for the metastasis of many cancers, and the extent of lymphangiogenesis is an important indicator in tumor progression. The promiscuity of immune cells and LVs suggests that immune cells modulate this biological process during inflammation and cancer. Those, cross talks between LVs and the immune system can be used for therapeutic strategies for cancer and other pathologies.

This Research Topic brings together eight articles that provide insights into the various biological functions of the LECs, the ways they regulate the immune responses, and the therapeutic strategies that can be developed.

In their mini review, Yee et al. focused on the role of microRNAs (miRNAs) as regulators of LECs' function. The authors discuss the role of miR-31 and mi-R181a that targets PROX1, the transcription factor controlling the upregulation of LECs' markers. As a result, LECs' specific genes expression is repressed, therefore miR-31 and mi-R181a control LECs' differentiation and plasticity. Moreover, elevated levels of these miRNAs are found during inflammation suggesting a possible role of these miRNAs in inflammatory lymphangiogenesis. The authors suggest that they can be used as new therapeutic tools in inflammation and cancer.

Chemokines and their receptors are key factors in LVs' function. In their mini review, Bonavita et al. discuss the role of atypical chemokine receptors (ACKRs), and in particular the role of ACKR2 in lymphatic biology. The authors reported data showing the essential role of ACKR2 expressed

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by LECs, in regulating chemokine concentration and leukocyte migration, promoting therefore resolution of inflammatory responses in infection, allergy, and cancer. Finally, they speculate that ACKR2 could be considered as a potential therapeutic target to attenuate inflammation during psoriasis and lung infection, or by influencing cancer cell dissemination to metastatic tissues.

Lymphatic vessels are key players in the cellular migration that accompanies lymphocytes patrolling during homeostasis and inflammation. In their review, Hunter et al. discuss in great details T cell migration within and between peripheral tissues and secondary lymphoid organs. While T cell migration within the lymph node occurs in a one manner where recirculating lymphocytes exit through efferent LVs to return to the blood circulation, in peripheral tissues T cells exit through afferent lymphatics to migrate to draining lymph node, before joining the blood circulation. The authors discuss further the relevance of T cell migration through afferent LVs in immune surveillance and resolution of local inflammation, and the use of this migration process for the development of immunomodulatory therapies.

Number of publications reported the function of LECs as APCs. In their review, Humbert et al. discussed how this property shapes the immune response. LECs express a large range of peripheral tissue-restricted antigens (PTAs) and present class I restricted PTA-derived antigens to $CD8^+$ T cells, leading to the deletional tolerance of self reactive $CD8^+$ T cells. The authors discuss further the contribution of LECs as regulators of peripheral T cell responses in autoimmunity and cancer.

While these papers cover the regulation of the peripheral immune response by LECs through different biological processes, LECs' function is itself regulated by T cells during inflammation and cancer. We have reported that temporal inflammatory lymph node lymphangiogenesis is regulated by a mixed Th1/Th2/Th17 response (1). Yeo and Angeli discuss further the cross talks between LECs and T cells and their implications in cancer, and the use of lymph node LECs as a potential therapeutic target in addition to immunotherapy strategies for cancer progression and metastasis.

Tumor vasculature plays a crucial role in shaping the tumor microenvironment and contributes to cancer immune evasion. In their review, Hendry et al. described the mechanical and molecular mechanisms underlying tumor-promoting properties of tumor vasculature. They also explained the design of combined antiangiogenic and immunotherapeutic treatments and summarized the drug combinations explored in preclinical and clinical settings. Promising results have been reported for antiangiogenic therapy combined with immune checkpoint inhibitors in different types of cancer, suggesting that each treatment may potentiate the effect of the other. We suggest that endothelial colony-forming cells isolated and cultured from blood may represent a tool for studying the endothelial compartment in cancer patients (2, 3), and assessing the impact of combined treatments during patient follow-up.

Dieterich et al. delineated the differential role played by lymphatic and blood vascular vessels in the tumor microenvironment, highlighting a role for LVs in promoting cancer immune tolerance. By using two different *in vivo* models, the authors showed that, during cancer development, tumor-associated LVs—but not blood vessels—upregulate the checkpoint inhibitor PD-L1. Notably, this effect is dependent on IFNγ production by tumor stromal cells. T cell interactions with tumor-associated LVs, and T cell inhibition upon contact with PD-L1-expressing LECs, suggest that characteristics ascribed to LECs in secondary lymphoid organs are shared by LECs present in the tumor microenvironment. As reported in Lukacs-Kornek's review (Lukacs-Kornek), LECs' functions are shared by liver LECs. Our knowledge on liver LECs is limited to the notion that they are increased in chronic liver diseases and cancer. Availability of markers for LEC identification will clarify the contribution of these cells to liver disease pathogenesis.

AUTHOR CONTRIBUTIONS

SE organized the Research Topic; wrote and edited the Editorial. SDB helped in Research Topic management and contributed to writing the Editorial.

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MINI REVIEW
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microRNAs in the Lymphatic Endothelium: Master Regulators of Lineage Plasticity and Inflammation

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microRNAs (miRNAs) are highly conserved, small non-coding RNAs that regulate gene expression at the posttranscriptional level. They have crucial roles in organismal development, homeostasis, and cellular responses to pathological stress. The lymphatic system is a large vascular network that actively regulates the immune response through antigen trafficking, cytokine secretion, and inducing peripheral tolerance. Here, we review the role of miRNAs in the lymphatic endothelium with a particular focus on their role in lymphatic endothelial cell (LEC) plasticity, inflammation, and regulatory function. We highlight the lineage plasticity of LECs during inflammation and the importance of understanding the regulatory role of miRNAs in these processes. We propose that targeting miRNA expression in lymphatic endothelium can be a novel strategy in treating human pathologies associated with lymphatic dysfunction.

Keywords: lymphatic endothelial cells, microRNA, inflammation, lineage plasticity, lymphangiogenesis

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INTRODUCTION

The lymphatic system is a transport network that regulates tissue fluid homeostasis, the absorption of macromolecules, and the trafficking of immune cells (1). Lymphatic vessels are made up of a single layer of partly overlapping lymphatic endothelial cells (LECs). Embryonic studies on development of lymphatic vasculature have identified key transcription factors required for development and maintenance of the lymphatic system. The same transcription factors regulate lymphangiogenesis, the process of new lymphatic vessel growth from pre-existing vessels, which has crucial roles in wound healing, inflammation, infection, and cancer. In addition to transcriptional regulation, post-transcriptional mechanisms play a key role in LEC responses to inflammation. In particular, several microRNAs (miRNAs) have emerged as key determinants of LEC differentiation and inflammatory responses. This review will discuss our current understanding of the role of individual miRNAs and components of the miRNA biogenesis machinery in LEC immune function.

miRNA-MEDIATED SILENCING

microRNAs are a class of highly conserved, small non-coding RNA (~20–24 nt) that regulate gene expression at the posttranscriptional level of all biological pathways including cell development, differentiation, and function (2). In mammals, the canonical process of miRNA biogenesis encompasses

the generation of primary miRNA (pri-miRNA) transcripts that are transcribed by RNA polymerase II in the nucleus. Stem-loop structures of pri-miRNA transcripts are processed by the RNAse III endonuclease, Drosha, to form hairpin-shaped precursor miRNA (pre-miRNA) (3, 4). Following this, pre-miRNA is exported into the cytoplasm where it is further processed by another RNAse III endonuclease, Dicer, which cleaves off the hairpin structure. The resultant double-stranded miRNA is separated into two strands with the mature miRNA strand packaged onto the miRNA-induced silencing complex that includes an Argonaute (AGO) effector protein. The miRNA guides RISC to specific target sites, primarily the 3′ untranslated region (UTR) of target mRNAs, leading to repression of target gene expression (5). Binding sites are generally 8mers or canonical sites that enable high miRNA regulation of mRNA expression (6). Due to this short target sequence, miRNAs can have multiple targets, and it is predicted that 30% of all protein-coding genes is under miRNA regulation in mammals (7).

EMBRYONIC DEVELOPMENT AND SPECIFICATION OF THE LYMPHATIC VASCULATURE

Sabin hypothesized the venous origin of the lymphatic system (8), which became increasingly supported by developmental studies around the beginning of the twenty-first century (9). Specific genes for lymphatic differentiation and identity were identified, and these included vascular endothelial growth factor receptor-3 (VEGFR-3), lymphatic vessel hyaluronan receptor-1 (LYVE-1), podoplanin, and prospero-related homeodomain protein 1 (PROX1) (10). VEGFR-3 is a receptor tyrosine kinase for lymphatic-specific VEGF-C and VEGF-D (11). LYVE-1 is a widely used lymphatic-specific marker, implicated in cellular trafficking and a homolog of the CD44 glycoprotein (12, 13). Both VEGFR-3 and LYVE-1 are expressed during early endothelial cell development and become restricted to LECs at later stages. Genetic deletion of VEGFR-3 or VEGF-C in mice leads to defective lymphatic vascular development (14, 15). In contrast, LYVE-1 gene-deficient mice develop normal lymphatic vasculature (16).

The murine lymphatic system begins to form in a subpopulation of venous endothelial cells, LEC precursors, at embryonic day (E) 8.5 that express PROX1, LYVE-1, and VEGFR-3 (14). At E9.75, a lymphatic bias signal upregulates PROX1, LEC budding, and formation of primary lymph sacs (10). PROX1-deficient embryos lack lymphatic vasculature, VEGFR-3, or LYVE-1 expression and are embryonic lethal at E14.5 (10). Two upstream transcriptional regulators of PROX1, SOX18 (17), and COUP-TFII promote the lymphatic bias signal until E13.5 (18, 19). PROX1 and VEGFR-3 continue to be expressed only in postnatal and adult lymphatic vasculature (20). Constant levels of PROX1 are required to maintain LEC lineage, which is supported by VEGF-C/VEGFR-3 signaling (21). Postnatal LECs have lower PROX1 expression compared with embryonic lymphatic endothelium, suggesting low expression of PROX1 is sufficient to maintain LEC identity (22). Additional transcription factors and regulators of lymphatic development have been reported, including neuropilin 2 (23, 24), FOXC2 (25, 26), integrin-9α (27, 28), NOTCH (29, 30), C-MAF (31), and GATA2 (32).

miRNAs AND ENDOTHELIAL CELL DEVELOPMENT

microRNA biogenesis is essential for vertebrate development, and tissue-specificity of miRNAs has been demonstrated in angiogenesis (33–36). Loss of Dicer in mice leads to poor vascular formation and embryonic lethality (33). The highest expressed miRNA in endothelial cells, miR-126 mediates angiogenesis and maintenance of vascular integrity (37–40). Deletion of miR-126 results in vascular leakage, hemorrhaging, and embryonic lethality in a subset of mice (38). Surviving mice lived to adulthood without noticeable abnormalities, suggesting additional regulatory factors after birth. Accordingly, miR-126 targets sprout-related protein-1 (SPRED-1), phosphoinositol-3 kinase regulatory subunit 2 (PIK3R2 also known as P85β), and VCAM-1 in human and murine cells (37–39). By targeting VCAM-1, miR-126 can inhibit leukocyte adherence and potentially regulate vascular inflammation (37). SPRED-1 is an intracellular inhibitor of angiogenic and MAP kinase signaling, and its repression by miR-126 correlated with the increase of pro-angiogenic genes VEGF and fibroblast growth factor in mice (38). Additionally, VEGF can induce miR-132 and promote angiogenesis by suppressing p120RasGAP in human vascular endothelial cells (41).

REGULATION OF THE miRNA BIOGENESIS MACHINERY IN LECs

In addition to individual miRNAs, the miRNA biogenesis machinery is regulated during activation of LECs. AGO2 levels are controlled by miR-132 in human LECs (42). Inhibition of miR-132 in activated LECs results in increased AGO2 and the anti-angiogenic miR-221, providing further support for the function of miR-132 in endothelium. Furthermore, activation of TIE-2 by angiopoietin-1 (ANG-1) results in phosphorylation of TRBP (43), a DICER co-factor, which facilitates miRNA processing (44). Through this mechanism, ANG-1 treatment increases levels of miRNAs, including miR-126 and miR-21, which could contribute to the antiapoptotic function of ANG-1 (45, 46) in LECs.

LEC PLASTICITY

Altering the levels of PROX1 expression during embryonic, postnatal, or adult stages can reprogram LEC phenotype into blood endothelial cell (BEC) (28, 47, 48). PROX1 deletion results in the upregulation of BEC-specific markers in human and murine LECs (47). Conversely, BECs can be transcriptionally reprogramed by overexpression of PROX1 *in vitro*, resulting in upregulation of VEGFR-3 and podoplanin and suppression of BEC-specific transcripts, such as the transcription factor STAT6 (48, 49). These studies represent that endothelial cell differentiation is reversible and highlight the plasticity of LECs.

miRNAs AND LEC LINEAGE COMMITMENT

The 3′-UTR of PROX1 is remarkably long (5.4 kb) and conserved among vertebrates (50), which suggests PROX1 expression may be posttranscriptionally regulated by miRNAs. In contrast, the 3′ UTR length of *SOX18* (585 bp) is short and likely to have less miRNA regulation. Profiling of miRNAs in human LECs and BECs led to the discovery that lymphatic development can be regulated by BEC miRNA signatures (40). Overexpression of miR-31 was shown to repress FOXC2 and several other LEC-signature genes (40). Both miR-31 and miR-181a can target PROX1 and as a result repress LEC-specific genes, including VEGFR-3, and vascular development in embryonic LECs (22, 40). Furthermore, signaling from bone morphogenetic protein (BMP) 2, a member of the TGF-β family, inhibited Prox-1 expression and lymphatic differentiation during zebrafish and murine development (51). Interestingly, BMP2 signaling upregulated miRNAs: miR-194, miR-186, miR-99a, miR-92a and also miR-31, and miR-181a (51). Knockdown of SMAD4 by siRNA downregulated the expression of miR-31 and miR-181a indicating a possible involvement of BMP2 as a negative regulator of LEC identity (51). Recently, miR-466 was shown to suppress PROX1 expression and tube formation in human dermal LECs, and both miR-466 and miR-181a induced inhibition of corneal lymphangiogenesis in rats (52).

LECs IN INFLAMMATION AND LYMPHANGIOGENESIS

The lymphatic vessels serve as a conduit for transport of leukocytes and antigen-presenting cells to lymph nodes (LNs), which orchestrate initiation of adaptive immune response (11). LECs express the chemokine ligand, CCL21 that attracts and guides the interactions of CCR7-positive T, B, and dendritic cells (DCs) to LNs *via* the afferent lymphatics (53). Not all LECs are equal, reportedly, LN–LECs express different levels of CCL21 forming chemokine gradients that facilitate directional migration into the LNs through an atypical chemokine receptor, CCRL1 (54). The role of LECs in immune regulation has been demonstrated in a series of papers showing LECs contributing to the induction of peripheral tolerance of DC and T cells. In human LECs, tumor necrosis factor alpha (TNFα) induces vascular and intercellular cell adhesion molecule 1 (VCAM-1, ICAM-1) and E-selectin, facilitating adherence of DCs to the endothelium (55). TNFα-stimulated lymphatic endothelium can interact with DCs *via* cell-to-cell contact to suppress human DC maturation and function by an ICAM-1–Mac-1 (CD11b) interaction (56). Notably, murine LECs lack expression of co-stimulatory ligands but can express the inhibitory checkpoint ligand, programed cell death ligand-1 (PD-L1) to negatively regulate $CD8^+$ T cells (57–60). LECs can also express MHC II *in vivo* and may induce tolerance of $CD4^+$ and $CD8^+$ T cells either by acting as an antigen reservoir for DCs or through cross-presentation of antigens (60–64). The mechanism of antigen transfer from LEC to DCs and whether LECs can induce similar levels of tolerance as DCs remains to be further understood.

During inflammation, the lymphatic system becomes activated and lymphatic remodeling is induced in both peripheral tissues and the draining LN (65). The increase in lymphangiogenesis may aid in the resolution of inflammation. Inflammation-induced lymphangiogenesis is commonly regulated by pathways involving VEGF-C/VEGFR-3 and VEGF-A/VEGFR-2 signaling (11). Studies in mice demonstrated that lymphangiogenesis is driven by increased VEGF-C, VEGF-D, and VEGF-A from macrophages during acute skin inflammation and chronic airway infection, reported to promote antigen clearance and prevent lymphedema (66, 67). Lymphatic vessels are impaired during chronic skin inflammation, which can be alleviated by the overexpression of VEGF-C (68). Interestingly, VEGF-C stimulation in skin inflammation instigated LECs to produce anti-inflammatory prostaglandin synthase, which led to higher levels of IL-10 on DCs leading to suppressed DC maturation (69). B cells can enhance the growth of LN lymphatic vasculature through VEGF secretion and increase DC migration to the LN (70). However, interferon-gamma (IFN-γ) secretion from T cells suppressed growth of LN-lymphatic vasculature *in vivo* and downregulated the expression of PROX1, LYVE-1, and podoplanin *in vitro* in a JAK/STAT-dependent mechanism (71). IFN-γ knockout mice express a higher baseline of lymphatic vasculature in the LN. Expression of PROX1, VEGFR-3, and LYVE-1 are also downregulated during acute skin inflammation (72, 73). In human dermal LECs, transforming growth factor-β (TGF-β) or TNFα stimulation results in loss of PROX1 and LYVE-1 expression (74, 75). In contrast, studies in mice suggest that NF-κB induces PROX1 and VEGFR-3 in a lipopolysaccharide (LPS)-induced peritonitis model, increasing sensitivity of pre-existing lymphatic vessels to VEGF-C and VEGF-D-expressing leukocytes (76). Additionally, IL-3 in LECs can induce PROX1 and podoplanin expression and maintain the differentiated LEC phenotype *in vitro* (77). LECs are also a major source of IL-7 *in vivo* which is required for remodeling and homeostasis of the LN microenvironment (78).

miRNAs IN LECs DURING INFLAMMATION AND INFECTION

Studies have demonstrated miRNAs in the regulation of inflammation including miR-146a/b, miR-155, and miR-132 in both immune and non-immune cell types (79–82). Several activities have been reported for miR-155 across the immune system, including Th1 differentiation of murine $CD4^+$ T cells by inhibiting IFN-γ signaling (83) and production of immunoglobulin class-switch differentiation of B cells by targeting transcription factor PU.1 (84). A wide range of inflammatory stimuli induce miR-155 expression including LPS, poly (I:C), IFN-β, and TNFα in human and murine macrophages, monocytes, and endothelial cells (79, 80, 85, 86). In addition, miR-155 regulates angiogenesis and inflammation by negatively regulating ETS-1, upstream of VCAM-1, and angiotensin II type 1 receptor (87).

microRNA profiling of rat mesenteric LECs treated with TNFα for 2, 24, and 96 h indicated a distinct miRNA signature at

various time points (88). Several miRNAs involved in angiogenesis, endothelial sprouting, and cell migration were upregulated, while miRNAs associated with cell survival and proliferation were downregulated at 24 and/or 96 h. Of those upregulated, miR-9 was shown to directly target NF-κB, downstream of TNFα signaling, and regulate TNFα-mediated inflammatory mechanisms. In addition, overexpression of miR-9 increases VEGFR-3 expression and tube formation, indicating a possible role in lymphangiogenesis. VEGFR-3 was also shown to be regulated by a mirtron miR-1236, arising from a spliced-out intron that is processed independently of Drosha, in human LECs (89). IL-1β can induce miR-1236 and downregulate VEGFR-3 protein which is similarly reported in inflammatory lymphangiogenesis. Although miR-1236 is lowly expressed in human LECs, it may be upregulated during inflammation-induced lymphangiogenesis to control the expression of VEGF-C/VEGFR-3 signaling.

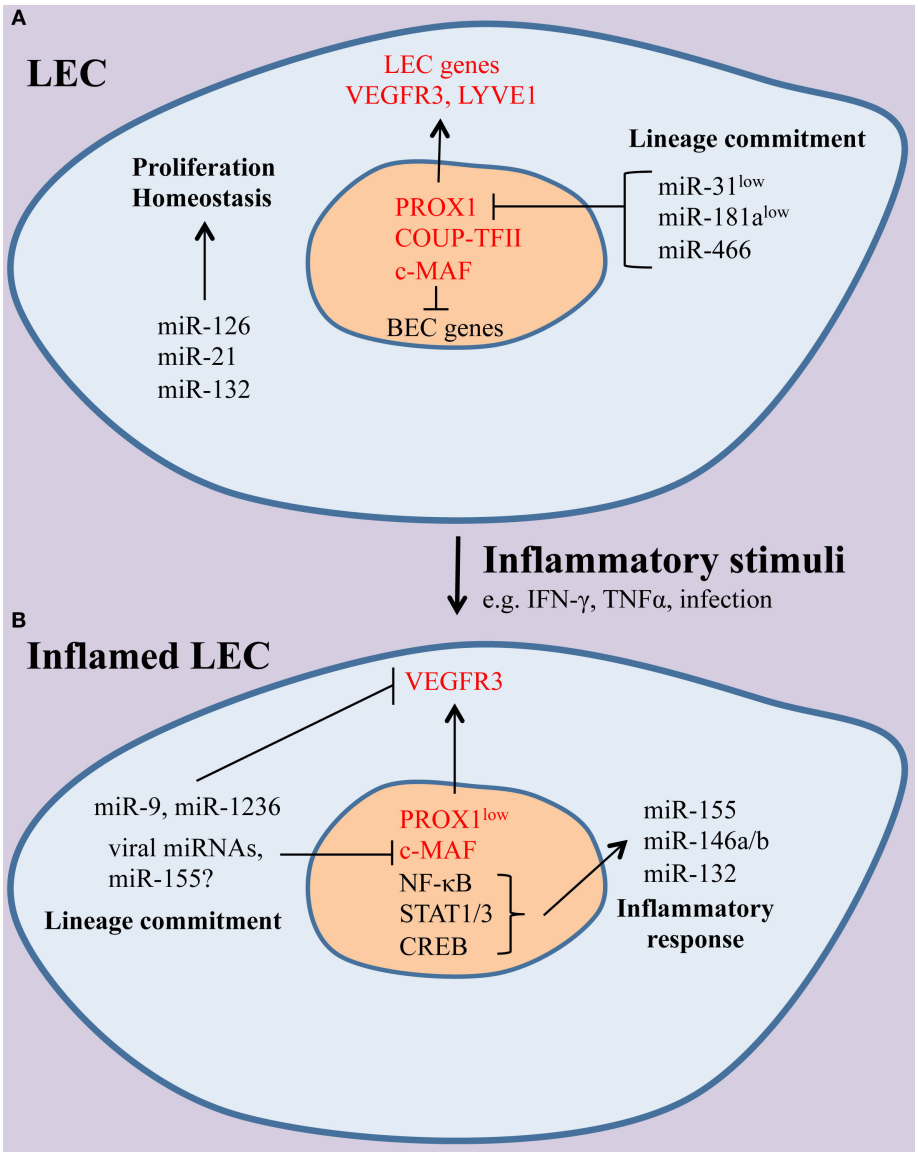

FIGURE 1 | The effect of inflammation on the microRNAs (miRNA) landscape of lymphatic endothelial cells (LECs). (A) Under homeostatic conditions, miRNAs, including miR-126, miR-21, and miR-132, contribute to normal LEC function. Lymphatic identity is maintained through suppression of the blood endothelial cell (BEC)-enriched miRNAs miR-31 and miR-181a, which can repress LEC-specific genes, including the master LEC fate regulator PROX1 and the receptor tyrosine kinase vascular endothelial growth factor receptor-3 (VEGFR-3). **(B)** During inflammation, a set of immunologically active miRNAs (miR-155, miR-132, miR-146a) are induced and shape LEC immune responses. In addition, LEC-specific genes are downregulated and miRNAs, including miR-9, miR-1236, and miR-K12-11, a viral ortholog of miR-155, contribute to the loss of LEC identity. It is likely that other miRNAs may modulate immune gene expression and lineage plasticity in LECs.

LESSONS FROM KAPOSI'S SARCOMA HERPESVIRUS (KSHV)

Our understanding of gene regulation in LECs has advanced significantly by studying infectious diseases that directly involve LECs. Kaposi's sarcoma (KS) is a tumor from lymphatic endothelial origin and is the most common cancer in untreated HIV-positive patients (90). KSHV infects both LECs and BECs to induce transcriptional reprograming giving rise to mixed phenotypes of LECs and BECs (91, 92). Phenotypically, KS is most similar to LECs and occurs at sites rich in LECs such as skin, LN, and mucosa (92). KSHV infection of human LECs induces an early antiviral miRNA response from miR-132 and miR-146a and inhibition of these miRNAs suppressed viral gene expression (82). Overexpression of miR-132 negatively regulates inflammation by impairing the expression of IFN-β and interferon-stimulated gene 15. Upon KSHV infection, miR-132 targets the transcriptional co-activator EP300 and downregulates the interferon response, increasing viral gene expression. In addition, KSHV can influence endothelial cell motility by downregulating the miR-221/miR-222 cluster and upregulating miR-31 (93). Whether upregulation of miR-31 can regulate PROX1 during KSHV infection is unknown. A KSHV latent gene, kaposin B was found to stabilize PROX1 mRNA and drive lymphatic reprograming of BECs (50). An additional target of KSHV infection is the transcription factor c-MAF, which represses BEC-specific identity in human LECs (31, 91). Downregulation of MAF occurs early and is maintained throughout viral infection. The miR-155 KSHV ortholog, miR-K12-11 (94), was shown to regulate MAF in human LECs (31). Interestingly miR-155 has been shown to suppress MAF expression in murine $CD4^+$ T cells (95).

CONCLUDING REMARKS AND FUTURE DIRECTIONS

Our understanding of miRNAs in LEC activation has greatly increased from recent reports but this area remains understudied (**Figure 1**; **Table 1**). LEC plasticity is under miRNA regulation that allows the rapid response of lymphatic endothelium to inflammatory and angiogenic stimuli. LECs display heterogeneity, and there are different types of lymphatic vessels and LECs that have organ-specific functions (96). Studying miRNAs in certain types of lymphatic vessels and niches, such as the skin, LN, or subpopulations within these contexts, can introduce new tools to understand the different functions that LECs regulate in these tissues.

Targeting miRNAs such as miR-126, miR-9, and miR-132 (**Table 1**) presents a novel opportunity to deliver localized therapy for treating disease. This can be either to inhibit or mimic the function of the miRNA. Anti-miR-132 was shown to inhibit angiogenesis and decrease tumor burden in a mouse model of human breast carcinoma (41). Antagonism of miR-122 to treat hepatitis

TABLE 1 | microRNAs (miRNAs) in the lymphatic endothelium.

miRNA	Primary role	Function and target	Model system	Reference
miR-126	Angiogenesis Inflammation	Highest expressed miRNA in endothelial cells, which regulates angiogenesis through SPRED1 and VCAM-1	Human primary ECs, murine ECs	Wang et al. (38), Harris et al. (37), and Fish et al. (39)
miR-132	Angiogenesis	Acts as an angiogenic switch by targeting p120RasGAP	Human umbilical vein ECs	Anand et al. (41)
	Inflammation	Regulates anti-viral immunity through EP300	Kaposi's sarcoma herpesvirus (KSHV)-infected lymphatic endothelial cell (LECs)	Lagos et al. (82)
miR-9	Inflammation	Regulates vascular endothelial growth factor receptor-3 (VEGFR-3), lymphangiogenesis, and NF-κB signaling	Rat LECs and human primary LECs	Chakraborty et al. (88)
miR-1236	Inflammation	Induced by IL-1β and regulates VEGFR-3 and lymphangiogenesis	Cultured human dermal LECs	Jones et al. (89)
miR-181a	Lineage commitment	Blood endothelial cell (BEC)-expressed miRNA, which inhibits PROX1 in LEC development	Murine LECs	Kazenwadel et al. (22)
miR-31	Lineage commitment	BEC-expressed miRNA which inhibits PROX1 and FOXC2 in LEC development	Human primary LECs, xenopus, and zebrafish	Pedrioli et al. (40)
miR-466	Lineage commitment	Inhibits PROX1 and tube formation	HDLECs and corneal lymphatic vessels	Seo et al. (52)
miR-K12-6, miR-K12-11 (ortholog of miR-155)	Lineage commitment	Viral miRNAs that target c-MAF contributing to virus-induced LEC reprograming	KSHV-infected LECs	Hansen et al. (31) and Hong et al. (91)
miR-146a/b	Inflammation	Early-response miRNA involved in TLR4 signaling and innate immunity	KSHV-infected LECs	Lagos et al. (82)
miR-155	Inflammation Angiogenesis	Targets ETS-1 upstream of endothelial adhesion molecules such as VCAM-1	Human umbilical vein ECs	Zhu et al. (87)
miR-221/miR-222	Angiogenesis	Targets transcription factors ETS-2 and ETS-1, respectively, regulating EC motility	Human primary LECs, KSHV-infected LECs	Wu et al. (93)

C virus infection is the first miRNA-targeting therapy in Phase II clinical trials (97). A challenge for miRNA-based therapies is ensuring effective delivery. Targeting miRNAs that drain into the LN through lymphatics vessels can lower the chances of off-target effects, drug resistance, and toxicity (98, 99). Lymphatic flow is unidirectional and the vessels can act as a bypass for absorption of compounds, such as lipophilic small molecule drugs, to avoid hepatic first-pass metabolism and enhance bioavailability (100). There are several routes that can be exploited for therapeutic delivery, including mucosal, intestinal, and parenteral (101). The lymphatic system is also thought to link the brain and the immune system (102). Although, lymphatic drug delivery is in its infancy, this approach may serve as a platform for accurately delivering miRNA-modifying compounds to target sites, providing new therapeutic opportunities for chronic inflammatory diseases.

AUTHOR CONTRIBUTIONS

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ACKR2: An Atypical Chemokine Receptor Regulating Lymphatic Biology

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The lymphatic system plays an important role in the induction of the immune response by transporting antigens, inflammatory mediators, and leukocytes from peripheral tissues to draining lymph nodes. It is emerging that lymphatic endothelial cells (LECs) are playing an active role in this context *via* the expression of chemokines, inflammatory mediators promoting cell migration, and chemokine receptors. Particularly, LECs express atypical chemokine receptors (ACKRs), which are unable to promote conventional signaling and cell migration while they are involved in the regulation of chemokine availability. Here, we provide a summary of the data on the role of ACKR2 expressed by lymphatics, indicating an essential role for this ACKRs in the regulation of the inflammation and the immune response in different pathological conditions, including infection, allergy, and cancer.

Keywords: chemokine, chemokine receptor, atypical chemokine receptor, lymphatic vessels, inflammation

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INTRODUCTION

Chemokines and Chemokine Receptors

Chemokines are small chemotactic cytokines secreted by different cell types (e.g., immune cells, cancer cells, and endothelial cells) mainly involved in the regulation of immune cell migration during routine immune surveillance, inflammation, and development (1). According to the position of conserved cysteine residues in their N-terminus, chemokines are classified into four subfamilies: CXC, CX_3C, CC, and C (2). In addition, they can be classified, on the basis of the conditions during which they are produced, in homeostatic or inflammatory chemokines (3, 4). Homeostatic chemokines (e.g., CCL19, CCL20, and CCL21) are constitutively produced and regulate leukocytes migration in basal conditions. Inflammatory chemokines (e.g., CXCL8, CCL2, and CCL3) are produced under pathological conditions, and they can act as secondary mediators induced by primary pro-inflammatory factors, such as IL-1 and TNF-α. Inflammatory chemokines actively participate in the inflammatory response attracting immune cells to the site of injury. Beyond their unequivocal role in regulating leukocyte recruitment, other activities, such as regulation of angiogenesis, fibrosis, proliferation, homeostasis, and cancer cell dissemination, have been attributed to chemokines (5–8).

The specific effects of chemokines on their target cells are mediated by chemokine receptors, members of a family of 7-transmembrane G-protein-coupled receptors (1). Chemokine receptors have a highly conserved structure, consisting of a single polypeptide chain with three intracellular and extracellular loops, an external N-terminus domain essential for the specificity of ligand binding, and an intracellular carboxy-terminus that, in concert with other motifs, such as the

Asp–Arg–Tyr–Leu–Ala–Ile–Val (DRYLAIV) motif between the third transmembrane domain and the second intracellular loop, is involved in receptor signaling. As a general rule, chemokine binding to the receptor causes conformational changes that trigger intracellular signaling pathways involved in cell activation and migration toward increasing chemokine gradients (9). Depending on the type of chemokine they bind, chemokine receptors can be classified as CXCR, CCR, CX3CR, or XCR1.

Beyond canonical chemokine receptors, a smaller family of atypical chemokine receptors (ACKRs) has been identified (10). These receptors are called atypical because they share structural features with canonical chemokine receptors and bind ligands with high affinity, yet, they are not able to induce cell migration. Indeed, these receptors have an altered DRYLAIV motif and, differently from the canonical chemokine receptors, upon chemokine engagement, they do not induce any GPCR signaling. Rather, ACKRs internalize and transport chemokines to the degradative compartment, modulating chemokine concentration and bioavailability. The family of ACKRs includes four receptors named according to the new nomenclature: ACKR1 (previously called DARC), ACKR2 (D6), ACKR3 (CXCR7), and ACKR4 (CCX-CKR) (11).

Role of Chemokine and Chemokine Receptor Expression in Lymphatic Function

Chemokine and chemokine receptors expressed by lymphatic vessels (LVs) have been mainly studied in the context of leukocyte traffic. Indeed, the lymphatic system represents an important transport network for leukocytes, in particular antigen-presenting cells that migrate, through afferent LVs, from the periphery to the lymph nodes (LNs). This trafficking is mainly dictated by CCL21. CCL21 is constitutively expressed by LEC and can be upregulated by inflammatory stimuli, such as TNF-α, which induce the release of CCL21 intracellular stores (12). CCL21 promotes the recruitment of CCR7-positive dendritic cells (DCs) but also of neutrophils and T cells to draining LNs (13–15).

Interestingly, lymphatics produce many other chemokines in a stimulus-specific manner, indicating that they can fine-tune leukocyte recruitment (16). CXCL12 and CX3CL1 were found to induce DC migration to LNs (17, 18) while the function of other inflammatory chemokines produced by inflamed LEC, such as CCL2 and CXCL8, is not fully understood (12, 19).

Besides, in keeping with chemokine receptor expression on cancer cells, leukocyte-like homing toward LVs and LNs plays an important role in promoting cancer cell migration and metastasis. For example, CCR7-positive cancer cells metastasize to LNs where CCL19 and CCL21, the ligands for CCR7, are produced (20).

In addition to producing chemokines, lymphatic endothelial cells (LECs) express canonical chemokine receptors. Primary culture of murine LEC was found to be positive for the expression of CCR5, CCR9, CXCR4, and CXCR6, whereas they weakly expressed CCR4, CCR6, CCR8, CCR10, CXCR3, and CX3CR1 (21). The function of these receptors expressed by LEC is still unknown, with the exception of CXCR4 that has a role in promoting lymphangiogenesis, similar to its angiogenic role in vascular endothelial cells (21).

Interestingly, LECs express ACKRs: ACKR1 is expressed by human podoplanin low LECs (22); ACKR2 by many human tissues on afferent lymphatic (23); ACKR3 by human LECs, with restricted expression in the tonsil and kidney and increased expression during renal allograft rejection (24); and finally, ACKR4 is expressed by LEC in the LN capsule (25, 26). Here, we are summarizing data on the expression and role of ACKR2 by LECs. Increasing evidence suggests a crucial role for this receptor in the regulation of inflammation and immune response (12, 27).

ACKR2/D6

The atypical chemokine receptor ACKR2, also known as D6 or CCBP2, is a highly promiscuous receptor capable of binding the majority of inflammatory CC-chemokines (28). Initially, according to its "atypical" features, it was assumed that ACKR2 was a non-signaling chemokine receptor. However, it was later demonstrated that not only is ACKR2 capable of internalization and scavenging of its ligands but that it also activates a β-arrestin-dependent signaling pathway, promoting receptor internalization and recycling to the cell membrane (29, 30).

ACKR2 Expression by Lymphatics

ACKR2 is expressed by trophoblasts in the placenta, by some leukocytes (31), and by LECs. Indeed, Nibbs et al., in a seminal paper (23), demonstrated that within non-inflamed tissues, human LECs, but not vascular endothelial cells, express ACKR2. Moreover, they found that the receptor was expressed on a subset of lymphatics, thus suggesting the existence of functional heterogeneity within the lymphatic vasculature. Specifically, ACKR2 expression was found in human skin sections only in afferent LVs, both in regions near the epidermis but also deeper within the dermis. ACKR2 was found in small lymphatics in the villi of small and large intestine and in the lamina propria mucosae of colon and large collective lymphatics located in the muscular layer. The appendix also showed ACKR2-positive lymphatics in lymphoid tissue of lamina propria and in the lamina muscolaris externa. ACKR2 was also detected in other secondary lymphoid organs such as tonsils, spleen, and LNs on sinus-like channels and vessels in the parafollicular areas of the tonsils and the red pulp of the spleen. ACKR2 was not detected in heart, kidney, liver, skeletal muscle, brain, cerebellum, pancreas, prostate, and thyroid, whereas it was found in liver, lung, and placenta, but not on LECs (23).

The regulation of ACKR2 expression in LECs was only studied *in vitro* using human dermal LECs. McKimmie et al. found that ACKR2 is upregulated by the lymphangiogenic cytokine vascular endothelial growth factor-D, by the immunosuppressive cytokine transforming growth factor-β, and by the inflammatory mediators IL-6, type-I IFNs, and IFN-γ. On the contrary, the pro-inflammatory cytokine IL-1α induced a significant downregulation of ACKR2 (32).

The Function of ACKR2 Expressed by LECs

Extensive evidence indicates that ACKR2 is involved in the regulation of chemokine levels around afferent LVs and in the removal of chemokines from inflamed tissues, thus acting as scavenger and "gatekeeper" by limiting the access, the interaction, and the inappropriate accumulation of inflammatory leukocytes in the lymphatic system, in particular in the subcapsular sinus region of LNs (33, 34).

McKimmie et al. observed a variable expression of ACKR2 on individual cutaneous LVs and a biased distribution toward the luminal face of the LECs, thus suggesting an involvement of ACKR2 in suppressing inflammatory leukocyte binding to lymphatic endothelial surfaces (32). Specifically, they demonstrated that ACKR2 contributes to the efficiency of antigen presentation of DCs, which is crucial in maintaining immune surveillance. Indeed, ACKR2, by scavenging pro-inflammatory chemokines, suppresses inflammatory chemokine binding to the LEC surface, thus increasing the availability and contributing to selective presentation of CCR7 ligands that attract $CCR7^+$ mature DCs (32).

Recently, it was found that ACKR2 regulates LV density, competing with the canonical chemokine receptor CCR2 for the binding of CCL2, a chemokine produced in the skin during inflammation that drains into the LNs where it induces monocyte infiltration (**Figure 1**). By immunostaining of LV networks, Lee et al. found that *Ackr2*$^{-/-}$ mice displayed an increased LV density in the ears, diaphragms, and popliteal LNs in resting and regenerative conditions, compared to WT mice. Further investigation on E15.5 embryonic mice showed the presence of macrophages in proximity of developing LVs and involved in developmental lymphangiogenic processes. These macrophages, recruited by CCL2 and phenotyped as $CD11b^{high}$ $F4/80^{low}$ $Lyve\text{-}1^+$, were significantly higher in *Ackr2*$^{-/-}$ mice whereas they were reduced in *Ccr2*$^{-/-}$ mice. Together, this evidence demonstrated that ACKR2 and CCR2 reciprocally regulate macrophage proximity to LVs and contribute to control lymphangiogenesis in inflammatory conditions. Accordingly, *Ackr2*$^{-/-}$ mice displayed relatively inefficient antigen presentation (35).

INSIGHTS INTO LYMPHATIC ACKR2 EXPRESSION AND FUNCTION DURING DISEASE

Several studies using ACKR2-deficient mice or human samples have demonstrated an important role of ACKR2 expressed by LVs in inflammatory conditions and in cancer. The general emerging picture is that ACKR2 acts as a negative regulator of inflammation, but contrasting results were published on its role in the control of adaptive immune responses and in autoimmune disease development.

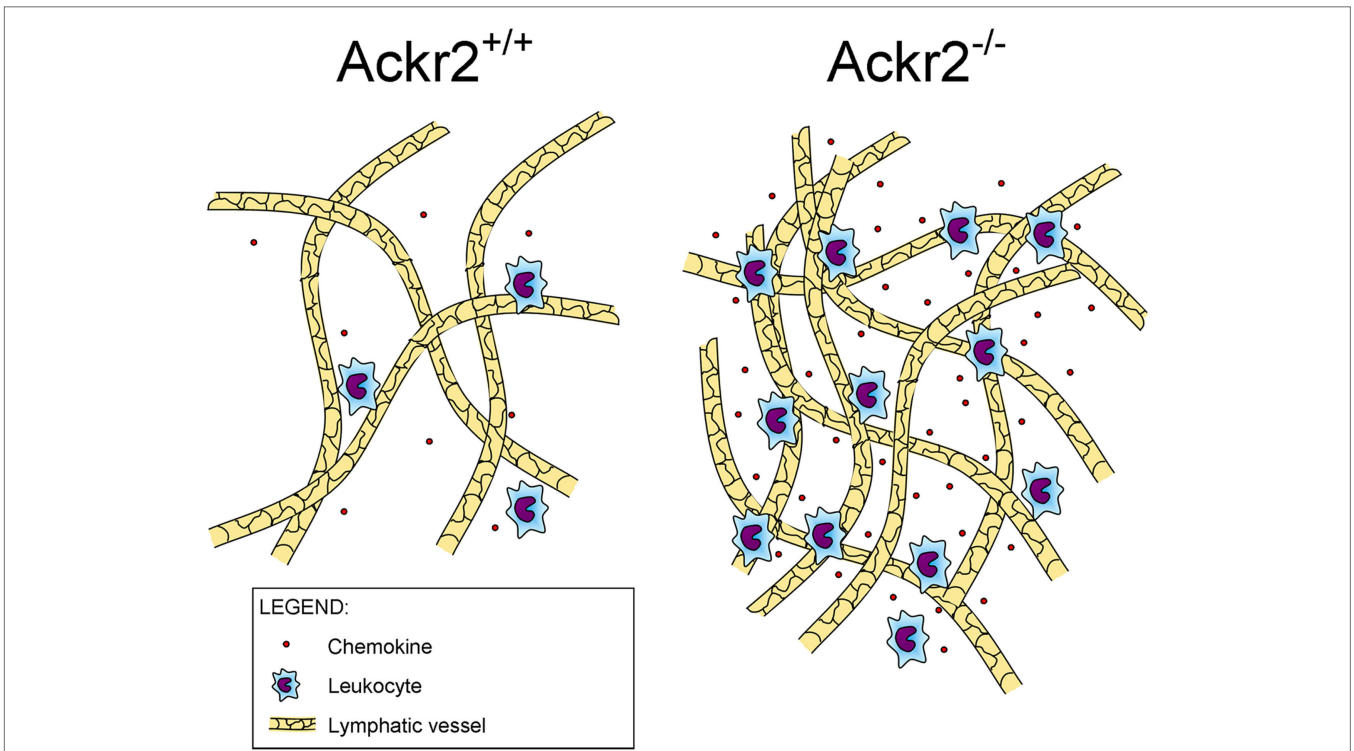

FIGURE 1 | Role of ACKR2 expression on lymphatic vessels. ACKR2 expression on lymphatic endothelial cells, by scavenging inflammatory CC chemokines, shapes functional gradients promoting appropriate leukocyte recruitment into inflamed tissues, regulates their trafficking into lymph nodes, and controls lymphatic vessel density.

