REGULATION OF IMMUNE FUNCTION BY THE LYMPHATIC VASCULATURE

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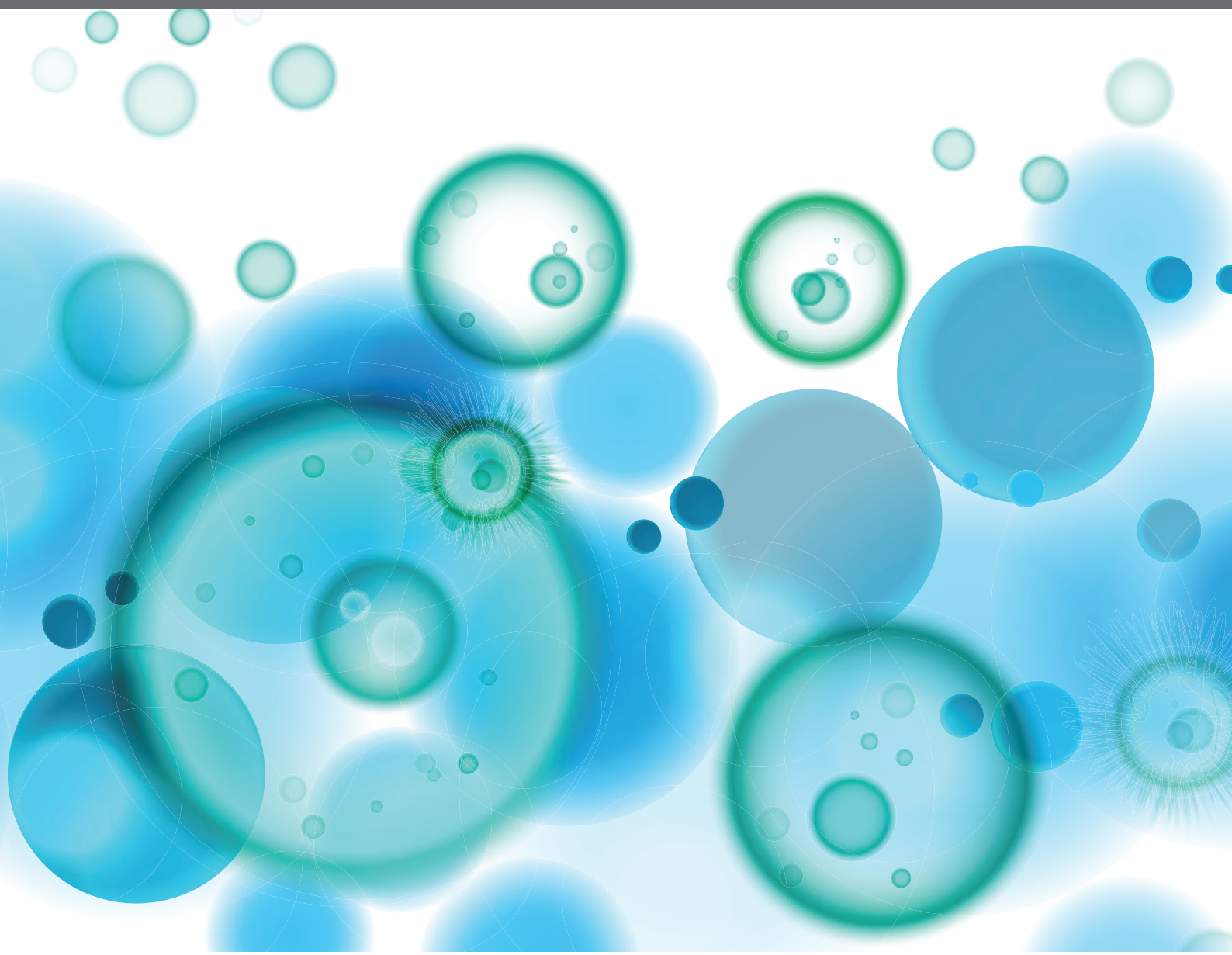

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REGULATION OF IMMUNE FUNCTION BY THE LYMPHATIC VASCULATURE

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EDITORIAL
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Editorial: Regulation of Immune Function by the Lymphatic Vasculature

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Editorial on the Research Topic

Regulation of Immune Function by the Lymphatic Vasculature

The lymphatic vasculature is composed of a hierarchy of vessels that extend from peripheral tissues into lymph nodes (LN) and provide the critical route for leukocyte migration and antigen presentation that drive innate and adaptive immune responses. However, beyond physically connecting peripheral tissue to LNs, there is an expanding view of how the lymphatic vasculature influences immune responses. The lymphatic vasculature can modulate immune responses ranging from peripheral tolerance during homeostasis to protective immunity following infection or vaccination to tumor immune escape. Recent work demonstrates that lymphatic vessels actively regulate transport functions, express and secrete chemo-attractants to initiate leukocyte migration, and directly interact with leukocytes to inform their behavior, activation, and establishment of memory. The immunological impact of how the lymphatic vasculature is influenced by tissue microenvironments and ongoing regional inflammatory processes is only beginning to be appreciated, but is of critical relevance to the role lymphatic vessels play in diseases. This Research Topic, "Regulation of Immune Function by the Lymphatic Vasculature," brings together 17 articles that present our current understanding of and provide new insight into the necessary role the lymphatic vasculature plays in regulating inflammation and immunity.

Lymphatic vessels mediate the exit of multiple leukocyte types from peripheral tissue and LNs, including dendritic cells, neutrophils, T cells and B cells. A comprehensive review by Jackson presents the molecular mechanisms that determine leukocyte homing and transmigration to and through peripheral lymphatic vessels while Hampton and Chtanova describe the immunological consequences of leukocyte exit in disease. Farnsworth et al. complement these insights with an extensive review of the interplay between lymphatic vessels and chemokines, and how the dynamic display of various chemokine ligands in response to microenvironmental stimuli influences the migratory nature of leukocytes. Interesting new data presented by Campbell et al. describe novel MHC-dependent interactions between T cells and dendritic cells within peripheral lymphatic capillaries. These exciting data suggest that within lymphatic capillaries, dendritic cells may interact with circulating T cells prior to entry into the LN. How the kinetics of leukocyte egress from peripheral tissues contributes to disease remains a critical question moving forward and raises the possibility that lymphatic vessels may help to pattern both the initiation and resolution phases of peripheral tissue inflammation.