The Role of Lymphatic ACKR2 Expression in Inflammation

Using murine models of cutaneous inflammation, such as phorbol ester skin painting and subcutaneous injection of complete Freund's adjuvant, it was demonstrated that *Ackr2*$^{-/-}$ mice develop an exacerbated inflammatory response with increased necrotic areas, angiogenesis, and a significantly higher leukocyte infiltration compared to WT mice (36–38). In both models, ACKR2 promoted the resolution of cutaneous inflammation by chemokine clearance.

The cutaneous lesions developing in ACKR2-deficient mice after phorbol ester skin painting resemble human psoriasiform pathology, indicating a possible role of ACKR2 in the control of this pathology (36). Using a psoriasis-like skin inflammation model induced by imiquimod, it has been shown that localized inflammation and IFN-γ induce the upregulation of ACKR2 in remote tissues that control the spread of psoriasiform inflammation inhibiting T cell epidermal influx (39). Similar results were found in psoriatic patient lesions in which ACKR2 is highly expressed in the surrounding skin in comparison with healthy controls, while it is downregulated in lesional and perilesional sites (36, 37, 40).

Several papers have also described an important role for ACKR2 expressed by lung LVs in the regulation of pulmonary inflammation. ACKR2 expression is upregulated in the LVs and in alveolar macrophages in the lung of patients with chronic obstructive pulmonary disease. In this context, the increased expression of ACKR2 could regulate the trafficking of leukocytes from the lungs to draining LNs (41, 42). ACKR2 was also found on LECs in lungs and LNs from patients with pulmonary tuberculosis (43). *Ackr2*$^{-/-}$ mice infected by *Mycobacterium tuberculosis* show reduced survival, compared to WT mice, due to an increased number of lung and LN-infiltrating mononuclear cells and an abnormal production of pro-inflammatory cytokines and CC chemokines (43). Interestingly, inhibition of inflammatory chemokines in *Ackr2*$^{-/-}$ mice led to less controlled growth of *M. tuberculosis*, indicating that ACKR2 has an important role also in immune activation. The role of ACKR2 in the lung was also studied in an allergen-induced airway disease model. Allergen-challenged ACKR2-deficient mice had more lung inflammation compared to WT counterparts, having more DCs, T cells, and eosinophils in the lung parenchyma and more eosinophils in airways. Surprisingly, ACKR2-deficient mice had reduced airway responses to methacholine compared to WT mice, indicating that ACKR2 has opposing effects on allergic inflammation and airway reactivity (44).

Contrasting results have been published on the role of ACKR2 expressed by LVs in intestinal inflammation. ACKR2 is overexpressed by LVs in the gut in inflammatory bowel disease, and it was found to have a protective role in a dextran sulfate sodium (DSS)-induced colitis mouse model (45). Indeed, *Ackr2*$^{-/-}$ mice have increased levels of inflammatory chemokines and infiltrating leukocytes, and increased intestinal inflammation, weight loss, and disease activity index, compared to WT mice (45). On the contrary, using the same murine model of intestinal inflammation, reduced clinical symptoms and tissue pathology in response to DSS in ACKR2 deficient were observed compared to WT mice. This protection is due to increased secretion of IL-17A by γδ-T cells in the lamina propria (46).

The role of ACKR2 in autoimmune diseases is also debated. ACKR2-deficient mice were described to be protected from the development of an experimental model of autoimmune encephalomyelitis (EAE) due to impaired migration of DCs and inhibition of T cell priming (47). More recently, using the same EAE model, it was described that ACKR2-deficient mice are not protected from the development of the disease but, on the contrary, they develop worse clinical symptoms compared to WT mice due to increased innate B cell-dependent production of IL-17 (48). Finally, in a murine model of graft versus host disease, we have found that ACKR2-deficient mice are protected from the development of the disease due to increased number of inflammatory monocytes with enhanced immunosuppressive activity (49).

In conclusion, ACKR2 expressed by LVs has an anti-inflammatory function by clearing chemokines present in inflamed tissues. This activity promotes the migration of DC to LN through LVs that is necessary for the induction of the adaptive response but that can be detrimental in autoimmune diseases. Conflicting phenotypes published could possibly be explained by the fact that ACKR2 is also controlling IL-17 production, a critical cytokine for inflammatory and autoimmune diseases.

The Role of LV ACKR2 in Cancer and Metastasis

In human cancer lesions, ACKR2 was found to be expressed by peritumoral LVs in oral squamous cell carcinomas and in colon cancer. Accordingly, murine models of inflammation-induced cancer in the skin and in the gut revealed that ACKR2 protects mice from the development of tumors by dampening inflammation (38, 45).

ACKR2 was found to be protective in cancer progression also when expressed by tumor cells, by inhibiting inflammatory chemokines and protumoral leukocyte infiltration. ACKR2 is expressed by vascular tumors with lymphatic origin or differentiation (23) and is highly expressed by Kaposi's sarcoma spindle cells (32, 50–52). In this latter tumor, we have found that ACKR2 expression is downregulated in more aggressive tumors by the activation of the KRAS/BRAF/ERK pathway, thus unleashing chemokine-mediated macrophage recruitment and their acquisition of an M2-like phenotype that sustains angiogenesis and tumor growth (52, 53).

ACKR2 was also found to be expressed in human breast cancer, and its expression predicts relapse-free survival (RFS) (54) while it is inversely correlated with axillary lymph node metastasis (55). Of note, a functional non-synonymous single nucleotide polymorphism of ACKR2 is associated with lymph node metastasis and RFS in breast cancer, indicating that the expression and function of ACKR2 in the host could also affect tumor progression (54, 56).

In conclusion, while it is clear that in inflammation-induced cancer ACKR2 expression by tumor cells inhibits cancer progression by decreasing macrophage infiltration and angiogenesis,

further studies are necessary to understand the exact nature of the role of ACKR2 expressed by the host and how it can affect tumor progression and metastasis.

CONCLUDING REMARKS

Lymphatic vessels have been traditionally considered as an inert drainage system, which passively transports fluids, proteins, and leukocytes. However, an increasing number of studies show that lymphatics play a much more active role, especially in the context of inflammation and ongoing immune responses. The expression of chemokine and chemokine receptors by LECs can be seen as evidence in support of an active role for lymphatics in regulating immunity. By the expression of ACKRs, LECs create and shape functional gradients of chemokines and modulate leukocyte recruitment. Moreover, they avoid inappropriate accumulation of chemokines and immune cells into inflamed tissues.

Here, we reported data demonstrating the essential role of ACKR2, expressed by LECs, in regulating chemokine concentration and leukocyte migration. This promotes the resolution of inflammatory responses in different pathological conditions including infection, allergy, and cancer. This evidence enables the speculation that ACKR2 could be considered as a potential therapeutic target to be induced in order to attenuate inflammation, e.g., during psoriasis and lung infection.

Even if the role of ACKR2 in inflammatory conditions has been clarified, further experimental studies are required to better understand its role in tumors. In this context, although an inverse correlation between ACKR2 expression and tumor stage was observed, it is unclear whether this correlation can be utilized as a clinical prognostic marker. Another challenging issue yet to be resolved is to understand whether ACKR2 could be a putative target for cancer immunotherapy. Indeed, it remains to be investigated if the activity of ACKR2 on lymphatics promotes or inhibits adaptive immune responses and whether ACKR2, by shaping chemokine gradients, can influence cancer cell dissemination to metastatic organs.

AUTHOR CONTRIBUTIONS

All the authors have contributed to this review by writing and critically evaluating the literature.

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Corrigendum: ACKR2: An Atypical Chemokine Receptor Regulating Lymphatic Biology

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REVIEW
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T Cell Trafficking through Lymphatic Vessels

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T cell migration within and between peripheral tissues and secondary lymphoid organs is essential for proper functioning of adaptive immunity. While active T cell migration within a tissue is fairly slow, blood vessels and lymphatic vessels (LVs) serve as speedy highways that enable T cells to travel rapidly over long distances. The molecular and cellular mechanisms of T cell migration out of blood vessels have been intensively studied over the past 30 years. By contrast, less is known about T cell trafficking through the lymphatic vasculature. This migratory process occurs in one manner within lymph nodes (LNs), where recirculating T cells continuously exit into efferent lymphatics to return to the blood circulation. In another manner, T cell trafficking through lymphatics also occurs in peripheral tissues, where T cells exit the tissue by means of afferent lymphatics, to migrate to draining LNs and back into blood. In this review, we highlight how the anatomy of the lymphatic vasculature supports T cell trafficking and review current knowledge regarding the molecular and cellular requirements of T cell migration through LVs. Finally, we summarize and discuss recent insights regarding the presumed relevance of T cell trafficking through afferent lymphatics.

Keywords: T cells, migration, trafficking, afferent, efferent, lymphatic vessels, lymph node

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INTRODUCTION

In an antigen-inexperienced host, the frequency of naïve T cells specific for any given antigen is extremely low, several thousand at most (1, 2). Given that the diversity of possible antigens is almost countless and that T cell activation requires direct contact with antigen, naïve T cells constantly circulate through secondary lymphoid organs (SLOs) in pursuit of antigen (1, 2). Upon encountering antigen in SLOs, antigen-specific naïve T cells proliferate and become activated effector T cells (T_{eff}) that egress from SLOs and enter peripheral tissue at sites of inflammation (2, 3). Most T_{eff} die after antigen is cleared but a few antigen-experienced T cells remain for long-term protection and either develop into tissue-resident memory T cells (T_{RM}), into central memory T cells (T_{CM}) that recirculate between SLOs and blood, or into effector-memory T cells (T_{EM}) that circulate through blood and home to inflamed tissue (1, 2). In addition to the abovementioned antigen-experienced cell types, regulatory T cells (T_{regs}) also circulate between blood, tissue, and SLOs (2–4).

Throughout the life of a T cell, the blood and lymphatic vasculature act as highways for T cell circulation. While much is known about T cell migration across and within the blood vasculature, much less is known about T cell migration into and within the lymphatic vasculature. Since the late 1950s, cannulation studies in sheep and rats have helped develop our current understanding of the cell subsets that circulate through lymphatic vessels (LVs). More recent technical advances

(summarized in **Box 1**) have helped to further improve our understanding of the cellular and molecular mechanisms of T cell migration through LVs. In this review, we first introduce the structure of the lymphatic vascular system and summarize current knowledge of the cellular composition of efferent and afferent lymph. We then review the mechanisms by which T cells exit from lymph nodes (LNs) into efferent lymphatics as well as emerging knowledge of T cell entry and migration within afferent lymphatics. Finally, new insights regarding the overall relevance of T cell circulation through the afferent lymphatic vasculature are discussed.

STRUCTURE OF THE LYMPHATIC VASCULATURE

The lymphatic system consists of central and peripheral lymphoid organs and a LV network that permeates most tissues of the body (32, 33). In peripheral tissues, extravasated fluid, macromolecules, and leukocytes, i.e., the main constituents of lymph, are taken up by a network of blind-ended lymphatic capillaries, which converge into larger collecting vessels that drain into and through LNs (33). Upon passage through chains of tissue-draining LNs (dLNs), connected by adjoining collecting LVs, lymph is finally returned to the blood vasculature through the thoracic ducts, which merge into the subclavian vein (33) (**Figure 1A**).

Tissue fluid uptake and immune cell entry/transport into LVs is thought to mainly occur at the level of the initial lymphatic capillaries, where characteristic structural features support these processes. Lymphatic capillaries are composed of partially overlapping, oak leaf-shaped lymphatic endothelial cells (LECs) that are connected by discontinuous button-like cell–cell junctions (**Figure 1B**). Moreover, lymphatic capillaries are surrounded by a thin, highly fenestrated basement membrane (34, 35). Tissue fluid and leukocytes [as best shown for dendritic cells (DCs)] enter through the characteristic flaps between overlapping LECs (34, 35). Collecting LVs are structurally more specialized for fluid and immune cell transport (**Figure 1C**). Lymphatic collectors are composed of cuboidal LECs connected by continuous zipper-like cell–cell junctions and are surrounded by a continuous basement membrane and smooth muscle cell layer (34, 35). Intraluminal valves prevent the backflow of lymph, while contraction of smooth muscle cells helps to propagate lymph toward the dLN (36).

BOX 1 | Tools to study T cell trafficking *in vivo*.

Tool	Description	Selected reference
Cannulation studies	This procedure involves the surgical insertion of cannula (tube) directly into an afferent or efferent vessel or into the cisterna chyli, to collect lymph fluid. The cellular composition of lymph is subsequently analyzed, typically by flow cytometry or microscopy methods	(5–9)
Adoptive transfer	In adoptive transfer experiments, cells are isolated from donor mice, fluorescently labeled (unless already marked by endogenous expression of a fluorophore or a congenic marker) and intravenously or subcutaneously injected into a recipient mouse. In some cases, T cells are subjected to an *in vitro* culturing step (e.g., *in vitro* activation) prior to injection. At defined time points after transfer, T cell numbers in lymph nodes (LNs) (or other tissue) are quantified by flow cytometry, LN sectioning and microscopy, or other means. While this experimental setup is technically straightforward, the transferred cells may differ from the endogenously migrating populations. Also, typically only a small fraction of cells injected subcutaneously actually migrate to dLNs or beyond	(7, 10–13)
Intravital microscopy (IVM)	This technique allows the study of migratory processes at the single-cell level and in real time. It involves fluorescence-based time-lapse imaging by, e.g., confocal-/multiphoton- or stereomicroscopy. Several mouse reporter lines expressing a fluorescent protein in lymphatic vessels (LVs) have been generated (14–18). In the case of T cells, most studies have been performed with fluorescently labeled and adoptively transferred T cells, but endogenous models are also available (19–21)	(22–26)
Intralymphatic injection	Microinjection of T cells directly into a LV upstream of a draining lymph node. Similar to adoptive transfer but permits the study of T cell entry specifically across the LN subcapsular sinus. This represents an elegant yet technically challenging method complementing IVM studies	(25)
LN egress studies	This experimental setup allows quantifying dwell time of T cells in LNs. In a typical experiment, fluorescently labeled T cells are first transferred intravenously into a recipient mouse. After an equilibration phase, further T cell ingress into LNs is blocked by administration of entry-blocking antibodies (e.g., directed against the integrin subunit α4 or against L-selectin). Antibody treatment allows the uncoupling of T cell entry from exit, which continues to occur. Exit rates, for example, can be calculated by comparing fluorescent T cell numbers in LNs at the time of antibody injection to a later time point (e.g., 24 h later; flow cytometry-based quantification)	(9, 22, 27, 26)
Photoconvertible transgenic mice	The use of photoconvertible transgenic mice permits monitoring the migration of endogenously labeled cells *in vivo*. It requires transgenic mice expressing a photoconvertible fluorescent protein in all cell types [e.g., Kaede protein (28) or Kikume Green–Red protein (29)]. Upon illumination with violet light, fluorescent proteins undergo irreversible changes that alter their fluorescent spectrum (typically a green to red shift). By selectively illuminating the tissue at a particular site (e.g., skin), one can subsequently quantify the appearance of photoconverted T cells in other tissues (e.g., dLNs) to gain insight about their trafficking behavior. The system can easily be combined with pharmacologic blockade of genes of interest. Alternatively, backcrossing onto a genetic knockout can be done	(28, 30, 31)

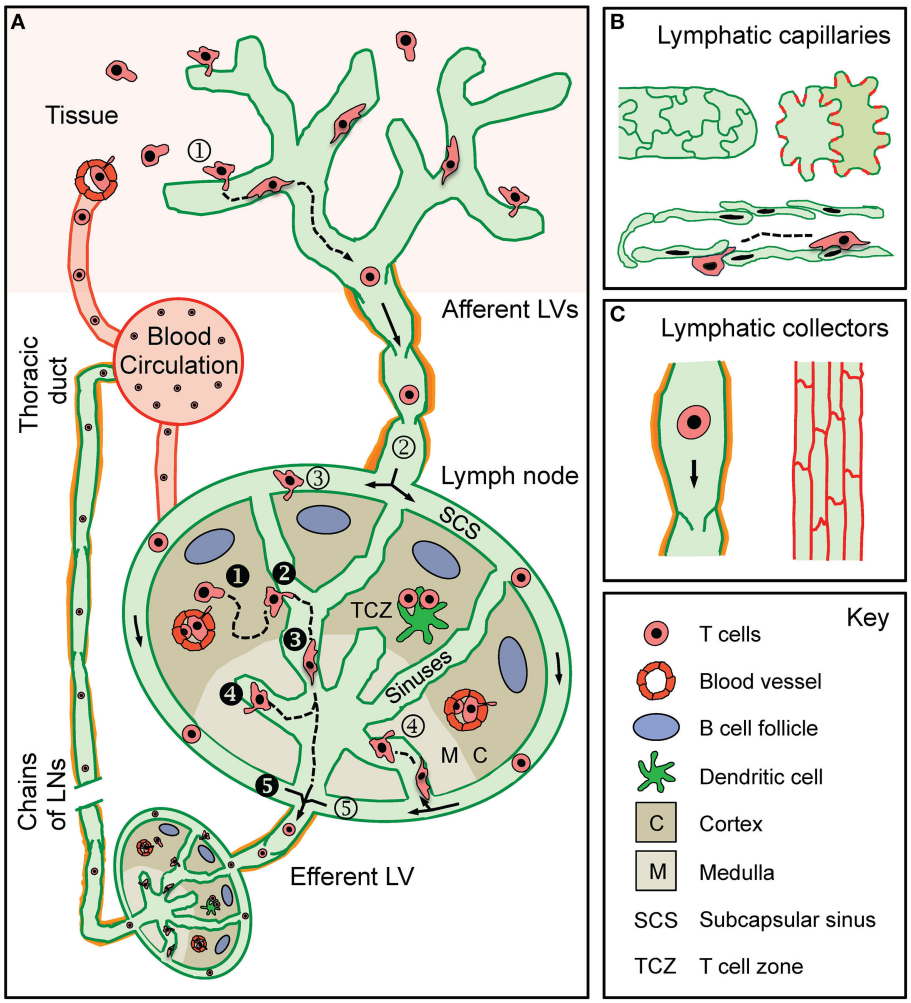

FIGURE 1 | **T cell traffic through the lymphatic vascular system**. **(A)** Recirculating effector-memory T cells in peripheral tissues ① enter afferent lymphatic vessels (LVs). The exact point of entry or the mode of intralymphatic movement has not been investigated so far. T cells that ② arrive in the lymph node (LN) subcapsular sinus (SCS) have been shown to cross the lymphatic endothelium into the LN parenchyma at the level of the ③ SCS or of the ④ medullary sinuses. Some T cells do not enter the LN parenchyma but ⑤ directly exit through the efferent LV located at the hilus region of the LN. Recirculating naïve and central memory T cells arrive in the LN either *via* the blood (high endothelial venules) or *via* the afferent LV draining from an upstream LN (i.e., efferent lymph). ❶ T cells within the LN ❷ make random contact with the sinuses before entering and ❸ actively crawling or passively flowing within the sinuses. T cells were observed to ❹ cross the sinuses several times before finally being ❺ passively carried away into the efferent LV. T cells in the efferent LV circulate through downstream LNs before being returned to the blood circulation via the thoracic duct. **(B)** Lymphatic capillaries are composed of oak leaf-shaped lymphatic endothelial cells (LECs), which partially overlap and are held together by button-like associated junctional adhesion molecules (red lines). This setup creates open flaps through which leukocytes, fluid, and macromolecules enter into the vessel lumen. **(C)** LECs in collecting vessels have a cuboidal shape and are connected by continuous cell-cell junctions (red lines). Collecting vessels contain intraluminal valves and are surrounded by a basement membrane and contracting smooth muscles cells (orange).

Collecting vessels enter the LN and convey lymph along the subcapsular sinus (SCS) and through the LN sinuses toward the efferent LV in the hilus region (37) (**Figure 1A**). Efferent lymph is then transported in the efferent collecting vessel to downstream LNs and is finally returned to the blood vasculature. Considering that LNs in mice and humans are typically arranged in chains (38), the efferent LV of a tissue-draining LN is conjointly the afferent LV of the next downstream LN. In this review, we will consider afferent lymph as lymph that has not previously passed through a LN, i.e., lymph that is derived solely from non-lymphoid tissue (as designated in **Figure 1A**).

CELLULAR COMPOSITION OF LYMPH

Most of our current knowledge on the cellular composition of lymph extends from cannulation studies (see **Box 1**). This relatively simple surgical model allows collection of lymph under physiologic conditions from a defined area of drainage over long periods of time (6, 39, 40)—and therefore most accurately reflects the composition of cells circulating through LVs. In rodents, efferent lymph can be collected from the cysterna chili in mice (8, 9, 41), or by cannulation of the thoracic duct in rats (5, 42). However, due to the small size of afferent LVs in mice

and rats, cannulation of afferent LVs in rodents is very difficult. Correspondingly, most experimental studies comparing the composition of efferent and afferent lymph have been performed in larger animals like sheep (6, 7, 39, 40, 43–45).

Efferent Lymph

Cannulation studies have revealed that thoracic duct lymph (46–48) as well as efferent lymph collected after passage through one or more LNs is mainly constituted by T lymphocytes (6, 43, 44). More than 90% of lymphocytes in efferent lymph were shown to have initially entered the LN through high endothelial venules (HEVs) (39, 43). $CD4^+$ T cells enter and recirculate through LNs more rapidly than $CD8^+$ T cells (27). Accordingly, $CD4^+$ T cells constitute the major cellular fraction in efferent lymph and outnumber $CD8^+$ T cells at a ratio higher than that in blood (49, 50). Most T cells in efferent lymph collected from sheep exhibit a naïve phenotype, with a reported increase in the proportion of memory T cells in older animals (44, 51, 52).

Antigenic stimulation of LNs often leads to distinct phases in the efferent lymph response: an initial "LN shutdown" where lymphocyte output is decreased; a "recruitment phase" where lymphocyte output rises above resting levels; and a "resolution phase" where lymphocyte output and cellular composition return to resting levels (53–55). While in most cases a sequential egress of $CD4^+$ and then $CD8^+$ T cells has been reported (56–58), the dominance of a particular lymphocyte subset in efferent lymph appears to be dependent on the antigenic stimulus (45, 59–61).

Afferent Lymph

Compared to efferent lymph, the cellularity of afferent lymph is much lower (5–10%) under homeostatic conditions (6, 43, 44). While αβ T lymphocytes represent the most abundant cell type of afferent lymph (80-90%), DCs (5–15%), monocytes, B cells, and few granulocytes are also routinely found in steady-state afferent lymph (39, 43). $CD4^+$ T cells in afferent lymph collected from sheep outnumber $CD8^+$ T cells by approximately fourfold to fivefold (6, 43, 44). As reported in sheep, $CD4^+$ T cells are the dominant cell type in afferent lymph collected from superficial dermal LVs of healthy humans (62–64). T cells in afferent lymph of both humans and sheep exhibit an effector-memory (T_{EM}) phenotype, characterized by elevated expression of common T cell activation markers, adhesion molecules, and effector cytokines (44, 45, 63, 64). Although γδ T cells are present in large numbers in afferent lymph from sheep (65), they are almost non-existent in lymph or blood in humans (63, 64) and so are not further discussed here.

As cannulation of LVs is difficult in mice, a lot of our current knowledge of the T cell populations migrating through afferent LVs in mice has come from other experimental techniques used to investigate leukocyte trafficking (see **Box 1**). Specifically, these include adoptive transfer experiments or experiments performed in transgenic mice in which migrating leukocytes can be tracked by photoconversion of endogenously expressed fluorescent proteins [e.g., Kaede mice (28)—see **Box 1**]. Conclusions drawn from these approaches in mice are in accordance with earlier cannulation studies in larger animals. Moreover, they have revealed that the $CD4^+$ T cell dominance in afferent lymph results from more efficient $CD4^+$ T cell migration from the skin to the dLN (7, 31). In Kaede mice, the majority of $CD4^+$ T cells that migrated from the skin to the dLN expressed the common T cell activation marker CD44 as well as the skin-homing molecules C–C chemokine receptor type 4 (CCR4) and E-selectin ligands (30, 31). Approximately 25% of $CD4^+$ T cells that migrated from the skin to the dLN were also found to express the T_{reg} transcription factor $FOXP3^+$ (30). Similarly, others have reported that adoptively transferred T_{regs} enter afferent LVs and migrate from the skin to dLN in mice (66–68). Notably, T_{regs} are phenotypically similar to T_{EM} and are only distinguishable when specific T_{reg} markers are used. The fact that FOXP3, the most widely used T_{reg} marker, was only described approximately 13 years ago might explain why T_{regs} have thus far not been reported from cannulation studies performed in sheep and humans (which frequently date back to earlier times).

In contrast to the conventional viewpoint that naïve T cells exclusively recirculate between blood and SLOs, low numbers of naïve T cells have also been found in both homeostatic and inflamed non-lymphoid tissues and have been suggested to circulate through afferent LVs (20, 69, 70). Indeed, in adoptive transfer experiments in mice, naïve T cells were shown to avidly migrate from the skin to dLN (7, 10). However, it is important to consider that the majority of endogenous $CD4^+$ T cells in the skin have an effector/memory-like phenotype (10, 71). Correspondingly, cannulation studies in humans and sheep, and studies in Kaede mice, suggest that naïve T cells constitute only a minor subset of T cells in afferent lymph under both steady-state (30, 44, 64) and inflammatory conditions (12, 30, 72).

Impact of Tissue Inflammation on Afferent Lymph Composition

Cannulation studies in sheep have revealed that acute skin inflammation, e.g., elicited by injection of complete Freund's adjuvant (CFA), induced a dramatic increase in granulocyte numbers in skin-draining afferent lymph, whereas $CD4^+$ and $CD8^+$ T cells initially remained fairly stable (12, 53, 72, 73). By contrast, chronic inflammation, resulting from CFA-induced granuloma formation, was shown to lead to a substantial increase in $CD4^+$ and $CD8^+$ T cell output in skin-draining afferent lymph (12, 72). Contrastingly, in Kaede mice, an acute contact hypersensitivity response elicited a striking increase in the number of T cells that migrated from the skin to the dLN (30). However, it needs to be considered that numbers of T cells in steady-state lymph of laboratory mice might be unnaturally low, because of the sterile housing conditions that lead to the formation of a reduced pool of effector-memory T cells populating peripheral tissues (74).

RECIRCULATION OF T CELLS THROUGH EFFERENT LYMPHATICS

Seminal studies performed in the late 1950s by Sir James Gowans were the first to show that lymphocytes constantly circulate between blood and SLOs (42, 46). Naïve T cells in the blood extravasate through HEVs into the LN *via* a multistep adhesion cascade and subsequently migrate to T cell areas in the paracortex

(75). Following entry into the LN, intranodal position, migration, and motility of T cells are mediated by C–C chemokine receptor type 7 (CCR7) and its two chemokine ligands, CCL19 and CCL21 (75, 76). Naïve T cells spend approximately 6–12 h surveying a LN for specific antigen and if undetected, transmigrate into cortical or medullary sinuses and exit through the efferent LV (28, 75). Below and in **Table 1**, we briefly review the chemotactic cues, adhesion molecules, and cellular processes involved in T cell egress from the LN into the efferent LVs.

T Cell Egress vs. Retention: Interplay of $S1P_1$, CD69, and CCR7

Early findings that pertussis toxin (a natural inhibitor of $G\alpha_i$-protein-coupled receptors, such as chemokine receptors) inhibited the export of mature T cells from the thymus (84), suggested that egress of T cells from the LN could also be an active process. Studies on the immunosuppressive activity of Fingolimod (FTY720), a now approved treatment for multiple sclerosis (85), incited further research on the molecular mechanism of T cell exit from LNs. FTY720 induces sequestration of lymphocytes in SLOs through retention and "log jamming" of lymphocytes on the abluminal side of the lymphatic sinuses, thereby inhibiting lymphocyte egress into circulation and migration to sites of disease (86–88). Besides histologic analysis of lymphatic sinuses, efferent lymph cannulation studies and LN egress experiments, in which T cell homing into LNs is first blocked and T cell numbers subsequently quantified over time, have been instrumental for studying T cell exit into efferent LVs (see **Box 1**).

Role of S1P

Several studies have shown that the egress-blocking activity of FTY720 can mainly be attributed to the action of FTY720 on sphingosine-1-phosphate (S1P) receptors, in particular, S1P receptor 1 ($S1P_1$) expressed on T cells (8, 9, 89, 90). The natural ligand of $S1P_1$ is S1P, an endogenous sphingolipid that mediates diverse cellular processes, including cell survival, cytoskeletal rearrangements, and cellular chemotaxis (91, 92). S1P levels in tissues are tightly controlled by sphingosine kinase 1 and 2 (Sphk1/2)-mediated production and S1P degradation, which depends on S1P lyase and other enzymes (77, 93). While erythrocytes, red blood cells, and the blood endothelium constitute major cellular sources of plasma S1P, lymph S1P is derived independently from the blood (91, 94). In fact, LECs were identified as the major source of S1P in lymph (41).

S1P levels in the blood and in lymph are much higher than in lymphoid organs (77, 95). Low concentrations of S1P in lymphoid tissues and S1P abundance in lymph was shown to create a gradient across LECs, which induces transmigration of $S1P_1$-expressing T cells into the lymphatic sinuses and egress into efferent lymph (93, 96): acting as a functional antagonist, FTY720 induces downregulation and degradation of $S1P_1$ in T cells, thereby inhibiting S1P-mediated chemotaxis across the lymphatic sinuses (8). Similar to FTY720 treatment, adoptively transferred $S1P_1$-deficient T cells were found to "log jam" around medullary and cortical sinuses and failed to egress into efferent lymph (8, 9, 23, 41). An analogous egress defect could also be evoked when the S1P gradient in LNs was experimentally destroyed, by inhibiting S1P lyase (77), or upon genetic deletion of Sphk1 and Sphk2 in LECs (41).

Modulation of $S1P_1$ for Fine-Tuning T Cell Transit Time through LNs

Similar to FTY720, high concentrations of S1P are capable of inducing $S1P_1$ internalization in T cells (92, 97). Consequently, T cells in blood express low levels of $S1P_1$ (95). Following entry into LNs *via* HEVs, T cells begin to upregulate $S1P_1$ (95). Given that entry into LN sinuses, and subsequent egress from the LN, is $S1P_1$ dependent, T cell transit time through the LN is in some manner dependent on $S1P_1$-mediated resensitization to S1P in lymph. In addition to S1P-induced receptor internalization, the C-type lectin CD69 has also been reported to regulate $S1P_1$ surface expression in T cells. CD69 is an early T cell activation marker and is upregulated in T cells by various inflammatory mediators, such as type I interferons (78, 93). CD69 has been shown to interact with $S1P_1$, thereby inducing a receptor conformation similar to the ligand bound state, leading to $S1P_1$ internalization

TABLE 1 | Molecules regulating T cell exit from lymph nodes (LNs) through efferent lymphatic vessels (LVs).

Molecule	Selected reference	Comment
$S1P_1$/S1P	(8, 9, 41, 77)	$S1P_1$-deficient T cells are retained in LNs; disruption of S1P gradient in LNs prevents T cell egress
CD69	(78, 79)	CD69 expression induces $S1P_1$ internalization and degradation in T cells resulting in T cell retention in LNs
C–C chemokine receptor type 7 (CCR7)	(22)	$CCR7^{-/-}$ T cells egress more rapidly from LNs whereas CCR7 overexpressing T cells are retained
CXCR4	(80)	Synergizes with CCR7 in retaining T cells in LNs
Leukocyte function-associated antigen 1 (LFA-1)/ intercellular adhesion molecule 1 (ICAM-1)	(26)	$CD4^+$ $LFA\text{-}1^{-/-}$ T cells egress more rapidly from LNs.
Common lymphatic endothelial and vascular endothelial receptor-1 (CLEVER-1)	(81)	Blockade of CLEVER-1 reduces T cell binding to LN sinuses *in situ* *In vivo* involvement not confirmed thus far
Mannose receptor (MR)/L-selectin	(82)	Blockade of MR/L-selectin reduces T cell binding to LN sinuses *in situ* *In vivo* involvement not demonstrated thus far
α9 integrin	(83)	Blockade of LEC-expressed α9 reduces T cell egress from LNs

and degradation (78, 79). CD69 expression by recently activated T cells therefore serves to inhibit the egress promoting function of $S1P_1$ (24, 78, 79). However, activated T cells only transiently express CD69 (98). Accordingly, once activated T cells have undergone several rounds of division and have downregulated CD69, they start to re-express $S1P_1$ and appear in circulation (8, 22). Akin to CD69 regulated surface expression of $S1P_1$ on recently activated T cells, T cell receptor signaling (the first signal of T cell activation) has been reported to induce transcriptional downregulation of $S1P_1$ (8). Transcriptional restoration of $S1P_1$ is also likely to regulate T cell egress during an immune response.

Role of CCR7

In addition to $S1P_1$, CCR7 expression levels in T cells also impact the time T cells spend in LNs. Upon antigen recognition, activated T cells downregulate CCR7 (22). Fibroblastic reticular cells within the LN produce CCL21 and help generate a gradient where CCL21 levels are highest toward the LN center and decrease toward the peripheral medullary areas (25, 99). In addition to mediating intranodal positioning, migration, and motility (75), CCR7 also confers T cell retention within LNs (22). T cells devoid of CCR7 ($CCR7^{-/-}$) egressed more rapidly than their wild-type (WT) counterparts, whereas transgenic T cells overexpressing CCR7 were retained in the LN for longer periods of time (22). Treatment with pertussis toxin restored egress competence of $S1P_1$-deficient lymphocytes and in mixed bone marrow chimeras FTY720 treatment increased the number of $CCR7^{-/-}$ T cells found in efferent lymph relative to their WT counterparts (22). Collectively, these findings suggest that CCR7 on T cells promotes their retention in LNs and that egress signals through $S1P_1$ in part overcome CCR7-mediated retention (22). Interestingly, more $CCR7^{+/-}$ than WT T cells entered sinuses, suggesting that the interplay between CCR7-mediated retention and $S1P_1$-mediated egress occurs at the level of entry into sinuses (22). More recently, it has also been reported that C–X–C chemokine receptor 4 (CXCR4) on T cells synergizes with CCR7 to retain both naïve and activated T cells in LNs (80).

Adhesion Molecules Involved in Egress across Sinuses

While it is well established that adhesion molecules and their integrin ligands play an important role in T cell entry into LNs through HEVs (100), not much is known about their role in T cell egress across lymphatic sinuses. A role for leukocyte function-associated antigen 1 (LFA-1) in delaying egress of T cells across lymphatic sinuses has recently been suggested. Following the probing of the surface of LN sinuses, $CD4^+$ T cells devoid of LFA-1 had a greater tendency to egress across sinuses and spent less time in the LN than their WT counterparts (26). This distinction was lost in mice lacking the major LFA-1 ligand intercellular adhesion molecule 1 (ICAM-1) (26).

In addition to LFA-1, the common lymphatic endothelial and vascular endothelial receptor-1 (CLEVER-1), as well as the macrophage mannose receptor (MR) or its ligand L-selectin have been implicated in T cell migration across lymphatic sinuses: when performing adhesion assays on LN sections, antibody-mediated blockade of CLEVER-1 or MR reduced binding of lymphocytes to sinus endothelium (81, 82). However, the *in vivo* involvement of these receptors in LN egress has not been demonstrated thus far. On the other hand, a possible role for the integrin α9 subunit in lymphocyte egress from inflamed LNs has recently been reported (83). Integrin α9β1 is a well-described binding partner of the extracellular matrix component tenascin-C, and both α9 and tenascin-C reportedly are upregulated in medullary and cortical LN sinuses during inflammation. The study revealed that tenascin-C binding to LEC-expressed α9β1 induced S1P production in LECs, establishing a mechanistic link between α9 integrin expression and $S1P_1$-mediated T cell egress. In fact, antibody-based blockade of α9 or tenascin-C deficiency resulted in impairment of T cell egress from inflamed LNs, reminiscent of treatment with FTY720 (83).

Cellular Insights into Egress from Intravital Microscopy (IVM)

T cell egress from LNs has not only been studied at the population level but also at the single-cell level using IVM (see **Box 1**). Such studies have confirmed previous histology-based studies showing that T cell migration and egress occurs both at the level of the cortical and medullary sinuses (23, 101). T cells were observed entering sinuses at multiple locations, however, occasionally two or more T cells entered at specific entry "hot spots" (23, 101). In cortical sinuses without flow, T cells migrated at the same speed as those in the parenchyma and occasionally exited sinuses back into the LN parenchyma (23, 24). In larger cortical sinuses with flow, T cells were more rounded, shared fairly uniform velocities, and had a lower frequency of exit back into the parenchyma (23, 24). T cells in the macrophage-rich medullary sinuses appeared to become poorly mobile and occasionally exited the sinuses and returned to the T cell zone (23). Following migration of T cells through cortical and medullary sinuses, T cells were released into the subcapsular region near the efferent vessel and moved off rapidly with lymph flow (23). Overall, T cell transit time through the LN appears to be determined by random walk encounters with lymphatic sinuses (24). Only at the level of the sinus do $S1P_1$-expressing T cells start to sense S1P in lymph, which triggers their exit into the lymphatic compartment (22–24).

T CELL ENTRY AND MIGRATION WITHIN AFFERENT LVs

In comparison to T cell egress from LNs, little is known about T cell migration from peripheral tissue into afferent LVs. As already suggested by the dominance of $CD4^+$ over $CD8^+$ T cells in afferent lymph (43, 44, 62), $CD4^+$ T cells migrate more efficiently through afferent LVs. Indeed, adoptive transfer studies (7), crawl-out experiments from murine skin explants (102), and studies in Kaede mice (31) uniformly demonstrate that $CD4^+$ T cells more efficiently exit the tissue *via* afferent LVs. This is also reflected by emerging findings from many laboratories showing that under steady-state conditions most $CD8^+$ T cells in peripheral organs form part of a slow-moving, skin-resident memory population [T_{RM}; reviewed in Ref. (103, 104)]. Although recent

studies indicate that a similar tissue-resident population also exists for CD4+ T cells (31), many CD4+ memory T cells seem to rapidly traffic through the dermis, forming part of a recirculating memory population (31, 102).

Although several molecules involved in T cell egress through afferent LVs have recently been identified, we still know fairly little about this process, particularly at the single-cell level. In fact, thus far only DC, but not T cell, migration through afferent LVs has been visualized using IVM (see **Box 1**). Interestingly, these findings have revealed that migration into and within afferent LVs occurs in a stepwise fashion: DCs enter LVs at the level of lymphatic capillaries and then crawl in a semi-directed manner within lymphatic capillaries (105–107). Only once they have reached contracting lymphatic collectors do cells switch from an active to passive mode of movement, i.e., they are passively carried away with the lymph flow toward the dLN. Similarly, neutrophils were recently found to actively crawl within dermal lymphatic capillaries (108). The reason why intralymphatic DCs and neutrophils only flow in lymphatic collectors is likely linked with the low flow conditions in lymphatic capillaries [reportedly ranging from 1 to 30 μm/s; (109, 110)], which are several orders of magnitude lower than blood flow in blood vascular capillaries (111) or peak lymph flow velocities measured in large contracting lymphatic collectors (112, 113). Although not demonstrated so far, it is therefore likely that T cell migration through lymphatic capillaries also involves an active, intraluminal crawling step (**Figure 1A**). In the following section, important molecules involved in T cell migration from the skin to the dLN will be discussed in greater detail (see also **Table 2**).

Chemotactic Exit and Retention Cues: CCR7, $S1P_1$ and Others

Classical definitions outline that non-lymphoid tissue homing T_{EM} are devoid of CCR7 (119). However, in humans, CCR7 is expressed on the majority of T cells in blood, including those that express adhesion molecules required for homing to non-lymphoid tissue (120). Consistent with these findings, 40–50% of all skin-associated CD4+ T cells in humans (121) and mice (31) express CCR7. Several studies have identified CCR7 and its ligand CCL21, which is constitutive expressed by LVs (107, 122), as one of the most important drivers of T cell migration to dLNs: adoptive transfer experiments (7, 10) and experiments performed in Kaede mice (31) have shown that compared to WT T cells, significantly fewer (in the order of 10–20%) $CCR7^{-/-}$ CD4+ or CD8+ T cells migrated from the skin to the dLN. Moreover, in a model of allergic airway inflammation, $CCR7^{-/-}$ CD4+ T_{EM} cells accumulated in the lung and airways (114). Similarly, CD4+ T_{EM} have been shown to accumulate within the epithelial tissues of $CCR7^{-/-}$ mice (123), and $CCR7^{-/-}$ T_{regs} accumulated in inflamed skin (124). Although CCR7 appears to be crucial for T cell exit from homeostatic and acutely inflamed skin, its contribution to T cell exit from chronically inflamed skin appears to be more limited (11, 12). In the case of DCs, IVM studies have recently revealed that the CCR7/CCL21 axis mediates DC migration toward and into LVs (106, 122) and also impacts the directionality of DC crawling within lymphatic capillaries (107). By contrast, the exact contribution of CCR7 to T cell migration through afferent lymphatics has not been addressed so far.

Besides CCR7/CCL21, the second best described chemotactic pathway involved in T cell exit from skin is $S1P_1$/S1P. As mentioned, LECs are considered the major contributor to S1P levels in lymph (41). Overexpression of $S1P_1$ in CD8+ T cells prevented "settling" of T_{RM} in the intestine, kidney, salivary gland, and skin, suggesting $S1P_1$ enhanced exit *via* afferent LVs (125). Similar to $S1P_1$-overexpressing CD8+ T cells, CD69-deficient CD8+ T cells failed to persist in skin after HSV infection, and treatment with an $S1P_1$ agonist restored their retention within the skin (126). Correspondingly, surface expression of CD69 and transcriptional loss of $S1P_1$ is a hallmark for CD8+ T_{RM} (127–130).

In contrast to CD8+ T_{RM}, tissue-resident CD4+ T cells have been less well characterized and studied. In a study using Kaede mice (see **Box 1**), Bromley and colleagues identified one population of CD4 memory T cells that remained in the skin and a second population, termed recirculating memory CD4+ T cells (T_{RCM}), that migrated from the skin to the dLN (31). T_{RCM} expressed a novel cell surface phenotype ($CCR7^{int/+}$, $CD62L^{int}$, $CD69^{-}$, $CD103^{+/-}$, $CCR4^{+/-}$, and E-selectin ligands+) and migrated in a CCR7-dependent manner (31). These cells displayed a trafficking behavior distinct from classical T_{EM} or T_{CM} cells in such that T_{RCM} migrated from skin to dLNs, and from circulation back into sites of unspecific cutaneous inflammation (31). The role of S1P in CD4+ T cell egress from skin has been addressed by two

TABLE 2 | Molecules regulating T cell migration through afferent lymphatic vessels (LVs) into lymph nodes (LNs).

Molecule	Selected reference	Comment
CCR7	(7, 31, 114)	Adoptively transferred or endogenous $CCR7^{-/-}$ T cells have reduced migration from peripheral tissues to dLNs
$S1P_1$/S1P	(10, 12)	Treatment of adoptively transferred CD4+ T cells or recipient mice with FTY720 or S1P significantly reduces T cell migration to dLNs
CD44/mannose receptor (MR)	(115, 116)	T cell-expressed CD44 interacts with LEC-expressed MR during CD4+ and CD8+ T cell migration into afferent LVs
Common lymphatic endothelial and vascular endothelial receptor-1 (CLEVER-1)	(117)	CLEVER-1 blockade decreases CD4+ and CD8+ T cell migration from the skin to the dLN
LT and VCAM-1	(67)	Shown to mediate migration of nT_{reg} from skin to dLNs
Macrophage scavenger receptor 1	(118)	Regulates lymphocyte entry into the LN parenchyma
PLVAP (MECA-32)	(13)	Mediates lymphocyte entry across the subcapsular sinus into the LN parenchyma

other recent studies (10, 12). Treatment of adoptively transferred T cells or of recipient mice with FTY720 or S1P significantly reduced T cell migration to the dLN (10, 12). Interestingly, acute inflammation was shown to increase S1P levels in the skin and also resulted in reduced migration of $CD4^+$ T cells to the dLN (10). This suggests that acute inflammation might induce T cell retention in the tissue.

T cells that have migrated from the skin to the dLN display high expression of CCR7, CXCR4, and $S1P_1$ (7, 10). In contrast to the involvement of CCR7 and $S1P_1$, CXCR4 was reported to have no role in T cell migration from homeostatic (10) or inflamed skin to the dLN (11). By contrast, in a pancreatic islet transplantation model, CCR2, CCR5, and CXCR3 reportedly contributed to the migration of natural T_{regs} (nT_{regs}) from the allograft to the dLN (66, 68). While LECs constitutively produce CXCL12, CCL21, and S1P (41, 131), they are also able to upregulate inflammatory chemokines under conditions of tissue inflammation (131, 132). This upregulation occurs in a stimulus-specific manner (131) and may serve to fine-tune leukocyte recruitment into LVs. Although not specifically studied so far, changes in the chemokine expression profile of LECs might also explain the reduced CCR7 and S1P dependence of T cell tissue exit observed from chronically but not from acutely inflamed skin (12). On the other hand, it has to be considered that most studies investigating T cell tissue exit have been performed using adoptively transferred T cells, which might not completely reflect the chemokine (or adhesion molecule) requirements of endogenous T cells.

Adhesion Molecules Involved in Entry and Migration within Afferent LVs

MR and CLEVER-1

Few adhesion molecules have thus far been implicated in T cell exit from skin. The MR (82), which has been shown to mediate T cell binding to lymphatic sinuses in LNs (82), is also expressed on efferent and afferent LVs (133, 134). Interaction of MR with its T cell-expressed binding partner CD44 reportedly mediates $CD4^+$ and $CD8^+$ T cell exit from the skin (115, 116). Similarly, CLEVER-1 is expressed on both efferent and afferent LVs and has been shown to mediate T cell entry into afferent LVs (81, 117, 135). Blockade of CLEVER-1 markedly decreased $CD4^+$ and $CD8^+$ T cell migration from the skin to the dLN in both mice and rabbits (117).

VCAM-1, Selectins, and Their Ligands

A recent study suggested a role for LEC-expressed VCAM-1 in homeostatic migration of nT_{reg} but not of naïve $CD4^+$ or $CD8^+$ T cells from skin to the dLN (67). VCAM-1 is a known target of LTβR (136) and blockade of LTβR reduced nT_{reg} exit from the skin (67). Similarly, fewer nT_{reg} devoid of the LTβR ligand, LTα, exited from the skin (67). As with ICAM-1, VCAM-1 expression is induced on afferent LVs during inflammatory conditions (131, 132). Whether VCAM-1 might more broadly support T cell migration through afferent LVs in the context of tissue inflammation remains to be determined. With regard to the involvement of selectins, T cell migration from homeostatic skin to dLNs was found to occur normally in mice lacking the ligands for P-, E-, and L-selectins or upon adoptive transfer of $CD62L^{-/-}$ T cells (10). However, it is noteworthy that P-selectin is also upregulated on afferent LVs during contact hypersensitivity-induced inflammation (131). This raises the question whether inflammation-induced selectins and their ligands might play a role in T cell exit under inflammatory conditions.

Insights into T Cell Entry into the LN from Afferent LVs

While several studies highlight the entry of T cells through HEVs or the migration of T cells within LNs (75, 137), few have focused on the entry of T cells into LNs from afferent LVs. Braun and colleagues investigated this entry pathway by performing time-lapse imaging in the popliteal LN following microinjection of T cells directly into the cannulated afferent LV (25). This study revealed that most naïve $CD4^+$ T cells were passively transported in the SCS to peripheral medullary sinuses where they either directly transmigrated, or first crawled within the peripheral medullary sinuses before transmigrating into the LN parenchyma at the level of the medullary sinuses (25). As reported for T cell egress from the LN parenchyma into lymphatic sinuses (23, 101), several T cells occasionally crossed the sinus floor at specific transmigration "hot spots" (25). Interestingly, naïve $CD4^+$ T cells entered across the medullary sinuses in a CCR7-independent manner, but subsequently preferentially migrated within the medulla toward the paracortical T cell zone by means of a CCR7-skewed random walk (25).

In contrast to T cells, injected DCs were able to directly transmigrate the SCS floor of the LN, allowing for a more direct access of the LN parenchyma (25). On the other hand, T cells injected after pre-injection of DCs now transmigrated the SCS floor on the afferent side of the LN and avidly migrated inward at sites of DC transmigration (25). These findings suggested that DCs induced local changes in the SCS floor during transmigration that facilitated direct entry of T cells into the LN parenchyma. Considering that afferent lymph typically contains both T cells and DCs that arrive simultaneously in the subcapsular space, it will be interesting to further explore LN entry from afferent LVs in an endogenous setup.

Other studies have suggested that T cells might enter the LN parenchyma directly through the SCS: as early as 4 h after adoptive transfer into the footpad of mice, T cells could be detected within the LN parenchyma in close proximity to the SCS (13, 118). Moreover, macrophage scavenger receptor 1, a molecule expressed on LECs of the SCS, but not on the medullary or cortical sinuses, was recently found to regulate lymphocyte entry into the LN parenchyma (118). Furthermore, the same group previously reported the involvement of plasmalemma vesicle-associated protein (PLVAP, also known as MECA-32) in lymphocyte entry across the SCS into the LN parenchyma (13). PLVAP is expressed by LECs in lymphatic sinuses where it forms diaphragms that overlay the entry to the FRC conduit system. This generates a sort of molecular sieve that restricts the access of soluble antigen into the conduit system and hence into the LN parenchyma. Interestingly, PLVAP also appeared to regulate

T cell entry into the LN, supposedly by supporting transcellular diapedesis across the SCS (13).

PURPOSE OF T CELL MIGRATION THROUGH AFFERENT LVs

As we gain more insight into T cell trafficking through LVs, our knowledge regarding the biological significance of this migratory process continues to grow. In the case of migration through efferent LVs, there is overwhelming evidence that this migratory step is crucial for immune surveillance: naïve T cells and T_{CM} constantly recirculate through blood, SLOs, and lymphatics in pursuit of antigen (1–3). Blocking this important migratory step, e.g., with FTY720, inhibits T cells recirculation and represents a powerful strategy for inducing immunosuppression, e.g., in the context of autoimmunity. On the other hand, recent data indicate that T cell trafficking through afferent LVs may not only occur to promote immune surveillance but may additionally have immune-dampening effects and serve to avoid overshooting T cell-mediated inflammatory responses. In the following section, these hypotheses shall be discussed in greater detail.

Role of T Cell Circulation through Afferent LVs in Immune Surveillance

T cell recirculation through afferent LVs is thought to contribute to immune surveillance by constantly replenishing the T cell pool in peripheral tissues with new antigenic specificities. However, increasing evidence suggests that recirculating T cells do not provide complete protection of peripheral tissues, and that T_{RM} play a more important role in this process (104, 138). Although mainly studied for $CD8^+$ T cells and in a limited number of infection models, T_{RM} (typically $CD69^{hi}$, $CD103^{hi}$, E-cadherin^{hi}, $S1PR_1^{lo}$, and $CCR7^{lo}$) have been shown to provide immediate protection against reinfection (104, 139). Current evidence suggests that T_{RM} differentiate from T_{eff}, remain resident within the tissue for long periods of time (>1 year in mice) and predominate at sites of infection or inflammation (104, 140, 141). Although there is some evidence that T_{RM} proliferate locally, it is unknown whether T_{RM} are ever replaced by circulating T cells (139, 142, 143). The protective mechanisms of T_{RM} are not yet fully known, but evidence suggests that T_{RM} functionally delay pathogen spread and further act as an antigen-specific sensor that "sounds the alarm" for the recruitment of circulating T cells (104). The relative contribution of resident and circulating T cells in pathogen clearance remains unknown and might be highly context dependent, e.g., dependent on the type of infection and the specific requirement for $CD4^+$ or $CD8^+$ T cells for immune control (104, 139).

Role of T_{reg} Tissue Exit in Controlling Immune Responses in dLNs

Previous studies have shown that the local ratio of T_{regs} to T_{eff} at inflamed sites is a critical determinant for the outcome of inflammation (144–146). In support of this notion, adoptively transferred $CCR7^{-/-}$ T_{regs} that accumulated in the skin of mice controlled Th1-mediated inflammation more efficiently than WT T_{regs} (124). While these findings suggest that retention of T_{regs} within peripheral tissue promotes resolution of inflammation, large numbers of T_{regs} reportedly exit the skin during a cutaneous immune response in mice (30).

$CD4^+$ T_{regs} control both priming and expansion of T_{eff} in SLOs and the activation of T_{eff} in the skin (147–150). Several islet allograft survival studies highlight T_{reg} migration to dLNs as a prerequisite for efficient downregulation of the ongoing allograft response (66–68, 151). Only T_{regs} within the skin, or having previously exited the skin *via* afferent LVs, reportedly displayed an activated phenotype (66). Upon adoptive transfer of egress-incompetent T_{reg} into the graft, graft survival was shorter than that for WT T_{regs} (66–68). Similarly, in a study using Kaede mice, T_{regs} that migrated from inflamed skin had an activated phenotype, inhibited immune responses more robustly than LN-resident T_{regs}, and were able to recirculate back to the skin (30). These findings suggest that T_{regs} that have exited the skin *via* afferent LVs restrict LN immune responses (and consequently tissue inflammation) and recirculate back to inflamed tissue to help further control local immune responses.

Role of Tissue Exit of Bystander T Cells in Resolving Local Inflammation

The extent of tissue inflammation often correlates with the number and composition of infiltrating T cells, which itself is dependent on T cell recruitment from blood, survival in the tissue, and, last but not least, on T cell exit through afferent LVs. Interestingly, two recent studies have shown that the ability of T cells to exit inflamed tissues has an impact on the degree of tissue inflammation. In mouse models of delayed-type hypersensitivity and TNF-driven Crohn's-like ileitis, reduced exit of $CCR7^{-/-}$ T cells from the site of inflammation translated into enhanced and prolonged inflammation (152, 153). Similarly, T cells overexpressing CCR7 had an enhanced capacity to exit from inflamed skin and accelerated resolution of inflammation (152). However, depending on the experimental setup, these experiments might have to be interpreted with caution because of the confounding influence of autoimmunity observed in $CCR7^{-/-}$ mice, which might be due to other factors in addition to limited exit from peripheral tissues (76).

While recruitment into tissue is independent of the antigen specificity of T cells (154, 155), exit of T cells from inflamed tissues appears to be at least in part antigen dependent (152, 156). In a mouse model of delayed-type hypersensitivity, transgenic $CD4^+$ Th1 cells, co-injected with DCs that were pulsed with cognate antigen, displayed reduced migration from inflamed skin to the dLN relative to polyclonal $CD4^+$ Th1 cells (152). Similarly, a significantly reduced number of antigen-specific cytotoxic $CD8^+$ T cells (Tc1), in comparison to antigen-unspecific Tc1 cells, migrated from the lung to the dLN in influenza-infected animals (156). These findings suggest that upon recognition of antigen, T cells have an impaired "tissue exit program" and are retained at the effector site, while antigen non-specific bystander T cells continue to exit *via* the afferent LVs in a CCR7-dependent manner (156). This mechanism is likely in place to reduce unnecessary tissue damage through bystander T cells.

CONCLUSION AND OUTLOOK

In addition to cannulation studies, which have for more than six decades provided insights into the cellular composition of lymph, newer techniques such as adoptive transfer studies, LN egress studies or experiments performed in Kaede mice have considerably accelerated our recent gain of knowledge regarding the molecular mechanism of T cell trafficking through LVs. At the same time, IVM studies have provided further insight into the dynamics of these processes, by visualizing the single-cell behavior and anatomic location of T cell migration toward, across, and within LVs. While T cell egress from LNs into efferent LVs has been quite intensively studied, we still know comparably little about T cell migration into and within afferent LVs, or about the subsequent T cell entry step into the parenchyma of a dLN. In the future, it will be important to better characterize the distinct T cell subsets migrating through afferent LVs and the molecules involved in their trafficking. Moreover, the importance of tissue-resident vs. recirculating memory T cells will need to be addressed in more models. Given that $CD4^+$ T cells constitute the main cell types recirculating through afferent LVs, this will be particularly relevant in the case of $CD4^+$ T cell-dependent immunity. At the same time, it will also be important to carefully chose the right animal models when studying these processes: the fact that only few memory T cells are present in peripheral tissues of laboratory mice held under optimized hygienic conditions, and that these mice respond differently to immunologic challenges in comparison to mice housed under less hygienic environments (74), indicate that our preferred experimental setups might not represent a faithful model for studying the importance of recirculating vs. tissue-resident T cells in immune recall responses.

On the other hand, we also still know very little about the potential function of recirculating T cells in dampening acute immune responses, possibly *via* tissue exit of T_{regs} or of T cells with irrelevant antigenic specificities. Recent observations that migration of DCs and neutrophils through afferent LVs involves active semi-directed migration within lymphatic capillaries suggest that the migratory process itself is more complex and might serve other purposes than the mere transport of cells to the dLN. Thus, in spite of recent advances regarding the molecular control of T cell traffic through LVs, we still know little about the biological relevance of these processes, particularly with regard to migration through afferent LVs. Ultimately, more insight into both the molecular mechanisms and the relevance are expected to contribute to identifying new targets for immunomodulatory therapies.

AUTHOR CONTRIBUTIONS

All the authors jointly wrote the manuscript and prepared the figures.

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REVIEW
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Shaping of Peripheral T Cell Responses by Lymphatic Endothelial Cells

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Lymph node stromal cells (LNSCs) have newly been promoted to the rank of new modulators of T cell responses. The different non-hematopoietic cell subsets in lymph node (LN) were considered for years as a simple scaffold, forming routes and proper environment for antigen (Ag)-lymphocyte encountering. Deeper characterization of those cells has recently clearly shown their impact on both dendritic cell and T cell functions. In particular, lymphatic endothelial cells (LECs) control lymphocyte trafficking and homeostasis in LNs and limit adaptive immune responses. Therefore, the new role of LECs in shaping immune responses has drawn the attention of immunologists. Striking is the discovery that LECs, among other LNSCs, ectopically express a large range of peripheral tissue-restricted Ags (PTAs), and further present PTA-derived peptides through major histocompatibility class I molecules to induce self-reactive $CD8^+$ T cell deletional tolerance. In addition, both steady-state and tumor-associated LECs were described to be capable of exogenous Ag cross-presentation. Whether LECs can similarly impact $CD4^+$ T cell responses through major histocompatibility class II restricted Ag presentation is still a matter of debate. Here, we review and discuss our current knowledge on the contribution of Ag-presenting LECs as regulators of peripheral T cell responses in different immunological contexts, including autoimmunity and cancer.

Keywords: lymphatic endothelial cells, peripheral tissue antigens, antigen presentation, immunomodulation, tolerance

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INTRODUCTION

The lymphatic system comprises a network of vessels together with lymphoid tissues all over the body that drain the extracellular compartment from most of the tissues. It transports lymph fluid, which is composed of immune cells and proteins drained from interstitial tissues, and helps to dispose of toxins and other unwanted components from the body. Lymphocytes follow the lymphatic system to migrate to infection sites, which supports and facilitates immune responses against potential harms. Frequently underestimated by scientists, the importance of lymphatics in controlling the immune system beyond the regulation of leukocyte trafficking has reached a new level with recent discoveries.

The initial observations of the lymphatic system date back to the Ancient Greece, referred to as "white blood." However, it was in the seventeenth century that Asellius formally discovered the lymphatic vessels or, what he called, the "milky veins" from mesenteries in dogs (1). Several diseases have been described to result from failures in the lymphatic system, some of them having life-threatening consequences, such as lymphedema (2). Even more strikingly, the role of lymphatics in

tumor spreading is known since the eighteenth century. Despite the ancient knowledge in the lymphatic system organization, our understanding in its multiple functions has rapidly evolved thanks to the unveiling of lymphatic endothelial cell (LEC) specific markers, such as the surface protein Lyve-1 or the transcription factor Prox-1, which are lacking in other endothelial cells. Several studies have subsequently demonstrated that LECs impact immune responses in many ways, including the modulation of immune cell migration and encounter, effector functions, and survival. In this review, we discuss our current understanding of the imunoregulatory properties of LECs. We specifically discuss the ability of LECs to directly impact T cell responses by presenting endogenous or exogenous antigens (Ags) to T cells in lymph nodes (LNs), and therefore to shape Ag-specific peripheral T cell responses in the context of autoimmunity and cancers.

ORIGIN AND TYPES OF LYMPHATICS

LEC Development

Nowadays, it is well accepted and documented that, during embryogenesis, LECs differentiate from specialized angioblasts in the developing veins (3, 4). Nevertheless, this has been controversial for long until just few decades ago due to, in particular, the lack of knowledge on lymphatic-specific markers. Two different hypotheses raised in early twentieth century debated the possible origin of the lymphatic system. On one hand, studies on embryonic cats suggested that primary lymph sacs arised from mesenchymal progenitors (5). On the other hand, intravenous injection of ink in pig embryos revealed that lymph sacs developed from budding of embryonic veins (6, 7). The identification of the vascular endothelial growth factor receptor-3 (VEGFR-3) (8) reinforced the latter hypothesis of a common origin for both lymphatic and blood endothelial cells (BECs). In adulthood, VEGFR-3 expression is restricted to LECs (8, 9). However, it is also expressed by angioblasts and developing veins during embryonic development (8, 10, 11). Impaired development of both lymphatic and blood endothelium in VEGFR-3-deficient mice suggested a common progenitor for LECs and BECs (11). Further ratification of VEGFR-3 requirement for lymphatic development was provided by studies modulating the expression of its main ligand, the vascular endothelial growth factor C (VEGF-C). Overexpression of VEGF-C induced lymphatic sprouting and lymphangiogenesis (12–14).

The identification of the homeobox gene Prox-1 in 1993 led few years later to the final confirmation of the theory proposing the venous origin of lymphatics. Deletion of Prox-1 in mice results in the absence of early lymphatic endothelial differentiation and, as a consequence, Prox-1 knockout mice totally lack the lymphatic system (10, 15). Prox-1 expression in particular cells of the embryonic veins at E9.5 starts the lymphatic polarization and imprints the LEC signature (10, 15, 16). Transcriptome studies showed high proximity in LECs and BECs gene expression profiles. However, Prox-1 acts as the specific regulator of genes that are inversely regulated in a type-specific manner (17, 18). Indeed, potentially all venous endothelial cells may give rise to blood or lymphatic endothelium as demonstrated by Prox-1-induced reprograming when overexpressed in BECs (16). After development, functional Prox-1 is required to maintain the lymphatic phenotype (19). The molecular mechanisms of Prox-1-driven lymphatic differentiation have been reviewed recently (4). In addition, recent studies in zebrafish validated the molecular mechanisms governing lymphatic development, further demonstrating that the vast majority of cells contributing to LECs in thoracic ducts of zebrafish raised from primitive veins (3, 20). Later in development, however, the origin of organ-specific lymphatic vasculature might be slightly different. Using cell-fate mapping technologies, a recent publication suggested a combination of venous- and non-venous-derived LECs in the developing cardiac lymphatics (21). This spatiotemporal discrepancy may explain the difficulties experienced in obtaining a fully convincing explanation in the origin of LECs.

The specification of LECs during development entails structural and functional differences between blood and lymphatic systems. In sharp contrast to the circular and closed blood vasculature, lymphatic circulation appears as a linear- and blind-ended circuit. Capillaries of the lymphatic system drain interstitial fluids from peripheral organs and tissues thanks to the particular organization of LECs in the terminal lymphatics. The uptake of interstitial fluid, macromolecules, and cells is possible due to the highly permeable thin-walled capillary vessels composed of a single layer of LECs, which are not covered by pericytes or smooth muscle cells and have little or no basement membrane (22). Lymphatic capillaries exhibit discontinuous or "button-like" junctions where the interjunctional gaps act as sites of leukocyte entry into the vessels (23, 24). Terminal lymphatic capillaries are linked to the surrounding extracellular matrix by anchoring filaments that sense changes in interstitial pressures during inflammation. This results in vessel lumen and junction aperture, therefore facilitating the uptake of tissue-derived fluids. Deeper, lymphatics change from a drainage-prone phenotype to a collector vessel morphology specialized in lymph transport. Collecting lymphatics are surrounded by pericytes and smooth muscle cells and possess a basement membrane, displaying continuous "zipper-like" junctions. The presence of valves (22, 23) ensures the lymph circulation while preventing retrograde flow.

Main LEC Types

Lymphatic vessels are present in almost all the vascularized organs, with the exception of the bone marrow. LEC immune modulatory properties represent a growing research area. LN LECs being the most characterized subset and representing the objective of this review is not discussed in this section.

However, lessons taken from studies performed during the last decade clearly establish different functions and possible roles for LECs from different anatomic locations. Deeper and careful future analyses will identify specific immunoregulatory features of distinct LEC populations.

For decades, lymphatic drainage was suggested to be involved in local immune responses (25). Dendritic cells (DCs) draw all the attention in initiating and eliciting tolerance or activation of the immune system. However, the role of lymph drainage in modulating adaptive immunity and tolerance remained largely unexplored. K14-VEGFR-3-Ig mice express soluble VEGFR-3-Ig

via the keratin 14 promoter, resulting in a lack of lymphatic growth, which is restricted to the skin, and in a drop in fluid clearance (26). In these mice, local lymphatic drainage appeared to be critical for humoral immunity and acquired tolerance, while T cell responses remained delayed but mostly unaffected. There is no doubt that additional mechanisms and functions of dermal LECs will be discovered in the future.

LSECs could be seen as LEC counterparts in the liver. First described in 1970 (27), LSECs possess a high ability to filter fluids, solutes, and particles from hepatic circulation, occupy a large surface area exposed to blood that carries external food and commensal bacterial Ag, and are known to cross-present exogenous Ag to T cells (28).

A traditional dogma states the immune privilege and lack of lymphatic system in the central nervous system (CNS). This idea has persisted despite the notion of immune surveillance of T cells in the brain (29). A recent and elegant study identified for the first time the lymphatic vasculature in a specific area of the meninges lining the dural sinuses (30). The vessels express LEC-specific markers such as Lyve-1, Prox-1, or Podoplanin and drain the cerebrospinal fluid to deep cervical LNs. These findings provide new insights in the establishment and progression of some neurological diseases involving immune cell contribution, such as multiple sclerosis or Alzheimer's. Moreover, CNS-resident stromal fibroblastic and endothelial cells were shown to guide antiviral $CD8^+$ T cell responses in a model of virus-induced neuroinflammation (31). The production of CCR7 ligands CCL19 and CCL21 by CNS stromal cells was found critical for the induction of viral-specific T cell recruitment and the support of local T cell reactivation. Whether newly discovered CNS lymphatics (30) similarly contribute to neuroinflammatory immunopathologies remains to be determined.

Lymphatic development in the tumor microenvironment, known as tumor lymphangiogenesis, has been extensively studied. The participation of tumor lymphatics in the spread of the disease, or metastasis, has been studied for many years. In fact, most human melanomas and carcinomas metastasize through the lymphatic system (32). The presence of tumor-associated LECs correlates with bad clinical outcome in several types of cancer (33) and therapies aiming the blockade of tumor lymphangiogenesis are being considered for treatment of such malignancies (34). Growing evidence highlight the impact of tumor-associated LECs in dampening antitumor immunity. How interactions between lymphatics and T cells in the context of tumor development will further alter T cell responses is discussed below.

Ag PRESENTATION INDEPENDENT IMPACT OF LECs ON PERIPHERAL T CELL RESPONSES

Hallmarks of T cell immunity include the generation of pathogen-specific effector responses to confer protection against a large range of invaders, without causing unwanted self-tissue damage. Naïve T cells constantly scan for their cognate Ag. However, given the extremely low frequency of T cells being specific for a particular peptide–major histocompatibility (MHC) complex (35, 36), this challenging task is strictly located into highly organized secondary lymphoid organs (SLOs), such as LNs, Peyer's patches (PPs), and the spleen. These SLOs contain both tissue-derived and blood-borne Ags, therefore facilitating naïve T cell-Ag encounter, and subsequent T cell activation and differentiation into T cell effectors. This part summarizes the different pathways by which LECs will impact T cell outcome inside and after exiting LNs.

Ag Delivery to LNs

As described before, LNs are connected to lymphatics, which drain peripheral tissue-derived fluids. By connecting tissues to draining LNs, LECs facilitate the passive entry of tissue-derived Ags that can thereby be captured, processed, and presented by resident DCs to T cells entering LNs through high endothelial venules (37, 38). Soluble Ags are immediately sampled by LN DCs, whereas particles carrying Ags, such as exosomes, apoptotic bodies or microvesicles, which have not been captured by subcapsular sinus macrophages, flow to LN medullary sinuses where they can be sampled by DCs (39). LECs also support the active migration of tissue-resident DCs into LNs. DC migration from tissues to draining LNs *via* lymphatic vessels is an important way to present Ags and activate naïve T cells. DCs enter afferent lymphatics through preformed portals (40), independent of integrin-mediated adhesion (41). However, LECs upregulate adhesion molecules upon inflammation, further favoring DC access to lymphatic vessels (42). In addition, expression of CLEC2 (a C-type lectin receptor) by DCs promotes their migration to LNs *via* lymphatics through interaction with its ligand gp38 (Podoplanin), which is expressed by both LECs and fibroblastic reticular cells (FRCs) (43).

Modulation of DC Functions

Tissue-resident DCs having acquired peripheral Ags subsequently migrate through afferent lymphatics into LNs in a CCR7-dependent manner. However, the lymphatic system does not only support DC migration from tissues to LNs. Indeed, close interactions between migrating DCs and LECs induce phenotypic and functional changes in DCs. First, contacts between TNF-α-stimulated LECs and DCs lead to decreased expression of costimulatory molecules by DCs *in vitro*, thus impairing DC ability to induce T cell proliferation (44). LEC-mediated regulation of DC functions is dependent on interactions between CD11b (Mac-1) on DCs and ICAM-1 on LECs (44). Interestingly, LECs are able to inhibit the function of LPS-activated DCs, suggesting once again a regulatory role for LECs in the resolution phase of inflammation. A recent report demonstrated that LECs function as reservoirs of peripheral tissue-restricted Ags (PTAs), which are subsequently acquired and presented by DCs to induce T cell anergy, therefore contributing to peripheral $CD4^+$ T cell tolerance (45).

T Cell Homeostasis

While T cell migration inside LNs is mainly driven by CCL19 and CCL21 produced by FRCs (46), naive and memory T lymphocyte maintenance in SLOs is highly dependent on IL-7. Together with FRCs (47), LECs represent an important source of IL-7 *in vivo*, regulating lymphocyte homeostasis and access to SLOs. IL-7-GFP

knock-in mice exhibit moderate GFP expression in LN-FRCs, whereas high levels were detected in both LN LECs and tissue LECs (48, 49). Similarly, LECs were shown to be the major source of IL-7 in both human and murine LNs (50). Furthermore, LECs not only produce IL-7 but also express the IL-7 receptor chains IL-7Rα and CD132, suggesting a possible role for IL-7 as an autocrine mediator of lymphatic drainage. IL-7-stimulated LECs induced lymphangiogenesis in the cornea of mice *in vitro*, whereas in IL-7Rα$^{-/-}$ mice, lymphatic drainage was compromised (51). In addition, IL-7 upregulation by both FRCs and LECs is essential for LN reconstruction and remodeling following viral infection or avascular transplantation (50). This suggests that IL-7 production in LN after resolution of an infection could be involved in memory T cell homeostasis. Accordingly, IL-7 promotes the development, the proliferation, and the survival of memory CD8$^+$ T cells (52, 53).

T Cell Egress from LNs

T cell egress from LNs is dependent on their expression of the sphingosine-1-phosphate (S1P) receptor (S1PR1). Using mice lacking S1P selectively in LECs while maintaining normal blood S1P, Cyster and collaborators have shown that LECs are an *in vivo* source of S1P in LNs, allowing T cell egress from LNs and PPs (54). S1PR1 expression is downregulated by blood circulating lymphocytes, and upregulated in LNs. Interactions between S1P-producing LECs and S1P1R-expressing T cells promote LN egress by overcoming retention signals mediated by CCR7 (55, 56). Although steady-state LECs express low levels of S1P, its production is upregulated in medullary sinus LECs upon PAMP/DAMP-mediated inflammation, suggesting that high S1P-expressing LECs can promote effector T cell egress from LNs in pathogenic situations. In contrast, in non-infectious sterile inflammatory contexts, low S1P-producing LECs would rather dampen T cell effector functions by favoring T cell retention in LNs.

T Cell Migration in Tumor-Associated Lymphatics

Increasing evidence suggest that tumor-associated lymphatics not only simply function as tumor cell transporters but also play additional important roles impacting tumor development. Accordingly, not only metastatic but also primary tumor progression can be affected by modulating tumor-associated lymphatic expansion. In the context of solid tumors, lymph flow from tumors is elevated, driving intense interstitial flow in the tumor stroma and increasing lymphatic drainage from the tumor to the draining LN (57). Combined with a suppressive cytokine environment, it is therefore possible that increased tumor Ags drainage could promote tumor-specific T cell dysfunction, including anergy and apoptosis. In addition, the lymph supports cells migrating from tissues, in particular CCR7$^+$ DCs, a phenomenon shown to be critical for initiating antitumor immune responses (58).

Tumor infiltration by T cells is one of the key steps in antitumor immunity. While cytotoxic T lymphocyte infiltration correlates with good prognosis, accumulation of T regulatory cells (Treg) or naïve T cells is detrimental for the clinical outcome (59, 60). Likewise, expression of CCL21 in the tumor promotes immune escape and tumor progression (61), which may be explained, at least in part, by the enhancement of naïve T cell recruitment. Although T cell receptor (TCR)-transgenic tumor-infiltrating naïve T cells may be activated *in situ* (62), it is unlikely, given the immunosuppressive tumor-related environment, that this will lead to fully competent effector T cell differentiation. In this regard, it is still to be demonstrated whether CCL21-producing LECs contribute to this effect. How LECs contribute to the overall tolerogenic properties of the tumor microenvironment is still an open question.

We have demonstrated that the lymphangiogenic growth factor VEGF-C produced in the tumor promoted immunological tolerance in murine melanoma (63). VEGF-C protected tumors against preexisting antitumor immunity and promoted local deletion of tumor-specific CD8$^+$ T cells (63, 64). Our findings introduce a new role for lymphatics in promoting tumor development and suggest that lymphatic endothelium in the local microenvironment may be a novel target for immunomodulation. Supporting those hypotheses there is a recent publication demonstrating that following exposure to tumor-derived factors, FRCs of the tumor-draining LNs adapt on multiple levels to exhibit features associated with immunosuppression, such as decreased production of IL-7 and CCL19/21 (65). Whether a similar profound reprograming occurs to LECs in tumor-draining LNs remains to be determined.

Ag PRESENTATION-DEPENDENT IMPACT OF LECs ON PERIPHERAL T CELL RESPONSES

In addition to their ability to modulate T cell responses by impacting immune cell migration, interactions, and homeostasis, LECs can also function as Ag-presenting cells through several mechanisms and directly influence peripheral T cell outcome.

Presentation of Endogenously Expressed PTAs to T Cells by LECs

In order to prevent autoimmunity, thymocytes go through a process of negative selection, part of the so-called central tolerance, allowing the deletion of autoreactive T cell clones before they exit from the thymus to enter into the periphery [reviewed in Ref. (66, 67)]. In the thymus, medullary thymic epithelial cells (mTECs) promiscuously express PTAs, Ag that are normally expressed in the periphery (68, 69). The expression of a vast majority of PTAs in mTECs is regulated by transcription factors (70), including the autoimmune regulator (Aire), mutations in Aire leading to severe autoimmune disorders (71, 72). PTAs can be either directly presented by mTECs to the thymocytes, acquired from mTECs by thymus-resident DCs or acquired in tissues by migrating DCs or plasmacytoid DCs (pDCs), and cross-presented to the thymocytes (73–76) (**Figure 1A**). Thymocytes expressing a TCR with a too high affinity for self-Ag/MHC complexes undergo clonal deletion (73–75). A fraction of the CD4$^+$ thymocytes having a TCR with a high affinity differentiates into thymus-derived Tregs (tTregs), previously called natural Tregs (nTregs), and expresses

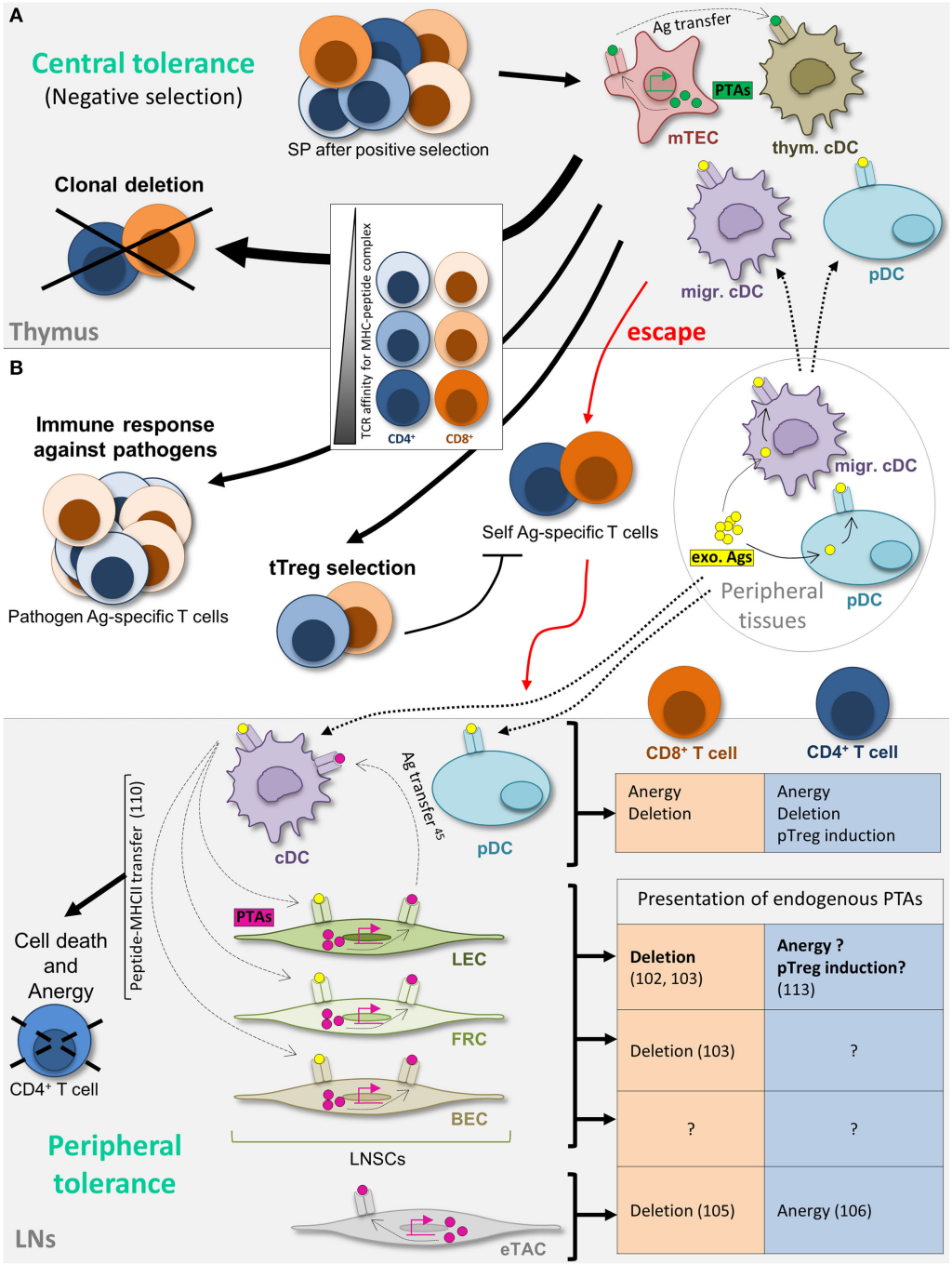

FIGURE 1 | **Maintenance of T cell tolerance**. **(A)** Schematic view of thymic central tolerance, reviewed in Ref. (67). After positive selection (not depicted), simple positive (SP) thymocytes undergo a process of negative selection. Thymus-resident conventional dendritic cells (cDCs) and peripheral tissue-restricted antigens (Ags) (PTA) (green)-expressing medullary thymic epithelial cells, as well as peripheral plasmacytoid DCs (pDCs) and cDCs, that have acquired Ag (yellow) in the periphery and migrate to the thymus, present self-peptide major histocompatibility complex (MHC) complexes to SP thymocytes. Thymocytes expressing a T cell receptor (TCR) with high affinity for self (dark colors) are clonally deleted. SP expressing a TCR with intermediate affinity differentiate into thymus-derived T regulatory cell (tTreg) (medium colors). Low-affinity TCR-expressing SP (light colors) exit from the thymus and enter the periphery, however comprising some self-reactive T cells (dark colors) that escaped central tolerance. **(B)** Peripheral T cell tolerance in the lymph nodes (LNs). References related to lymph node stromal cell contributions are indicated (numbers). Self-Ag-specific T cell tolerance is further maintained in the periphery in LNs. cDCs and pDCs acquire Ag from peripheral tissues (yellow) and migrate to LNs to present Ag to autoreactive T cells. cDCs also acquire Ag expressed by lymphatic endothelial cells (LECs). LECs, fibroblastic reticular cells, and blood endothelial cells present endogenously expressed PTAs (pink), as well as peptide–MHC-II complexes acquired from cDCs, therefore contributing to peripheral T cell tolerance *via* distinct mechanisms. Extrathymic autoimmune regulator (Aire)-expressing cells (eTACs) present endogenously expressed PTAs. The outcome of Ag presentation by each cell subtype is depicted in the figure. Cell migration and Ag transfer are represented by dotted and dashed arrows, respectively. exo Ags, exogenous antigens; migr. cDC, migratory cDC; pTreg, peripherally induced Treg; thym. cDC, thymus-resident cDC.

the transcription factor Foxp3 (77). A population of $CD8^+$ $Foxp3^+$ tTregs has also been described (78–81). However, some autoreactive—non-Treg—T cells do escape thymic central tolerance mechanisms and reach the periphery (82, 83), as a result from either an absence of specific self-Ag presentation in the thymus, or a lack of deletion due to a TCR exhibiting an affinity for self-Ag/MHC complexes below the negative selection threshold (84) (**Figure 1A**).

Therefore, additional mechanisms, called peripheral tolerance mechanisms, have evolved to maintain T cell tolerance apart from the thymus [reviewed in Ref. (66, 85)]. Cross-tolerance induction by peripheral DCs has been extensively studied and reviewed over the past two decades (86); immature DCs acquire Ag through the phagocytosis of apoptotic cells in peripheral tissues to present them to T cells in SLOs (87–89). In the absence of costimulatory signals, Ag presentation leads to $CD4^+$ and $CD8^+$ T cell clonal deletion (physical elimination) or anergy (functional inactivation) and/or to the induction of peripherally induced Tregs (pTregs), previously called induced Tregs (iTregs) in the presence of anti-inflammatory factors (77, 90–92). Both resident and migratory DCs, including pDCs, contribute to this process in the LNs (93–96) (**Figure 1B**). Nevertheless, emerging evidence demonstrates that peripheral tolerance does not exclusively rely on DCs. Lymph node stromal cells (LNSCs), and in particular LECs, also play an important role in the maintenance of peripheral tolerance (**Figure 1B**).

PTA-specific Expression by LECs

The discovery of the ectopic PTA expression by mTECs in the thymus was the first example that cells of non-hematopoietic origin present endogenously expressed self-Ag to T cells (68, 69). Using GFAP-HA or iFABP-tOVA transgenic mouse models, in which hemagglutinin (HA) or a truncated form of ovalbumin (tOVA) are expressed as self-Ag in enteric glial cells (EGCs) or mature intestinal epithelial cells (IECs), respectively, it was shown few years ago that the EGC-associated HA or IEC-associated tOVA proteins were unexpectedly expressed not only by EGCs or IECs but also by CD45-negative stromal cells, in all LNs and not exclusively in mesenteric LNs. Those LNSCs were able to process endogenously expressed self-proteins into antigenic peptides to directly present these Ag to $CD8^+$ T cells in SLOs, making them functionally similar to mTECs in the thymus (97–100). Moreover, it was shown in non-transgenic mouse models that LNSCs naturally express PTAs and directly present them to $CD8^+$ T cells. Among other examples, LNSCs ectopically express tyrosinase (tyr), while its expression is normally confined to melanocytes (101). It was later shown that LECs are the only cells ectopically expressing this Ag in the LN (102, 103). Indeed, using CD31 and gp38 (Podoplanin) as markers to distinguish the LNSC subtypes, it was observed that each subtype expresses a distinct set of PTAs, with some PTAs exclusively expressed in one specific LNSC subset and some others redundantly expressed (102, 103) (**Figure 1B**). This suggests a non-redundant role for the different LNSC subtypes in the tolerization of various self-specific T cells. In addition, the expression of PTAs by LECs is subanatomically compartmentalized, with a high expression of PTAs observed only in LN medullary sinus LECs (104).