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Given the multiple roles lymphatic vessels play in regulating leukocyte trafficking and function, the field has long considered how lymphatic function, or dysfunction, contributes to the perturbation of immune homeostasis. Intriguingly, lymphatic vessel expansion is associated with autoimmune, chronic inflammatory, and malignant contexts. However, it remains unclear whether lymphatic remodeling is a cause of or rather a biproduct of tissue inflammation. Schwager and Detmar provide an extensive review of the mechanisms that regulate inflammatory lymphangiogenesis and discusses implications for disease and Schwartz et al. review the current literature surrounding the contribution of lymphatic dysfunction to autoimmunity. Providing new insights, Stephens et al. demonstrate that TLR4 signaling during intestinal inflammation is required for mesenteric lymphatic vessel expansion and leakiness, which leads to associated changes in dendritic cell migration to draining LNs. Though inhibition of TLR4 signals restored lymphatic function, there was no change in tissue inflammation, suggesting lymphatic vessel dysfunction lies downstream of inflammatory insult. Interestingly, Tamburini et al. also describe disease-dependent changes in lymphatic endothelial cell (LEC) transcriptional states in human chronic liver disease. Whether these changes cause inflammation or are the response to inflammation remains to be carefully determined.

The interplay between lymphatic and immune dysfunction is perhaps most apparent in the context of lymphedema. As lymphedema progresses, tissue fluid accumulation, and stasis cause multiple tissue changes, including fibrosis, adipose tissue deposition, and chronic inflammation. These tissue changes, along with impairment in the ability to transport antigen and antigen presenting cells to lymph nodes, drives progressive deterioration in local immune function. Kataru et al. and Yuan et al. provide back to back reviews highlighting the cellular and molecular mechanisms that occur during lymphedema and their impact on immunity in mouse models and outcomes for patients.

In this Research Topic, and in the field in general, much emphasis is placed on peripheral lymphatic vessel remodeling, however, Lucas and Tamburini discuss the molecular mechanisms of LEC expansion and contraction in LNs draining inflamed tissues and the role of LN LECs in regulating antigen presentation vs. antigen exchange. Louie and Liao further review the role of subcapsular macrophages in the LN, whose position and survival depend on LN LECs, in innate pathogen defense. Interestingly, the LN LEC population may also be altered in the context of chronic disease. Tay et al. present new data that describes reduced lymphocyte egress from LNs during hypercholesterolemia, which results in LN hypertrophy. These changes in lymphocyte trafficking were likely driven at least in part by altered CCL21 and S1P gradients, signaling molecules generated by LN LECs.

LECs, particularly those found in LNs, were recently demonstrated to harbor intrinsic antigen-presentation capabilities and to archive antigens in order to influence T cell activation. Antigen-dependent and independent interactions between LECs, T cells and DCs may both preserve peripheral tolerance and promote protective immunity depending on context. These observations have continued to fuel the concept that LECs directly influence immunity. This hypothesis is now further supported by transcriptional data from Berendam et al. that describe modules of immune transcripts within LN LECs that support the novel functionality of LN LECs and provide the basis for future mechanistic studies. These data are summarized and integrated with the existing knowledge by Santambrogio et al. in order to describe the machinery found within LECs that may facilitate antigen scavenging and presentation.

Finally, the contribution of lymphatic vessels to tumor progression remains an area of intense interest. While historical paradigms associate lymphatic vessels with regional tumor metastasis, new work integrates the potential effects of lymphatic vessel remodeling on anti-tumor immunity and immunotherapy. Garnier et al. provide a comprehensive review of the existing framework for lymphatic vessel involvement in anti-tumor immunity, describing both the impact of lymphatic vessel remodeling on immunity and responses to local inflammation. Tamburini et al. demonstrate that the microenvironment of post-partum mammary gland involution potentiated immunotherapy leading to reduced tumor volume, increased immune cell activation and decreased lymphatic vessel density compared to tumors in nulliparous hosts. Lymphatic vessels are high expressors of PD-L1 in involuting mammary glands and may contribute to this response, consistent with recent published data also demonstrating a functional role for PD-L1 on tumor-associated lymphatic vessels.

With this cumulative work, our field continues to show that lymphatic vessels are active components of host immunity. The mechanisms through which lymphatic vessels synchronize immune expansion and contraction in both LNs and peripheral tissues provide therapeutic opportunities for immunomodulation and will ultimately make a significant impact on our understanding of health and disease.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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MINI REVIEW
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Lymph Node Lymphatic Endothelial Cell Expansion and Contraction and the Programming of the Immune Response

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Lymphatic endothelial cells (LECs) form the structure of the lymphatic vessels and the sinuses of the lymph nodes, positioning them to be key players in many different aspects of the immune response. Following an inflammatory stimulus, LECs produce chemokines that recruit immune cells to the lymph nodes. The recruitment of immune cells aids in the coordination of both LEC and lymph node expansion and contraction. More recent data has demonstrated that to coordinate LEC division and death, cell surface molecules, such as PD-L1 and interferon receptors, are required. During homeostasis, LECs use PD-L1 to maintain peripheral tolerance by presenting specific peripheral tissue antigens in order to eliminate tissue specific responses. LECs also have the capacity to acquire, present, and exchange foreign antigens following viral infection or immunization. Here we will review how lymph node LECs require immune cells to expand and contract in response to an immune stimulus, the factors involved and how direct LEC-immune cell interactions are important for programming immunity.