In mTECs, the expression of most, but not all, PTAs is regulated by Aire (70, 71). In the LN, a rare bone marrow-derived population was described to express Aire and was called extrathymic Aire-expressing cells (eTACs). Consequently, eTACs express various PTAs in an Aire-dependent manner, and present them through major histocompatibility complex class I (MHC-I) and MHC-II molecules to induce $CD8^+$ T cell deletion (105), and $CD4^+$ T cell anergy (106), respectively (**Figure 1B**). On the contrary, PTAs expressed by non-hematopoietic LNSCs, including LECs, are not dependent on Aire (103). The regulation of the expression of the pancreatic self-Ag Ppy by LECs in pancreatic LNs depends on the transcriptional regulator Deaf1, which, together with Aire, belongs to the SAND gene family (107, 108). Interestingly, variant isoforms of Deaf1 in mice and human display an impaired Ppy expression, and were linked to autoimmune type I diabetes (107). The fact that LNSCs do not express Aire may explain the low overlapping PTA expression in mTECs and LNSCs (109), therefore suggesting a complementary contribution of mTECs and LNSCs in T cell tolerance induction and maintenance. Future investigations will identify other transcription factors, selectively or commonly expressed by LNSC subsets, which promote different PTA expression.

PTA Presentation by LECs to T Cells

LNSCs not only endogenously express PTAs but also the direct presentation of PTA-derived peptides in the context of MHC-I molecules to $CD8^+$ T cells leads to their clonal deletion and subsequent tolerance induction (97, 98, 101) (**Figure 1B**). In the GFAP-HA or iFABP-tOVA models mentioned above, the lack of presentation of HA or tOVA by enteric stromal cells to HA- or tOVA-specific $CD8^+$ T cells was associated with enteric autoimmunity. Among other LNSC subsets, LECs are involved in this $CD8^+$ T cell deletional tolerance and are necessary and sufficient for the induction of peripheral tolerance to some self-Ag, like Tyr, an autoantigen associated with autoimmune vitiligo (102, 103, 107). These studies show a crucial role for LECs in the maintenance of peripheral tolerance.

Nevertheless, the ability of LNSCs, and in particular LECs, to directly present endogenously expressed PTAs in the context of MHC-II molecules to $CD4^+$ T cells is still a matter of debate, as well as the subsequent impact on $CD4^+$ T cell outcome. We have previously shown that the endogenous expression of MHC-II molecules is regulated in LECs, BECs, and FRCs by the promoter IV (pIV) of the master regulator CIITA (110). One study has however demonstrated that the adoptive transfer of HA-specific TCR transgenic $CD4^+$ T cells (6.5) in GFAP-HA transgenic mice, in which HA is expressed as an autoantigen by EGCs, did not dampen lethal enteric autoimmunity (98). However, as mentioned by the authors, the absence of direct presentation of HA peptide by LNSCs to HA-specific $CD4^+$ T cells in their model does not rule out a possible upregulation of MHC-II molecules in LNSCs and a direct presentation under pro-inflammatory conditions (98). Indeed, several studies that will be discussed later in this review have suggested that LNSCs, among which LECs, upregulate MHC-II molecules at their surface upon inflammation (110, 111).

For their part, Engelhard and colleagues claim that LECs are unable to present endogenously expressed PTAs (β-galactosidase, membrane-bound HA or I-Eα in their models) to $CD4^+$ T cells, not related to Ag localization but due to a lack of H2-M expression in LECs, which would prevent the loading of peptides onto MHC-II molecules (45). However, this study was carried out in the steady state, whereas LECs, BECs, and FRCs, that express IFN-γ inducible-CIITA pIV, might require IFN-γ to upregulate H-2M molecules, as they do for MHC-II expression, these two genes being co-regulated by CIITA (112). Moreover, Mebius and colleagues observed the presence of mRNA transcripts for H2-M in LECs, among other MHC-II-related molecules (113).

Mebius and colleagues identified that in transgenic mice expressing OVA under the control of the keratin 14 promoter (K14mOVA mice), OVA was unexpectedly expressed in LECs. In addition, OVA^+ LEC were able to present OVA peptides through MHC-II to OTII cells *in vitro*, leading to an increased $Foxp3^+$ OT-II cells Treg homeostasis (113). Using LN transplantation experiments, the authors further suggested that the presentation of endogenously expressed self-Ag by LNSCs, and especially by LECs, contribute *in vivo* to the maintenance of $Foxp3^+$ $CD4^+$ Tregs in the periphery (**Figure 1B**) (113). Finally, lentiviral vectors allowing the selective transduction of $MHC\text{-}II^+$ non-hematopoietic cells with MHC-II- and MHC-I-restricted HY male-derived epitopes induced T cell hyporesponsiveness/anergy of HY-specific $CD4^+$ and $CD8^+$ T cells in female mice (114). Moreover, in Marilyn TCR transgenic mice expressing HY-specific $CD4^+$ T cells, increased conversion of effector $CD4^+$ T cells into $CD25^+$ $Foxp3^+$ pTregs was observed (114). Whether these effects were due to a direct Ag presentation of endogenously expressed HY to $CD4^+$ T cells by $gp38^+$ stromal cells, i.e., LECs and FRCs in the LN, remains to be determined. Indeed, as stated by the authors, they cannot rule out that other, non-DC, hematopoietic cell types could contribute to the presentation of HY Ags, due to undesired transduction and subsequent direct Ag presentation and/or Ag transfer to stromal cells (110, 114). Despite a lack of demonstration of direct Ag presentation by $gp38^+$ stromal cells and the lack of distinction between the contribution of the different stromal cell subtypes in this model, these data are in accordance with the results of Baptista et al., as mentioned above (113).

Molecular Pathways Involved in LEC-Mediated Peripheral T Cell Tolerance

The molecular pathways involved in the clonal deletion of $CD8^+$ T cells by LNSCs, and in particular by LECs, are not fully elucidated. Using the iFABP-tOVA transgenic mouse model described above, in which tOVA is expressed as a self-Ag in the intestinal epithelium, it was shown that the induction of $CD8^+$ T cell tolerance requires PD-1:PD-L1 interaction, as the disruption of this pathway leads to severe intestinal enteric autoimmune disorder (115). More specifically, in a model of adoptive transfer of Tyr-specific TCR transgenic $CD8^+$ T cells (FH T cells) into Tyr-expressing bone marrow chimeric mice, in which either radiosensitive hematopoietic or radioresistant non-hematopoietic cells lacked PD-L1 expression, FH T cells were deleted only when PD-L1 was expressed by the non-hematopoietic LN compartment (116). Moreover, among the LNSC subsets, LECs were the ones expressing the highest level of PD-L1, with medullary sinuses LECs being the highest expressers. In addition, LECs do not express costimulatory molecules at their surface. The administration of agonistic anti-4-1BB antibodies prevented the deletion of FH $CD8^+$ T cells. The lack of costimulation through 4-1BB by LECs would lead to PD-1 upregulation by FH T cells, as Tyr presentation by LECs led to a higher expression of PD-1 by FH T cells, an effect that was suppressed upon agonistic anti-4-1BB antibody administration. This would, in turn, prevent CD25 upregulation, which is necessary for $CD8^+$ T cells survival. Indeed, CD25 expression on FH T cells was upregulated only in the presence of agonistic anti-4-1BB or blocking anti-PD-L1 antibodies after Tyr presentation by LECs (116). Hence, in this model, LECs are responsible for the presentation of the endogenously expressed Tyr, which, together with a combination of a lack of costimulation and a provision of co-inhibitory signal, leads to Tyr-specific $CD8^+$ T cell deletion (116). The high expression of PD-L1 in LECs is likely regulated by lymphotoxin β receptor (Ltβr), as the treatment of mice with anti-Ltβr antibodies led to decreased PD-L1 expression in LECs (104). Using $\mu MT^{-/-}$, $CD3\varepsilon^{-/-}$, and $Rag1^{-/-}$ mice, it was further shown that B cells are required for the expression of the adhesion molecule MadCAM-1 at the surface of LECs in the medulla, itself necessary for the expression of PD-L1. On the contrary, T cells seemed to suppress PD-L1 expression in LECs through mechanisms that have not been deciphered yet (104). Finally, it was suggested that the expression of MHC-II on LECs would be involved in the induction of $CD8^+$ T cells tolerance to endogenously expressed self-Ag in LECs by engaging the inhibitory molecule LAG-3. Indeed, after adoptive transfer of β-gal-specific TCR transgenic $CD8^+$ T cells (Bg1 cells) into Prox-1xβgal mice, in which β-gal is selectively expressed by LECs, the proliferation of Bg1 cells was increased following administration of blocking anti-LAG-3 antibodies, which was acting in synergy with anti-PD-L1 blocking antibodies (45).

We previously showed that high PD-L1 expression by LECs correlate with their unique ability, compared to other LNSC subsets, to induce $CD4^+$ T cell apoptosis after presentation of DC-acquired peptide–MHC-II complexes (110). Although the molecular mechanisms accounting for the induction of tolerance to MHC-II-restricted self-Ag endogenously expressed and directly presented by LECs to $CD4^+$ T cells have not been elucidated so far, they are thus likely to involve PD-L1 expression by LECs, as in the case of $CD8^+$ T cells.

Ag Acquisition and Presentation by LECs to T Cells

The lymphatic system, by controlling Ag availability, constitutes one of the first checkpoints for immune responses (100). It is not surprising then that LECs, which have early access to any given Ag, display different mechanisms for Ag uptake and processing (**Figure 2**). Indeed, recent work revealed that Ag trafficking can be observed at more levels than the classical concept of LECs as lymph carriers. Complex interactions between

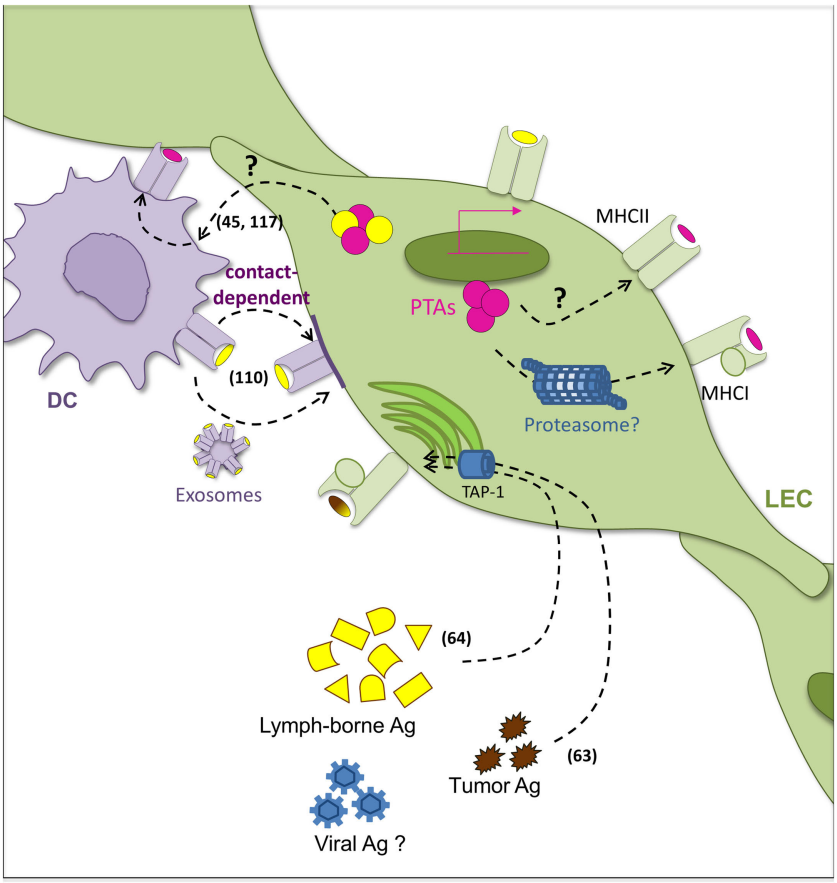

FIGURE 2 | Pathways of Ag acquisition and presentation by LECs. Several pathways of antigen (Ag) acquisition and loading coexist in lymphatic endothelial cells (LECs). Interactions with dendritic cells (DCs) underlie complex mechanisms of Ag transfer in both directions. On one hand, LECs act as Ag reservoirs for DCs which can uptake LEC-derived Ag. The mechanisms accounting for this phenomenon remain however unclear. On the other hand, LECs acquire peptide–MHC-II complexes from DCs in a cell–cell contact dependent manner (DC-derived Ag is depicted in yellow). DC-derived exosomes might also be implicated. Peripheral tissue-restricted Ags (PTA) (in pink) expressed by LEC can be loaded into MHC-I molecules. Intracellular pathways of degradation of such PTAs have however been not investigated. Moreover, whether PTA can be incorporated in MHC-II compartments is still a matter of debate. Alternatively, LECs possess the ability to uptake exogenous lymph-borne and tumor-derived Ag that can be incorporated in MHC-I pathway in a TAP-1-dependent manner. Related references are indicated in numbers.

LECs and DCs (45, 110, 117) depict an exciting picture of Ag bidirectional exchange that ultimately may serve to modulate the overall magnitude of the immune response (**Figure 2**).

Uptake of Exogenous Ag

It has been extensively demonstrated in several mouse and human models that LECs exhibit an active endocytotic capacity (38, 118). They are able to uptake exogenous molecules and, depending on their location, process Ag for cross-presentation and cross-priming of Ag-specific $CD8^+$ T cells (63, 64) (**Figure 2**). Interestingly, Ag-loaded primary LN LECs were shown to be capable of cross-priming Ag-specific $CD8^+$ T cells in a TAP1-dependent manner (64). As described above for endogenous PTA presentation, Ag-loaded LECs induced T cell apoptosis, the lack of expression of costimulatory molecules being the most extended explanation. LECs neither express nor upregulate the costimulatory molecules CD40, CD80, and CD86 following TLR engagement or in presence of IFN-γ or TNF-α (110, 116). While LECs upregulate the immunostimulatory molecules HVEM, CD48, and MHC-II under such conditions (116), they also upregulate PD-L1 (102, 110, 119). Pointing at the same direction, Ag cross-presentation by LSECs induces tolerized $CD8^+$ T cells in the liver. In this context, PD-L1 expression was also relevant for such outcome (120). Interestingly, in the absence of inflammation, surviving LSEC-educated T cells had an Ag-experienced central memory-like phenotype in SLOs (121). Furthermore, LSEC-primed memory T cells could be reactivated *in vitro* and *in vivo* in an Ag-specific manner, and they could contribute to a viral challenge (121).

The direct contribution of Ag presentation by LECs to $CD4^+$ viral immunity is still a matter of debate. As mentioned above, LECs serve as Ag reservoir during viral infections (117) (**Figure 2**). Nonetheless, genetic ablation of MHC-II in radioresistant stromal cells in LNs resulted in longer maintenance of Ag-specific $CD4^+$ T cells (122). Specific impact of LN LECs and mechanisms accounting for such effects should be yet clarified.

Cellular Ag Transfer

The hallmark of professional APCs is the constitutive cell surface presence of MHC-II and their ability for Ag processing and presentation (123). Constitutive MHC-II expression is restricted to a small number of cells of the immune system. Nonetheless, there are many different cell types from both hematopoietic and non-hematopoietic origins that can indeed express MHC-II and interact with $CD4^+$ T cells in the periphery (100, 124, 125).

As mentioned above, LECs constitute such non-professional APC cell types that express MHC-II in an IFN-γ-dependent manner. Indeed, MHC-II expression in LN LECs has been reported at both transcriptional and protein expression levels (102, 110, 111). By using transgenic mouse models lacking the different CIITA promoters, we have previously demonstrated that steady-state levels of MHC-II molecules on the surface of LECs and other stromal subsets in LNs reflect a combination of IFN-γ-inducible basal activity and acquired peptide:MHC-II complexes from DCs (110). The acquired MHC-II molecules were loaded with DC-derived Ags, licensing LECs to induce anergy and increased cell death Ag-specific $CD4^+$ T cells (**Figures 1B** and **2**). Lack of measurable productive T cell responses has been one of the major difficulties preventing the clarification of the impact of Ag presentation by LECs on $CD4^+$ T cell outcome. As for $CD8^+$ T cell responses, the absence of costimulatory signals, such as CD80 or CD86 and the constitutive expression of PD-L1 by LECs, preclude the possibility of functional effector $CD4^+$ T cell priming. In this regard, it has been shown that human LN-derived LECs fail to induce allogeneic $CD4^+$ T cell proliferation even after IFN-γ stimulation (119). In these particular *in vitro* settings, LECs were unable to induce proliferation of either naïve or memory $CD4^+$ T cells.

Membrane exchange between cells is not uncommon in immunology (126). Peptide:MHC-I and MHC-II complexes have been shown to be transferred between DC and tumor cells (127) or infected cells (128), as well as between DCs (129). Ag transfer can occur as peptide exchange on cell surfaces. Peptide epitopes can bind directly on cell surface or early endosomal MHC molecules (130), where both MHC-I and MHC-II are receptive for lymph-borne peptide binding. This might be particularly relevant in the context of self-tolerance, since recent analyses showed that the human lymph peptidome contains predominantly self-peptides, including products derived from extracellular processing of proteins (131). Exosomes were also implicated in the transfer of peptide:MHC-II complexes from DCs to LNSCs (110), and they cannot be excluded to contribute to alternative Ag trafficking (**Figure 2**).

Antigen transfer between LECs and DCs is, however, not restricted to one direction. Indeed, the transfer of PTAs specifically expressed in LECs to hematopoietic cells has been described (45) (**Figure 2**). Neither membrane-bound nor cytoplasmic PTAs were directly presented by LECs to prime Ag-specific $CD4^+$ T cell responses. As mentioned above, this was attributed to the lower expression of H2-M in LECs compared to professional APCs, which is required for peptide binding into the MHC-II groove. Instead, peptides derived from PTAs expressed by LECs were found to be loaded onto MHC-II in DCs (45). While the exchange mechanism is still open to examination, it was reported not to be dependent on recognition of apoptotic cells or DC phagocytosis. These complementary bidirectional observations highlight the close relationship and communication between professional APCs and LECs to enable MHC-II presentation.

CONCLUDING REMARKS

Increasing evidence suggest that lymphatics are much more than simple pipes that drain tissue-derived fluids containing proteins, particles, and cells. Through the expression of different surface molecules and the production of soluble factors, LECs indeed modulate immune responses in many ways, including the active regulation of cellular migration, interactions, and functions. Recent studies have highlighted a possible role for LECs as direct instructors of T cell immunity. Indeed, the discovery that LNSCs, including LECs, ectopically express tissue-derived Ags, a feature thought to be restricted to mTECs and thymic central T cell tolerance, has pushed forward LECs to potentially function as Ag-presenting cells. Accordingly, the selective expression of model Ags in LECs leads to an Ag-specific recognition by T cells, which, after an early step of activation and proliferation, are either inactivated or deleted. Therefore, the presentation of endogenously expressed Ags by LECs seems to contribute to peripheral T cell tolerance. Studies have also suggested that LECs acquire exogenous Ags by distinct pathways, including direct uptake, or cell-membrane transfer, and present them to induce T cell dysfunction. The molecular mechanisms contributing to LEC ability to inactivate T cells are still not fully elucidated. However, a consensus candidate, PD-L1, the ligand for program-cell death 1 receptor expressed by T cells, emerged from several recent studies to be highly expressed by LECs, and important to mediate T cell tolerance. Although pioneering studies suggest that Ag-presenting LNSCs are sufficient to maintain peripheral T cell tolerance, the specific contribution of LECs remains to be addressed. Likewise, substantial differences among LECs from distinct anatomical locations entail different functions. Specific roles of local LECs should be carefully dissected in order to fully understand how they differentially impact T cell responses. In addition, most studies so far have been performed in steady state, and the contribution of Ag presentation by LECs under different pathological conditions in shaping of peripheral T cell responses remains to be determined. In addition, future studies will assess how current therapies for cancer or autoimmune diseases aiming at modulating immune cell functions, specifically alter the ability of LECs to impact T cell responses.

AUTHOR CONTRIBUTIONS

SH, JD, and MH have developed the concept, wrote the manuscript, prepared the figures, and critically read, revised, and approved the manuscript.

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Bidirectional Crosstalk between Lymphatic Endothelial Cell and T Cell and Its Implications in Tumor Immunity

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Lymphatic vessels have been traditionally considered as passive transporters of fluid and lipids. However, it is apparent from recent literature that the function of lymphatic vessels is not only restricted to fluid balance homeostasis but also extends to regulation of immune cell trafficking, antigen presentation, tolerance, and immunity, all which may impact the progression of inflammatory responses and diseases such as cancer. The lymphatic system and the immune system are intimately connected, and there is emergent evidence for a crosstalk between T cell and lymphatic endothelial cell (LEC). This review describes how LECs in lymph nodes can affect multiple functional properties of T cells and the impact of these LEC-driven effects on adaptive immunity and, conversely, how T cells can modulate LEC growth. The significance of such crosstalk between T cells and LECs in cancer will also be discussed.

Keywords: lymphatic endothelial cells, T cell, lymph node, cancer, inflammation, tolerance, cytokine

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LYMPH NODE (LN) ARCHITECTURE

Lymph nodes are strategically positioned and highly organized organs that serve as "rendezvous" points for dendritic cells (DCs), T cells, and B cells. The maintenance of LN structure and compartmentalization are essential for the elicitation and development of effective immune response. LN can be subdivided into three main regions, namely, the cortex, the paracortex, and the medulla. Encapsulated LNs receive lymph from peripheral tissue and organs through the afferent lymphatic. Molecules, antigens, microorganisms, and cells such as lymphocytes and antigen-presenting cells (APCs) within the lymph are emptied into to the subcapsular sinus (SCS) of the LN. Subcapsular and medullary sinuses are directly interconnected, and hence, lymph-borne cells, fluid, and soluble molecules can pass through LN without percolating through the cortex (1). Within the SCS resides CD169-expressing macrophage and DC; these cells capture large molecules, particles, and microorganisms; and then display antigens to the lymphocytes (2–4). Densely packed B cells and follicular dendritic cells (FDCs) are organized into discrete B cell follicles in the cortex. FDCs cluster in the center of the follicles and form a dense network in which B cells contact with the antigens. Lymphocytes mainly enter LNs from the blood *via* high endothelial venules (HEVs) (5). T cell zones of the paracortex contain $CD4^+$ and $CD8^+$ T cells and subsets of DCs in close contact with a network of conduits formed by fibroblastic reticular cells (FRCs). The medulla is composed of a three-dimensional labyrinthine structure of sinus channels starting as cortical sinusoids and expands to become wider medullary sinuses that finally drain collectively into the efferent lymphatic vessel (6).

Lymph nodes consist of not only hematopoietic cells ($CD45^+$) but also heterogeneous populations of non-hematopoietic cells ($CD45^-$). Currently, there are five major stromal cell subsets that have been characterized, namely, the marginal reticular cells (MRCs), FRCs, lymphatic endothelial cells (LECs), blood endothelial cells (BECs), and FDCs. They can be identified by their anatomical location within the LN and by the expression of CD31, podoplanin (also known as Gp38), CD35 (complement receptor 1), and mucosal addressin cell adhesion molecule-1 (MadCAM-1). MRCs and FRCs express Gp38 but not CD35 and CD31. MRCs can be delineated from FRCs not only by their expression of MadCAM-1 but also by their localization in the outer follicular region immediately underneath the SCS (7). LECs express both CD31 and Gp38, whereas BECs express only CD31. FDCs are centrally located within B cell follicles and are often classified based on the expression of CD21/CD35 (8), FDC-M1 (9), and FDC-M2 (complement C4) (10). Conventionally, stromal cells have long been perceived to provide structural support to the LNs during homeostasis and inflammation. Emerging evidence also indicates that stromal compartments of LNs play active roles in the immune response through their interactions with hematopoietic cells. We will briefly discuss here the role of FRCs as it has been covered recently in excellent reviews (11–13), and this review focuses on LECs.

FIBROBLASTIC RETICULAR CELLS

Fibroblastic reticular cells are resident mesenchymal cells, primarily residing in the T cells zone and capable of secreting and forming an elaborate reticular network within the LN. Single layer of FRCs enwrap extracellular matrix (ECM) that consists of a central core formed by 20–200 parallel bundles of fibrillar collagens (I and III) and intervening matrix of fibrils (14–16). These collagen bundles are surrounded by a layer of fibrillin-constituted microfibrils that are further ensheathed by a unique basement membrane-type structure (15, 16). In addition, stabilizing and cross-linking molecules such as fibromodulin, decorin, and lumican are also associated with the collagen fibers (17). FRCs also express other ECM component including ER-TR7 and common basement membrane component such as laminin and fibronectin (13). Integrin subunits and adhesion ligands such as intercellular adhesion molecule 1 (ICAM-I) and vascular cell adhesion molecule 1 are also found in FRCs (13). The three-dimensional tubular conduit system formed by FRCs extend the SCS throughout the T cell zone and form a contiguous lumen with fluid channels around the HEVs (18). Small lymph-borne molecules including chemokines and antigens from upstream periphery are transported within the core of FRC conduits from the SCS toward the HEVs. Molecules of high molecular mass (>70 kDa) cannot gain access to the conduit lumen and hence circumvent the lymphoid compartment and drained along the sinuses into the efferent lymphatic vessels (1, 4). Large particles including whole virus particles can also be captured by SCS macrophages and presented to migrating B cells in the underlying follicles (2, 4, 19).

In addition to acting as a key structural component in the LNs, FRCs are actively engaged in functional interactions with hematopoietic cells by forming conduits for antigens and inflammatory stimuli (1, 18), maintaining T cell survival (20), providing "tracks" and chemokines cue to guide cellular movement (21, 22), and supporting DC–T–B cell interactions during immune response (23) and peripheral tolerance (24–26). Disruption of FRC integrity and organization in the LNs during viral infection leads to profound loss of immunocompetence (27) strongly underscoring the roles of FRCs in maintaining proper immune response.

LYMPHATIC ENDOTHELIAL CELLS

Lymphatic vessels are present in most tissues and are important for maintenance of fluid homeostasis, immune cells trafficking, and movement of soluble antigens (28). Lymph from upstream peripheral tissues first passes through the SCS, a space underneath the collagen-rich fibrous capsule that covers the LN. The floor of SCS is lined by LECs expressing lymphatic vessel endothelial hyaluronan receptor 1 (LYVE-1) and is interspersed with $CD169^+$ macrophages and DCs. From there, lymph percolates through the highly branched medullary sinuses and blind-ended cortical sinuses before leaving the LNs *via* the efferent lymphatic vessel (6). Cortical LECs form the vessels and branch into the T cell zone and have been indicated to facilitate B and T cell egress (29–31). Medullary sinuses lined by $LYVE\text{-}1^+$ endothelium are found at LN exit within the medulla. Recently, the markers to delineate the LECs located in the SCS, cortex, and medulla have been reported and include programmed death ligand 1 (PD-L1), ICAM-1, MadCAM-1, and lymphotoxin β receptor (32).

Research on LN LECs in the past decades has demonstrated that lymphatic vessels are not "inert conduits" but rather plastic structures that actively sense and respond to changes in the peripheral tissue environment. For example, inflammation induced by bacterial pathogen, immunization in the presence of complete Freund's adjuvant, and contact sensitization have been shown to promote the growth of lymphatic vessels from preexisting ones, a process named lymphangiogenesis, in LNs (33–37). Furthermore, it becomes apparent that such lymphatic remodeling in LN can have important biological consequences including modulation of inflammation and adaptive immune responses (38–41). Indeed, a growing body of evidence is now demonstrating that LECs themselves can help shape adaptive immune responses through their interactions with key immune cells including DCs, macrophages, and lymphocytes. Owing to their migration through and within lymphatic vessels and their anatomical distribution in LNs, T cells frequently encounter LECs. This review focuses on the crosstalk between T cells and LECs in LNs and its immunological consequences.

LN LECs CONTROL T CELL POOL

LECs Regulate T Cell Migration to, within, and out of LN

We will briefly discuss in this section how LECs attract and facilitate the trafficking of T cells from the periphery to LN

and within the LN since this topic has been covered in depth in excellent reviews (42, 43). Although LECs have been shown to express a large number of chemokines that attract T cells (38), the role of CCL21 is the most established in the homing of naïve, memory, and T regulatory (Treg) T cells to LNs. The signaling induced by CCL21 binding to its receptor, CCR7, on the surface of migratory T cells is critical for T cell trafficking from the periphery to the LN as shown in mice deficient for CCR7 ligands (44). Then, LECs in the cortical sinuses regulate intranodal lymphocyte trafficking by collecting lymphocytes for further transit to medullary sinuses (45). Moreover, lymphocytes can frequently move from the lymphatic sinuses back to the LN parenchyma (45). In line with these findings, it was reported that lymph-borne lymphocytes are passively transported into the peripheral medullary sinuses. Subsequently, they enter the LN parenchyma independently of CCR7 signals by migrating into adjacent peripheral medullary cords (46).

Medullary sinuses are directly connected to the efferent lymphatic vessel and have been proposed in addition to cortical sinuses as exit routes for the egress of lymphocytes from LNs (29, 30, 45). The molecular mechanisms of lymphocyte egress mediated by LECs remain elusive, and further investigations will be needed to explain how medullary sinuses can serve as both entry and egress structures for T cells. Most work on T cell egress has focused on mechanisms that lymphocytes uses to reach efferent lymphatic vessels and has identified sphingosine-1-phosphate (S1P)/S1P1 as a critical signal axis in promoting T cell egress (47). S1P levels are low in LN parenchyma but high in lymph fluid, thus creating a gradient. This S1P gradient guides T cells exhibiting decreased CCR7-retention signals from LN parenchyma into medullary and cortical sinuses and ultimately facilitates T cell egress (48). Notably, S1P in cortical sinuses and efferent lymph has been shown to be produced by LYVE-1$^+$ LECs. Mice lacking specifically S1P kinase, the enzyme responsible for S1P synthesis, in LECs show compromised T cell egress (49). It is well established that local immune responses and inflammation are accompanied by alterations in the trafficking of lymphocytes through LNs. Specifically, the entry of lymphocytes into LNs increased, whereas their egress into efferent lymph is temporarily inhibited for few hours to days, depending on the nature of the stimulus (50–52). Few years ago, we reported that inflammation in LN, as it evolves from early to late phases, can induce a biphasic remodeling of lymphatic network, with the SCSs being expanded first, followed by the cortical and medullary sinuses. We showed that the early expansion of SCSs enhances the migration of DCs from the periphery, whereas the preferential expansion of cortical and medullary sinuses at later stages of inflammation supports the restoration of lymphocyte egress to steady-state levels (53).

LN LECs Support the Survival of T Cells

Several emerging evidence indicates that LECs may not only regulate the homeostasis of T cells in LNs through the modulation of their migration but also their survival. Interleukin (IL)-7 binds to IL-7Rα chain in combination with the common-γ chain and is essential for T lymphocyte homeostasis within the secondary lymphoid organs. IL-7 expression *in vivo*, which appears to limit the size of the lymphocyte pool, was thought to be regulated by IL-7 receptor α (IL-7Rα)-mediated consumption rather than the rate of IL-7 expression (54, 55). However, this concept has been recently challenged by a study showing that IL-7 expression can be induced in the liver in response to Toll-like receptor signaling and can directly control T cell responses (56). In line with this latter study, an earlier report by the same group demonstrated that excessive IL-6 expression increases IL-7 expression, which in turn was associated with the development of autoimmune reaction (57). These studies underscore that production of IL-7 by non-hematopoietic cells is tightly and dynamically regulated. In LNs, IL-7 provides antiapoptotic and proliferative signals to naïve and memory T cells (58–61). Although FRCs have been shown to be a major producer of IL-7 in LNs (20), it appears now evident that LECs are also an important source of IL-7 in murine and human LNs (62, 63). Interestingly, during inflammation-induced LN remodeling that influences intranodal lymphocyte dynamics, IL-7-expressing cortical sinus LECs have been shown to be essential for LN remodeling (63). In line with the role of IL-7 in maintaining memory T cells, a recent study revealed that LECs in lungs from mouse and humans can support the survival of memory T-helper cells through the production of IL-7 and IL-33 during allergic airway inflammation (64). IL-33 is a pro-inflammatory cytokine that initiates chronic inflammation in the lung, and its receptor is highly expressed on memory Th2 cells. IL-33 has been shown to directly induce memory Th2 cells to produce IL-5 and induces eosinophilic inflammation. Although this study focuses on lung LECs, it raises the possibility that LECs through the production of diverse cytokines may control the survival of pathogenic T cells during chronic inflammation, which in turn may have serious pathological consequences. Furthermore, the fact that IL-7 has been shown to mediate the transition from effector into memory T cells (65, 66) may also suggest the potential implication of LECs in shaping T cell differentiation in LNs during immune response.

LN LECs REGULATE T CELL ACTIVATION

LN LECs Function as APCs for Peripheral T Cell Tolerance

Peripheral immune tolerance is generally ascribed to quiescent tissue-resident DCs cross-presentation of tissue-associated antigens to self-reactive T cells that have escaped thymic negative selection (67). More recently, accumulating evidence demonstrates that direct presentation of self-antigens by LN stromal cell subsets including FRCs and LECs can also mediate peripheral tolerance (25, 26, 68). Among LN stromal cell populations, LECs are likely the first cells that are in direct contact with the antigens, danger signals, and immune cells that carry peripheral blueprint to the draining LN. LECs express MHC class I (68–70) and MHC class II (41, 71, 72) and are capable of inducing T cell tolerance directly and suppressing DC-mediated T cell activation. In addition, T cell activation is also affected by the cytokine environment and relative balance between costimulatory and inhibitory signals from the APCs (41, 71, 73–75).

There are several potential pathways by which LECs can induce T cell tolerance. For instance, LN LECs express multiple peripheral tissue antigens (PTAs) (25, 69). In steady state, LECs lack costimulatory molecules such as CD80, CD86, or 4-1BBL that normally drive immunogenic T cell response. Instead, high expression of PD-L1 on LECs and engagement with its receptor on T cells predispose them to promote peripheral T cell tolerance (41). In a model of LEC-induced tolerance of melanocytes differentiation protein tyrosinase-specific $CD8^+$ T cells, lack of stimulation through 4-1BB led to rapid and increased expression level of PD-1. Signaling through PD-1 inhibits upregulation of IL-2R on $CD8^+$ T cells, culminating in apoptotic death associated with the loss of IL-2 prosurvival signaling (41). On the other hand, rescue of tyrosinase-specific $CD8^+$ T cells by interfering PD-1 signaling or providing costimulatory signals gain effector function and induce autoimmune vitiligo, demonstrating that LECs are important and specialized APCs for peripheral T cell tolerance (41). This latter finding is in line with the observation in severe enteric autoimmunity that loss of PD-1/PD L1 inhibitory pathway blocks $CD8^+$ T cell tolerance to intestinal self-antigens (76). It is worth to note that tyrosinase and PD-L1 are expressed at higher levels in LN LECs as opposed to LECs in periphery (diaphragm or colon), indicating that the LN microenvironment endows LN LECs with tolerogenic properties not found in tissue LECs (32). Given that LECs express various PTAs, dysregulation of LEC-associated tolerance is likely expected to contribute to the development of several autoimmune disorders.

In addition to transcriptionally expressed PTAs, LN LECs have also been shown to scavenge and cross-present exogenous antigen to naïve $CD8^+$ T cells in the model of B16 F10 melanoma expressing the foreign antigen ovalbumin (OVA) and overexpressing vascular endothelial growth factor (VEGF)-C (70). VEGF-C-induced LN lymphangiogenesis suppresses anti-tumor immunity by local deletion of OVA-specific $CD8^+$ T cells, which in turn drives disease progression and metastatic outgrowth. Similar observation was also reported under homeostatic conditions whereby intradermal injection of fluorescently labeled OVA protein was engulfed by LN LECs, processed, and presented on MHC class I to cognate $CD8^+$ T cells in a TAP1-dependent manner (77). Such T cell/LEC interaction was shown to lead to decreased cytokine production and increased expression of Annexin V and exhaustion markers (PD-1, CD80, and CTLA-4) *in vitro* (77). These experimental findings suggest that regardless of the source of antigen (exogenous or endogenous), constitutive expression of inhibitory molecules and lack of costimulatory molecules on LECs will predominantly induce peripheral tolerance.

Furthermore, LECs express intermediate levels of MHC class II molecules suggesting that they might also tolerize $CD4^+$ T cells (41, 71). MHC class II on LECs has shown to be either acquired from the DCs or endogenously expressed (24, 72). Rouhani et al. employed transgenic systems where antigens β-galactosidase (β-gal) and hemagglutinin (HA) were conditionally expressed in LECs under the control of Prox-1 and LYVE-1 promoters (72). Both $CD8^+$ and $CD4^+$ T cell receptors are available in these models and hence allowing comparative evaluation of the ability of LECs to drive tolerance to epitopes from the same protein presented by either MHC class I or MHC class II molecules. The authors demonstrated that PTA β-gal and HA epitopes on MHC class I were directly presented to $CD8^+$ T cells, whereas these epitopes on MHC class II molecules were not presented to $CD4^+$ T cells both *in vivo* and *in vitro*. Instead, these antigens were transferred to DC and then presented to $CD4^+$ T cell to induce anergy. Therefore, LECs serve as a reservoir and repertoire of PTAs in the LN that may be acquired by DCs to induce tolerogenic $CD4^+$ T cells. Similarly, Dubrot et al. showed that LECs acquire peptide: MHC class II complexes from DCs (24). However, in contrast to Rouhani et al., these complexes were not observed to be transferred back to LECs in sufficient quantities to induce $CD4^+$ T cells recognition and subsequent antigen-specific T cells apoptosis.

LN LECs Modulate DC Functions

LECs may also regulate T cell activation indirectly by modulating antigen-presenting functions of DCs. Under steady state, immature DCs typically capture autoantigens from apoptotic cells, migrate to LNs, and promote T cell tolerance (78–80). Exposure of DCs to danger signals during inflammation or infection increases the expression of MHC class II molecules, costimulatory molecules, and cytokine that ultimately can trigger immunity and prevent tolerance. LECs have been shown to attenuate T cell response by suppressing DC maturation (73, 74, 81). Direct contact of immature DCs with an inflamed, TNF-α-stimulated LECs decreases expression of CD86 on DCs, dampening their ability to stimulate T cell proliferation (81). This interaction was mediated by the binding of ICAM-1 on LECs to Mac-1 on DCs and was observed in the absence of PAMPs (81). LECs also restrain T cell proliferation through upregulation of nitric oxide synthase-2 and production of NO in response to interferon (IFN)-γ and TNF-α released from activated T cells (73). Furthermore, IFN- γ-stimulated cultured human LN LEC produces inhibitory indoleamine 2,3 dioxygenase that in turn impairs $CD4^+$ T cell proliferation (74). Interestingly, in different contexts such as viral challenge and subunit vaccination, viral antigens are captured and archived in LECs and subsequently transferred to DCs for the maintenance of memory T cells and enhancement of protective immunity (82). Therefore, crosstalk between LECs and DCs within the LN can either drive tolerogenic or immunogenic responses depending on the antigenic stimuli, immune cells encountered, and the type of inflammatory challenges.

LN LECs Archive Antigens

Several studies have reported that persistence of virally associated antigens after acute infection and subsequent viral clearance or so-called the reservoir of antigens was localized within the LNs draining the site of initial infection (82–86). A recent report demonstrated that LN LECs retain persisting antigens for weeks after vaccination (82). This antigen archiving was dependent on the induction of LN lymphatic proliferation. However, LECs did not present directly the archived antigen to T cells but instead required hematopoietic APCs. The number and percentage of $CD8^+$ T cell-producing IFN-γ and IL-2 were significantly increased when antigen was retained in LECs.

Notably, we previously reported that LN lymphangiogenesis persists during prolonged inflammation (53). Thus, it is plausible that the persistence of an expanded LN lymphatic network after viral infection or vaccination may allow the long-term storage of viral antigens. As a consequence, ongoing antigen presentation and recognition by memory T cells may lead to selective enrichment of virus-specific memory T cells in the draining LN even after the clearance of the infectious agent. This enriched population of antigen-specific T cells may provide more rapid effector responses in the periphery and better control of secondary infections.

T CELLS CONTROL LEC GROWTH UPON INFLAMMATION

Because lymphangiogenesis in LN has been shown to have diverse functional consequences on inflammation and immune responses depending on the context and timeframe of its occurrence (39, 40), this process is expected to be highly regulated. Indeed, a large number of studies have identified cellular and molecular mechanisms promoting the growth of lymphatic vessels. In contrast, little knowledge is currently available on pathways counter-regulating lymphangiogenesis. Both non-immune and immune cells have been described to orchestrate the expansion of lymphatic vessel network within LN. Interestingly, among immune cells, B and T cells have been shown to have opposite effects, namely, B cells support inflammatory lymphangiogenesis in LNs, whereas T cells have antilymphangiogenic effects. The first evidence supporting a role for T cells as negative regulators of LEC growth arises from a mouse study in which T cells were ablated using athymic mice (37). This antilymphangiogenic effect of T cells in the athymic mice was restored by the adoptive transfer of $CD4^+$ or $CD8^+$ T cells. This study suggests that both $CD4^+$ and $CD8^+$ T cells may harbor an antilymphangiogenic property. Other studies in different mouse models of inflammatory lymphangiogenesis have further confirmed the regulatory function of $CD4^+$ T cells on LEC growth (34, 87). In the model of LN lymphangiogenesis induced by bacterial lipopolysaccharide, the authors demonstrated that the secretion of IFN-γ by T cells accounts for the inhibitory effect of T cells on LN lymphangiogenesis (37). Moreover, in line with an earlier study (88), they showed using *in vitro* cultured LECs that IFN-γ can act directly on LECs and affect their proliferation and survival (see **Table 1**) (37, 88).

These two latter studies provided the first evidence for a role of cytokines in controlling the expansion of lymphatic vessels. Since then, this notion has been further validated by several recent studies reporting the effect of other cytokines including IL-10, IL-17, TGF-β, and IL-4/IL-13 on LEC growth *in vitro* and/or in diverse models of inflammatory or *de novo* lymphangiogenesis induced in LN or other tissues (**Table 1**). From these studies, it becomes apparent that (i) cytokines are not always antilymphangiogenic; (ii) one given cytokine may have prolymphangiogenic or antilymphangiogenic properties depending on the context in which lymphatic growth occurs; and (iii) modulation of lymphatic proliferation, survival, and migration by cytokines can be mediated by a direct effect on LECs or indirectly by controlling the expression of lymphangiogenic factors such as VEGF-A, -C, and -D. Interestingly, all these cytokines can be secreted by different $CD4^+$ T subsets including Th1, Th2, Th17, and Treg cells raising the possibility that different T cell subsets recruited to LN may affect LEC growth. Although this notion is indirectly supported by the studies cited in **Table 1** and a recent study reporting the effect of Treg on lymphatic transport in a mouse model of lymphedema (100), direct evidence for a role of these T cell subsets and their cytokines in controlling LN lymphangiogenesis is lacking.