Keywords: lymphatic endothelial cell, lymph node expansion, PD-L1, apoptosis, immune tolerance, lymph node contraction, dendritic cell, interferon

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INTRODUCTION

Lymphatic endothelial cells (LECs) are a specialized subset of endothelial cells that comprise lymphatic vessels in the tissue and lymph node (LN). LECs interact with innate and adaptive immune cells both in the tissue and in the LN. LECs have the capacity to produce chemokines in order to recruit immune cells to the LN. Of the chemokines that LECs produce, CCL21 has been implicated in the recruitment of dendritic cells (DC), which in turn promotes LN expansion (1–4). Regulation of LN LEC division and death during LN expansion and contraction is a complicated process to which innate immune cells, adaptive immune cells, and specific signaling molecules contribute. Furthermore, LN LECs not only receive signals from immune cells, but also provide signals to the adaptive immune system to regulate peripheral tolerance and protective immunity. In this review we will highlight how LN LEC interactions and signaling regulate LECs in the LN in response to an inflammatory insult and how LECs program the immune response.

REGULATION OF LN LEC DIVISION BY THE INNATE IMMUNE SYSTEM DURING INFLAMMATION

During an inflammatory response, the LN must expand to allow for the rapid influx and division of responding lymphocytes. To do this, several coordinated processes in the LN occur: (1) the secretion of chemokines and cytokines and thus the recruitment of innate immune cells; (2) the relaxation of the fibroblastic reticular cell (FRC) network; (3) the division of the stromal cells in the lymph node; (4) the adaptive immune response and (5) the contraction of the LN.

Between 0 and 24 h following an inflammatory stimulus both type 1 and type 2 interferon (IFN) production is increased, which inhibits LEC division (5) (**Figure 1A**). Why LEC division is inhibited at this time point is unclear, however this time point coincides with increased expression of CCL19 and 21 by LN stromal cells (17, 18). Dendritic cells (DC) are recruited to the LN through interactions between CCR7 and CCL19 and 21 (19–21) (**Figure 1A**). Following DC recruitment to the LN, LEC division is initiated. CD11c+ DCs have been shown to lead to LEC proliferation through LEC-DC contact, a process that is ablated following CD11c+ cell depletion (6). Further work showed that DCs regulate the relaxation of the FRC network through the interaction of C-type lectin like 2 (CLEC-2) with podoplanin (PDPN) on the FRCs (22–24). CLEC-2 binding inhibits PDPN signaling, resulting in FRC elongation and increased LN elasticity (24). PDPN expression by LECs and binding by CLEC-2 also elicits the expansion of LECs in the LN (25, 26). In addition to PDPN engagement, DC initiation of LEC proliferation also occurs by inducing vascular endothelial growth factor (VEGF) production by FRCs in the lymph node (8, 9). Early LEC proliferation appears to be independent of T and B cells, as transfer of bone marrow derived dendritic cells into a mouse that lacks T or B cells can elicit LEC expansion at early timepoints following an immune stimulus (6). Thus, DC-stromal interactions are part of the initial step in the expansion of the LN following an inflammatory insult (**Figure 1B**).

In addition to DCs, macrophages have an important role in regulating lymphangiogenesis in response to inflammation through the production of VEGF, specifically, VEGF-C, VEGF-D, and to a lesser extent, VEGF-A (7). These CD11b+ macrophages accumulate around lymphatic vessels in the draining LN following inflammation in the skin, resulting in LEC proliferation (7). Importantly, following clodronate macrophage depletion, lymphangiogenesis was markedly decreased in the draining LN, demonstrating that macrophages participate in inflammation-induced LEC proliferation (7) (**Figure 1B**). These data are consistent with macrophages inducing lymphangiogenesis in non-lymphoid organs such as the cornea, peritoneum, and skin (27–29). Neutrophils have been shown to participate in LEC division in the skin (30), both through the production of VEGF-D and by increasing the bioavailability of VEGF-A via MMP-9 and heparanase. However, whether neutrophils or other innate cells contribute to LN LEC expansion is still unclear (30).

Although the mechanisms by which innate immune cells influence LN LEC expansion during an inflammatory response have been fairly well-studied, less is known about the transcription and signaling that occur within the LEC. The primary signals that LECs receive to induce division include VEGF receptor (VEGFR) engagement as described above. However, other factors are involved, including IL-7 which is important for LEC remodeling (11) (**Figure 1C**). Perhaps not surprisingly, the transcriptional program that ensues in LECs sorted from LNs at 12 h after lipopolysaccharide stimulation suggests that the LECs recruit immune cells through chemokine expression (CXCL9 and CCL5) (10). LECs also increase IFN inducible gene expression (Mpeg1, Lcn2, Irf7, IFI44, and Ly6a among others) at this time point (10). How LN LECs are transcriptionally regulated during inflammation to directly control LEC division could be through the immediate downregulation of genes that regulate cell division, Ccna2 and Klhl9 (10). This downregulation of genes involved in division could be due to the response to IFNγ induced by lipopolysaccharide and may be part of the mechanism behind how IFNα or IFNγ inhibit LEC division (5, 14) (**Figures 1A,C**). Furthermore, following sorting of LEC populations 6 days after polyI:C injection, non-dividing [(programed death ligand 1(PD-L1^{hi})] LECs express more CXCL4 (an angiostatin), while dividing (PD-L1^{lo}) LECs express more growth and differentiation factor 10 (GDF10) and integrin beta 1 (ITGB1), both of which are important for angiogenesis (5, 31–36). Intriguingly, VEGFR3 expression by LECs in the LN was unchanged. However, as LECs generally express high levels of VEGFR3 (10, 37), it seems likely that upregulation of VEGFR3 may not be required to induce LEC division, but instead that LECs require the upregulation of VEGF as described in detail above.

PD-L1 has also been shown to be involved in determining which LECs divide. In both mice that are PD-L1 deficient and mice in which the non-hematopoietic cells lack PD-L1, LEC division was significantly increased at 6 days after polyI:C injection (5). The mechanism behind how PD-L1 could regulate division was at least partially attributed to lost CXCL4 expression in *Pdl1*$^{-/-}$ LECs (5) (**Figure 1C**). Although CXCL4 was identified as a potential downstream target of PD-L1, much work needs to be done to determine the signaling pathway of PD-L1, and how it regulates division. These new findings indicate that PD-L1 may have a primary function in coordinating LN LEC expansion and survival during inflammation. Therefore, LECs recruit immune cells, receive signals from DCs and macrophages to divide (**Figure 1B**), change their transcriptional profile and divide based on expression of PD-L1 (**Figure 1C**) during the early phase (0–48 h) of an inflammatory response.

REGULATION OF LEC EXPANSION BY THE ADAPTIVE IMMUNE SYSTEM DURING INFLAMMATION

While DCs and macrophages contribute to LEC division at early timepoints during an immune response (**Figure 1B**), B cells have been shown to influence LEC division at the peak of the immune

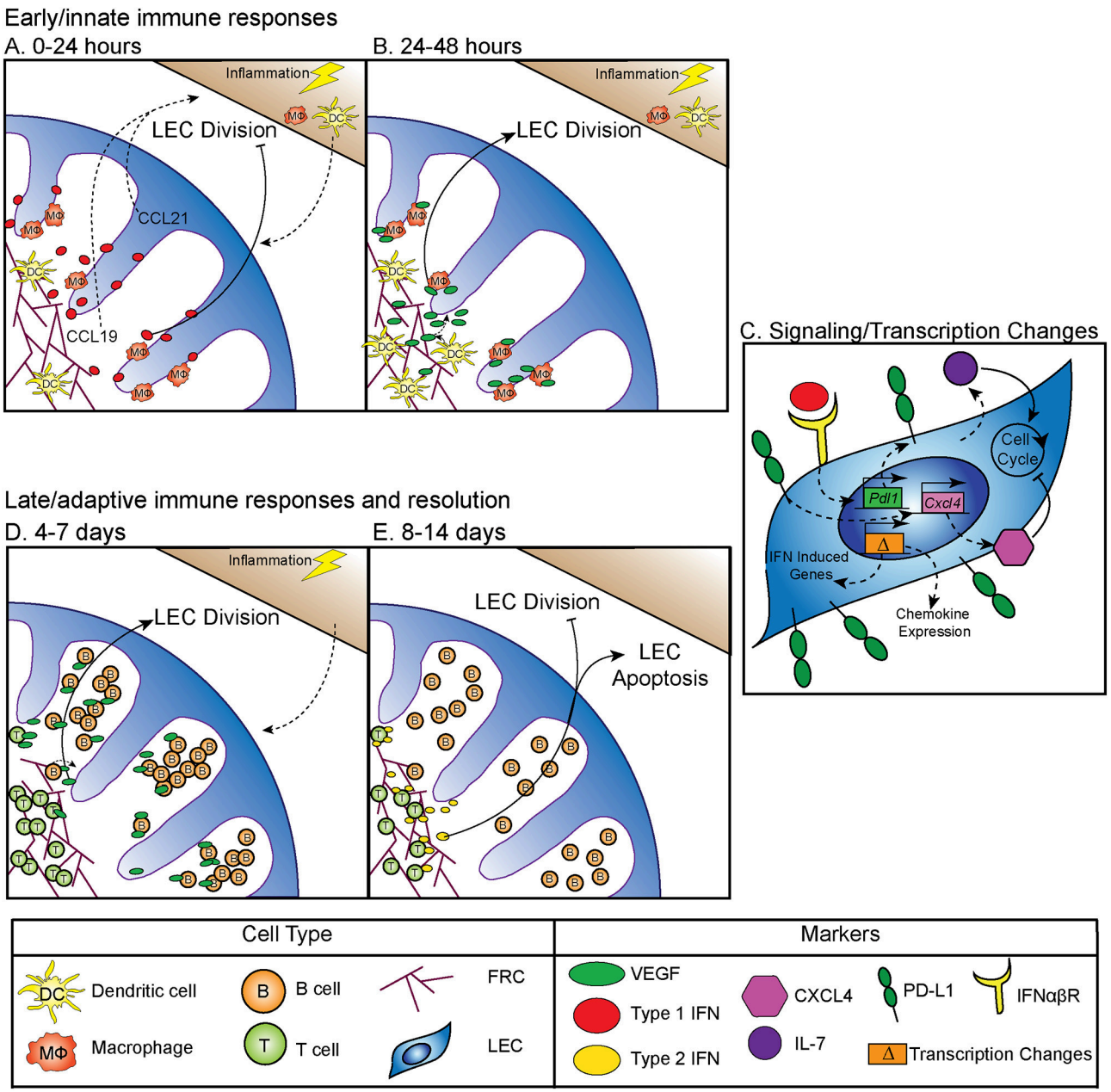

FIGURE 1 | Regulation of LN LEC expansion and contraction during an immune response. **(A)** Control of initiation of LEC expansion by innate immunity: 0–24 h. At early timepoints following an immune stimulus, type 1 IFN production inhibits LEC expansion. DCs traffic to the LN in response to CCL19 and CCL21. **(B)** Control of initiation of LEC expansion by innate immunity: 24–48 h. DCs induce the production of VEGF from FRCs to initiate LEC division. CD11b+ macrophages accumulate along lymphatic vessels in the LN, producing VEGF (5–9). **(C)** Intrinsic mechanisms controlling LEC expansion. LECs produce IL-7, which increases LEC division. PD-L1 expression on LECs inhibits LEC expansion, likely through increasing expression of CXCL4 which is a negative regulator of cell division. PD-L1 expression is controlled by type 1 IFN, resulting in PD-L1 upregulation at early timepoints following an immune stimulus, as well as the expression of other IFN inducible genes. LECs induce expression of chemokines, including CXCL9 and CCL5 following an immune stimulus (5, 10, 11). **(D)** Control of LEC expansion by adaptive immune cells: 4–7 days. At later timepoints during an immune response B cells interact with FRCs, and then produce VEGF to increase LEC expansion. T cells contribute to LEC expansion. **(E)** Control of LEC contraction by adaptive immune cells: 8–14 days. T cells produce type 2 IFN to inhibit LEC expansion and induce LEC apoptosis (8, 12–16).