IMPLICATIONS OF LEC IMMUNOMODULATORY PROPERTIES IN CANCER PROGRESSION

The ever-growing research on tumor biology, immunology, and lymphatic biology has recently highlighted the multifaceted roles of lymphatic vessels in shaping tumor immunity and in cancer progression. One of the cardinal functions of lymphatic vessel is to transport components of the local tissue containing interstitial solutes, cytokines, growth factors, and immune cells to the downstream LN for the maintenance of tissue fluid homeostasis and peripheral immune tolerance. Tumor cells can "hijack" the lymphatic and induce the expansion of lymphatic vessels for their dissemination, colonization, and the formation of metastasis in the tumor-draining LNs (101, 102) (**Figure 1**). *Via* the lymphatic route, tumor cells can also modify the microenvironment of the metastatic organs from the distal sites before their arrival—referred to premetastatic niche. LN lymphangiogenesis preceding metastasis is an important mechanism and is associated with cancer progression (103–106).

Moreover, LN LECs express several chemokines that can attract cancer cells expressing the cognate chemokine receptors. For instance, constitutive CCL21 expression by LEC can serve as a guide for CCR7-expressing breast cancer and melanoma cells invading the LNs (107). Overexpression of CCR7 in melanoma has been shown to promote LN metastasis in mice (108), and CCR7 expression in human cancer samples correlates positively with LN metastasis (109–111). Upregulation of CXCL12 expression has been reported to enhance LN metastasis of $CXCR4^+$ tumor cells (112). CCL1 is another chemokine produced by the SCS LEC, which has been shown to control $CCR8^+$ tumor cell entry and subsequent migration and colonization in the LN cortex (113). Blocking of CCL1-CCR8 signaling results in the arrest of tumor cells at the junction of the afferent lymphatic vessels and the LN.

As discussed earlier, LN LECs can profoundly affect T cell survival, fate, and activation that can be of significant importance in tumor immune responses (**Figure 1**). The primary tumor is connected to the downstream afferent lymphatic vessel and draining LNs, and this connection may allow the entry of tumor-derived factors to the draining LNs and consequently may alter regional immune responses. Such alterations were reported to occur even before LN metastasis (114). Moreover, owing to the lack of costimulatory molecules

TABLE 1 | Cytokines regulating lymphatic endothelial cell (LEC) growth.

Cytokine	Mechanism	Model system	Reference
Interferon (IFN)-γ	Inhibits proliferation and migration	Cultured pig thoracic duct LEC	(88)
	Increases apoptosis		
	Inhibits lymph node (LN) lymphangiogenesis	LPS-induced LN lymphangiogenesis in mouse; Lewis Lung carcinoma cell implantation in mouse	(37)
	Inhibits proliferation and tube formation; downregulates Prox-1 LYVE-1and podoplanin expression	Cultured murine thoracic duct LEC	
Interleukin (IL)-4/IL-13	Inhibits LN lymphangiogenesis	CFA/ovalbumin-induced LN lymphangiogenesis	(34)
	Inhibits corneal lymphangiogenesis	Mouse model of suture-induced corneal neovascularization	(89)
	Inhibits proliferation, tube formation and migration; increases apoptosis	Cultured human dermal LEC	
	Inhibits lung and trachea lymphangiogenesis	Mouse model of allergen-induced asthma	(90)
	Inhibits proliferation, tube formation and downregulates Prox-1 and LYVE-1 expression	Cultured murine LN LEC and human dermal LECs	
	Increases skin lymphangiogenesis and promotes recruitment of macrophages and vascular endothelial growth factor (VEGF)-C expression	K14-IL-4 transgenic mouse	(91)
IL-17	Increases corneal lymphangiogenesis *via* VEGFR-3/VEGF-C/-D pathway	Mouse model of cornea micropocket and Th17-dominant autoimmune dry eye disease	(92)
	Increases proliferation and tube formation *via* VEGFR-3-dependent pathway	Cultured human dermal LECs	
IL-10	Increases lymphangiogenesis and promotes VEGF-C production by macrophages	Mouse model of suture-induced corneal neovascularization	(93)
	No direct effect on LEC	Cultured human dermal LECs	
TGF-β	Inhibits lymphangiogenesis	Mouse model of chronic peritonitis	(94)
	Inhibits proliferation, tube formation, and migration; downregulates Prox-1 and LYVE-1 expression	Cultured human dermal LEC	
	Inhibits proliferation and tube formation	Cultured human dermal LEC	(95)
	Independent of VEGF-C/-D		
	Inhibits lymphangiogenesis	Mouse lymphedema model	(96)
	Promotes lymphangiogenesis and upregulates VEGF-C expression	Rat model of unilateral ureteral obstruction	(97)
	Promotes lymphangiogenesis and upregulates VEGF-C expression	Rat model of peritoneal fibrosis	(98)
	Enhances branching and sprouting of lymphatic network in embryonic skin	E13.5–15.5 mouse embryos	(99)
	Attenuates LEC proliferation	Cultured human dermal microvascular LECs	

expression and high levels of inhibitory ligand PD-L1 on LN LECs, lymphatic antigen presentation *via* MHC-I can induce deletional tolerance, a mechanism by which tumor cells may evade host immunity (41, 69, 70). VEGF-C-induced LN lymphangiogenesis can further promote immune tolerance in B16 melanoma-implanted mouse model (70). However, these studies suggest that manipulating LEC-associated tolerance or cancer dissemination may create opportunities for a new generation of antitumor immunotherapy. Importantly, cancer immunotherapies targeting the immune checkpoints, PD-1 and PD-L1, are revolutionizing current cancer treatments (115, 116). In humans, anti-PD-1 antibodies that target tumor-specific T cells (117–119) and anti-PD-L1 antibodies that bind to ligand expressed by the tumor and intratumor immune cells (120, 121) show promising clinical benefits. One can speculate that targeting this PD-1/PD-L1 immune checkpoint *via* systemic administration may also interrupt the tolerogenic signaling pathway between LN LECs and $CD8^+$ T cells. Perhaps, a more LN-specific delivery of these blocking antibodies or other anticancer vaccine may lead to a greater impact on antitumor immune responses (122).

Although LN LECs may contribute to immune suppressive environment within the tumor-draining LNs (whether by direct interaction with $CD8^+$ T cells or by draining the immunosuppressive cytokines from the upstream tumors), their roles in tumor immune surveillance cannot be neglected (**Figure 1**). Indeed,

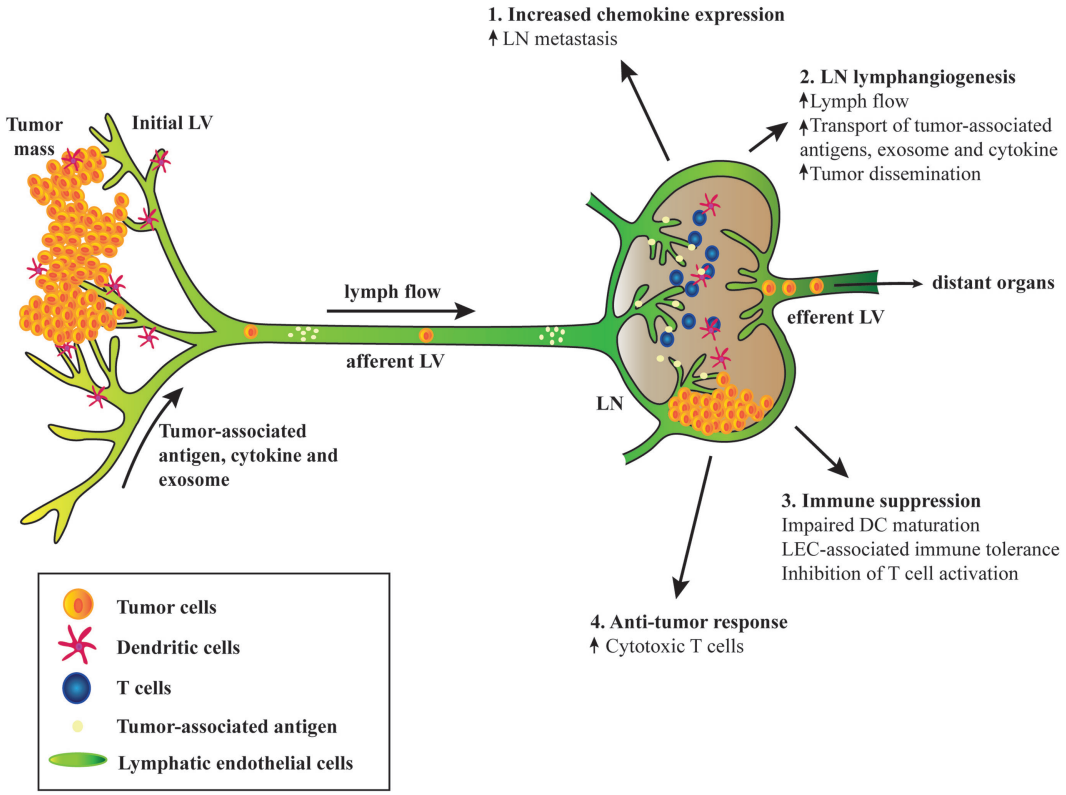

FIGURE 1 | Schematic diagram depicting the involvement of tumor-associated lymphatic endothelial cell (LEC) in cancer. (1) Tumor-associated upregulation of chemokine expression in lymph node (LN) LECs mediates metastasis of tumor cells expressing the cognate chemokine receptors. (2) Tumor-associated factors, cytokines, and exosome draining from the upstream tumors and afferent lymphatic induce LN lymphangiogenesis, leading to increased lymph flow, transport of tumor-derived factors, and enhanced tumor cell dissemination. (3) Tumor-associated LECs can suppress immunity and promote tolerance. Interaction between LN LECs and dendritic cells (DCs) *via* intercellular adhesion molecule 1 and Mac-1 inhibits DC maturation and hence limiting effective T cell activation. Tumor antigen presentation to naïve $CD8^+$ T cells by LN LECs induces dysfunctional T cell activation and tolerance due to expression of inhibitory receptor programmed death ligand 1 and lack of costimulatory molecules on LEC surface. LECs activated by T cell-derived pro-inflammatory cytokines produce factors such as NO and indoleamine 2,3 dioxygenase that inhibit T cell proliferation. (4) Robust $CD8^+$ T cells priming occurs in tumor-draining LN. Although tolerogenic LN microenvironment may dominate and sustain immune suppression, immune checkpoint blockades can reverse T cell exhaustion and increase effector T cell activities that may lead to tumor regression.

circulating tumor-specific T cells in metastatic melanoma patients are functional although those isolated from tumor-draining LNs exhibit exhausted characteristics (decreased IFN-γ and increased CTLA-4 and LAG-3 expression) (123). Interestingly, co-administration of anti-CTLA-4 and PD-1 antibodies reverses T cell exhaustion by increasing effector T cell activity and cytokine production and hence augmenting tumor inhibition (124). Tumor immunity was examined in the context of impaired lymphatic function using a *kCYC* transgenic mouse model expressing Kaposi's sarcoma-associated herpes virus latent-cycle gene, *k-cyclin*, and under the control of VEGFR-3 promoter (101). In this model, antigen-presenting ability of DCs and cytotoxicity of $CD8^+$ T cells isolated from the draining LNs of *kCYC* mice were attenuated. Furthermore, adoptive transfer of $CD8^+$ T cells derived from *kCYC* mice to naïve WT mice show impaired antitumor function (101). In another model of dermal lymphatic insufficiency (K14-VEGFR3-Ig mice), implanted melanoma grew robustly and exhibited marked reduction in leukocyte infiltration compared with those implanted in control mice, suggesting that lymphatic vessels are essential for the generation of tumor immune responses (125). In addition, we showed in a spontaneous mouse model of uveal melanoma that early resection of TDLNs promotes primary tumor growth, cancer cell dissemination, and metastasis (102). Even though we did not examine the role of immune responses in the absence of tumor-draining LNs, it is plausible that uncontrollable growth of primary tumor may be due to the lack of antitumor immunity since the depletion of $CD8^+$ T cells accelerates tumor growth and dissemination in the same model (126). These reports strongly indicate that functional lymphatic and presence of tumor-draining LNs are required for cancer immune surveillance. To further support this, current cancer immunotherapies targeting the immune checkpoints have demonstrated and supported the evidence that antitumor immunity exists even in the most advanced stages of cancer (116, 127–129).

CONCLUDING REMARKS

The ever-growing research on lymphatic biology has clearly identified LECs as key players in regulating adaptive immunity particularly by affecting T cell functions. However, the dynamics of T cells/LECs interactions and their immunological consequences in the context of cancer need to be further delineated. LN LECs are intricately affected by peripheral tumor, tumor-associated factors, and immune cells that in turn enhance tumor cell dissemination and drive the balance between host immunity and tolerance. Hence, LN LECs may represent a potential therapeutic target in addition to immunotherapy strategies for cancer progression and metastasis. Although tumor-associated LN lymphangiogenesis can contribute to tumor dissemination and increased immune tolerance, LN LECs are also important for the communication between tumors and immune cells to mount antitumor immune responses. For these reasons, combined research on immunology, lymphatic, and tumor biology is essential to further elucidate the immunological roles of LN LECs in cancer and their impact on disease progression.

AUTHOR CONTRIBUTIONS

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Tumor-Associated Lymphatic Vessels Upregulate PDL1 to Inhibit T-Cell Activation

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Tumor-associated lymphatic vessels (LVs) play multiple roles during tumor progression, including promotion of metastasis and regulation of antitumor immune responses by delivering antigen from the tumor bed to draining lymph nodes (LNs). Under steady-state conditions, LN resident lymphatic endothelial cells (LECs) have been found to maintain peripheral tolerance by directly inhibiting autoreactive T-cells. Similarly, tumor-associated lymphatic endothelium has been suggested to reduce antitumor T-cell responses, but the mechanisms that mediate this effect have not been clarified. Using two distinct experimental tumor models, we found that tumor-associated LVs gain expression of the T-cell inhibitory molecule PDL1, similar to LN resident LECs, whereas tumor-associated blood vessels downregulate PDL1. The observed lymphatic upregulation of PDL1 was likely due to IFN-g released by stromal cells in the tumor microenvironment. Furthermore, we found that blocking PDL1 results in increased T-cell stimulation by antigen-presenting LECs *in vitro*. Taken together, our data suggest that peripheral, tumor-associated lymphatic endothelium contributes to T-cell inhibition, by a mechanism similar to peripheral tolerance maintenance described for LN resident LECs. These findings may have clinical implications for cancer therapy, as lymphatic expression of PDL1 could represent a new biomarker to select patients for immunotherapy with PD1 or PDL1 inhibitors.

Keywords: peripheral tolerance, immune checkpoint, tumor vasculature, lymph node, PD1, abortive proliferation, T-cell exhaustion, tumor-induced immunosuppression

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INTRODUCTION

The lymphatic system comprises lymphatic capillaries and collecting vessels, as well as lymph nodes (LNs), and it exerts several essential functions in the body. Lymphatic vessels (LVs) take up interstitial fluid in peripheral tissues and transport it back to the blood circulation, thus maintaining basic tissue fluid homeostasis. At the same time, the lymphatic system provides a route for the recirculation of immune cells, such as memory T-cells, which constantly patrol the body, shuttling between the blood circulation and peripheral tissues. Similarly, dendritic cells and other antigen-presenting cells (APCs) use LVs to transport antigen taken up in the periphery to draining LNs, where they make contact with naive T- and B-lymphocytes to initiate adaptive immune responses. Consequently, the lymphatic system is closely connected to the immune system and the regulation of immune responses (1).

In pathological conditions, such as acute and chronic inflammation or cancer, peripheral LVs, and also draining LNs, undergo a dramatic expansion which is mediated by the enlargement of existing vessels as well as induction of *de novo* LV formation (lymphangiogenesis) (2–4). These effects are predominantly mediated by lymphangiogenic growth factors such as VEGF-C, produced at the site of inflammation or neoplastic growth. VEGF-C acts locally on nearby LVs, but may also be transported *via* the lymph to the draining LNs (5). Depending on the type of the inflammatory insult, the outcome of this expansion (and the concomitant increase in fluid drainage) may have beneficial or negative effects for the patient. For example, we and others have found that activation of LV expansion by administering VEGF-C decreases acute and chronic skin inflammation as well as rheumatoid arthritis (6–8), likely due to increased drainage of inflammatory factors and activated immune cells away from the site of inflammation. On the other hand, in cancer patients, an increased LV density in and around the tumor facilitates the lymphogenous spread of tumor cells and consequently correlates with LN metastasis and a poor prognosis (3, 4). At the same time, deficient lymphatic drainage in experimental tumor models reduces tumor inflammation and infiltration by immune effector cells, probably due to a lack of tumor-derived antigen reaching the local LNs which results in a state of "immunologic ignorance" of the tumor (9, 10).

Apart from these drainage-related effects, lymphatic endothelial cells (LECs) are also increasingly recognized as direct regulators of the immune system. LECs may act as non-professional APCs, expressing both MHC class I and class II molecules, which enable them to directly interact with T-cells and to modulate their activation status. This immune-regulatory function of LECs is particularly well studied in the case of LN resident LECs. Victor Engelhard and coworkers reported that LN LECs, but not LECs in peripheral LVs, express various self-antigens, including the melanocyte-specific antigen tyrosinase. Furthermore, LECs present peptides derived from these self-antigens on MHCI complexes to CD8+ T-cells and inhibit their activation in an antigen-dependent manner, thus eliminating autoreactive T-cells and maintaining peripheral tissue tolerance (11–13). LN LECs have also been found to take up free antigen from the lymph and to cross-present it to CD8+ T-cells, which may result in blunted T-cell responses to exogenous antigens (14). Taken together, the current data point to LN LECs being broadly inhibitory for CD8+ T-cells, both toward endogenous and exogenous antigens, at least under steady-state conditions. Whether LN LECs similarly interact with and inhibit CD4+ T-cells has remained somewhat controversial. On the one hand, LN LECs do express MHCII, but their ability to load it with antigen-derived peptides appears to be impaired due to a lack of H2-M expression (15). On the other hand, transfer of peptide-loaded MHCII complexes and/or antigen between LN LECs and other APCs, such as dendritic cells, has been reported, indicating that LN LECs may indeed play a role in the regulation of CD4+ T-cell responses (15, 16).

Various mechanisms how LN LECs control T-cells have been suggested, including a relative lack of co-stimulatory molecules and inhibition of T-cells *via* interaction of MHCII with LAG3 on the T-cell surface (11, 12, 15). In addition, LN LECs have been found to constitutively express the immune checkpoint molecule PDL1 (also called CD274 or B7H1), which inhibits T-cells *via* activation of the PD1 receptor, typically inducing a state of T-cell unresponsiveness termed "T-cell exhaustion" (17). However, in the case of peripheral tolerance induced by LN LECs *in vivo*, the effect on transferred autoreactive T-cells was reported to differ substantially from classical exhaustion, as those T-cells initially proliferated but subsequently became eliminated from the recipient mice (12), a process which has been termed "abortive proliferation." In any case, the precise role of PDL1 expression in this process has not been entirely elucidated.

Steady-state PDL1 expression in LN LECs has been reported to be dependent on lymphotoxin signaling in the LN microenvironment (11). Additionally, PDL1 expression is inducible in various cell types, such as myeloid cells and endothelial cells (ECs), by inflammatory cytokines, particularly by IFN-g (18, 19). Therefore, PDL1 acts as a negative feedback regulator of Th1/CD8+ T-cell immune responses, which are characterized by high IFN-g release. Correspondingly, acute skin inflammation induced by repeated application of the contact sensitizer oxazolone, which triggers a Th1-biased immune response, resulted in a strong upregulation of PDL1 mRNA in isolated LECs (20). Similarly, PDL1 was upregulated in LECs upon antigen-specific interaction with CD8+ T-cells *in vitro* (14).

With regards to cancer, the role of LECs in regulating T-cell immunity is incompletely understood. Overexpression of VEGF-C in the B16F10 mouse melanoma model has been reported to decrease endogenous CD8+ T-cell responses against a model antigen (ovalbumin) and to turn these tumors refractory to adoptive T-cell transfer with OT-1 T-cells. Furthermore, these authors observed presentation of tumor antigen by peripheral and LN LECs, suggesting that LECs may contribute directly to the inhibition of T-cell-mediated antitumor immune responses (21). However, the mechanisms behind the T-cell inhibition by tumor-associated LECs have not been investigated so far.

We hypothesized that tumor-associated LECs might upregulate PDL1 in response to tumor-derived signals, and might thus contribute to the inhibition of tumor specific T-cells. Using two distinct syngeneic tumor models in different mouse strains, namely intradermal injection of VEGF-C overexpressing B16F10 melanomas and an orthotopic breast cancer model (4T1), we found that PDL1 is significantly upregulated in peripheral, tumor-associated LVs, presumably in response to IFN-g secreted by cells present in the tumor stroma. Using ovalbumin as model antigen, we provide direct evidence that PDL1 indeed reduces the stimulation of CD8+ T-cells by antigen-presenting LECs.

RESULTS

PDL1 Is Upregulated in Tumor-Associated LVs

Previously, LVs in B16F10 melanomas overexpressing VEGF-C were reported to inhibit specific T-cell immunity (21), but the molecular mechanisms behind this inhibition have remained unclear. To investigate whether PDL1 is expressed in

tumor-associated LVs, we implanted VEGF-C overexpressing B16F10 melanoma cells [B16F10-VEGFC (22)] intradermally into syngeneic C57BL/6 mice, and analyzed the expression of PDL1 by immunofluorescence staining of tumor sections 2 weeks later. Using LYVE-1 staining to identify LVs within the tumor mass and in the tumor periphery, we found increased PDL1 staining within the lymphatic endothelium of tumor-associated LVs, compared to LVs in the back skin of naive C57BL/6 mice (**Figure 1A**). In addition, diffuse PDL1 staining in a subset of tumor cells and in single, tumor-infiltrating cells could be observed. Image-based quantification of the PDL1 staining intensity in LYVE-1+ LVs confirmed a significant upregulation of the protein in intratumoral LVs but not in peritumoral LVs (**Figure 1B**).

To test whether lymphatic PDL1 expression is dependent on the tumor type, high expression of VEGF-C, or on the background mouse strain used, we next investigated PDL1 expression in a second, unrelated tumor model. 4T1 mammary carcinoma cells were implanted orthotopically in syngeneic hosts (Balb/c), and immunofluorescence stainings for PDL1 were performed 3 weeks later. Similar to what we found in melanomas, intratumoral LVs in 4T1 tumors also expressed PDL1, whereas PDL1 was not expressed by peritumoral LVs and LVs of the abdominal skin and the mammary fat pad of naive Balb/c mice (**Figures 1C,D**).

As PDL1 might be expressed by various cell types, including immune cells which sometimes reside in very close proximity to LVs and may thus confound the microscopic analysis, we next performed FACS analyses of primary B16F10-VEGFC and 4T1 tumors compared to the corresponding control tissues. Antibodies against CD45, CD31, and podoplanin (PDPN) were used to differentiate between immune cells, blood vascular endothelial cells (BECs), and LECs (**Figure 2A**). Analysis of the fluorescence intensity confirmed that in both tumor models,

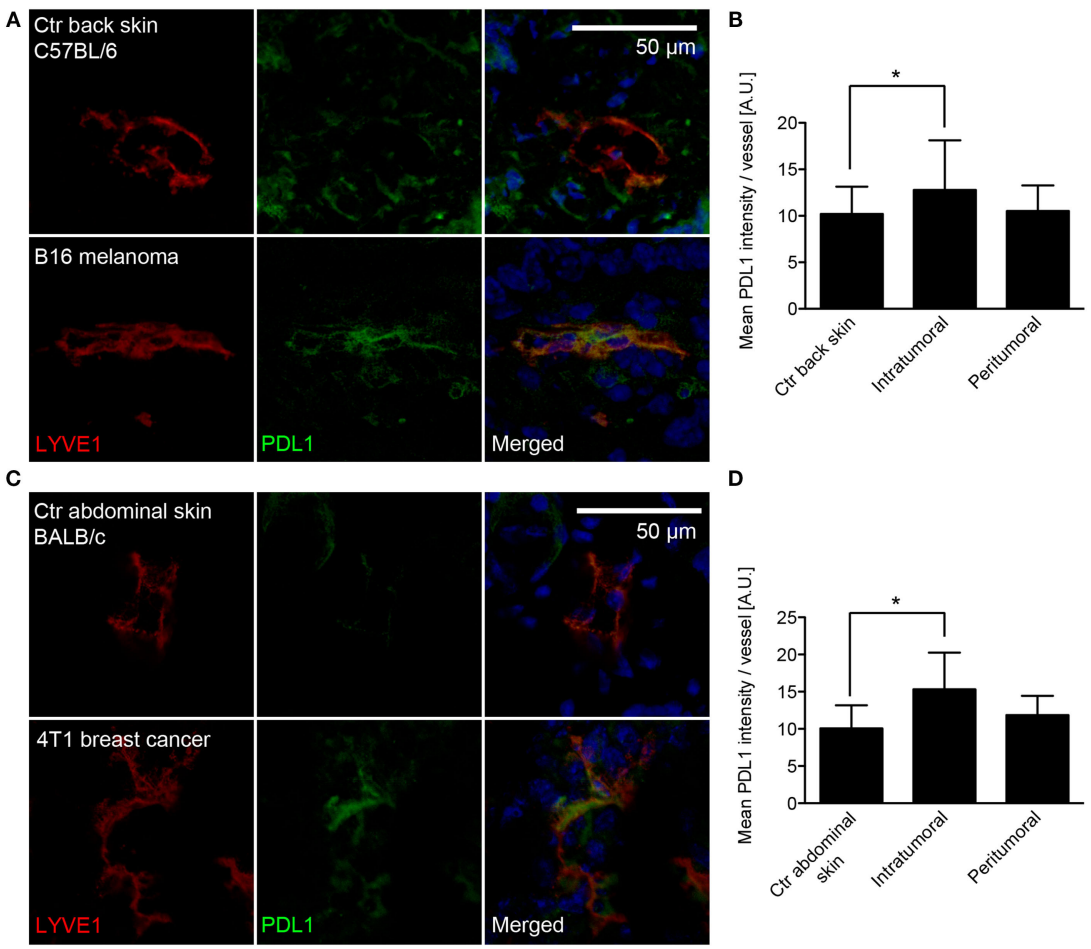

FIGURE 1 | PDL1 is expressed in tumor-associated lymphatic vessels (LVs). **(A)** Representative images of LVs (stained for LYVE-1, red) in control (Ctr) back skin (C57BL/6 background, top row) and B16F10-VEGFC melanoma (bottom row) co-stained for PDL1 (green). **(B)** Quantification of PDL1 staining intensity within the LYVE-1+ area of LVs in control back skin (*N* = 98 vessels from 10 individual mice), in the inner tumor mass of B16F10-VEGFC tumors (*N* = 17 vessels from six individual mice) and in the tumor periphery (*N* = 34 vessels from seven individual mice). **(C)** Similar to the B16F10-VEGFC model, PDL1 staining in LYVE-1-positive LVs was absent in the abdominal skin of BALB/c mice (top row) but was observed in 4T1 breast cancer-associated LVs (bottom row). **(D)** Quantification of PDL1 staining intensity within the LYVE-1+ area of LVs in control abdominal skin (*N* = 42 vessels from five individual mice), in the inner tumor mass of 4T1 tumors (*N* = 40 vessels from six individual mice), and in the tumor periphery (*N* = 35 vessels from five individual mice).

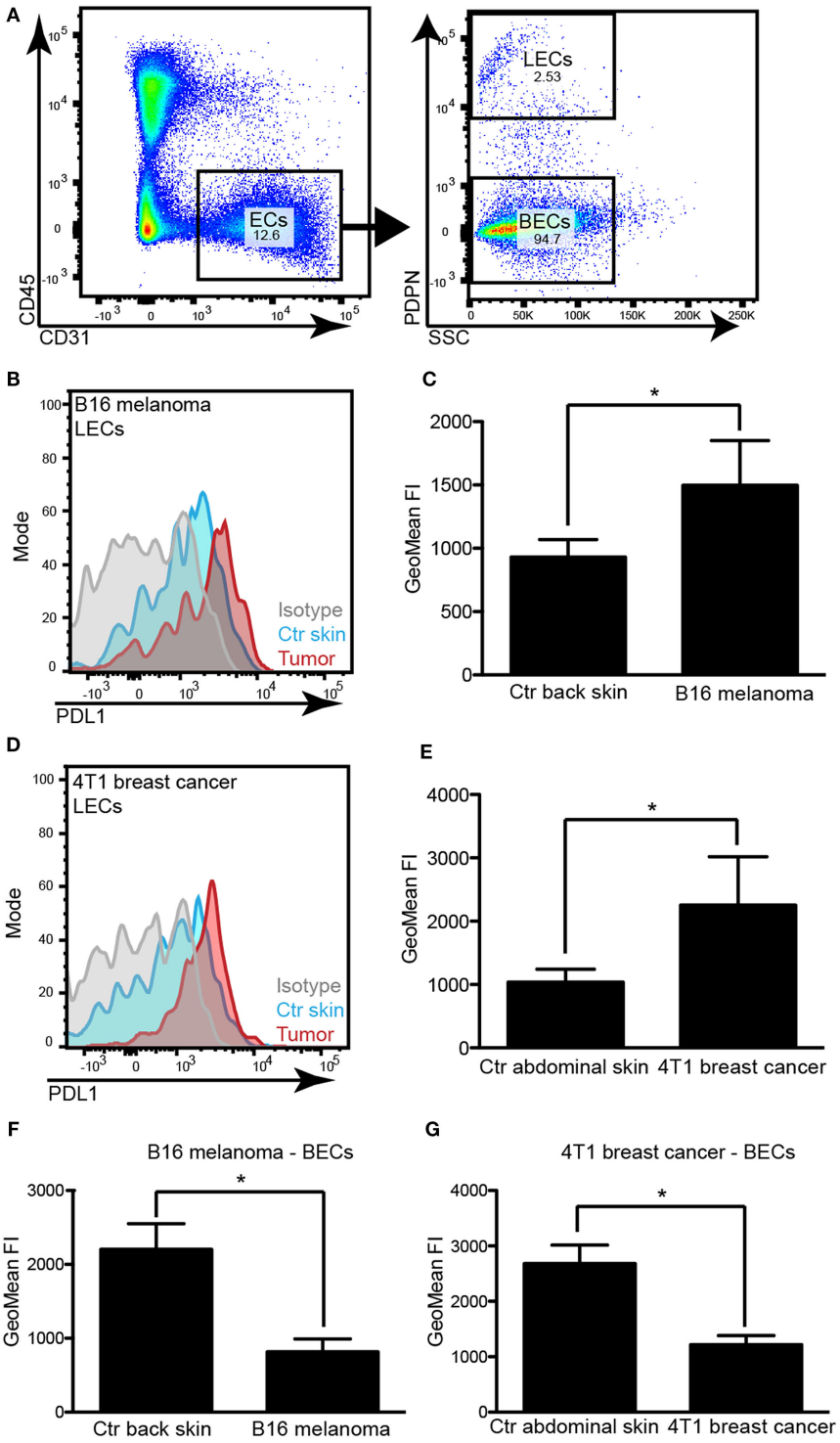

FIGURE 2 | **PDL1 is upregulated in tumor-associated lymphatic vessels but down-regulated in tumor-associated blood vessels**. **(A)** Example FACS plots to illustrate the gating strategy used to identify lymphatic endothelial cells (LECs) and blood vascular endothelial cells (BECs) in skin samples and tumors. Left panel: total endothelial cells (ECs) were identified as CD31+CD45− cells in a control skin sample (pre-gated for living singlets). Right panel: LECs were differentiated from BECs by staining for podoplanin (PDPN). **(B)** Example histogram of PDL1 staining intensity measured by FACS in LECs of control (Ctr) back skin and B16F10-VEGFC tumors. **(C)** Quantification of staining intensity in control (Ctr) back skin and B16F10-VEGFC-associated LECs ($N = 5$ mice/group). **(D)** Example histogram of PDL1 staining intensity measured by FACS in LECs of control abdominal skin and 4T1 tumors. **(E)** Quantification of staining intensity in control abdominal skin and 4T1-associated LECs ($N = 4$ mice/group; one out of two experiments with similar results is shown). **(F,G)** Quantification of PDL1 staining intensity in BECs of B16F10-VEGFC tumors [**(F)**, $N = 5$ mice/group) and of 4T1 tumors [**(G)**, $N = 4$ mice/group; one out of two experiments with similar results is shown].

tumor-associated LECs expressed higher levels of PDL1 than LECs in normal skin (**Figures 2B–E**). It is of interest that PDL1 expression on BECs showed the opposite behavior, with a significant reduction in PDL1 expression in tumor-associated BECs compared to normal skin BECs (**Figures 2F,G**), indicating that BECs react very differently to stimuli derived from the tumor microenvironment. To investigate whether PDL1 expression by LVs might be induced only in very late stages of tumor growth, and might thus not be relevant for the inhibition of T-cell responses, which are likely triggered during the early growth phase of tumors, we next analyzed lymphatic PDL1 expression already 8 days after 4T1 implantation. Similar to our observation at the 3-week time point, we found PDL1 induced in LECs and reduced in BECs (Figure S1 in Supplementary Material).

PDL1 Expression in LN Stromal Cells Is Not Affected by the Presence of an Upstream Tumor

Lymph node LECs have previously been reported to express PDL1 under steady-state conditions, depending on lymphotoxin signaling (11). Cytokines and growth factors drained from an upstream inflammatory site or a tumor mediate expansion of the lymphatic vasculature of the draining LN [reviewed in Ref. (2)], but their effect on the PDL1 expression by LN LECs has not been investigated. We therefore analyzed the PDL1 expression in the major LN stromal cell subsets [CD31+/podoplanin+ LECs, CD31+/podoplanin− BECs, CD31−/podoplanin+ follicular reticular cells (FRCs), and CD31−/podoplanin− double negative (DN) cells; **Figure 3A**] in tumor draining inguinal LNs and in inguinal LNs of naive mice. In agreement with previous reports (12), we found that LECs expressed the highest PDL1 levels among those cell types. The surface levels of PDL1 did not change significantly in response to the presence of an upstream tumor (**Figure 3B**). BECs also expressed considerable amounts of PDL1 on their cell surface. In line with our observations in primary tumors, PDL1 expression on LN BECs was reduced in tumor bearing mice, especially in the 4T1 breast cancer model (**Figure 3C**). PDL1 expression in FRCs and in DN cells was generally very low and was only slightly increased by the presence of an upstream tumor (**Figures 3D,E**). Taken together, these data indicate that LN LEC expression of PDL1 is constitutively high and is not affected by cytokines or other factors drained from upstream tumors.

PDL1 Expression in LECs Is Regulated by IFN-g

To elucidate how PDL1 expression is regulated in tumor-associated LECs, we treated immortalized mouse LECs (imLECs) with several (lymph-) angiogenic growth factors (VEGF-A, VEGF-C) and inflammatory cytokines (IFN-g, TNF-a), all of which are commonly expressed in the microenvironment of various tumors. Using qPCR, we found a strong upregulation of PDL1 mRNA expression in imLECs treated with IFN-g (up to 40-fold after 24 h). TNF-a had only very minor effects on PDL1 expression (up to twofold induction), whereas VEGF-A and VEGF-C had no effect at all (**Figure 4A**). IFN-g mediated induction of PDL1 expression was also reflected at the protein level: using FACS analysis, we found that imLECs constitutively express PDL1 on their surface and dramatically upregulated it upon treatment with IFN-g for 24 h (**Figures 4B,C**). To test whether tumor cells can directly induce PDL1 expression in LECs *via* secretion of one or several soluble factors (e.g., IFN-g), we treated imLECs with conditioned media from B16F10-VEGFC and 4T1 cells. Using qPCR and FACS, we only detected a minor induction of PDL1 by tumor cell conditioned media (**Figures 4D–F**). In line with this, IFN-g expression was not detectable in cultured B16F10-VEGFC cells and in 4T1 cells (data not shown), whereas IFN-g expression in total tumor tissue was readily detectable and was increased compared to the skin of corresponding naive mice in both tumor models (**Figures 4G,H**). Taken together, these findings suggest that cells present in the tumor stroma, for example infiltrating immune cells, are responsible for the induction of PDL1 expression in tumor-associated LVs *via* secretion of IFN-g.

T-Cells Physically Interact with Tumor-Associated LVs *In Vivo*

Previously, it has been reported that in VEGF-C overexpressing B16F10 melanomas, adoptively transferred T-cells were often clustering around tumor-associated LVs, indicating a physical interaction between T-cells and LECs (21). Using double immunofluorescence stainings for the lymphatic marker LYVE-1 and for CD4 or CD8 to identify helper T-cells and cytotoxic T-cells in B16F10-VEGFC tumors, we found that endogenous T-cells, although relatively few in number, occasionally interacted with LECs, both at the luminal and the abluminal side of the endothelium (**Figure 5A**). Very similar results were obtained when we analyzed 4T1 breast cancer tissue (**Figure 5B**). Given that tumor-associated LVs express elevated levels of PDL1, this finding suggests that tumor-infiltrating T-cells may receive inhibitory signals from LECs either while still residing in the tumor stroma, or upon exiting it *via* the lymphatic system in route toward the draining LNs.

PDL1 Inhibits Antigen-Dependent Activation of T-Cells by LECs

Previously, it has been reported that presentation of the ovalbumin-derived, MHCI-restricted peptide SIINFEKL by cultured imLECs to OT-1 CD8+ T-cells reduces their activation as compared to peptide-presenting dendritic cells, with a blunted upregulation of CD25 and reduced expression of IFN-g by OT-1 cells (14). Whereas upregulation of PDL1 by peptide-presenting imLECs was observed, its role in imLEC–OT-1 interaction was not investigated (14). We hypothesized that PDL1 expressed (and upregulated) by antigen-presenting imLECs might at least in part be responsible for the reduced OT-1 stimulation. Using the same *in vitro* model system, we confirmed that imLECs upregulate surface PDL1 upon SIINFEKL peptide presentation to OT-1 T-cells, whereas PDL1 was not upregulated in absence of a specific antigen (**Figure 6A**). Next, we used a PDL1-blocking antibody to determine the role of PDL1 during imLEC-mediated OT-1 activation. FACS analyses revealed that blockade of PDL1

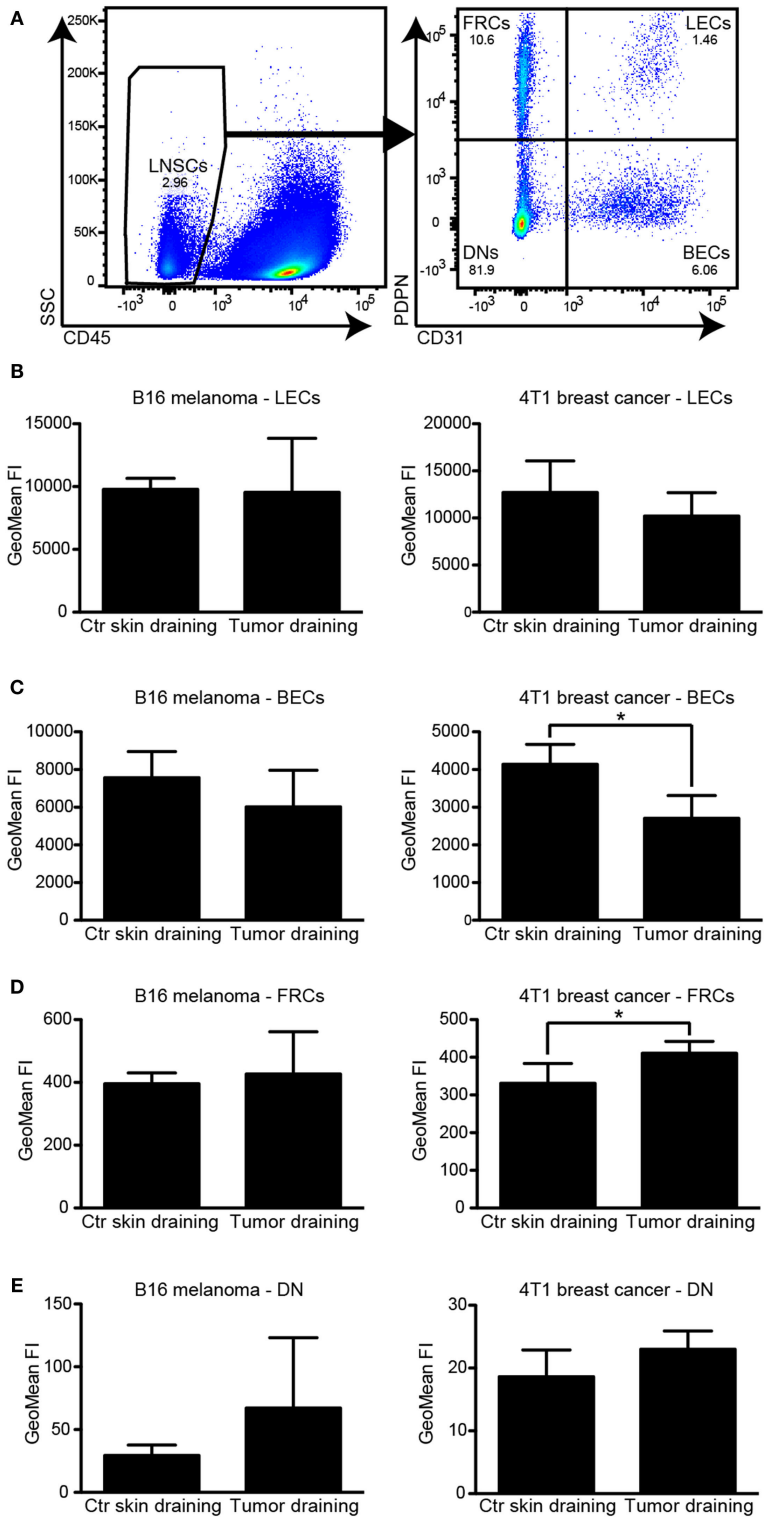

FIGURE 3 | **PDL1 is constitutively expressed in lymph node (LN) lymphatic endothelial cells (LECs) and is not affected by the presence of an upstream tumor**. **(A)** Example FACS plots of a LN (pre-gated for living singlets). CD45− LN stromal cells (left, LNSC) were separated into podoplanin (PDPN)+/CD31− follicular reticular cells (FRCs), PDPN+/CD31+ LECs, PDPN−/CD31+ BECs, and PDPN−/CD31− "double negative" (DN) cells (right panel). **(B–E)** Quantification of PDL1 staining intensity by FACS in LN LECs **(B)**, BECs **(C)**, FRCs **(D)**, and DNs **(E)** in B16F10-VEGFC draining inguinal LNs (left panels, *N* = 5 mice/group) and 4T1 draining inguinal LNs (right panels, *N* = 4 mice/group; one out of two experiments with similar results is shown), compared to control (Ctr) LNs in naive mice. LECs expressed the highest levels of PDL1. Only minor changes in PDL1 expression levels of BECs and FRCs in 4T1 draining LNs were observed.