response (**Figure 1D**). Following immunization with complete Freund's adjuvant, B cell recruitment to the lymph node was required for LEC expansion. In a mouse model where B cells lack L-selectin, an adhesion molecule necessary for lymphocyte migration across high endothelial venules in the LNs, LEC expansion was impaired due to the loss of VEGF-A production in the follicle (12). Intriguingly, utilizing *in vitro* modeling, this group also showed that activated B cells likely produce VEGF-A

in the LN only during inflammation (12). Indeed, another study found that inducing the expression of VEGF-A by B cells led to an increase in LN lymphangiogenesis, as well as enlargement of the LN (13). Recently, Dubey et al. showed B cells interact with lymphotoxin-beta receptor (LTβR) on FRCs which results in the production of B cell activating factor (BAFF). In combination with IL-4, production of BAFF causes B cells to produce VEGF-A and C (16). Together, these data suggest B cell production of VEGF-A or C can influence LN LEC expansion, but may not be required (15) (**Figure 1D**).

Others have shown that in addition to B cells, T cells are also involved in LN and LEC division. First, the lack of both B and T cells led to an almost complete loss of vascular-stromal expansion at later timepoints following complete Freund's adjuvant (8). When only T cells were absent, LEC proliferation was impaired, but surprisingly the absence of T cells did not affect total LEC numbers after complete Freund's adjuvant (8). Other work has also shown a role for T cells in regulating LEC expansion. In a mouse lacking endogenous T or B cells, T cell receptor transgenic T cell transfer did not lead to LEC expansion after immunization, unless the transferred T cells were activated with their cognate antigen (15). Thus, a functional T cell response, in the absence of B cells, is enough to induce LEC expansion following immunization. These data highlight the importance of the adaptive immune response in regulating LEC expansion during late time points (4–7 days) after an inflammatory stimulus (**Figure 1D**).

LEC APOPTOSIS AND LN CONTRACTION DURING RESOLUTION OF THE IMMUNE RESPONSE

While LEC expansion is important for coordinating the immune response, LEC contraction must also occur during the resolution of the immune response. Very little has been done to understand how this process occurs, however, in an athymic mouse, LN lymphatic vessel density is dramatically increased (14). This hypertrophy of lymphatic vessels is reduced by IFNγ production by T cells (14). Furthermore, when IFNγ was absent, lymphatic vessel regression did not occur as it normally does during LN contraction (14). This suggests that the production of IFNγ by T cells may be important for inhibiting lymphatic growth and/or promoting LEC apoptosis (**Figure 1E**). Interestingly, recent data looking at stromal cells, including LECs, 15 days after lymphocytic choriomeningitis virus, showed increased expression of the chemokines CXCL9 and CXCL10, as well as the activation marker Nur77 (38). While lymphocytic choriomeningitis virus was cleared by this time, LECs remain activated. This could be a process in which LECs recruit IFNγ producing cells until the regression of the lymphatic vasculature and LN size returns to normal.

While not directly regulating LEC contraction, PD-L1 does appear to specifically control LEC survival. These findings predict that PD-L1 may determine which LECs undergo apoptosis during LN contraction (5) (**Figure 2A**). This is consistent with other data showing that PD-L1 can act as a negative regulator of apoptosis in other endothelial cells (43), a process which may be hijacked by cancer cells (44–46). As such, loss of the cytoplasmic domain of PD-L1 in cancer cells resulted in increased apoptosis, from either T cell mediated killing, administration of a chemotherapeutic agent, or interferon beta cytotoxicity (44–46). Although the cytoplasmic domain of PD-L1 is relatively short, it appears that there are at least two signaling domains that help regulate inhibition to apoptosis in response to type 1 interferon, and mutation of these domains can sensitize cancer cells to interferon alpha/beta cytotoxicity (46). While these studies were done in the context of cancer cells which hijack normal cellular functions, recent data suggests that the expression of PD-L1 by LECs, and the regulation of cellular division and survival, may be a normal physiologic role for PD-L1. Further work is needed to determine the precise signaling pathways by which PD-L1 regulates survival, and if this process differs between endothelial cells and cancer cells (**Figure 2A**).

LECS BALANCE OPPOSING ROLES DURING AN IMMUNE RESPONSE

While LEC expansion and contraction in the lymph node is important for the immune response, LECs also have a major role in programming the adaptive immune response. As stated above, some LECs in the lymph node express PD-L1 at high levels (47). The role of PD-L1 expression by other cells has been well-described as being inhibitory for T cell activation when programmed death-1 expressed on T cells binds to PD-L1 (48–51). LECs are involved in the maintenance of peripheral T cell tolerance, via expression and presentation of tissue specific antigens, such as tyrosinase (39, 40, 42, 47). Loss of PD-L1 expression by LECs that express tyrosinase results in autoimmune vitiligo (39, 40). In addition, LECs are capable of inducing CD4+ T cell tolerance, through major histocompatibility complex (MHC) class 2. Interestingly, while LECs do not express functional MHC class 2 (42), they are able to acquire loaded MHC class 2—self antigen peptide complexes from DCs (41). These LECs then present the self-antigen to CD4+ T cells which results in anergic self-antigen specific T cells (41). Loss of PD-L1 or loss of LEC acceptance of MHC class 2 complexes leads to autoimmunity (41, 42) (**Figure 2A**). Furthermore, in tissue lymphatics, PD-L1 also plays a major role in inducing tumor tolerance by LECs (52, 53).