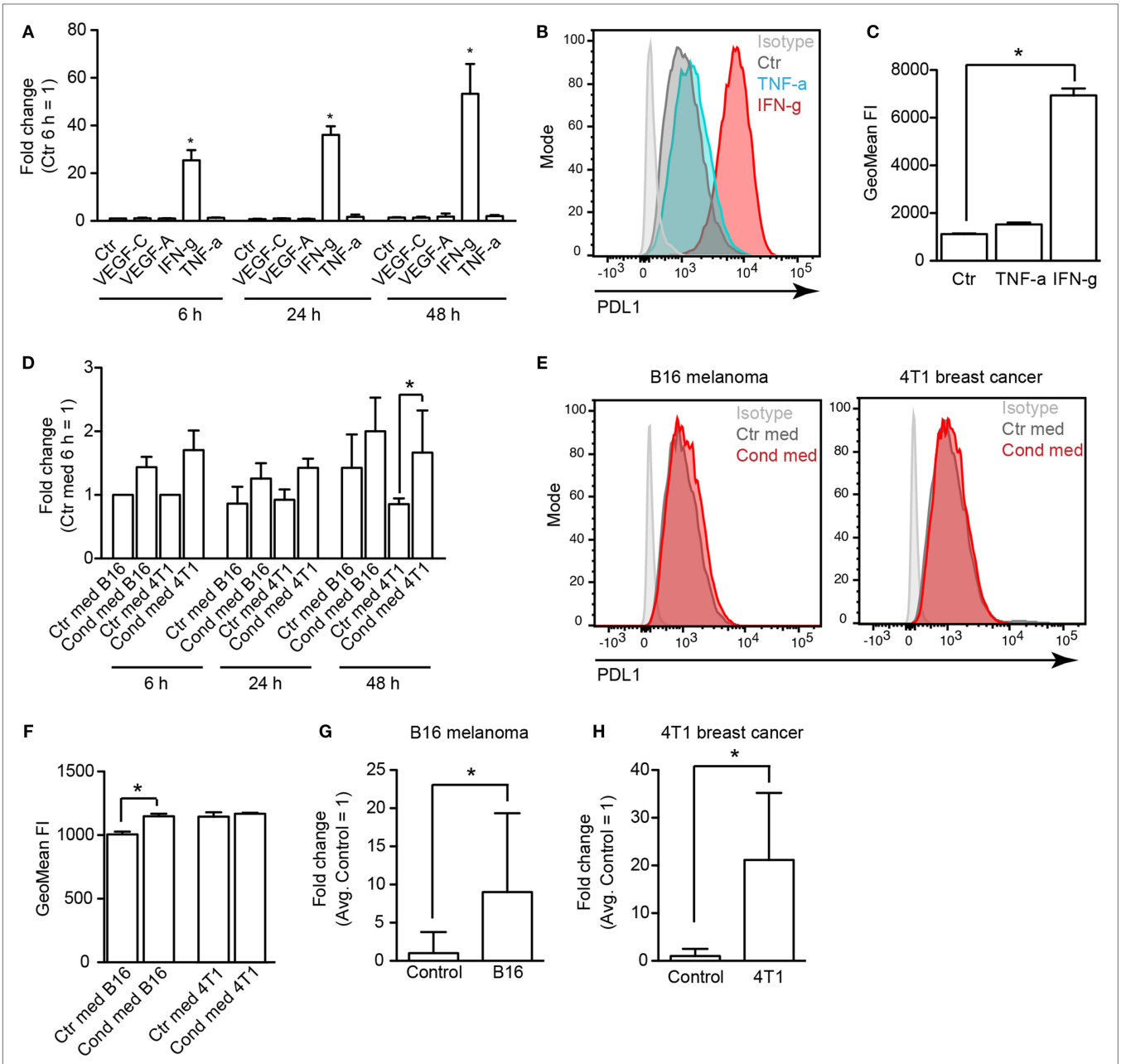

FIGURE 4 | **Lymphatic endothelial cells (LECs) upregulate PDL1 in response to IFN-g**. **(A)** Immortalized mouse LECs (imLECs) were treated with VEGF-A (20 ng/ml), VEGF-C (200 ng/ml), TNF-a (40 ng/ml), or IFN-g (100 ng/ml). Expression of PDL1 was assessed by qPCR after 6, 24, and 48 h. Incubation with IFN-g resulted in a significant upregulation of PDL1 mRNA (pooled data of three individual experiments are shown). **(B)** Example histogram of surface PDL1 expression assessed by FACS in imLECs treated with TNF-a or IFN-g or not (Ctr). **(C)** Quantification of surface PDL1 in untreated (Ctr), TNF-a treated, and IFN-g treated imLECs ($N = 3$). **(D)** qPCR showing PDL1 expression upon imLEC treatment with tumor cell conditioned media (Cond med) derived from B16F10-VEGFC or 4T1 cells, compared to control media (Ctr med) (pooled data of three individual experiments are shown). **(E)** Example histograms of surface PDL1 expression assessed by FACS in imLECs treated with B16F10-VEGFC (left) or 4T1 cell conditioned medium (right). **(F)** Quantification of surface PDL1 expression in imLECs treated with control (Ctr med) or tumor cell conditioned media (Cond med) ($N = 3$). **(G,H)** qPCR data showing IFN-g expression in B16F10-VEGFC **(G)** and 4T1 tumor tissue **(H)** compared to control tissue (back skin resp. abdominal skin) ($N = 7$–9 for B16F10-VEGFC and 4–7 for 4T1).

increased the expression of CD25 and IFN-g in OT-1 cells upon coculture with SIINFEKL-presenting imLECs (**Figures 6B–D**). Increased activation of OT-1 cells after PDL1 blockade also resulted in an elevated capacity to kill ovalbumin-expressing B16F10 tumor cells *in vitro* (Figure S2A in Supplementary Material). Interestingly, we also observed a strong upregulation of PDL1 by antigen-stimulated OT-1 T-cells themselves (Figure S2B in Supplementary Material), likely due to a paracrine effect of

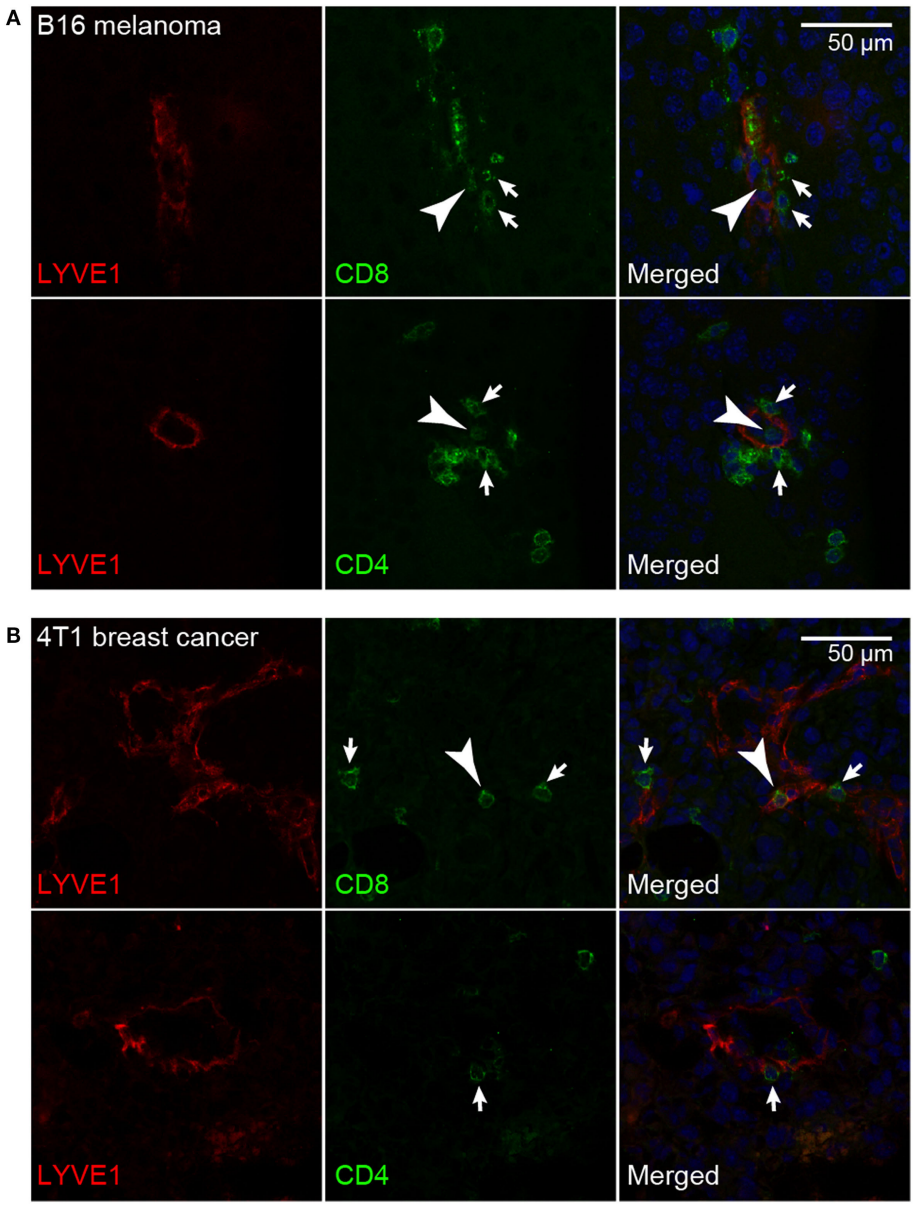

FIGURE 5 | **T-cells interact with tumor-associated lymphatic vessels**. Representative images of CD8+ and CD4+ T-cells (green) interacting with LYVE-1+ lymphatic endothelial cells (red) in B16F10-VEGFC melanoma **(A)** and 4T1 breast cancer **(B)**. T-cells interacting with the abluminal (arrows) and the luminal (arrowheads) surface of the lymphatic endothelium were observed in both tumor models.

T-cell secreted IFN-g. Thus, it is possible that the increased activation of OT-1 T-cells by antigen-presenting imLECs after PDL1 blockade is partly due to inhibition of OT-1 expressed PDL1, in addition to imLEC expressed PDL1. Therefore, we performed additional experiments in which PDL1 was specifically blocked on the surface of imLECs before coculture with OT-1 cells. Also in this setting, we found a potentiating effect of PDL1 blockade on OT-1 stimulation, resulting in elevated CD25 expression (Figure S2C in Supplementary Material), strongly suggesting that LEC expressed PDL1 indeed contributes to LEC-mediated T-cell inhibition.

DISCUSSION

Adaptive immune responses require the cooperation between cells of the immune system and the lymphatic vasculature. LNs are the principal site where naive T- and B-lymphocytes encounter antigen and where the decision is taken whether these lymphocytes become primed to proliferate and develop into effector cells, or whether they enter anergy, or even become eliminated (peripheral tolerance). Foreign and self-antigens are transported to the LNs *via* the lymphatic system, either as "cargo" transported by professional APCs, or as free molecules or complexes carried with

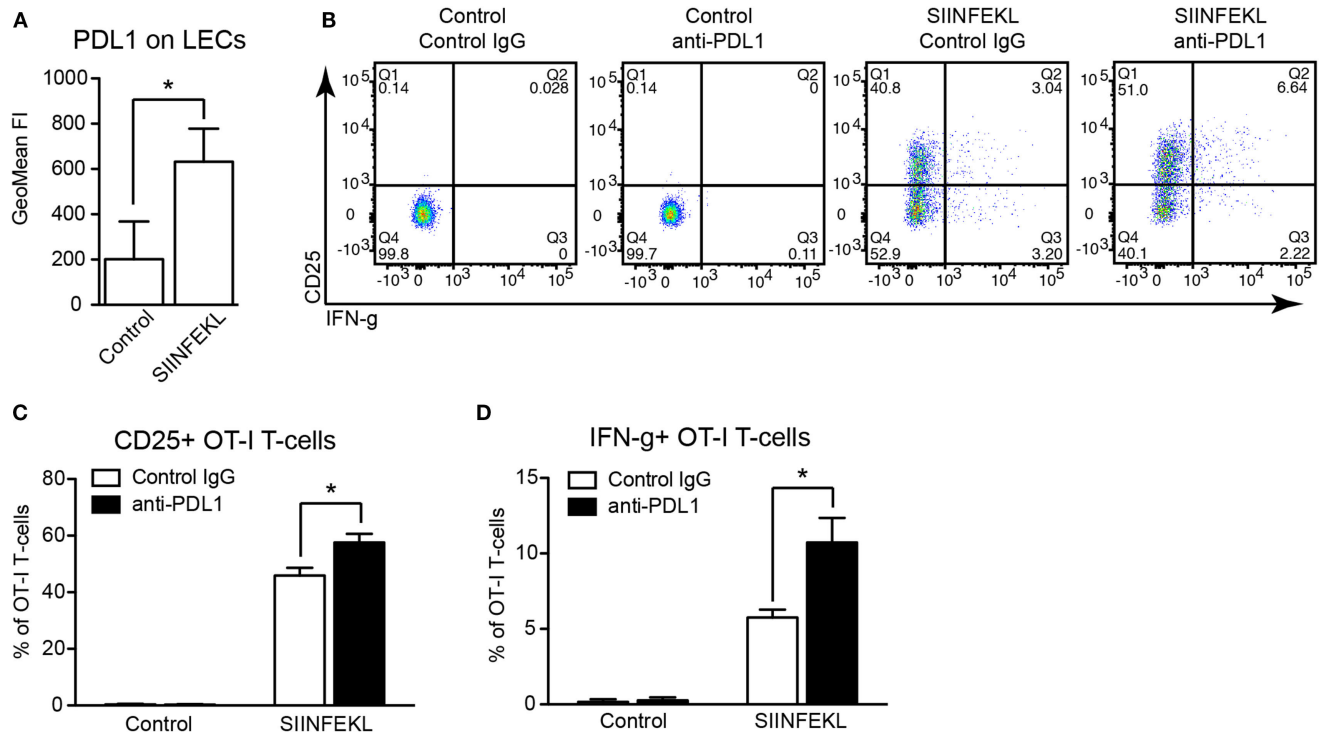

FIGURE 6 | PDL1 blockade increases antigen-specific stimulation of CD8+ T-cells by lymphatic endothelial cells (LECs). **(A)** PDL1 surface expression on immortalized mouse LECs (imLECs) cocultured with OT-1 CD8+ T-cells and pulsed with SIINFEKL peptide or not (Control) was determined by FACS ($N = 3$). **(B)** Representative FACS plots of CD25 and IFN-g expression by OT-1 CD8+ T-cells (pre-gated for living singlets) after coculture with control or SIINFEKL-pulsed imLECs, in the presence of PDL1-blocking antibodies or control IgG. **(C,D)** Quantification of CD25+ OT-1 cells **(C)** and IFN-g+ OT-1 cells **(D)** by FACS gated as in panel **(B)** ($N = 3$).

the lymph flow. Once arrived at the LN, antigens are transferred into the T- and B-cell zones, either by the LN conduit system, or by LN resident or migratory APCs.

With regards to tumor immunology, some priming of naive T-cells has been reported to occur within the tumor microenvironment [reviewed in Ref. (23)]; yet, draining LNs are still the primary site for the mounting of adaptive immune responses. Consequently, using transgenic mice, a lack of or disturbed lymphatic draining from the tumor site resulted in reduced immune activation, immune cell infiltration into the tumor, and cytokine release (9, 10), indicating that antigen transport by LVs is required for efficient antitumor immune responses. On the other hand, induction of lymphangiogenesis by forced expression of VEGF-C resulted in a blunted T-cell response in the B16F10 melanoma model (21), suggesting that the lymphatic endothelium, within the tumor and/or the draining LNs, has a direct, T-cell inhibitory function. Interestingly, tumor-associated LECs have been reported to present tumor-derived antigen (ovalbumin) on MHCI (21). Using the same tumor model (VEGF-C expressing B16F10 melanoma), we demonstrate here that tumor-associated LECs upregulate the T-cell inhibitory immune checkpoint molecule PDL1. Our data also show that this is neither dependent on the tumor model (B16F10) nor on the genetic background (C57BL6) or the experimental overexpression of VEGF-C, as PDL1 was also upregulated in a completely unrelated tumor model, the 4T1 breast cancer model in the Balb/c background.

Presentation of antigen on MHCI in combination with PDL1 expression has been found to be a specific feature of LN LECs (11, 12). This phenomenon has been linked to the maintenance of peripheral immune tolerance, as LN LECs express and present various self-antigens, at least under steady-state conditions, reminiscent of the thymic epithelium which is responsible for central tolerance (13). The fact that PDL1 is upregulated on tumor-associated LECs as found here, as well as in acutely inflamed LECs (20), suggests that under pathological conditions, a very similar T-cell inhibitory mechanism is activated in peripheral LVs as well. Of note, we and others have shown that induction of lymphangiogenesis in inflammatory conditions ameliorates inflammation and promotes resolution (6–8). It is tempting to speculate that direct inhibition of T-cells, in addition to increased lymph drainage, may contribute to this effect. In the tumor context, multiple pathways and factors have been identified that inhibit the activity of tumor-infiltrating lymphocytes, resulting in T-cell anergy or exhaustion. Peripheral LECs presenting tumor-derived antigens with concomitant PDL1 upregulation by tumor-associated LECs may to some extent contribute to overall T-cell inhibition in the tumor microenvironment, which is consistent with the observations made before (21). On the other hand, it is intriguing that PDL1 expression on tumor-associated LVs may particularly

affect antigen-experienced T-cells, including memory T-cells, which have the capacity to recirculate from the tumor site to the circulation *via* the lymphatic system. Therefore, further studies are highly warranted to elucidate whether tumor-associated LECs particularly inhibit the development of memory responses. Inhibition of recirculating T-cells entering LVs may also have a negative impact on the priming of additional naive T-cells in downstream LNs.

PDL1 is an IFN-g target gene in microvascular ECs (18), and our data reveal that IFN-g treatment induces PDL1 expression in cultured LECs as well. As we found IFN-g to be expressed in the tumor microenvironment of both tumor models that we studied, we suggest that the observed PDL1 upregulation in tumor-associated LECs is in fact mediated by IFN-g. Consistently, we found no major PDL1 induction by tumor cell conditioned media, which did not contain significant amounts of this cytokine. Therefore, the regulation of PDL1 in LVs in the periphery differs from that in the LNs, where it was reported to be dependent on lymphotoxin, a specific activator of non-canonical NF-kB signaling (11). As we found no significant effect of TNF-a on PDL1 expression, at least *in vitro*, it is likely that only the non-canonical, but not the canonical, NF-kB pathway regulates PDL1 expression in LN LECs. In line with this concept, the PDL1 expression in LN LECs was not affected by the presence of a tumor and thus, by tumor-derived factors drained to the LNs.

Surprisingly, we observed the opposite effect on tumor-associated blood vessels. In contrast to LECs, we found that peripheral BECs in the skin express PDL1 under steady-state conditions, but that they downregulate it in presence of a tumor. Possibly, PDL1 upregulation by IFN-g is blocked in tumor-associated BECs due to high expression of VEGF-A and the induction of angiogenesis, which may interfere with inflammatory activation of BECs (24, 25). Functionally, PDL1 expression on BECs has been reported to inhibit autoreactive CD8+ T-cells in a myocarditis model (26), indicating that reduced PDL1 expression in tumor-associated BECs might facilitate infiltration of activated effector T-cells into the tumor stroma. However, further studies are needed to test this hypothesis and to investigate whether the PDL1 expression level on tumor BECs is higher in tumor models that show no T-cell infiltration at all.

Despite the findings that PDL1 is constitutively expressed by LN LECs (11, 12) and that it is upregulated on tumor-associated LECs as identified here, its precise role in LEC-mediated T-cell inhibition is not entirely clear. Treating naive mice with PDL1-blocking antibodies resulted in the development of autoimmune vitiligo after transfer of T-cells specific for the melanocyte-specific antigen tyrosinase (12). Using chimeric mice carrying PDL1−/− bone marrow, it was furthermore reported that the inhibition of autoreactive T-cells was mediated by a PDL1+, radioresistant stromal cell type, consistent with LN LECs being responsible for the maintenance of peripheral tolerance toward tyrosinase in this model (12). Similarly, cultured LECs presenting the ovalbumin-derived SIINFEKL peptide to OT-1 CD8+ T-cells have been described to upregulate PDL1 and to activate OT-1 cells much less effectively than dendritic cells, resulting in reduced expression of the activation marker CD25 and production of IFN-g (14). Nonetheless, no direct evidence that PDL1 expression by LECs is indeed required and/or sufficient for T-cell inhibition has been published so far. Using the same *in vitro* system of cultured LECs presenting the SIINFEKL peptide to OT-1 cells, we reveal here that inhibition of PDL1 with a blocking antibody indeed increases CD25 and IFN-g expression by OT-1 cells, and harnesses them for killing of ovalbumin-expressing tumor cells. As OT-1 cells themselves strongly upregulate PDL1 in this setting (indicating that PDL1 expression might serve as a sensitive marker of T-cell activation), we also pre-blocked PDL1 specifically on the LEC surface before coculturing them with OT-1 cells. This way of PDL1 blockade is conceivably less efficient than adding the blocking antibodies throughout the coculture period, due to additional PDL1 upregulation by the imLECs during this time period. Furthermore, dynamic antibody binding and dissociation may still result in some PDL1 blockade on the OT-1 T-cells during the coculture. Nonetheless, our observation that the activation of OT-1 cells was increased even in this setting further supports the notion that LEC expressed PDL1 at least partially dampens the activation of T-cells by antigen-presenting LECs. However, based on our data, an additional role of T-cell expressed PDL1 acting as a negative feedback regulator of T-cell activation cannot be entirely excluded at this moment.

Clinically, the PDL1 expression in cancer has lately received considerable attention due to the development of highly potent PD1-blocking antibodies (nivolumab and pembrolizumab) as well as PDL1-blocking antibodies (e.g., atezolizumab and durvalumab) which show dramatic improvements of outcome in melanoma patients or are in advanced clinical studies, respectively (27). However, only a subset of patients profits from the treatment, and the search for predictive biomarkers to reliably identify those patients is ongoing. PDL1 expression on cancer cells is currently regarded as such a biomarker and has recently been approved by the US Food and Drug Administration as a companion or complementary diagnostic test for certain specific tumor entities. However, there are difficulties and inconsistencies in the clinical protocols to determine the rate of PDL1+ cancer cells in biopsy material as there is no fully standardized detection antibody, definition of cutoffs for the evaluation of the stainings, tissue preparation, and processing protocols available yet. Furthermore, it is debatable whether the currently widely used detection in cancer cells itself is the optimal predictive marker (28). In this regard, it is interesting that a recent study performed in patients with multiple different tumor types found that PDL1 expression in stromal cells, including tumor-infiltrating leukocytes, was superior in predicting patients who would benefit from treatment with a new PDL1-blocking antibody currently under development (29). Furthermore, there is growing evidence that at least for some malignancies, patients treated with anti-PD1 antibodies have a survival benefit independent of the expression of its ligand PDL1 on tumor cells (30). Thus, it is likely that additional (stromal) cell types are involved in the inhibition of antitumor T-cell responses *via* the PDL1-PD1 axis. In the light of our findings presented here, assessment of the vascular PDL1 expression may yield additional predictive accuracy and may further improve the selection of patients to undergo treatment with PD1 or PDL1 inhibitors in the future.

MATERIALS AND METHODS

Cell Lines

B16F10 cells expressing luciferase and human VEGF-C have been generated and described previously (22). Cells were cultured in DMEM containing Glutamax, pyruvate, 10% FBS, and penicillin/streptomycin (all from Gibco/Thermo Fisher). G418 (1.5 mg/ml, Roche) was added to the culture medium to ensure stable expression of the VEGF-C transgene. 4T1 mammary carcinoma cells expressing luciferase (Caliper Life Sciences) were maintained in DMEM supplemented with L-glutamine, 10% FBS, and penicillin/streptomycin under standard culture conditions (37°C, 5% CO_2). ImLECs isolated from *H-2Kb-tsA58* (Immorto) mice have been described previously (20) and were maintained on collagen type I (Advanced Biomatrix)/fibronectin (Millipore) coated dishes (10 µg/ml each) in Ham's F12/DMEM supplemented with 20% FBS, 56 µg/ml heparin (Sigma), 10 µg/ml EC growth supplement (Abd Serotec/BioRad), penicillin/streptomycin, and 1 U/ml recombinant mouse IFN-g (Peprotech) at 33°C, 5% CO_2. Before experiments, cells were cultured at 37°C without IFN-g for at least 72 h, which leads to a reduction of the polyoma T antigen in these cells.

Mice and Tumor Models

C57BL/6 and Balb/c wild-type mice were obtained from Janvier. OT-1 transgenic mice were kindly provided by Dr. Roman Spörri and Dr. Annette Oxenius, ETH Zurich. All mice were bred in house under SOPF conditions. For the B16F10-VEGFC melanoma model, C57BL/6 mice were depilated on the back and 2×10^5 tumor cells suspendend in 20 µl PBS were injected intradermally into the flank. For the 4T1 breast cancer model, 1×10^5 tumor cells suspended in 50 µl PBS were injected into the fourth mammary fat pad of Balb/c mice. The primary tumor growth was monitored for 2 weeks (B16F10-VEGFC) or 3 weeks (4T1) before tissues were prepared for analysis as described below. In case of the 4T1 model, some FACS analyses were already performed at day 8 after implantation. All tumor studies were performed in agreement with the regulations of the local ethical board (Kantonales Veterinäramt Zürich, license 12/15).

Immunofluorescence Staining and Image Analysis

Tumors were dissected, embedded in OCT compound, snap frozen, and stored at −80°C until preparation of cryosections (7 µm). For stainings, sections were air-dried, fixed in ice-cold acetone and 80% methanol, rehydrated in PBS, and subsequently blocked in PBS + 0.2% BSA, 5% donkey serum, 0.3% Triton-X100, and 0.05% NaN_3 (blocking solution). Primary antibodies [rabbit anti-LYVE-1 (1:600, Angiobio), rat anti-PDL1 (2 µg/ml, clone 10F.9G2, Biolegend), rat anti-CD4 (5 µg/ml, clone H129.19, BD), and rat anti-CD8 (5 µg/ml, clone 53.6-7, BD)] suspended in blocking solution were incubated at room temperature for 2 h or at 4°C over night, followed by extensive washing and incubation with Alexa488 or Alexa594-conjugated secondary antibodies (donkey anti-rat, donkey anti-rabbit, 10 µg/ml, Life Technologies/Thermo Fisher) together with Hoechst33342 (2 µg/ml, Sigma) for nuclear counterstaining. Finally, slides were washed extensively again and mounted using Mowiol.

Images were taken on a Zeiss Axioskop 2 mot plus with a 10× or a 20× objective, or a Zeiss LSM780 inverted confocal microscope at 20×. Image analysis was performed using ImageJ (NIH). To determine the staining intensity of PDL1 in LVs, LYVE-1+ vessels were selected by thresholding, using size exclusion to exclude single LYVE-1+ macrophages. Subsequently, the average PDL1 staining intensity within each LV was measured.

FACS Analysis of Tumor Tissue and LNs

For FACS analysis of tumors and control tissues (back skin and abdominal skin, respectively), the tissue was dissected, minced, and digested in a collagenase solution [5 mg/ml Collagenase II (Sigma), 40 µg/ml DNaseI (Roche)] for 30 min at 37°C. The digested tissue was passed through a cell strainer before erythrocyte lysis with PharmLyse (BD). After washing and a second filtration step, the cell suspension was labeled with fluorescently tagged antibodies [hamster anti-podoplanin-PE (1:400, clone 8.1.1, eBioscience), rat anti-CD31-APC (1:300, clone MEC13.3, BD), rat anti-CD45-APC/Cy7 (1:200, clone 30-F11, Biolegend), and rat anti-PDL1-PE/Cy7 (1:200, clone 10F.9G2, Biolegend)]. 7AAD (Biolegend) was used for life/dead discrimination. Inguinal LNs were processed essentially as described before (31). In brief, the capsule of dissected LNs was ruptured and the tissue was digested with 1 mg/ml Collagenase IV (Gibco)/40 µg/ml DNaseI for 20 min at 37°C to release the majority of the immune cells. The remaining stromal fragments were washed twice, digested with 3.5 mg/ml Collagenase IV/40 µg/ml DNaseI for 15 min at 37°C, and disaggregated by pipetting in presence of 0.5 mM EDTA. After filtration, the stromal cell enriched cell suspension was stained as described above. Data were acquired on a FACS CANTO (BD) and analyzed using FlowJo (Treestar Inc.).

LEC Stimulation *In Vitro*

imLECs were starved over night in Ham's F12/DMEM + 1% FBS and subsequently stimulated with VEGF-A (20 ng/ml, Cell Sciences), VEGF-C (200 ng/ml, R&D), IFN-g (100 ng/ml, Peprotech), or TNF-a (40 ng/ml, Peprotech). Tumor cell conditioned media were prepared by culturing 1×10^7 B16F10-VEGFC or 4T1 cells in medium supplemented with 1% FBS for 72 h. The conditioned media were centrifuged, filtered through a 0.45 µm filter, and stored at −80°C until use. ImLECs were stimulated with 50% conditioned media in Ham's F12/DMEM + 1% FBS. DMEM with Glutamax and pyruvate, or DMEM with L-glutamine (both with 1% FBS) served as control media. For FACS analysis, LECs were washed with PBS and trypsinized with 0.01% trypsin/EDTA. Cells were labeled with primary antibody (rat anti-PDL1, 2 µg/ml, clone 10F.9G2, Biolegend), washed and labeled with a secondary antibody (donkey anti-rat-Alexa488, 10 µg/ml, Life Technologies/Thermo Fisher). 7AAD was used for life/dead discrimination. Data were acquired on a FACS CANTO and analyzed using FlowJo.

RNA Extraction and qPCR

RNA from stimulated LECs was extracted at the indicated time points using the Nucleospin RNA kit (Macherey-Nagel)

according to the manufacturer's instructions. Total tumor and control tissue RNA was extracted from cryosections using the RNeasy Plus Micro kit (Qiagen). All RNA was reverse transcribed using the High Capacity cDNA kit (Applied Biosystems/Thermo Fisher). qPCR analyses were performed on a 7900HT FAST instrument (Applied Biosystems/Thermo Fisher) in triplicate using SYBRGreen (Roche). RPLP0 was used as internal reference gene. Relative expression (RE) calculated according to the formula $RE_{geneX} = 2^{-(Ct_{geneX} - Ct_{RPLP0})}$ and was expressed as fold change normalized to the control condition. The primer sequences used for qPCR were RPLP0 fwd: AGATTCGGGATATGCTGTTGG, rev: TCGGGTCCTAGACCAGTGTTC; PDL1 fwd: ACAAG CGAATCACGCTGAAAG, rev: GGCCTGACATATTAGTTCA TGCT; and IFNG fwd: ACACTGCATCTTGGCTTTGC, rev: CTGGCTCTGCAGGATTTTCA.

OT-1 T-Cell Activation and Tumor Cell Killing Assay

For OT-1 stimulation experiments, CD8+ OT-1 T-cells were isolated from the spleens of naive OT-1 mice using CD8+ MACS beads (Miltenyi) according to the manufacturer's instructions. ImLECs were starved over night in Ham's F12/DMEM + 1% FBS, pulsed with 1 ng/ml SIINFEKL peptide (AnaSpec) for 1 h, and washed three times with PBS. Subsequently, OT-1 and peptide-pulsed imLECs were cocultured for 24 h at a 10:1 ratio in the presence of 10 μg/ml PDL1-blocking antibody (clone 10F.9G2, Biolegend) or control rat IgG (Sigma). For analysis of T-cell activation, OT-1 cells were harvested and stained with rat anti-CD8-FITC (1:200, clone 53.6-7, BD), rat anti-PDL1-PE (1:200, clone MIH5, eBioscience), and rat anti-CD25-PerCP (1:200, clone PC61, Biolegend). Intracellular staining for IFN-g (1:200, clone XMG1.2 conjugated to APC, Biolegend) was done using the Cytofix/Cytoperm kit (BD) and fixable Zombi-NIR (BioLegend) was used for life/dead discrimination. For imLEC pre-blocking experiments, the PDL1-blocking antibody was incubated with the imLECs together with the SIINFEKL peptide pulse before washing and coculture with OT-1 cells. For tumor cell killing assays, OT-1 cells were stimulated with peptide-pulsed imLECs as described above and subsequently cocultured with B16F10 cells expressing ovalbumin at a 1:5 target:effector ratio for 8 h. Zombi-NIR was used to determine the ratio of dead and life tumor cells. All activation and killing assays were performed in triplicate. Data were acquired on a FACS CANTO and analyzed using FlowJo.

Statistical Analyses

All statistical analyses were performed with GraphPad Prism 5 (GraphPad Software Inc.). All bars indicate mean + SD. Student's *t*-test was used to compare two groups, one-way ANOVA with Tukey's post-test was used to compare more than two groups, and two-way ANOVA with Bonferroni post-test was used to compare data grouped by two variables. A p-value <0.05 was considered statistically significant (indicated by asterisks).

AUTHOR CONTRIBUTIONS

LD designed and performed experiments, analyzed and interpreted the data, and wrote the manuscript. KI, TC, KK, and CH performed experiments, and analyzed and interpreted the data. MD designed experiments, interpreted data, and revised the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2017.00066/full#supplementary-material.

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REVIEW
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The Role of the Tumor Vasculature in the Host Immune Response: Implications for Therapeutic Strategies Targeting the Tumor Microenvironment

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Recently developed cancer immunotherapy approaches including immune checkpoint inhibitors and chimeric antigen receptor T cell transfer are showing promising results both in trials and in clinical practice. These approaches reflect increasing recognition of the crucial role of the tumor microenvironment in cancer development and progression. Cancer cells do not act alone, but develop a complex relationship with the environment in which they reside. The host immune response to tumors is critical to the success of immunotherapy; however, the determinants of this response are incompletely understood. The immune cell infiltrate in tumors varies widely in density, composition, and clinical significance. The tumor vasculature is a key component of the microenvironment that can influence tumor behavior and treatment response and can be targeted through the use of antiangiogenic drugs. Blood vascular and lymphatic endothelial cells have important roles in the trafficking of immune cells, controlling the microenvironment, and modulating the immune response. Improving access to the tumor through vascular alteration with antiangiogenic drugs may prove an effective combinatorial strategy with immunotherapy approaches and might be applicable to many tumor types. In this review, we briefly discuss the host's immune response to cancer and the treatment strategies utilizing this response, before focusing on the pathological features of tumor blood and lymphatic vessels and the contribution these might make to tumor immune evasion.

Keywords: endothelial cells, lymphatic endothelial cells, angiogenesis inhibitors, tumor immune evasion, immunotherapy

INTRODUCTION

The interaction between tumor cells and the microenvironment in which they exist is increasingly recognized as a key player in the development and progression of cancer. The microenvironment of a tumor includes the blood and lymphatic vasculatures, stroma, nerves, and cells of the immune system, which may be resident in the involved tissue or recruited from the periphery. The hallmarks

of cancer include features of the tumor cells themselves, such as replicative immortality and resistance to cell death, as well as features relating to the microenvironment, such as induction of angiogenesis and evasion of the immune response (1). Successful reversal of this immune evasion by checkpoint inhibitors is now a clinical reality, with inhibitors of cytotoxic T lymphocyte-associated protein-4 (CTLA-4) as well as programed cell death protein-1 (PD-1) and programed death ligand-1 (PD-L1) delivering durable responses in a subset of patients with a range of cancer types including melanoma (2, 3), urothelial carcinoma (4), Hodgkin lymphoma (5), non-small cell lung carcinoma (6–8), Merkel cell carcinoma (9), and squamous cell carcinoma of the head and neck (10). In addition, decades of research into the use of adoptive cell transfer and genetic engineering of tumor killing T cells has resulted in breakthrough therapy designation of anti-CD19 chimeric antigen receptor (CAR) T cell transfer for use in B-acute lymphoblastic leukemia (11). However, there is marked variability in patient response to immune checkpoint blockade (12), and the use of CAR T cells against solid tumors has seen little success in the clinic (13).

Immunotherapy, particularly checkpoint inhibitors, differs from conventional cancer therapies. A complex intermediate step is introduced by activating the host's immune system, instead of a direct toxic effect on tumor cells or targeting of a tumor cell-specific mutation. Understanding the tumor microenvironment is critical to understanding the exact mechanisms of actions of these therapies and predicting response. There is a clear need for robust microenvironmental biomarkers to direct therapeutic strategies. The presence of tumor-infiltrating lymphocytes (TILs) is correlated with improved prognosis in many tumor types, as well as improved response to some conventional therapies and most immunotherapies (14). Tumors can exert direct effects to adapt to, escape, and suppress antitumor immunity, which is reviewed in Ref. (15). The access of immune cells to the tumor is a critical factor in the efficacy of both adoptive cell transfer and immune checkpoint inhibition, and the role of the tumor vasculature in providing or blocking access to the tumor is likely to prove an important consideration in immunotherapeutic strategies. In addition, blood vessels, lymphatic vessels, and the hypoxic tumor environment have important immunomodulatory roles, which contribute to the immune evasion of tumors. In this review, we provide a brief overview of factors affecting the host immune response to tumors and current immunotherapy approaches, which show exciting clinical results. We then focus on the molecular and mechanical features of the tumor vasculature that modulate the host antitumor immune response and consider the implications of these interactions for potential therapeutic approaches to enhance immunotherapy.

THE HOST IMMUNE RESPONSE TO TUMORS

For an effective host immune response, the tumor must be recognized as foreign and the immune effector cells must be able to access the tumor to destroy it. It is well established that tumors are antigenic and able to induce a systemic, tumor-specific immune response (16, 17). Unstable tumor genomes contain many mutations that generate altered protein products, which have the potential to be recognized as foreign by the host immune system during surveillance. The tumors must therefore develop mechanisms of evading this immune response in order to establish, grow, and eventually metastasize. For example, circulating T cells specific to tumor antigens can be demonstrated in patients with metastatic melanoma, yet the tumor progresses (18, 19).

There is wide variation in the immune cell infiltrate seen in solid tumors, both within and between different tumor types, which is illustrated in **Figure 1**. This can provide important prognostic and predictive information. The density of TILs correlates with improved survival in many tumors ranging from melanoma to colorectal cancer, renal cell carcinoma, and non-small cell lung carcinoma (20). However, specific immune cell subsets modify this association, including regulatory T cells (T_{regs}), myeloid-derived suppressor cells (MDSCs), and tumor-associated macrophages (TAMs) (20, 21). The presence of TILs has also been shown to be predictive of response to conventional anticancer treatment, for example, anti-HER2/neu therapy and trastuzumab and anthracycline chemotherapy in breast cancer (22). A classification of tumors based on their immune phenotypes has been proposed, both as a broad conceptual approach (23, 24) and as specific quantitative scoring (21). Broadly, tumors can be classified as "T-cell inflamed" or "non-inflamed" based on the presence or absence of $CD8^+$ cytotoxic T cells within the tumor (23). For example, **Figure 1A** shows a basal phenotype breast carcinoma with a

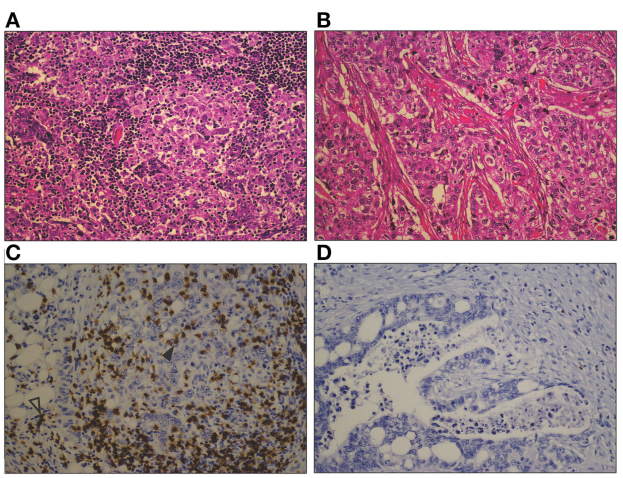

FIGURE 1 | Photomicrographs comparing a heavy lymphocytic infiltrate in a basal phenotype breast carcinoma (A), with a sparse infiltrate in a different basal phenotype breast carcinoma (B) (H&E, original magnification 200×). A similar contrast is seen between a marked $CD8^+$ T cell infiltrate in a mismatch repair-deficient colon cancer **(C)**, and the sparse infiltrate in a mismatch repair proficient colon cancer **(D)**. $CD8^+$ T cells are seen both within the tumor epithelium (closed arrowhead) and in the tumor stroma (open arrowhead) (CD8 immunohistochemical stain, original magnification 200×).

florid lymphocytic infiltrate, whereas **Figure 1B**, which is also a basal phenotype breast carcinoma, shows very few TILs. Even in melanoma, widely accepted as an immunogenic tumor and the solid tumor in which immunotherapy has had the most success, approximately 40% of tumors display a non-inflamed phenotype (24). The existence of an inflamed phenotype is supported by gene expression profiling of tumors, through which a subset of tumors rich in immune-related gene transcripts has been identified in pancreatic ductal adenocarcinoma, colorectal carcinoma, and melanoma (25–27). A multitude of different scoring systems and methodologies have been proposed to describe the immune infiltrate in tumors, with variable reproducibility and practicality (21, 28, 29). As such, use of these scoring systems is limited in routine pathology practice, despite the valuable information they could convey.

It is hypothesized that the mutational load of the tumor correlates with the presence of an immune infiltrate, due to the greater potential for neoantigen formation. In support of this hypothesis is the evidence that mismatch repair deficient tumors with vast mutational loads show higher immune cell infiltrates than mismatch repair proficient tumors (30) (for example, see **Figures 1C,D**, respectively). The tumor types showing high levels of response to immune checkpoint blockade—melanoma, smoking associated lung cancer, and urothelial cancer—are the tumor types with the highest overall mutational loads (31). However, this correlation is weak at an individual tumor level, as the presence of mutations does not necessarily result in neoantigen formation, and multiple factors are involved in the presentation of antigens to elicit an immune response (32, 33). In addition, the extent and composition of the immune infiltrate varies widely between individual tumors within these highly mutated types (29, 34). Features of the microenvironment, including blood and lymphatic vessel structure, stromal fibroblasts, and extracellular matrix, may contribute to this variation by modulating the access of immune cells to the tumor and their activation and function in the tumor microenvironment.

Trafficking of effector T cells to tumors is complex and tightly regulated. T cell migration, activation, and differentiation are intricately linked processes. Following activation by antigen-presenting cells (APCs), T cells upregulate chemokine receptors and ligands for endothelial adhesion molecules. Binding of inflammatory chemokines enhances adhesion and extravasation, allowing effector T cells to enter the tumor microenvironment (35, 36). Levels of chemokines within tumors, particularly the CXCR3 ligands CXCL9 and CXCL10, have been shown to correlate with T cell infiltration into tumors and enhanced antitumor responses (37, 38). Chemokine/chemokine receptor mismatching is postulated as an important mechanism of reduced T cell trafficking into tumors (35). Post-translational modification of chemokines can also affect immune cell infiltration. For example, nitration of CCL2 as a result of the intratumoral production of reactive nitrogen species can reduce T cell infiltration into tumors, while macrophages and MDSCs can still be attracted by nitrated CCL2 (39).