LECs also have a role in the maintenance of protective memory through the archiving of foreign antigens following both immunization and viral infection (15, 26). LECs that acquire antigens during an inflammatory response do not present the foreign antigen directly. Instead, LECs archive antigens, and hand off the antigen to migratory DCs either directly or via LEC apoptosis (**Figure 2B**). The migratory DCs (26) then present the antigen to CD8+ T cells, improving the effector T cell response upon re-challenge (15). Importantly, antigen archiving is decreased without an inflammatory signal, and other groups have shown that LECs can present foreign antigens in a tolerizing manner when inflammation does not occur, in a process called foreign antigen scavenging

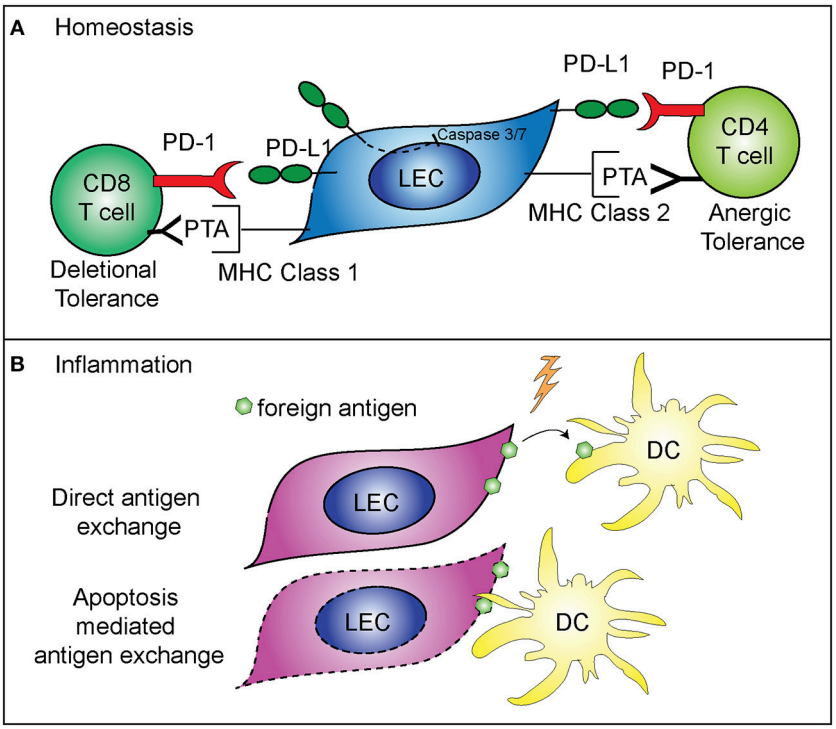

FIGURE 2 | Mechanisms of immune regulation by LECs. **(A)** PD-L1 on LECs inhibits LEC apoptosis and regulates peripheral immune tolerance. PD-L1 negatively regulates cleaved caspase 3/7 production, resulting in decreased apoptosis of LECs that express PD-L1 (5). LECs present peripheral tissue antigens to CD8 T cells on MHC class 1, leading to deletional tolerance via PD-L1 (39, 40). LECs express peripheral tissue antigens (PTA), which are either transferred to DCs for presentation to CD4 T cells or LECs acquire loaded MHC class 2 complexes from DCs, and present to CD4 T cells leading to anergic tolerance via PD-L1 (41, 42). **(B)** LECs archive foreign antigen during inflammation and transfer the antigens to DCs for presentation to memory T cells (15). Two mechanisms are involved in LEC-DC antigen exchange: direct antigen exchange between LECs and DCs, and DC acquisition of archived antigens via LEC apoptosis that occurs during LN contraction (26).

(54). In this case, LECs directly present the antigen to CD8+ T cells and the CD8+ T cells are deleted (54). These recent findings illustrate the flexibility of LECs in programming immune responses in the LN and highlight differences between inflammatory and non-inflammatory responses.

What mechanisms regulate the different functions of LECs during the transition from homeostasis to an immune response are not fully understood. An inflammatory signal is needed to prevent LECs from presenting foreign antigen in a tolerizing manner, and a role for LEC expansion has been described in antigen archiving. Following immunization, LECs will acquire, but will not archive antigen unless the LECs are expanding (15). These data indicate that there may be a role for LEC expansion in regulating the function of the lymphatic network during an acute, inflammatory response. What controls LEC expansion during a memory immune response is virtually unknown. However, it seems likely that the immediate production of cytokines, such as IFNγ by memory T cells, would inhibit LEC expansion, similar to the role of effector T cell production of IFNγ inducing LN contraction (14). Interestingly, PD-L1 expression is controlled by both type 1 and type 2 IFN (5), therefore it is possible that, in the absence of division, the upregulation of PD-L1 may be a mechanism to prevent improper activation of auto-reactive T cells and B cells while the LN is preparing to respond to the inflammatory insult (**Figure 2**).

SUMMARY

Further research is needed to fully explore novel regulators of LEC expansion and contraction, including PD-L1 and CXCL4. How LECs function to both maintain peripheral tolerance and promote protective immunity is also not well-understood. Understanding these processes, and how LECs can determine the fate of the immune response, are likely very important in the prevention of autoimmunity as well as the development of a strong memory response. Therefore, future studies of LN LECs during an active immune response may lead to novel therapeutic targets in a wide range of diseases.

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REFERENCES

1. Bromley SK, Thomas SY, Luster AD. Chemokine receptor CCR7 guides T cell exit from peripheral tissues and entry into afferent lymphatics. *Nat Immunol.* (2005) 6:895–901. doi: 10.1038/ni1240
2. Debes GF, Arnold CN, Young AJ, Krautwald S, Lipp M, Hay JB, et al. Chemokine receptor CCR7 required for T lymphocyte exit from peripheral tissues. *Nat Immunol.* (2005) 6:889–94. doi: 10.1038/ni1238
3. Tal O, Lim HY, Gurevich I, Milo I, Shipony Z, Ng LG, et al. DC mobilization from the skin requires docking to immobilized CCL21 on lymphatic endothelium and intralymphatic crawling. *J Exp Med.* (2011) 208:2141–53. doi: 10.1084/jem.20102392
4. Weber M, Hauschild R, Schwarz J, Moussion C, De Vries I, Legler DF, et al. Interstitial dendritic cell guidance by haptotactic chemokine gradients. *Science* (2013) 339:328–32. doi: 10.1126/science.1228456
5. Lucas ED, Finlon JM, Burchill MA, Mccarthy MK, Morrison TE, Colpitts TM, et al. Type 1 IFN and PD-L1 coordinate lymphatic endothelial cell expansion and contraction during an inflammatory immune response. *J Immunol.* (2018) 201:1735–47. doi: 10.4049/jimmunol.1800271
6. Webster B, Ekland EH, Agle LM, Chyou S, Ruggieri R, Lu TT. Regulation of lymph node vascular growth by dendritic cells. *J Exp Med.* (2006) 203:1903–13. doi: 10.1084/jem.20052272
7. Kataru RP, Jung K, Jang C, Yang H, Schwendener RA, Baik JE, et al. Critical role of CD11b+ macrophages and VEGF in inflammatory lymphangiogenesis, antigen clearance, and inflammation resolution. *Blood* (2009) 113:5650–9. doi: 10.1182/blood-2008-09-176776
8. Chyou S, Benahmed F, Chen J, Kumar V, Tian S, Lipp M, et al. Coordinated regulation of lymph node vascular-stromal growth first by CD11c+ cells and then by T and B cells. *J Immunol.* (2011) 187:5558–67. doi: 10.4049/jimmunol.1101724
9. Tan KW, Yeo KP, Wong FH, Lim HY, Khoo KL, Abastado JP, et al. Expansion of cortical and medullary sinuses restrains lymph node hypertrophy during prolonged inflammation. *J Immunol.* (2012) 188:4065–80. doi: 10.4049/jimmunol.1101854
10. Malhotra D, Fletcher AL, Astarita J, Lukacs-Kornek V, Tayalia P, Gonzalez SF, et al. Transcriptional profiling of stroma from inflamed and resting lymph nodes defines immunological hallmarks. *Nat Immunol.* (2012) 13:499–510. doi: 10.1038/ni.2262
11. Onder L, Narang P, Scandella E, Chai Q, Iolyeva M, Hoorweg K, et al. IL-7-producing stromal cells are critical for lymph node remodeling. *Blood* (2012) 120:4675–83. doi: 10.1182/blood-2012-03-416859
12. Angeli V, Ginhoux F, Llodra J, Quemeneur L, Frenette PS, Skobe M, et al. B cell-driven lymphangiogenesis in inflamed lymph nodes enhances dendritic cell mobilization. *Immunity* (2006) 24:203–15. doi: 10.1016/j.immuni.2006.01.003
13. Shrestha B, Hashiguchi T, Ito T, Miura N, Takenouchi K, Oyama Y, et al. B cell-derived vascular endothelial growth factor A promotes lymphangiogenesis and high endothelial venule expansion in lymph nodes. *J Immunol.* (2010) 184:4819–26. doi: 10.4049/jimmunol.0903063
14. Kataru RP, Kim H, Jang C, Choi DK, Koh BI, Kim M, et al. T lymphocytes negatively regulate lymph node lymphatic vessel formation. *Immunity* (2011) 34:96–107. doi: 10.1016/j.immuni.2010.12.016
15. Tamburini BA, Burchill MA, Kedl RM. Antigen capture and archiving by lymphatic endothelial cells following vaccination or viral infection. *Nat Commun.* (2014) 5:3989. doi: 10.1038/ncomms4989
16. Dubey LK, Karempudi P, Luther SA, Ludewig B, Harris NL. Interactions between fibroblastic reticular cells and B cells promote mesenteric lymph node lymphangiogenesis. *Nat Commun.* (2017) 8:367. doi: 10.1038/s41467-017-00504-9
17. Pietila TE, Veckman V, Lehtonen A, Lin R, Hiscott J, Julkunen I. Multiple NF-B and IFN regulatory factor family transcription factors regulate CCL19 gene expression in human monocyte-derived dendritic cells. *J Immunol.* (2006) 178:253–61. doi: 10.4049/jimmunol.178.1.253
18. Johnson LA, Jackson DG. Inflammation-induced secretion of CCL21 in lymphatic endothelium is a key regulator of integrin-mediated dendritic cell transmigration. *Int Immunol.* (2010) 22:839–49. doi: 10.1093/intimm/dxq435
19. Gunn MD, Tangemann K, Tam C, Cyster JG, Rosen SD, Williams LT. A chemokine expressed in lymphoid high endothelial venules promotes the adhesion and chemotaxis of naive T lymphocytes. *Proc Natl Acad Sci USA.* (1998) 95:258–63. doi: 10.1073/pnas.95.1.258
20. Forster R, Schubel A, Breitfeld D, Kremmer E, Renner-Muller I, Wolf E, et al. CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs. *Cell* (1999) 99:23–33. doi: 10.1016/S0092-8674(00)80059-8
21. Ohl L, Mohaupt M, Czeloth N, Hintzen G, Kiafard Z, Zwirner J, et al. CCR7 governs skin dendritic cell migration under inflammatory and steady-state conditions. *Immunity* (2004) 21:279–88. doi: 10.1016/j.immuni.2004.06.014
22. Acton SE, Astarita JL, Malhotra D, Lukacs-Kornek V, Franz B, Hess PR, et al. Podoplanin-rich stromal networks induce dendritic cell motility via activation of the C-type lectin receptor CLEC-2. *Immunity* (2012) 37:276–89. doi: 10.1016/j.immuni.2012.05.022
23. Acton SE, Farrugia AJ, Astarita JL, Mourao-Sa D, Jenkins RP, Nye E, et al. Dendritic cells control fibroblastic reticular network tension and lymph node expansion. *Nature* (2014) 514:498–502. doi: 10.1038/nature13814
24. Astarita JL, Cremasco V, Fu J, Darnell MC, Peck JR, Nieves-Bonilla JM, et al. The CLEC-2-podoplanin axis controls the contractility of fibroblastic reticular cells and lymph node microarchitecture. *Nat Immunol.* (2014) 16:75–84. doi: 10.1038/ni.3035
25. Maruyama Y, Maruyama K, Kato Y, Kajiya K, Moritoh S, Yamamoto K, et al. The effect of podoplanin inhibition on lymphangiogenesis under pathological conditions. *Invest Opthalmol Visual Sci.* (2014) 55:4813–22. doi: 10.1167/iovs.13-13711
26. Kedl RM, Lindsay RS, Finlon JM, Lucas ED, Friedman RS, Tamburini BJ. Migratory dendritic cells acquire and present lymphatic endothelial cell-archived antigens during lymph node contraction. *Nat Commun.* (2017) 8:2034. doi: 10.1038/s41467-017-02247-z
27. Maruyama K, Ii M, Cursiefen C, Jackson DG, Keino H, Tomita M, et al. Inflammation-induced lymphangiogenesis in the cornea arises from CD11b-positive macrophages. *J Clin Invest.* (2005) 115:2363–72. doi: 10.1172/JCI23874
28. Kim KE, Koh Y-J, Jeon B-H, Jang C, Han J, Kataru RP, et al. Role of CD11b + macrophages in intraperitoneal lipopolysaccharide-induced aberrant lymphangiogenesis and lymphatic function in the diaphragm. *Am J Pathol.* (2009) 175:1733–45. doi: 10.2353/ajpath.2009.090133
29. Shi VY, Bao L, Chan LS. Inflammation-driven dermal lymphangiogenesis in atopic dermatitis is associated with CD11b+ macrophage recruitment and VEGF-C up-regulation in the IL-4-transgenic mouse model. *Microcirculation* (2012) 19:567–79. doi: 10.1111/j.1549-8719.2012.00189.x
30. Tan KW, Chong SZ, Wong FH, Evrard M, Tan SM, Keeble J, et al. Neutrophils contribute to inflammatory lymphangiogenesis by increasing VEGF-A bioavailability and secreting VEGF-D. *Blood* (2013) 122:3666–77. doi: 10.1182/blood-2012-11-466532
31. Perollet C, Han ZC, Savona C, Caen JP, Bikfalvi A. Platelet factor 4 modulates fibroblast growth factor 2 (FGF-2) activity and inhibits FGF-2 dimerization. *Blood* (1998) 91:3289–99.
32. Bikfalvi A. Platelet factor 4: an inhibitor of angiogenesis. *Semin Thromb Hemost.* (2004) 30:379–85. doi: 10.1055/s-2004-831051