Once arriving within the tumor microenvironment, T cells must also proliferate locally, as evidenced by the enrichment of cancer-specific T cells in the tumor compared to the peripheral blood (40). A range of cellular, metabolic, and molecular features of the tumor microenvironment contribute to limit the proliferation and activation of antitumor immune effector cells. Activation of $CD8^+$ T cells requires APCs that can efficiently cross-present antigen. However, hypoxia in the tumor microenvironment can impair the maturation and differentiation of dendritic cells (DCs) and polarize macrophages to an immunosuppressive phenotype (41). Nutritional depletion, hypoxia, and reactive nitrogen species, features characteristic of the abnormal metabolic environment of tumors, can limit the activation of T cells and induce apoptosis [reviewed in Ref. (42)]. Enzymes contributing to immunosuppression are also found in the tumor microenvironment. Indoleamine 2,3-dioxygenase (IDO) is an intracellular enzyme preferentially expressed by subsets of APCs, which functions to catalyze catabolism of tryptophan to kynurenine (43). Depletion of tryptophan and accumulation of kynurenine in the tumor microenvironment impairs DC function and limits the clonal expansion of T cells (44), induces $CD8^+$ T cell anergy (45), and promotes T_{reg} induction and activation (46, 47). IDO has been implicated in resistance to immune checkpoint inhibitors (48), and blockade of the IDO pathway is under investigation in clinical trials (49). Depletion of L-arginine in the microenvironment can also result in the impairment of T cell function. Enzymes of the arginase and nitric oxide synthase (NOS) families control the metabolism of L-arginine [reviewed in Ref. (50)]. Expression of inducible NOS and arginase-1 has been demonstrated to limit T cell responses and promote the immunosuppressive microenvironment in different tumor types (51–53). These metabolic features of the tumor microenvironment combine with cellular mechanisms such as the expression of co-inhibitory immune checkpoint molecules [reviewed elsewhere (54)] to control the activity and proliferation of immune cells in the tumor microenvironment. Both exclusion of immune cells and inhibition of their function clearly contribute to the creation of an immunosuppressive microenvironment, which allows tumor immune evasion. The contribution of the tumor vasculature to T cell trafficking, the regulation of endothelial adhesion molecule expression, and the creation of an immunosuppressive microenvironment are discussed in the following sections.

CURRENT THERAPIES UTILIZING THE HOST IMMUNE RESPONSE

Tumors that do support T cell trafficking and show high levels of immune cell infiltration appear to use a range of immunosuppressive pathways to evade the host response. An important immune evasion strategy is the use of inhibitory signaling pathways, known as immune checkpoints, which are part of the physiological process of peripheral tolerance, designed to protect against autoimmunity (55). In this process, self-antigens taken up by APCs will be presented to T cells without the appropriate coactivation signals such as the binding of CD80 or CD86 to CD28, or in the presence of co-inhibitory signals such as the binding of PD-1 to PD-L1. This results in anergy

or deletion of the self-reactive T cell. Tumors can co-opt these signaling pathways to evade the immune response, by expressing high levels of co-inhibitory molecules such as PD-L1 (54). Release of these immune checkpoints through the use of inhibitory monoclonal antibodies targeting CTLA-4, PD-1, or PD-L1 can result in durable antitumor responses in a subset of patients (2–7, 56, 57). Responses have been demonstrated across multiple tumor types; however, the selection of patients likely to respond remains problematic (12). The presence of TILs is critical to the success of these immune checkpoint inhibitors (58).

An alternative approach that utilizes the host immune response to fight tumors is termed adoptive cell transfer. Here, TILs are isolated from the patient's tumor tissue, expanded *ex vivo* and reintroduced into the patient's blood stream. This approach has a number of limitations and to date has seen minimal success in the clinic (59). Genetic modification of the T cells can improve tumor cell specificity and enhance activation (59). CARs include a specific antigen-binding domain and an intracellular signaling domain, which allow MHC-independent activation of T cells. Limited success has been seen in the use of CAR T cell and adoptive cell transfer against solid tumors compared to impressive results in hematological malignancies (13).

A limiting factor in the efficacy of CAR T cells in solid tumors is the lack of infiltration into the tumor itself. This therapeutic approach has seen the most success in B cell leukemia, in which the tumor cells express a common and specific antigen (CD19) and are easily accessible, as they are circulating in the peripheral blood (11). Infiltration of solid tumors by the transferred T cells is required for efficacy (60); however, it has been demonstrated in both humans and mice that only a small fraction of transferred T cells reach the tumor tissue (35). Following transfer, CAR T cells may be readily identifiable in peripheral blood, but scant in the tumor tissue (61). It has also been shown that mesothelin-targeted CAR T cells demonstrated markedly superior efficacy in an orthotopic mouse model of mesothelioma when delivered regionally rather than systemically (62). Current clinical trials are investigating methods to overcome this suboptimal trafficking of CAR T cells, including altering the chemokine milieu of the tumor and expressing matched chemokine receptors on the engineered T cells (35, 63). Investigations into local delivery approaches are also ongoing (13).

IS THERE AN ACCESS ISSUE?

The existence of the non-inflamed tumor phenotype and the lack of success of CAR T cell therapy in solid tumors support the concept that exclusion of immune cells from the microenvironment plays an important role in the immune escape of tumors. It has been recognized that the tumor vasculature is part of the permissive microenvironment that prevents the immune rejection of tumors (64). Understanding the impact of the tumor vasculature's role in this exclusion will be important in selecting appropriate therapeutic strategies to enhance the potential of immunotherapy. The immunomodulatory effects of tumor blood vessels and lymphatics are also important targets in understanding and manipulating the tumor microenvironment.

ROLE OF THE TUMOR VASCULATURE IN IMMUNE CELL EXCLUSION

Molecular Mechanisms

Specialized endothelial cells line the blood and lymphatic vessels of the body and act in a variety of ways to control the delivery and removal of oxygen, nutrients, and circulating cells to the tissues. Endothelial cells are active participants in the immune response to inflammation (65), through their role in regulating the trafficking and activation of immune cells. A summary of the alterations in leukocyte–endothelium interactions seen in tumors is provided in **Figure 2**. Migration of leukocytes (lymphocytes, monocytes, and granulocytes) from the blood vessels into peripheral tissues is a multistep process involving rolling, slow rolling, activation, firm adhesion, adhesion strengthening, intraluminal crawling, and transcellular and paracellular migration (66). E-selectin and P-selectin on endothelial cells and L-selectin on granulocytes, monocytes, and most lymphocytes mediate rolling through interaction with P-selectin glycoprotein ligand-1 and other glycosylated ligands (66). Selectins require shear stress resulting from the flow of blood to support adhesion (67). Intercellular adhesion molecule-1 (ICAM-1) is a member of the immunoglobulin superfamily that plays an important role in the adhesion cascade, participating in rolling, firm adhesion, and transcellular migration (68). ICAM-1 and vascular cell adhesion molecule-1 (VCAM-1), another immunoglobulin superfamily member (69), are located on the luminal surfaces of endothelial cells and bind to the integrins such as lymphocyte function-associated antigen-1 (LFA-1) and very late antigen-4 (VLA-4), respectively (70, 71). LFA-1 is expressed on lymphocytes, monocytes, and neutrophils, whereas VLA-4 is expressed on lymphocytes and monocytes (72). Clustering of ICAM-1 and VCAM-1 is also a critical step in transendothelial migration, and blocking this clustering is sufficient to prevent migration of leukocytes expressing LFA-1 or VLA-4 (73). Expression of vascular adhesion molecules in intratumoral blood vessels is correlated with the number of TILs. E-selectin is required for T cell extravasation in skin, and expression of E-selectin in cutaneous squamous cell carcinoma and Merkel cell carcinoma correlates with infiltration by $CD8^+$ T cells and better prognosis (74, 75). Medullary breast carcinomas are defined in part by a florid lymphocytic infiltrate and showed a higher expression of ICAM-1 on intratumoral blood vessels than ductal breast carcinomas of no special type (76).

Inflammatory signals are required to upregulate expression of ICAM-1, which can be expressed by a range of cells in addition to endothelial cells, including fibroblasts, thymic epithelial cells, macrophages, and follicular DCs (70). In addition to mediating the adhesion of leukocytes to endothelial cells, ICAM-1:LFA-1 interactions also participate in the formation of an immune synapse between T cells and APCs (77). A mature immune synapse requires molecular interactions mediating adhesion, antigen presentation, and costimulation or inhibition. A synapse may also form within the docking structure forming the adhesion between endothelial cells and lymphocytes (78). Inflammatory cytokines IL-1, TNFα and, to a lesser degree, IFNγ, cause a rapid rise in the expression of ICAM-1 on cultured endothelial cells (79).

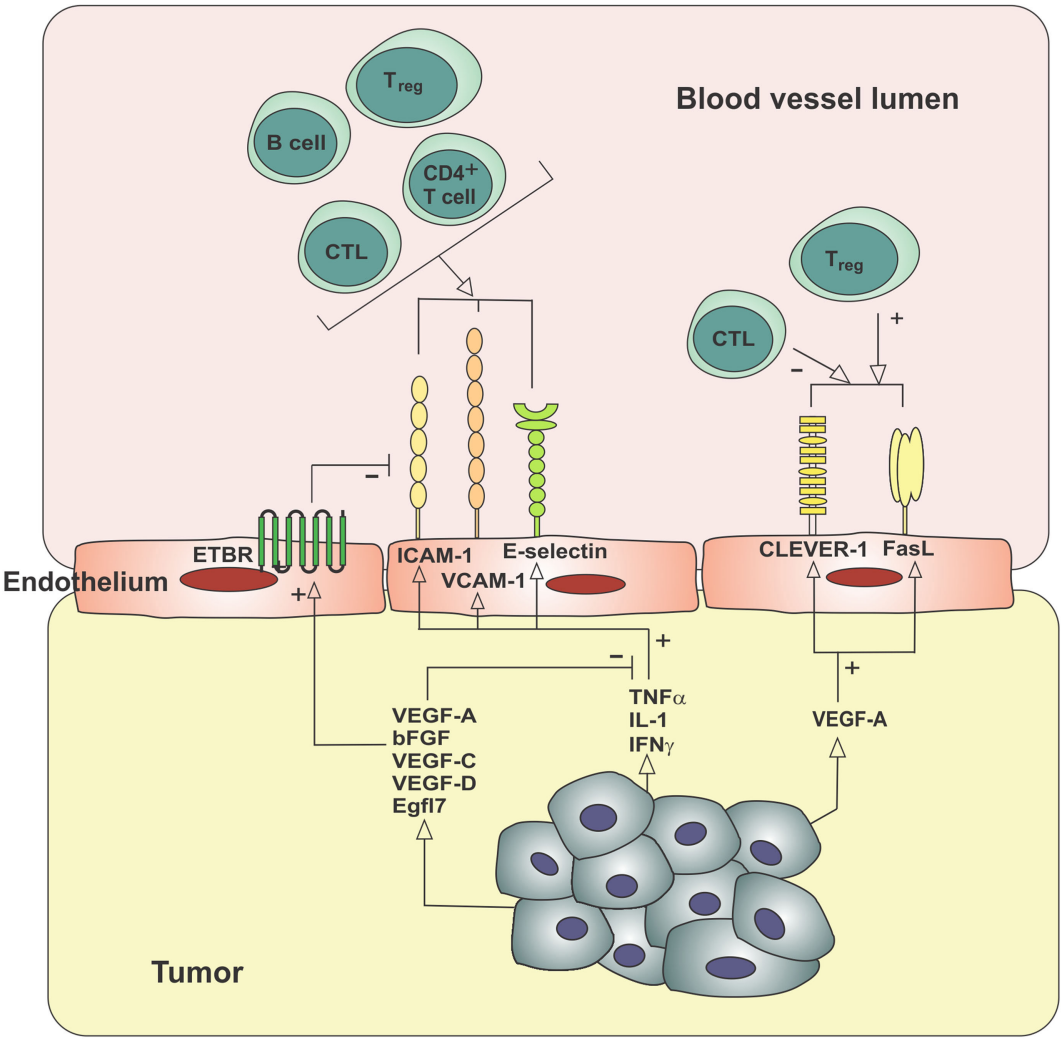

FIGURE 2 | Molecular mechanisms contributing to the exclusion of immune cells from the tumor microenvironment. Tumor-derived angiogenic factors can block the usual upregulation of cell adhesion molecules in response to inflammatory mediators (endothelial anergy), increase the expression of ETBR, which blocks the clustering of ICAM-1 required for lymphocyte adhesion, and increase expression of cell surface receptors, which selectively decrease CTL extravasation while increasing T_{reg} extravasation. bFGF, basic fibroblast growth factor; CLEVER-1, common lymphatic endothelial and vascular endothelial receptor-1; CTL, cytotoxic T lymphocyte; EGFL7, epidermal growth factor-like domain 7; ETBR, endothelin B receptor; FasL, Fas ligand; ICAM-1, intercellular adhesion molecule-1; IFNγ, interferon-gamma; IL-1, interleukin-1; TNFα, tumor necrosis factor-α; T_{reg}, regulatory T cell; VCAM-1, vascular cell adhesion molecule-1; VEGF, vascular endothelial growth factor.

Different cell types may vary as to which inflammatory signals are capable of inducing ICAM-1 expression (77).

Adhesion molecules including ICAM-1, VCAM-1, and E-selectin may be absent or expressed at low levels on tumor vasculature, despite the inflammatory microenvironment of the tumor. Pro-inflammatory pathways are induced in tumor cells by oncogenic activation of transcription factors such as HIF-1α and NFκB, resulting in the high levels of inflammatory mediators detected in most solid tumors (80). However, this inflammatory environment appears to fail to induce the expression of vascular adhesion molecules on intratumoral vessels. This has been demonstrated in experimental models of melanoma and carcinoma (81), as well as in human cutaneous squamous cell carcinoma, Merkel cell carcinoma, and metastatic melanoma tissue (74, 75, 82). This lack of responsiveness to inflammatory signals has been termed endothelial anergy (83) and may play an important role in the exclusion of antitumor immune effector cells from the tumor microenvironment.

Evidence suggests that endothelial anergy is due at least in part to angiogenic factors (84, 85), a range of molecules including vascular endothelial growth factor-A (VEGF-A), VEGF-C, VEGF-D, and basic fibroblast growth factor (bFGF), some of which are produced in response to tissue hypoxia. The tumor microenvironment is characteristically hypoxic due to disordered and loosely regulated angiogenesis that fails to adequately supply the expanding tumor mass (86). This hypoxia leads to stabilization

and nuclear accumulation of hypoxia-inducible factors (HIF-1α and HIF-2α), transcription factors that lead to upregulation of angiogenic factors, and other molecules that act to improve tissue oxygenation. VEGF-A can be secreted by tumor cells and TAMs and is overexpressed in the majority of solid tumors (87, 88). VEGF-A and bFGF, also a strong mitogenic factor for endothelium produced by tumor cells, contribute to the suppression of ICAM-1 in tumors (84). This downregulation of adhesion molecules in response to angiogenic factors has been demonstrated *in vitro* (83, 84, 89, 90) and in mouse tumor models (85, 91, 92). As described above, tumor vasculature appears unresponsive to inflammatory signals that mediate the expression of adhesion molecules through the NFκB signaling pathway. bFGF can block this stimulation by preventing the degradation of pathway inhibitor Iκβα, thus stopping the translocation of NFκB to the nucleus and activation of target gene transcription (93).

The concept of endothelial anergy and the downregulation of adhesion molecules mediated by angiogenic factors is supported by the evidence that antiangiogenic therapy results in increased expression of adhesion molecules on tumor vasculature (94). Angiostatic therapy using platelet factor 4, anginex, angiostatin, or endostatin results in upregulation of ICAM-1, VCAM-1, and E-selectin in animal models and *in vitro* (94, 95) and also reinstates the responsiveness of the endothelium to inflammatory signals (94). These anti-angiogenic peptides showed promising anti-tumor effects in initial pre-clinical trials, however have failed to demonstrate efficacy in human cancers and are no longer being clinically investigated (96). Multi-target tyrosine kinase inhibitors such as SU6668, sunitinib, and sorafenib are a more promising antiangiogenic treatment approach and are approved for the treatment of some human cancers such as the highly angiogenic renal cell carcinoma (96). These small molecules inhibit the activation of a range of tyrosine kinase receptors, including vascular endothelial growth factor receptor-1 (VEGFR-1), VEGFR-2, and fibroblast growth factor receptor (FGFR-1), receptors for angiogenic factors VEGF-A, VEGF-C, and VEGF-D, and bFGF, as well as growth factor receptors such as platelet-derived growth factor receptor-β (PDGFRβ) and c-kit. Use of SU6668, a small molecule inhibitor of VEGFR-2, FGFR-1, and PDGFRβ, blocked the actions of bFGF and showed reversal of adhesion molecule downregulation in a mouse model of metastatic breast cancer (89). A number of pre-clinical studies have shown that various antiangiogenic therapies, including tyrosine kinase inhibitors and inhibitory monoclonal antibodies against VEGF-A and VEGFR-2, may help to increase tumor infiltration by lymphocytes (97–108). These are summarized in **Table 1** and discussed further in Section "Implications for Treatment Strategies". It would be of interest to delineate the extent to which this increased infiltration is due to reversal of endothelial anergy or alternatively due to blockade of the direct effects of VEGF-A on tumor cells, stromal cells, or immune cells, or alteration of the hypoxic microenvironment. Initial clinical studies also support an increase in tumor infiltration by immune cells with the combination of immunotherapies and antiangiogenic agents, summarized in **Table 2** and discussed further in Section "Implications for Treatment Strategies" (109, 110). To the best of our knowledge, reversal of endothelial anergy in human tumors by antiangiogenic agents remains to be conclusively demonstrated. Further investigations of changes in adhesion molecule expression and lymphocyte infiltration resulting from antiangiogenic drugs currently approved for use in the clinic, which largely target the VEGF-VEGFR signaling pathway, may provide useful information and should be a high priority.

In addition to VEGF-A and bFGF, other angiogenic and tumor-associated factors may also contribute to the exclusion of TILs. VEGF-C and VEGF-D are closely related members of the VEGF family that promote angiogenesis, lymphangiogenesis, and cancer metastasis (118–122). These factors can be secreted by tumor cells, immune cells, and tumor-associated fibroblasts (123–125). In human breast carcinoma, higher levels of VEGF-C and VEGF-D were seen in ductal carcinomas compared to medullary carcinomas and correlated with decreased ICAM-1 expression and lower numbers of infiltrating lymphocytes (76). Other growth factors including placenta growth factor (PlGF) and epidermal growth factor have also been shown to downregulate ICAM-1 expression *in vitro* (126). Epidermal growth factor-like domain 7 (EGFL7) is secreted by normal blood endothelial cells, at sites of pathological angiogenesis, and by tumor cells (127, 128). Higher levels of EGFL7 have been correlated with poor prognosis in some tumor types such as colorectal cancer (127). Delfortrie et al. have shown that EGFL7 also functions to decrease levels of adhesion molecules ICAM-1 and VCAM-1, resulting in a reduction in TILs (128).

Endothelin-1 (ET-1) is a molecule that plays a role in both angiogenesis and controlling the trafficking of immune cells. ET-1 acts through two receptors, the endothelin A receptor (ETAR) and the endothelin B receptor (ETBR) (129). ET-1, ETAR, and ETBR expression is correlated with VEGF-A expression and microvessel density in breast and ovarian carcinoma (130). Messenger RNA profiling of microdissected endothelial cells from ovarian cancer showed overexpression of ETBR in tumors lacking infiltrating lymphocytes (131). The binding of ET-1 to ETBR prevented T cell adhesion to endothelium, even in the presence of the inflammatory cytokine TNFα, an additional mechanism of endothelial anergy (131). Findings suggesting selectivity in lymphocyte extravasation due to ETBR expression were reported for glial tumors (132). Glioblastomas with higher numbers of ETBR-expressing vessels showed lower infiltration by cytotoxic T cells and higher numbers of regulatory T cells. Cytotoxic T cells infiltrated around ETBR-negative blood vessels, but were absent around vessels expressing ETBR (132). Similar findings were seen in primary central nervous system lymphoma, in which both endothelial and tumor cells expressed ETBR (133). However, no correlation between ETBR expression and TILs was seen in oral squamous cell carcinoma (134). Blockade of ETBR increased T cell adhesion to endothelium through the upregulation and clustering of ICAM-1 (131). Blockade of ETBR was also shown to increase T cell homing to tumors and increase the effectiveness of cancer vaccines in mice (131).

Selective extravasation of different leukocyte subsets may also be mediated by additional molecules including common lymphatic endothelial and vascular endothelial receptor-1 (CLEVER-1) (135) and Fas ligand (FasL) (136). CLEVER-1, also known as stabilin-1 and FEEL-1, is a multifunctional scavenging receptor expressed constitutively on lymphatic endothelial cells

TABLE 1 | Summary of pre-clinical studies combining antiangiogenic therapies and immunotherapy.

Antiangiogenic therapy	Immunotherapy	Tumor model	Results of combination therapies compared with immunotherapy alone	Reference
Neutralizing anti-VEGF-A antibodies				
Anti-mouse VEGF-A antibody	Peptide-pulsed dendritic cell vaccination	MethA sarcoma and D549 xenograft in mice	– Decreased tumor growth – Improved survival	Gabrilovich et al. (111)
Anti-mouse VEGF-A antibody, B20-4.1.1-PHAGE	Adoptive transfer of tumor-specific T cells	B16 melanoma in syngeneic C57BL/6J mice	– Decreased tumor growth – Improved survival – Increased T cell infiltration into tumor – Different effects with different doses	Shrimali et al. (97)
Bevacizumab	Adoptive transfer of cytokine-induced killer cells (CIK)	Human lung adenocarcinoma xenografts (A549) in mice	– Improved CIK homing and infiltration	Tao et al. (98)
Ligand traps				
sVEGFR-1/R-2	GM-CSF secreting tumor cell vaccination	Melanoma (B16) and colon carcinoma (CT26) in mice	– Improved survival – Increased number of activated DCs and TILs – Decreased number of regulatory T cells	Li et al. (99)
Aflibercept	Recombinant TMEV Xho1-OVA8 antitumor vaccine	Glioma (GL261) in mice	– Delayed tumor progression – Improved survival	Renner et al. (112)
Neutralizing anti-VEGFR-2 antibodies				
Anti-VEGFR-2 antibody, DC101	HER2/Neu targeted vaccination	Spontaneous breast carcinoma in FVB and Neu-N mice	– Reduction in tumor growth and improved immune responses in FVB mice – Efficacy in Neu-N mice required depletion of T_{regs}	Manning et al. (100)
Anti-VEGFR-2 antibody, DC101	Whole cancer tissue cell vaccination	Breast carcinoma (MMTV-PyVT) in mice	– Improved survival – Polarized macrophages to M1 phenotype – Improved T cell infiltration	Huang et al. (101)
Angiostatic peptides				
Recombinant adenovirus expressing antiangiogenic factors endostatin and PEDF	Recombinant adenovirus expressing IL-12 and GM-CSF	Viral-induced woodchuck hepatocellular carcinoma	– Reduction in tumor volume – Increased apoptosis – Increased TILs	Huang et al. (102)
Recombinant adenovirus expressing antiangiogenic factors endostatin and PEDF	Recombinant adenovirus expressing IL-12 and GM-CSF	Implanted hepatocellular carcinoma (BNL) in mice and chemically induced HCC in rats	– Reduction in tumor volume – Increased apoptosis – Increased TILs – Immunotherapy alone was effective for smaller tumors, but combination therapy more effective against larger tumors	Chan et al. (103)
Recombinant human endostatin	Adoptive transfer of CIK	Lung adenocarcinoma xenografts (A549, SPC-A1, Lewis lung carcinoma) in mice	– Increased CIK homing – Increased TILs – Decreased immunosuppressive cells	Shi et al. (113)
Aginex, peptide targeting galectin-1	Adoptive T cell transfer	Melanoma (B16) in mice	– Restored adhesion molecule expression and T cell infiltration – Significant reduction in tumor growth	Dings et al. (105)
Multi-target tyrosine kinase inhibitors				
SU6668	B7.2-IgG/TC vaccination	Breast carcinoma (4T1) in mice	– Increased $CD8^+$ TILs – Decreased tumor growth – Decreased formation of distant metastasis	Huang et al. (106)
Sunitinib	IL-12 and 4-1BB activation	Colon carcinoma xenografts (MCA26) in mice	– Modulation of immune infiltrate composition and polarization toward effector phenotype – Improved survival	Ozao-Choy et al. (114)

(Continued)

TABLE 1 | **Continued**

Antiangiogenic therapy	Immunotherapy	Tumor model	Results of combination therapies compared with immunotherapy alone	Reference
Sunitinib or sorafenib	rMVA–CEA–TRICOM vaccine	Colon carcinoma (MC38-CEA) and breast cancer (4T1) in mice	– Marked reduction in tumor volume – Increase in tumor antigen-specific TILs	Farsaci et al. (107)
Sunitinib	Glucocorticoid-induced TNFR-related protein (GITR)	Liver metastasis of renal cell carcinoma (RENCA) in mice	– Reduction in number and size of tumors – Increased activation of immune cells	Yu et al. (115)
Others				
TNFα-RGR protein fusion	Adoptive T cell transfer and anti-Tag vaccination	RIP1-Tag5 transgenic mouse (pancreatic insulinomas)	– Improved survival – Increased TILs – Promotes M1 polarization of macrophages	Johansson et al. (108)
Trebananib (blocks interaction between angiogenic factors angiopoietin 1 and 2 with receptor Tie2)	Antigen-specific cytotoxic T cell transfer	Carcinoma cell lines MDA-MB-231 (breast), LNCaP (prostate), and OV17-1 (ovarian)	– Increased ICAM-1 expression – Improved CTL lysis	Grenga et al. (116)

(LECs) and type 2 macrophages and induced by inflammation on blood endothelial cells (137, 138). Functions have been demonstrated to include both lymphocyte trafficking and adherence of cancer cells to lymphatic endothelium (139, 140). In a mouse model of melanoma, levels of CLEVER-1 correlated with increased infiltration by FoxP3$^+$ T_{regs} and type II macrophages. Following administration of anti-CLEVER-1 antibody, numbers of T_{regs} and type II macrophages were reduced, and there was increased immune activation and decreased tumor growth (135). FasL mediates T cell apoptosis and can be induced on blood vascular endothelial cells in solid tumors by tumor-derived VEGF-A, prostaglandin E2, and IL-10 (136). Endothelial FasL is able to kill activated T lymphocytes, but CD4$^+$CD25$^+$ regulatory T cells are resistant to FasL-mediated killing due to high levels of antiapoptotic protein c-FLIP (136). Endothelial FasL expression correlated with lower numbers of CD8$^+$ T cells in a range of cancer types. Blockade of VEGF-A, prostaglandins, or FasL resulted in increased CD8$^+$ T cell infiltration and impaired tumor growth (136).

In addition to effects on the tumor vasculature, hypoxia and angiogenic factors such as VEGF-A also have direct immunomodulatory effects, which are summarized in **Figure 3**. As mentioned above, hypoxia-inducible factors are transcription factors activated by low tissue oxygen levels sensed by hydroxylase enzymes (141). HIFs control the transcription of various genes involved in the adaptation to hypoxic conditions, and also have a number of direct effects on immune cells. In hypoxic tumors, macrophages are polarized toward an immunosuppressive M2 phenotype, MDSCs accumulate and DC maturation and differentiation is impaired, inhibiting the activation of T cells (41). Cytotoxic T cells show increased lytic capacity under hypoxic conditions, but decreased proliferation and differentiation (41). Hypoxic stress increases secretion of CCL28 and CXCL12 by tumor cells, thereby attracting regulatory T cells (142, 143). HIF-1α also directly binds to a hypoxia response element in the promoter of the gene encoding immune checkpoint molecule PD-L1, and hypoxia thereby increases expression of PD-L1 on MDSCs, tumor cells, DCs, and macrophages (144). VEGF-A also directly enhances the expression of PD-1, TIM-3, and CTLA-4 on intratumoral CD8$^+$ T cells, contributing to T cell anergy (145). These data suggest an important role for hypoxia, angiogenesis, and the endothelium in creating a permissive microenvironment to prevent the immune rejection of tumors.

Mechanical Properties

The tumor vasculature may also contribute to the exclusion of effector lymphocytes from the tumor microenvironment by physical means. In normal immune responses, T cells exit the vasculature predominantly in the post-capillary venule, a site of low shear stress where adhesion molecules are preferentially expressed (78, 146). Newly formed blood vessels within tumors, however, are structurally and functionally abnormal, lacking the specialized organization of normal tissue vasculature (147). Tumor vessels are heterogeneous, tortuous, and irregularly branched (148, 149). The vessel walls are leaky with wide junctions between endothelial cells, increased fenestrations and loss, or abnormalities of the surrounding pericytes and basement membranes. Tumor endothelial cells lose polarity, can detach, and stratify (149). The normal laminar flow of blood is disrupted, and with it, the margination, rolling, and adhesion of lymphocytes. Areas of stagnation and increased interstitial fluid pressure are also present, resulting in heterogeneous tumor perfusion (150). The delivery of chemotherapeutic agents is hampered by this chaotic and inefficient tumor blood flow (149, 151), and access of antitumor lymphocytes may also be impaired.

Shear stress, the parallel force applied to the endothelial lining of blood vessels by laminar blood flow in normal vasculature, is a key regulator of vascular physiology (152). Endothelial cells respond to shear stress through mechanosensory molecules including CD31 (platelet endothelial adhesion molecule) and VE-cadherin, which can activate various signaling pathways leading to complex and context-dependent effects on endothelial

TABLE 2 | Summary of published and ongoing clinical trials combining antiangiogenic therapies and immunotherapy.

Antiangiogenic therapy	Immunotherapy	Tumor type	Results/status	Reference; trial number
Bevacizumab (anti-VEGF-A antibody)	Ipilimumab (CTLA-4 inhibitor)	Metastatic melanoma	– Increased $CD8^+$ TILs and macrophages – Changes in circulating immune cell composition – Mild increase in toxicity compared to level expected for ipilimumab alone – Overall response rate 11%	Hodi et al. (109); Phase I
Bevacizumab	Ipilimumab	Glioblastoma	– Partial response rate 31% – Stable disease 31% – Treatment well tolerated	Carter et al. (117); Phase I
Bevacizumab	Atezolizumab (PD-L1 inhibitor)	Metastatic renal cell carcinoma	– Partial response rate 40% – Stable disease 40% – Treatment well tolerated – Increased immune cell infiltrate and Th1 gene expression	Wallin et al. (110); Phase I
Bevacizumab	Ipilimumab	Metastatic melanoma	Completed	NCT01743157; Phase I–II
Bevacizumab	Ipilimumab	Unresectable stage III or IV melanoma	Active	NCT00790010; Phase I
Bevacizumab	Ipilimumab	Unresectable stage III or IV melanoma	Recruiting	NCT01950390; Phase II
Bevacizumab	Nivolumab (PD-1 inhibitor)	Metastatic renal cell carcinoma	Recruiting	NCT02210117; Phase I
Bevacizumab	Pembrolizumab (PD-1 inhibitor)	Brain metastasis in melanoma or non-small cell lung cancer	Recruiting	NCT02681549; Phase II
Bevacizumab	Pembrolizumab	Recurrent glioblastoma	Active	NCT02337491; Phase II
Bevacizumab	Pembrolizumab	Metastatic renal cell carcinoma	Active	NCT02348008; Phase Ib and II
Bevacizumab and hypofractionated stereotactic irradiation	Pembrolizumab	Glioblastoma	Recruiting	NCT02313272; Phase I
Bevacizumab or sunitinib	Atezolizumab	Metastatic renal cell carcinoma	Recruiting	NCT02420821; Phase III
Bevacizumab	Atezolizumab	Stage IV non-squamous, non-small cell lung cancer	Recruiting	NCT02366143; Phase III
Ziv-aflibercept (ligand trap)	Pembrolizumab	Advanced solid tumors	Recruiting	NCT02298959; Phase I
MEDI3617 (anti-angiopoietin-2 antibody)	Tremelimumab (CTLA-4 inhibitor)	Advanced solid tumors	Recruiting	NCT02141542; Phase I

adhesion molecule expression (153). In tumors, the disrupted and sluggish blood flow in tumors due to abnormal vasculature results in lower levels of shear stress (154). A threshold level of shear stress is required for the expression of E-selectin, P-selectin, and L-selectin, which mediate leukocyte rolling (67). Low shear stress can enhance expression of adhesion molecules on endothelial cells, particularly ICAM-1, but can also decrease the responsiveness of the endothelium to inflammatory signals such as TNFα, thus becoming an additional promoter of endothelial anergy (155). Low shear can also upregulate VEGF-A expression by tumor cells (154), which may modulate adhesion molecule expression and perpetuate angiogenesis. The direct effects of the mechanical properties of abnormal tumor blood vessels on immune cell extravasation remain to be fully elucidated.

Pericytes and vascular smooth muscle cells are contractile cells that surround and interact with the endothelial cell layer of blood vessels. Pericytes are required for vessel stabilization and maturation, and in tumor vessels they are often immature, less abundant, and loosely attached (156). Recruitment of pericytes to immature and proliferating blood vessels involves, among others, the PDGF/PDGFRβ and angiopoietin (Ang)-1/Tie2 signaling pathways (157). Disrupting pericyte coverage through targeting

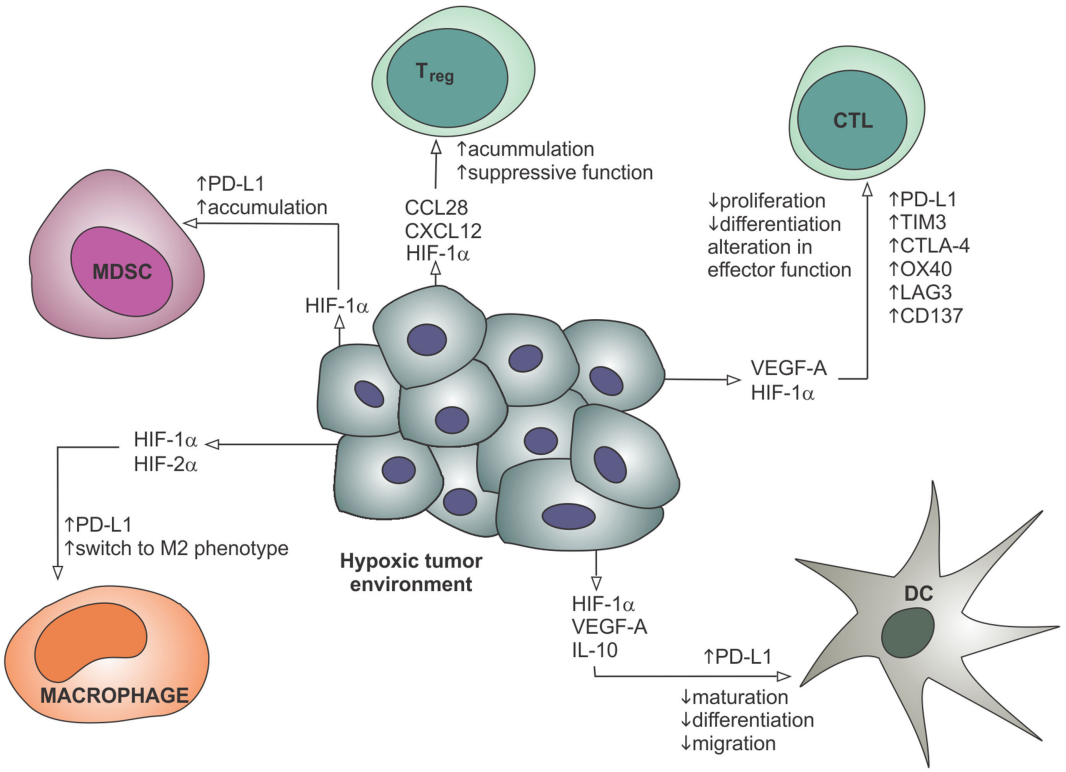

FIGURE 3 | Hypoxia contributes to the recruitment of suppressive immune cells, restricts the maturation and migration of dendritic cells, reduces proliferation and differentiation of effector CTLs, and leads to the upregulation of immune checkpoint molecules such as PD-L1. These effects are mediated through gene regulation by hypoxia-inducible factors and secreted factors such as VEGF-A. CTL, cytotoxic T lymphocyte; DC, dendritic cell; HIF, hypoxia-inducible factor; IL-10, interleukin-10; MDSC, myeloid-derived suppressor cell; T_{reg}, regulatory T cell; VEGF-A, vascular endothelial growth factor-A.

of the PDGF/PDGFRβ pathway results in increased vessel leakiness, decreased tumor vascularity, and decreased tumor growth, particularly when combined with anti-VEGF-A treatment (158–160). Conversely, promotion of pericyte coverage and pericyte–endothelial cell interactions through activation of VEGFR and PDGFRβ has been proposed to enhance vessel stabilization and normalization (160). During changes in oxygen availability, Ang2 can bind to Tie2 on endothelial cells, thus blocking the binding of Ang1, releasing the pericyte, and destabilizing the vessel (161). Inhibition of Ang2 can improve pericyte coverage and normalize tumor vessels in mouse models (162). Clinical trials of pericyte modulation by PDGFRβ inhibition alone have been largely disappointing (163, 164). Other approaches to modulate pericyte coverage require further investigation in the clinic. To the best of our knowledge, no clinical trials have yet examined the effect of vascular normalization due to pericyte modulation on lymphocyte infiltration. However, pericytes may however have additional immunomodulatory effects. Hong et al. demonstrated an increase in MDSCs in tumors grown in a pericyte deficient mouse model, due to IL-6 production in the hypoxic tumor microenvironment (165). MDSC levels decreased when pericyte coverage was restored (165). In human breast cancers, MDSC gene expression correlated with decreased pericyte gene expression and poor prognosis (165). Pericyte coverage is thus an important consideration in vascular normalization studies and may play a role in creation of the immunosuppressive tumor microenvironment. Rgs5, one of a family of molecules that inhibits signaling by G protein-coupled receptors, is expressed by pericytes and hypoxic endothelial cells and has been shown to be overexpressed in tumor vasculature (166, 167). Loss of Rgs5 in mice results in pericyte maturation, vascular normalization, improved oxygenation, and reduced vessel leakiness (166). Importantly, it was also found that tumor infiltration by both endogenous and adoptively transferred lymphocytes was increased in Rgs5-deficient mice (166). This finding supports the hypothesis that physical normalization of the blood vessels and their supporting cells improves immune cell extravasation. Human RGS5 shows high homology to the mouse gene and appears to perform similar functions (168), although data describing its role in human tumors are limited.

The abnormal, poorly organized structure of tumor blood vessel walls results in leakiness and extravasation of fluid into the tumor microenvironment (169). Angiogenic factors also contribute to this leakiness. VEGF-A was initially described as vascular permeability factor (170) due to its marked enhancement of vessel permeability and is found in high levels in malignant effusions (171). However, data appear to suggest that this permeability of tumor blood vessels does not result in increased lymphocyte extravasation. As discussed above, expression of angiogenic

factors instead correlates with reduced TILs (76, 172). Use of antiangiogenic therapy and vascular normalization can improve lymphocyte infiltration into tumors, discussed further below. Lymphocyte extravasation requires controlled molecular regulation and as such increased vessel wall permeability, and fluid extravasation alone may not increase the lymphocyte infiltration in the tumor.

HIGH ENDOTHELIAL VENULES AND THE RECRUITMENT OF NAÏVE T CELLS

High endothelial venules (HEVs) are specialized post-capillary venules normally found in secondary lymphoid organs including lymph nodes and Peyer's patches, characterized histologically by their cuboidal "high" endothelial lining. They are adapted to promote trafficking of naïve lymphocytes into the lymphoid organ, expressing specific addressins including peripheral node addressin (PNAd) and mucosal addressin (MAdCAM-1). Activated lymphocytes, including effector T cells and memory T cells, can also be recruited by HEVs into lymph nodes under inflammatory conditions through the upregulation of VCAM-1, E-selectin, and P-selectin (173). Blood vessels with morphological and immunohistochemical features of HEVs have been identified in a range of human tumors, including breast, ovarian, colorectal, and lung cancers (174). The presence of HEVs correlates strongly with the presence of $CD8^+$ effector T cells as well as B cells and Th1 cells (174), often organized as tertiary lymphoid structures, that is, ectopic lymphoid structures with all the characteristics of lymph nodes (175). Evidence suggests that these local tertiary lymphoid structures may play a role in recruitment and priming of naïve T cells and promote differentiation into tumor-specific effector T cells, within the tumor microenvironment itself (176). Interestingly, both positive and negative effects on antitumor immunity have been associated with tertiary lymphoid structures and lymph node-like vasculature (177, 178). The recruitment of naïve T cells and differentiation into effector T cells seen in some settings (177) contrasts with the recruitment of MDSCs and differentiation of T_{regs} seen in others (178). The inflammatory context in which these tertiary lymphoid structures develop may help to explain these findings.

LYMPHANGIOGENESIS, INTERSTITIAL FLUID PRESSURE, AND IMMUNE EVASION

Recent work has established a key role of LECs in inducing immune tolerance, both in peripheral tissues and the draining lymph node. Tumors and their microenvironments promote lymphangiogenesis and lymphatic remodeling through both molecular and mechanical means. VEGF-C and VEGF-D signaling *via* interactions with VEGFR-2 and VEGFR-3 are important drivers of tumor lymphangiogenesis, promoting intratumoral and peritumoral lymphatic growth and metastasis (179). These growth factors may be secreted by tumor cells, immune cells, and stromal cells (123–125).

As described in previous sections, loosely regulated angiogenesis in tumors results in abnormal, leaky blood vessels. In conjunction with alterations in the stroma and extracellular matrix surrounding the tumor, this results in increased interstitial fluid pressure within the tumor (180). Interstitial fluid pressure within tumors can measure up to 60 mmHg, whereas normal tissue has a range of −3 to +3 mmHg (180). This pressure gradient causes an increase in interstitial flow at the tumor margin, and increased lymphatic drainage by peritumoral lymphatics (181). Increased interstitial fluid and lymphatic flow has a number of effects on the tumor microenvironment, contributing to peritumoral lymphangiogenesis, altering the extracellular matrix and fibroblast differentiation, and promoting the development of lymphoid-like features (178, 181). These lymphoid-like stromal features such as CCL21 expression, required for the homing of naïve T cells, are important components of the tertiary lymphoid structures seen in tumors, which, as discussed above, can show both positive and negative associations with antitumor immunity. Lymphatic flow can also induce the upregulation of transforming growth factor beta (TGFβ) by fibroblasts, leading to myofibroblast differentiation, contraction, and matrix stiffening (182). TGFβ also dampens the innate immune response through effects on the maturation of DCs, natural killer (NK) cells, T cells, neutrophils, and macrophages and supports the differentiation and induction of regulatory T cells (183). TGFβ has been suggested as a link between the mechanics of interstitial fluid pressure, lymphatic flow, and the development of an immunosuppressive tumor microenvironment (181).

ROLE OF LECs IN IMMUNE SUPPRESSION AND TOLERANCE

Peripheral tolerance is the process by which self-reactive T cells that escape thymic selection are deleted or rendered anergic. Lymphatic flow and the delivery of lymph fluid to the lymph node are required for the induction of new peripheral tolerance (184, 185). Hence, the increased lymphatic flow seen draining tumors may play a critical role in the development of a permissive immune microenvironment. Induction of peripheral tolerance in the draining lymph node is a multistep process involving the transport of antigens and APCs to the lymph node, antigen presentation in the lymph node, and activation of inhibitory pathways including deletion of reactive T cells, anergy, and T_{reg} induction. LECs, both in peripheral tissues and in the lymph node, and lymph node stromal cells have important roles in the induction of tolerance, which is summarized in **Figure 4**.

The development of peripheral tolerance depends on the delivery of soluble antigens and tissue-resident APCs to the draining lymph node. Migration of tissue DCs into initial lymphatics is dependent on CCR7 expression by activated DCs and CCL21 expression on LECs (178). Antigens are carried in the interstitial fluid through the button junctions of the initial lymphatics. Once at the draining lymph node, DCs are guided to the paracortical T cell zone by CCL21 and CCL19. Small antigens are directed into the lymph node *via* intricate conduits, then taken up and

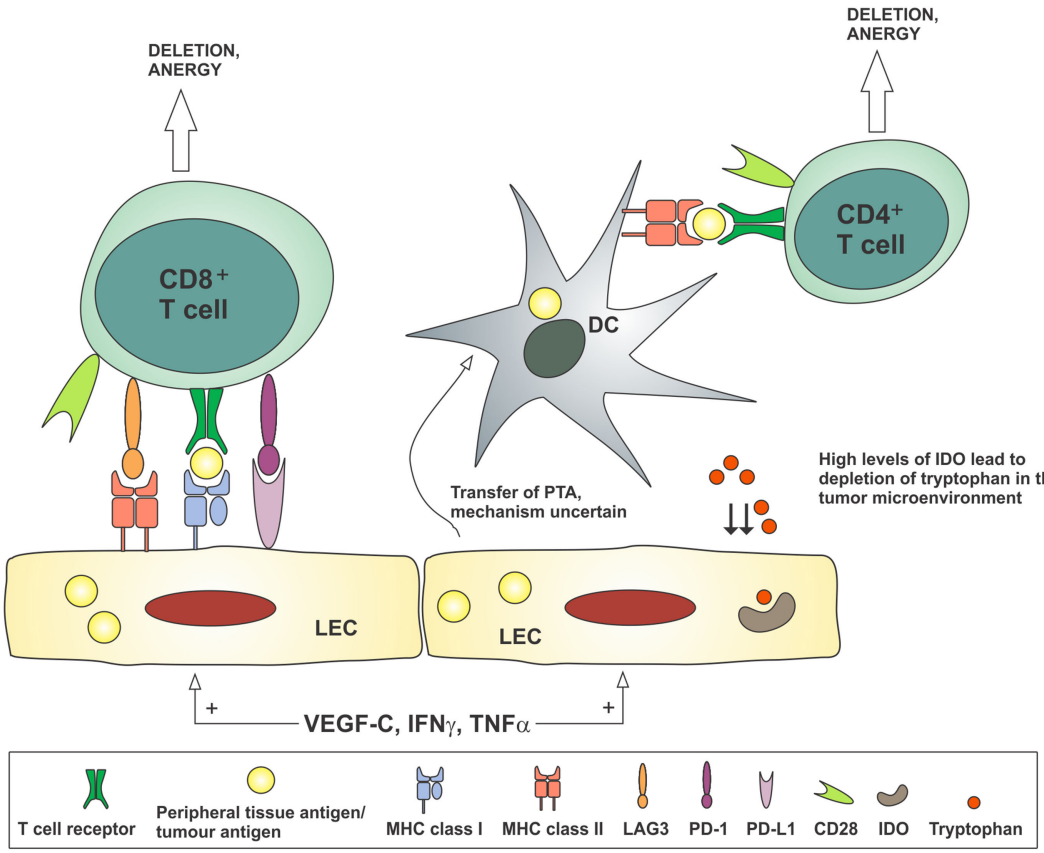

FIGURE 4 | Lymphatic endothelial cells may contribute to the development of tolerance to tumor antigens by antigen presentation to CD8⁺ T cells in the absence of costimulatory molecules such as CD80/CD86, or in the presence of co-inhibitory molecules such as PD-L1 and LAG3. Peripheral tissue antigens or tumor antigens may be transferred from LECs to dendritic cells, which present these antigens to CD4⁺ T cells in the absence of costimulatory molecules, thereby inducing anergy. Stimulation of LECs by VEGF-C and inflammatory cytokines TNFα and IFNγ can reduce CD86 expression on dendritic cells and produce IDO, which depletes tryptophan from the microenvironment, thereby preventing the activation of T cells. DC, dendritic cell; IDO, indoleamine 2,3-dioxygenase; IFNγ, interferon-gamma; LEC, lymphatic endothelial cell; PTA, peripheral tissue antigen; TNFα, tumor necrosis factor-alpha; VEGF-C, vascular endothelial growth factor-C.

processed by lymph node-resident DCs, while larger antigens are captured and processed by sinus macrophages (186, 187).

Stromal cells within the lymph node, including LECs and fibroblast reticular cells (FRCs), play important structural and physiological roles in the functions of the node. LECs and FRCs express MHC class I molecules as do nearly all nucleated cells (188). However, LECs and FRCs participate in the process of peripheral immune tolerance through ectopic expression of tissue-specific antigens on MHC class I, for example, antigens usually restricted to melanocytes, intestinal epithelium or pancreas, and presentation of these antigens to CD8⁺ T cells (188, 189). These antigens are not scavenged from the lymph fluid but directly expressed in both an autoimmune regulator (Aire)-dependent manner, as is seen in central tolerance in the thymus, and also in an Aire-independent manner (188). The costimulatory molecules CD40, CD80, and CD86 are not expressed on LECs and FRCs; however, the inhibitory molecule PD-L1 is expressed at high levels (190). Hence, presentation of antigens by LECs and FRCs can result in deletional tolerance of the reactive CD8⁺ T cells. In addition to this presentation of self-antigens, LECs activated by VEGF-C have also been shown to scavenge and cross-present tumor antigens, leading to the apoptosis of tumor-specific CD8⁺ T cells (181). MHC class II, expressed by professional APCs including DCs and B cells, is also expressed at low levels by lymph node LECs but not tissue LECs. LECs do not appear to present endogenous antigen on MHC class II molecules but instead act as a reservoir for transfer of antigen to DCs for effective presentation to CD4⁺ T cells (191). In addition, MHC class II may be a ligand for the co-inhibitory molecule LAG3, resulting in induction of CD8⁺ T cell tolerance through synergy with PD-1/PD-L1 signaling (191).

Lymphatic endothelial cells and FRCs also prevent the expansion of the activated T cell pool in lymph nodes by expression of NOS 2 and production of nitric oxide (192). LECs stimulated by inflammatory cytokines TNFα and IFNγ can also suppress the ability of DCs to activate and induce T cell proliferation by reducing the expression of the costimulatory molecule CD86 (193) and activating production of IDO (194), an enzyme of the innate immune system that depletes tryptophan, an amino acid essential for the activation of T cells. These features of lymph node stromal cells contribute to ongoing suppression of any immune

reactions to self-antigens and may contribute to suppression of responses to tumor antigens.

The contribution of lymphatic flow to tumor immune evasion is supported by the evidence that a permissive environment is created in tumor-draining lymph nodes, the so-called "metastatic niche" [reviewed elsewhere (195)]. The presence of tumor cells in the sentinel lymph node, that is, the first lymph node draining the region of the tumor, is associated with disease progression and often changes clinical management. It is now well established that the sentinel node undergoes changes in stromal and immune cell composition, even before the arrival of tumor cells (196). Lymphangiogenesis and lymphatic remodeling in the lymph node, driven by VEGF-A, VEGF-C, and VEGF-D, are important components of the pre-metastatic niche (197–199). HEVs, which normally support extravasation of naïve lymphocytes into the lymph node parenchyme, are also remodeled, becoming dilated and losing their typical "high" morphology and other molecular characteristics important for lymphocyte trafficking (199, 200). VEGF-D can suppress the proliferation of typical versus remodeled HEVs in the draining lymph node (199). In addition, the recruitment of naïve lymphocytes to the lymph node is impaired in tumor-draining nodes through loss of expression of CCL21 in HEVs, whereas recruitment of inflammatory cell subsets is enhanced in larger venules (201). While tumor-secreted factors such as VEGFs can act directly on LECs and HEVs in lymph nodes, HEV morphology and function are known to be dependent on lymphatic drainage, particularly the trafficking of DCs (202). Therefore, it is likely that lymphatic flow, HEV function, and immune cell composition in tumor-draining lymph nodes are strongly interrelated. The composition and function of immune cells is known to be altered in tumor-draining lymph nodes, with a lower percentage of effector T cells, loss or immaturity of DCs, and higher numbers of T_{regs} (196). In addition, effector T cells in tumor-draining lymph nodes may be functionally tolerant (203). In a mouse melanoma model, tumor cells implanted into lymph nodes unrelated to the primary tumor were rejected by a specific $CD8^+$ T cell response (204). However, tumor cells introduced into the tumor-draining lymph nodes were able to successfully implant following anergy of the reactive T cells due to MHC class I presentation of tumor antigens (204).

The relationship between tumor lymphangiogenesis, lymphatic remodeling, and the immune response is not yet fully elucidated with some apparently contradictory reports in the literature. Lymphatic vessel density at the invasive margins of tumors has been shown to correlate with metastasis and reduced overall survival in many tumor types, including melanoma, breast cancer, colorectal cancer, and lung cancer [reviewed elsewhere (179)]. Expression of lymphangiogenic factors and their receptors can also be prognostic and predictive of metastatic disease in these tumors. Interactions between VEGF-D and VEGFR-3 can promote the early events of lymphatic metastasis, as demonstrated in a VEGF-D-driven mouse tumor model (205). The proximity of tumor cells expressing VEGF-D to small lymphatic vessels can also be an important determinant of metastasis (206). For the reasons outlined above, increased lymphatic vessel density and lymphatic flow is thought to increase peripheral tolerance and enhance the immunosuppressive microenvironment of both the tumor site and the draining lymph node. Surprisingly, a recent study of human colorectal cancers found that lymphatic vessel density at the invasive margin correlated with the cytotoxic T cell density and inversely correlated with the risk of metastasis (207). Recent analysis of The Cancer Genome Atlas data of human metastatic melanoma samples has shown a correlation between levels of lymphatic gene expression and expression of genes associated with immune infiltration (208). In a mouse model of melanoma, it was found that mice lacking dermal lymphatics showed a lower immune cell infiltrate than mice with intact lymphatic drainage, but that adoptive T cell transfer was more effective in the absence of lymphatic vessels (208). This finding was hypothesized to be due to the lack of T_{regs} and suppressive macrophages in the tumor microenvironment, allowing the transferred T cells to exert their cytotoxic effects (208). Further investigation of the contribution of lymphatic vessels to the immune infiltrate in tumors and the development of an immunosuppressive environment is needed.

ROLE OF BLOOD VASCULAR ENDOTHELIAL CELLS IN IMMUNE SUPPRESSION AND TOLERANCE

Blood vessel endothelial cells (BECs) also function as semi-professional APCs and can modulate the T cell response. BECs constitutively express both MHC class I and MHC class II molecules and upregulate these in response to inflammatory signals (78). They possess antigen-processing machinery and have been shown to take up and present antigens *in vivo* and *in vitro* (209). Critical costimulatory molecules CD80 and CD86 are not expressed on cultured human endothelial cells, rendering them unable to stimulate naïve $CD8^+$ T cells (210). However, limited activation of memory $CD8^+$ T cells that have less stringent costimulatory requirements has been observed (210). Co-inhibitory molecules including PD-L1 and PD-L2 can be expressed by endothelial cells (209, 211). Expression of these immune checkpoint molecules is upregulated by TNFα and can inhibit $CD8^+$ T cell activation (211). Huang et al. demonstrated that endothelial cells derived from B cell lymphomas can express the co-inhibitory molecule TIM-3, which correlated with increased growth and dissemination of lymphoma in a mouse model (212). Expression of the immunosuppressive enzyme IDO has also been demonstrated in endothelial cells in renal cell carcinoma (213).

B7-H3 and B7-H4 are members of the B7 family of immune regulatory molecules, which includes PD-L1 (B7-H1) and PD-L2 (B7-DC) (214). Both molecules are thought to function as co-inhibitory signals limiting T cell activation (215, 216). Expression of B7-H3 on tumor cells and the endothelium of tumor-associated vasculature has been described in ovarian, endometrial, and cervical carcinomas and correlated with higher grade and poor prognosis (217–219). Interestingly, in cervical carcinomas, endothelial B7-H3 expression inversely correlated with $CD8^+$ T cell infiltration (219), whereas there was no correlation in endometrial carcinomas (218). Expression of B7-H3 and B7-H4 has also been demonstrated on tumor vasculature in renal cell carcinomas and is associated with poor prognosis (220, 221). Correlation with TILs has not been reported in this

setting. Clearly the endothelial lining of tumor blood vessels has immunomodulatory capabilities, but it remains to be demonstrated conclusively *in vivo* that tumor endothelial cells take up and present tumor-specific antigens and contribute to the immunosuppressive tumor microenvironment.

IMPLICATIONS FOR TREATMENT STRATEGIES

Current clinical therapeutic approaches targeting the tumor vasculature include neutralizing antibodies to VEGF-A (bevacizumab), neutralizing antibodies to VEGFR-2 (ramucirumab), ligand traps (aflibercept), and multi-target tyrosine kinase inhibitors such as sunitinib and sorafenib, which target a range of receptor tyrosine kinases including the VEGF receptors, PDGF receptors, Flt3, and c-kit (222, 223). The ligand trap aflibercept is a recombinant protein containing regions of the extracellular domain of VEGFR-1 and VEGFR-2 fused to the Fc portion of IgG and functions to prevent the binding of VEGF-A, VEGF-B, and PlGF to VEGF receptors, on the cell surface (96). In addition, tyrosine kinase inhibitors targeting the epidermal growth factor receptor (EGFR), now widely used in the treatment of EGFR-mutant lung adenocarcinoma, have also been shown to decrease production of VEGF-A, reduce tumor hypoxia, and possibly have a direct effect on tumor endothelial cells (224, 225). Bevacizumab is the most commonly used and well-studied agent, approved for use in combination with conventional chemotherapy in colorectal, lung, renal cell, and ovarian cancer [reviewed elsewhere (226)]. The mechanism of action of these antiangiogenic therapies is not yet fully understood. Rather than purely starving the tumor of nutrients, these antiangiogenic therapies are also thought to exert their effect by physical normalization of the tumor vasculature and alleviation of hypoxia (147). VEGF-A inhibitors have been shown to reduce the size and tortuosity of tumor vessels, enhance vessel maturation, recruit pericytes, and normalize the basement membrane (149). This results in improved oxygenation and drug delivery to tumors, in part through the ability of normalized vessels to sustain a pressure gradient (151). Vascular normalization has been difficult to demonstrate clinically, as effects may be transient, variable in response to different doses, and occur in only a proportion of tumors. However, studies using advanced magnetic resonance imaging techniques have demonstrated that antiangiogenic therapy can improve tumor perfusion in the clinical setting (227). In a study of cytotoxic chemotherapy combined with VEGF receptor inhibition for the treatment of glioblastoma, patients in whom this improved perfusion was demonstrated had an improved overall survival (227). This finding suggests that vascular normalization can indeed improve access of chemotherapeutic agents to tumors and therefore may also improve the delivery of immunotherapies and the trafficking of immune effector cells. Blocking the VEGF signaling pathway may also act to reduce immunosuppression in the tumor environment.

As outlined in previous sections, the tumor vasculature and the immune microenvironment are intricately linked, with the blood and lymphatic vessels both regulating access of immune cells to the tumor and showing direct immunosuppressive actions through angiogenic factors and endothelial cells. The combination of antiangiogenic therapy and immunotherapy has been explored in a variety of pre-clinical models (**Table 1**) and forms the basis for a number of current clinical trials (**Table 2**). Much of the pre-clinical evidence relates to adoptive cell transfer and vaccination strategies, in combination with a wide variety of antiangiogenic therapies including VEGF-A blockade (97, 98, 111), VEGFR-2 blockade (100, 101), ligand traps (99, 112), receptor tyrosine kinase inhibitors (106, 107, 114, 115), irradiation (166), and angiostatic peptides (102, 103, 105, 113). For example, Shrimali et al. demonstrated enhanced tumor infiltration, decreased tumor size, and improved survival when adoptive T cell transfer was combined with treatment with an anti-mouse VEGF-A antibody in a mouse model of melanoma (97). Results from these pre-clinical models suggest that vascular normalization can improve lymphocyte infiltration into tumors and combining antiangiogenic therapy and CAR T cell transfer in solid tumors may be worthy of further investigation in clinical trials.

In the clinical setting, interactions between immune checkpoint inhibitors and the tumor vasculature are beginning to be described. Ipilimumab, an anti-CTLA-4 antibody, shows durable responses in up to 30% of patients with metastatic melanoma (2) and can result in an immune-mediated lymphocytic vasculopathy with resultant vessel obstruction and tumor necrosis (228). In a cohort of patients with advanced melanoma, pre-treatment serum levels of VEGF-A correlated with poor overall survival and poor response to immune checkpoint therapy with ipilimumab (229). Initial promising results have been reported in phase I clinical trials combining ipilimumab and the anti-VEGF-A antibody bevacizumab in advanced melanoma and glioblastoma (109, 117). This combination appears safe and well tolerated (109, 117) and warrants further investigation and comparison to current treatment regimens. Tumor endothelial cells isolated from melanoma patients treated with this combination of ipilimumab and bevacizumab showed variable upregulation of adhesion molecules E-selectin, ICAM-1, and VCAM-1, with resulting enhancement of T cell infiltration into the tumor (109, 230). Changes in levels of circulating chemokines, cytokines, and growth factors were seen following treatment, including increased levels of chemoattractant IP-10 (CXCL10) and decreased levels of VEGF-A (230). Endothelial anergy induced by VEGF-A could be demonstrated in these samples and reversed by the addition of bevacizumab (230). A recent report describes results from a phase I study combining bevacizumab and the anti-PD-L1 antibody atezolizumab in the treatment of advanced renal cell carcinoma (110). Before the addition of atezolizumab, bevacizumab treatment increased the Th1 gene expression signature, which is associated with CD8 T^+ cells, NK cells, and Th1 chemokines (110). There was a pronounced increase in intratumoral T cells following combination therapy, suggested to be related to an increase in expression of both CX3CL1 (fractalkine) and its receptor (110). Although not a primary endpoint of this small single-arm study, clinical activity was higher with combination therapy than that has been previously reported with either bevacizumab or atezolizumab alone (110). Each drug may potentiate the effects of the other, controlling tumor angiogenesis and counteracting the immunosuppressive microenvironment.

These studies provide important clinical and laboratory data to support further investigation of the use of antiangiogenic agents to enhance immunotherapy.

Following the description of the role of lymphangiogenesis, lymphatic remodeling, and lymphangiogenic factors in promoting tumor metastasis, targeting this signaling axis has been suggested as an adjunct to conventional cancer treatments (231). Analogous to the targeting of angiogenesis through anti-VEGF-A antibody bevacizumab, monoclonal antibodies to VEGF-C (232), VEGF-D (233, 234), and VEGFR-3 (235) have been developed and are being evaluated in both pre-clinical models and clinical trials. Ligand traps that contain components of VEGFR-2 (236) and VEGFR-3 (237) have also been developed, which are designed to block the binding of VEGF-C and VEGF-D to cell surface receptors. Multi-target receptor tyrosine kinase inhibitors such as sunitinib and sorafenib, described above, can also block signaling through VEGFR-3 on LECs (238). As detailed in previous sections, LECs and lymphangiogenic factors can also influence the host immune response to cancer. Consideration should be given to the potential to enhance immunotherapy by targeting lymphangiogenesis through monoclonal antibodies or ligand traps. Blocking the immunomodulatory functions of VEGF-C and VEGF-D and decreasing lymphangiogenesis to reduce the tolerance-promoting effects of LECs may be effective ways to improve immunotherapy approaches such as checkpoint inhibitors or adoptive cell transfer. Pre-clinical evaluation of these combinations will help to delineate the contribution of the lymphatic vasculature to evasion of the host immune response and explore the potential benefit of targeting this component of the microenvironment.

CONCLUSION

Physiological processes such as the growth and remodeling of blood and lymphatic vessels and the immune response to foreign antigens are altered in the tumor microenvironment, and these alterations contribute to the establishment and progression of cancer. Significant interactions between endothelial cells and immune cells alter the extent and composition of the immune infiltrate in tumors, through both molecular and mechanical means. In addition, lymphangiogenesis and LECs have important roles in the development of tolerance to peripheral tissue antigens, including tumor antigens. The contribution of blood and lymphatic vessels to the modification of the antitumor host immune response in human cancer remains to be fully described. It is not known whether aspects of the tumor vasculature are different in tumors that respond to immunotherapy and those that do not, and if features such as hypoxia, production of angiogenic factors, or lymphatic vessel density may serve as predictive biomarkers. Immunotherapy and antiangiogenic therapy both target aspects of the tumor microenvironment rather than specifically targeting the tumor cells themselves. As such, combination approaches may be required to obtain the full benefit of these therapies. Further investigation of antiangiogenic and antilymphangiogenic therapy as a potential adjunct to immunotherapy may see improvement in the access of CAR T cell therapy to solid tumors and expand the benefits of immune checkpoint inhibition to non-inflamed tumors.

AUTHOR CONTRIBUTIONS

Conceived the topic and outlined the paper: SF, SH, SS, MA, and RF. Wrote and revised the paper: SH, SF, SS, MA, RF, and BS. Final approval of the version to be published: SH, SF, SS, MA, RF, and BS.

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The Role of Lymphatic Endothelial Cells in Liver Injury and Tumor Development

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Lymphatics and lymphatic endothelial cells (LECs) possess multiple immunological functions besides affecting immune cell migration, such as inhibiting T cell proliferation and antigen presentation by dendritic cells. Moreover, they control the trans-endothelial transport of multiple molecules and antigens. Emerging evidence suggest their active involvements in immunregulation, tumor, and metastases formation. In the liver, increased lymphangiogenesis, specifically at the portal area has been associated with multiple liver diseases in particular primary biliary cirrhosis, idiopathic portal hypertension, and liver malignancies. Nevertheless, the exact role and contribution of LECs to liver diseases are poorly understood. The review summarizes the current understanding of LECs in liver diseases.

Keywords: lymphatic endothelial cells, lymphatics, liver injury, HCC

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LIVER AS A LYMPHOID ORGAN

The liver primarily operates as a metabolic center to maintain homeostasis that includes processing of gut-derived nutrients, the clearance of toxins, and the production of the bile (1). Besides these well-known functions, it is also considered as a lymphoid organ (2). This is on one hand due to the fact that non-parenchymal cells, such as hepatic stellate cells (HSCs) and liver sinusoidal endothelial cells (LSECs), take on antigen presenting and immunomodulatory functions to create a tolerant microenvironment (2, 3). On the other hand, the liver encompasses large populations of resident immune cells, such as Kuppfer cells, NK, T, and NKT cells that shape the local immune response, respond to danger signals and closely interact with parenchymal and non-parenchymal liver cells (3). These resident immune cells are located within the sinusoids where the mixture of arterial and venous blood carrying oxygen and gut-derived metabolic products arrives into the liver. From the sinusoids blood flows toward the central vein and finally leaves the liver conveying blood to the vena cava inferior. It is less known about the lymphatic circulation of the liver despite of the fact that it produces between 25–50% of the total lymph received by the thoracic duct (4, 5). This review summarizes the current understanding of the lymphatics of the liver and their known functions under steady state and during liver injury. Liver injuries manifest in various diseases including autoimmune hepatitis, infectious [hepatitis C virus (HCV)- and hepatitis B virus (HBV)-induced liver hepatitis], and metabolic disorders. Major causes of metabolic injuries are alcoholic liver damage (manifesting

Abbreviations: CCL21, CC-chemokine ligand 21; CCl_4, carbon tetrachloride; CCR7, C–C chemokine receptor type 7; CD, cluster of differentiation; cDC1/2, conventional dendritic cells type 1/2; FACS, fluorescence-activated cell sorting; FRCs, fibroblastic reticular cells; HSCs, hepatic stellate cells; LECs, lymphatic endothelial cells; LNs, lymph nodes; MS, mechanosensors; SLO, secondary lymphoid organs; SR, scavenger receptors; TLRs, toll-like receptors.

in liver steatosis, hepatitis, and cirrhosis) and the diet-related non-alcoholic fatty liver disease (NAFLD). Biliary injuries involve primary sclerosing cholangitis and primary biliary cirrhosis that are considered as immune-mediated liver disorders. Independent of the diverse etiology, liver inflammation and damage trigger a wound healing process that progressively leads to liver fibrosis, cirrhosis, and end-stage liver disease (6).

THE LYMPHATIC SYSTEM OF THE LIVER

The hepatic lymphatic system is divided into a deep and a superficial fraction (5, 7). The former follows the hepatic vein and the portal tracts, and the later collects lymph from the convex and inferior surfaces of the liver. The lymph itself originates in the perisinusoidal space of Disse (4). At the hepatic sinusoids, the interstitial space contains collagen fibers that connect LSECs and hepatocytes and form the portal limiting plate. Thus, fluid from the sinusoids flows through this structure and moves toward the perilobular space (it is referred as the space of Mall) and finally enters the portal lymphatic vessels (4, 5, 7, 8). This fluid movement is attainable due to the hydrostatic pressure differences observed between the portal vein and the interstitial space (9). Additionally, because of pressure gradient between arterial capillaries and the interstitial space, some blood is filtered through the peribiliary capillaries that surround the interlobular bile ducts. Nevertheless, the contribution of this process for the total liver lymph output is less than 10% (10). Besides the above-described route, the interstitial fluid can also follow the interstitial space connected with the hepatic capsule that contains superficial lymphatic vessels (5, 7). Both, the deep and superficial lymphatics of the liver drain primarily to the hepatic/celiac lymph nodes (LNs) (7, 11).

LYMPHATIC CONTENT AND CELLULAR TRANSPORT

The lymph generated in the perisinusoidal space contains 80% of the proteins present in plasma (5). The content of the lymph gains increasing attention as it contains self-peptides derived from intracellular, membrane-associated, and matrix proteins (12, 13). Moreover, it carries apoptotic cellular materials, infectious agents and represents a remote communication system for small molecules (e.g., cytokines) and cell-derived vesicles between the organ and its draining LN (13–16). The relevance of small molecule/vesicle trafficking *via* the lymphatics to the etiology of liver diseases is entirely unexplored. Such self-antigen delivery can be a key in autoimmune liver diseases. Moreover, biliary content during bile obstruction leaks to the lymphatics at the portal tract (5, 17) and probably reaches the draining LNs. Since bile acids might trigger inflammatory responses and necroptosis, it could influence hepatic immune responses arising within the draining LN.

Due to the resident immune, parenchymal and non-parenchymal cells, a tolerogenic environment is created for immune responses within the liver (3). Nevertheless, if immunity is required as a response to for example pathogens either monocyte-derived DCs present in intrahepatic myeloid-cell aggregates for T cell population expansion (iMATEs) provide bases for efficient T cell responses (18) or cytotoxic T lymphocytes are generated by migratory DCs reaching the draining LN (19, 20). On the other side of the immune spectrum, migratory DCs are likely involved in the generation of regulatory T cells toward dietary antigens in the liver-draining LN (21). The lymphatics thus represent a crucial channel for a potential immunogenic and tolerogenic response outside of the liver suppressive environment (19, 22). To ensure this function, the lymph transports various immune cells. Accordingly, electron microscopy studies revealed the presence of DCs in between the limiting plate of hepatocytes and in the interstitial space of portal tract (4). This migratory process is more active after LPS injection (4). Not only liver resident but also circulating DCs can enter the lymphatic system in the liver, and this DC blood-lymph translocation seems to alter DCs and creates a more tolerogenic phenotype under steady state (23, 24). This could be due to DC interaction en route with liver non-parenchymal cells such as LSECs (25) or with lymphatic endothelial cells (LECs) along the lymphatics (26). Thus, the lymphatic circulation of liver-resident DCs and the circulating DC translocation might contribute to important peripheral tolerogenic responses under steady state. The major migratory cell population is the cDC1 ($CD11c^+CD103^+CD11b^-$) cells, and it remains to be elucidated whether monocyte-derived DCs or cCD2 ($CD11c^+CD103^-CD11b^+$) cells contribute to the migratory cell population under differing circumstances (20, 27).

DC migration is maintained by CCR7–CCL21 interaction, where CCL21 is secreted by LECs that are also positive for various adhesion molecules and glycoproteins that are involved in cellular transport, such as gp38, ICAM-1, and E-selectin (28, 29). Besides LECs, EM study revealed the presence of fibroblast-like cells close to collagen fibers at the portal area representing fibroblastic reticular cells (FRCs) (4). Migratory DCs display close correlation with FRCs near the portal tract (4). Accordingly, in human liver, a low number of $gp38^+$ FRCs are present at the portal area under steady state (30). FRCs secrete CCL19 that guides DC migration and provide survival factors for immune cell homeostasis (28, 31). Importantly, under pathological conditions, such as in primary biliary cirrhosis, the portal FRC and LEC network extends and is associated with structures similar to tertiary lymphoid organs (30). Similarly, in murine *P. acnes*-induced granulomatous hepatitis, portal tract-associated lymphatic structures, so called PALTs, are formed where T and B cell responses arise (32). Further studies are necessary to clarify that such tertiary lymphoid structure formation is related to migratory and lymphatic changes in liver diseases or represent a pathological structure where LN-independent immune responses influence disease progression.

Besides DCs, lymphocytes, plasma cells, and mast cells could be identified within the lymphatic vessels of the liver and near the portal tract under steady state (4, 5). While memory lymphocytes and plasma cells are common travelers within lymphatic vessels, the exact function of mast cells remains uncertain within the healthy liver. The later is especially intriguing, since mast cells release inflammatory mediators during various liver diseases and contribute as accessory cells to disease progression (33). The liver

is especially rich in lymphocytes involving not only conventional T cells but also innate lymphoid cells that express lymphoid homing markers, such as CCR7 (34, 35). Nevertheless, future studies are necessary to determine to which extent the various lymphocyte subpopulations travel *via* the lymphatics from the liver and what are the biological consequences of their migration.

LYMPHATIC ENDOTHELIAL CELLS OF THE LIVER

Lymphatic endothelial cells are the building blocks of lymphatic capillaries and vessels and express variety of molecules that distinguish them from blood endothelial cells (BECs) such as CCL21 or cadherin-13 (**Table 1**) (29, 36). Most of these molecules refer to LECs within secondary lymphoid organs (SLO); however, some differences due to the liver environment could be observed (**Table 1**). For example, lymphatic vascular endothelial hyaluronan (Lyve-1) is specific for LECs in lymphoid organs but is present in LSECs and in some liver macrophages (37). The best way is to identify liver LECs based on their expression of CD31 and gp38 (podoplanin). Liver LECs are $CD45^{-}CD31^{+}gp38^{+}$ and thus can be distinguished from FRCs ($CD45^{-}CD31^{-}gp38^{+}$), from LSECs ($CD45^{-}CD31^{+}gp38^{-}$), and from the recently described $gp38^{+}$ liver progenitor cells ($CD45^{-}CD31^{-}CD133^{\pm}gp38^{+}$) (38).

Lymphatic endothelial cells not only provide the structural unit for the vessels but also are involved in additional biological processes. As discussed already, *via* its expression of cytokines and adhesion molecules, LECs guide immune cell migration. Additionally, they are active participants in the nearby arising immune responses. They directly diminish DC maturation and T cell proliferation and thus function as a negative regulatory circuit during immune responses (26, 29, 39). A variety of immunoregulatory factors are expressed by LECs that enable these functions. For example, LECs secrete TGFβ and nitric oxide, all of which are immunosuppressive (39, 40). Additionally, LECs lack the expression of co-stimulatory molecules and instead are rich in co-inhibitory markers, such as PDL1 (29, 39, 41, 42).

Lymphatic endothelial cells also possess the ability to express self-antigens and induce CD8 T cell deletion and serve as antigen reservoir for CD4 T cell tolerance (41–43). They also possess surface receptors for endocytotic activity and able to sample from their environment (44). Importantly, most of these immunomodulatory potentials are connected with LECs present in SLO, thus raising the question what are the similarities and differences between SLO-associated LECs and LECs present along the lymphatic vessels. Unfortunately, such comparison studies have not been conducted. It is also uncertain whether liver LECs are able to acquire soluble antigens from the lymph and have antigen-presenting capacity.

Lymphatic endothelial cells are also actively involved in cholesterol homeostasis, and the removal of cholesterol by lymphatic vessels is dependent on the uptake of HDL by scavenger receptor class B type I expressed in LECs (45–47). In line with this, endothelial O-glycan deficiency led to disorganized lymphatic vessels and resulted in the development of fatty liver disease (NAFLD) due to the missing lymphatic removal of gut-derived lipid products (48). Since lipid metabolic changes are associated with various liver diseases, it will be interesting to evaluate in more details how this affects lymphatic function and *vice versa* how lymphatic changes are reflected in liver metabolic alterations.

TABLE 1 | Summary of surface markers for identifying murine and human lymphatic endothelial cells.

Endothelial markers (LECs and BECs)	Endothelial markers excluded from BECs
ICAM-1 (CD54)	Lyve-1[a]
CD44	Prox-1
	VEGFR3
CD31	CCL21
CD34	Desmoplakin
	Integrin α9, α1
E-, P-selectin	B-chemokine receptor D6
Plakophilin	Cadherin-13
	MMR
	Gp38 (podoplanin)

[a]*Present in liver LSECs and some liver macrophages.*
BECs, blood endothelial cells; MMR, macrophage mannose receptor.

LYMPHATICS AND LIVER DISEASES

Chronic Liver Diseases

Multiple studies have demonstrated that the lymphatic system is significantly altered during liver diseases. The number of lymphatic vessels as well as the lymphatic flow increases in fibrotic and cirrhotic livers (37, 49–52). This is in line with observations that VEGF-C and VEGF-D expression is elevated during fibrosis (51, 53, 54). More importantly, the increased lymphangiogenesis is positively correlated with disease severity (49, 52). Moreover, the higher flow observed within the lymphatics during liver diseases could have additional consequences. Increased interstitial flow elevates the expression of cell recruiting cytokines (e.g., CCL21) and thus influences immune cell migration toward the draining LN (29). The flow at the same time likely reduces the portal pressure *via* channeling the excess fluid in cirrhosis and in portal hypertension (55).

Increased number of LECs is present during idiopathic portal hypertension (56), HCV-associated cirrhosis (52), and primary biliary cirrhosis (50). Given the wide-range of biological processes where LECs are involved, it is likely that the increase in the number of lymphatic vessels possesses functions exceeding fluid handling. The inflammatory environment triggers cytokine production in LECs and therefore increases immune cell recruitment (29). Additionally, bacterial products such as LPS (that is increased in portal vein during cirrhosis) induce not only chemoattracting cytokine production but also can activate Nf-Kb in LECs and thus consequently upregulate Prox1 and VEGFR-3 (57). Both molecules raise the sensitivity to VEGF-C and VEGF-D and thus influence lymphangiogenesis (57, 58). Within the liver, this remains to be elucidated.

Liver Tumor and Metastases Development

One of the consequences of liver diseases is the development of hepatocellular carcinoma (HCC). Human HCC samples

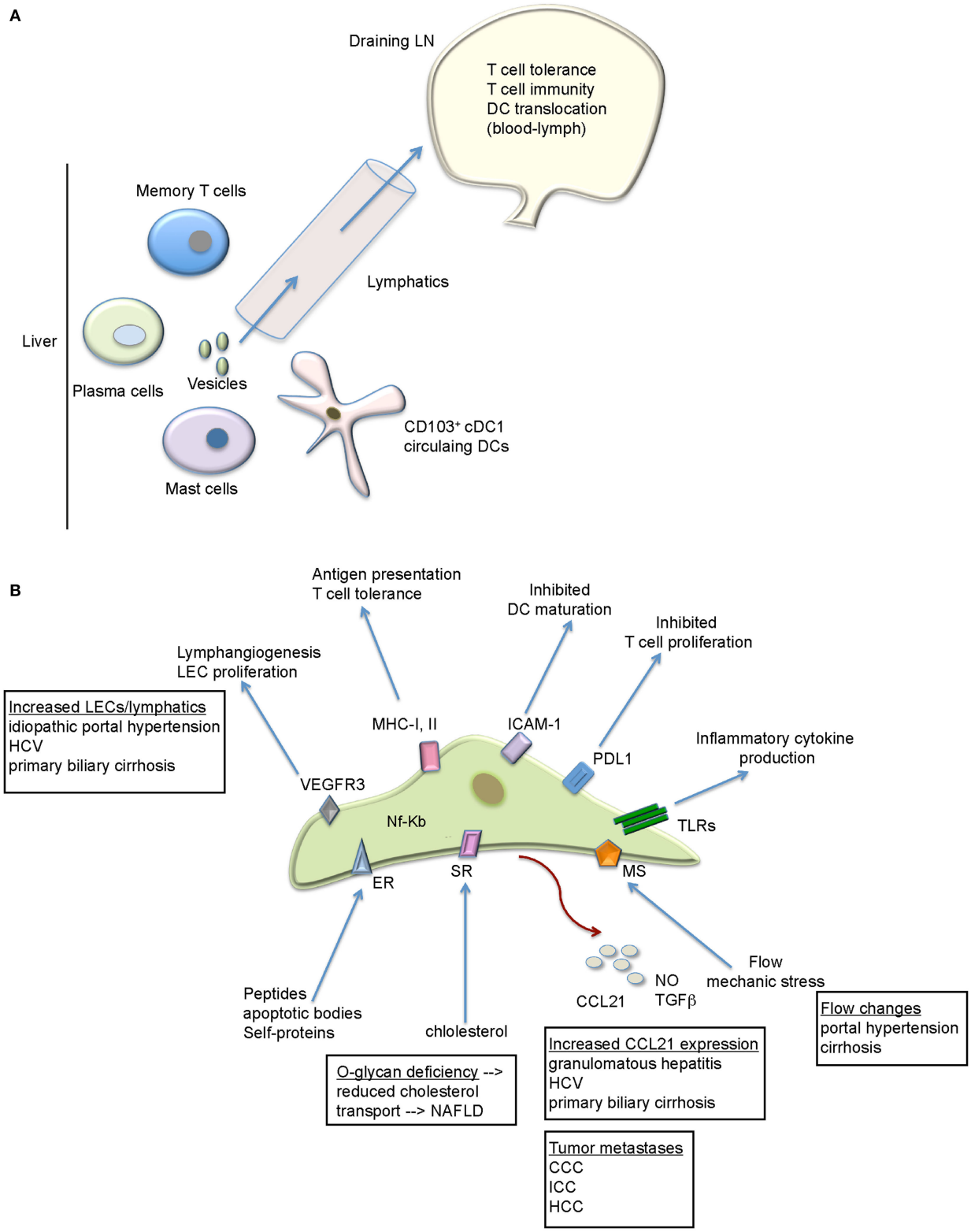

FIGURE 1 | **The functional role of the lymphatics within the liver**. **(A)** Besides fluid handling, the hepatic lymphatics transport various immune cell types, proteins, and vesicles to the draining LN. The LN provides the environment where not only tolerogenic but also immunogenic T cell response can arise that target liver pathogens/antigens. **(B)** LECs exhibit a variety of immunoregulatory functions that might be relevant for liver diseases. Future studies are necessary to evaluate all of these possibilities. Liver diseases with lymphatic/LEC alterations are depicted. CCC, colorectal carcinoma; ER, endocytic receptor; HCC, hepatocellular carcinoma; ICC, intrahepatic cholangiocytes carcinoma; MS, mechanosensors; SR, scavenger receptors, TLRs, toll-like receptors.

displayed Lyve-1$^+$ cells in the tumor-surrounding environment (37), and lymphatic vessels are present in the vicinity of metastatic liver tumors (37, 59, 60). In line with this observation, VEGF-C- or VEGF-D-expressing liver tumors are more prone to spread within the liver (60). The liver metastases of colorectal cancer also exhibit gp38$^+$ peri- and intra-tumoral lymphatic vessels that were correlated with tumor growth and metastases potential (61). Accordingly, intrahepatic invasion was the main prognostic marker for colorectal cancer and for intrahepatic cholangiocarcinoma and likely represents the main route of cancer dissemination in the liver (62–64). Indeed, intrahepatic cholangiocarcinoma is often associated with LN metastasis that translates to poorer outcome and reduced patient survival (63).

Lymphatic endothelial cells could facilitate such tumor cell spreading *via* CCL21–CCR7 interaction. Some colorectal cancer cells express CCR7 and thus could migrate toward the homeostatic chemokine CCL21 expressed by LECs (29). Additionally, lymphatic flow-induced chemokine gradient (e.g., CCl21 or CXCL12) could be sufficient to drive metastases of tumors positive for cytokines as observed in gliomas (29, 65). The exact mechanisms for HCC and other liver cancers are not well understood. Similarly, LECs display multiple immunomodulatory roles within the tumor microenvironment. LECs induce the recruitment of regulatory T cells, alter features of tumor-associated stroma, and contribute to the immunosuppressive milieu favoring tumor growth (29, 66, 67). Additional studies are necessary to evaluate these possibilities also for liver cancers and metastases.

SUMMARY AND OUTLOOK

Taken together, the liver is a unique metabolic and immunological niche within the body. Its lymphatic system represents a complex anatomical organization with a large lymph output. Based on the repertoire of the biological functions associated with lymphatics and LECs (**Figure 1**), it is suggested that LEC expansion is not only a passive accompanying event during liver diseases. This is particularly interesting since LEC changes seem to be reflective of the type of peripheral inflammation (68). Thus, this line of research urges more attention and studies that clarify its exact contribution to liver disease pathogenesis. This is possible, as improved marker combinations allow the flow cytometry detection and sorting of these cells from the liver. This, together with other techniques (e.g., histological analyses), provides solid basis for further functional investigations. This could raise our understanding of liver diseases and open novel therapeutic opportunities.

AUTHOR CONTRIBUTIONS

VL-K has designed and written the manuscript and prepared the table and the figure.

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