33. Bikfalvi A. Recent developments in the inhibition of angiogenesis: examples from studies on platelet factor-4 and the VEGF/VEGFR system. *Biochem Pharmacol.* (2004) 68:1017–21. doi: 10.1016/j.bcp.2004.05.030
34. Tanjore H, Zeisberg EM, Gerami-Naini B, Kalluri R. Beta1 integrin expression on endothelial cells is required for angiogenesis but not for vasculogenesis. *Dev Dyn.* (2008) 237:75–82. doi: 10.1002/dvdy.21385
35. Malan D, Wenzel D, Schmidt A, Geisen C, Raible A, Bolck B, et al. Endothelial beta1 integrins regulate sprouting and network formation during vascular development. *Development* (2010) 137:993–1002. doi: 10.1242/dev.045377
36. Li S, Nie EH, Yin Y, Benowitz LI, Tung S, Vinters HV, et al. GDF10 is a signal for axonal sprouting and functional recovery after stroke. *Nat Neurosci.* (2015) 18:1737–45. doi: 10.1038/nn.4146
37. Saharinen P, Tammela T, Karkkainen MJ, Alitalo K. Lymphatic vasculature: development, molecular regulation and role in tumor metastasis and inflammation. *Trends Immunol.* (2004) 25:387–95. doi: 10.1016/j.it.2004.05.003
38. Rodda LB, Lu E, Bennett ML, Sokol CL, Wang X, Luther SA, et al. Single-cell RNA sequencing of lymph node stromal cells reveals niche-associated heterogeneity. *Immunity*(2018) 48:1014–1028 e1016. doi: 10.1016/j.immuni.2018.04.006
39. Cohen JN, Guidi CJ, Tewalt EF, Qiao H, Rouhani SJ, Ruddell A, et al. Lymph node-resident lymphatic endothelial cells mediate peripheral tolerance via Aire-independent direct antigen presentation. *J Exp Med.* (2010) 207:681–8. doi: 10.1084/jem.20092465
40. Tewalt EF, Cohen JN, Rouhani SJ, Guidi CJ, Qiao H, Fahl SP, et al. Lymphatic endothelial cells induce tolerance via PD-L1 and lack of costimulation leading to high-level PD-1 expression on CD8 T cells. *Blood* (2012) 120:4772–82. doi: 10.1182/blood-2012-04-427013
41. Dubrot J, Duraes FV, Potin L, Capotosti F, Brighouse D, Suter T, et al. Lymph node stromal cells acquire peptide-MHCII complexes from dendritic cells and induce antigen-specific $CD4^+$ T cell tolerance. *J Exp Med.* (2014) 211:1153–66. doi: 10.1084/jem.20132000
42. Rouhani SJ, Eccles JD, Riccardi P, Peske JD, Tewalt EF, Cohen JN, et al. Roles of lymphatic endothelial cells expressing peripheral tissue antigens in CD4 T-cell tolerance induction. *Nat Commun.* (2015) 6:6771. doi: 10.1038/ncomms7771
43. Jin Y, Chauhan SK, El Annan J, Sage PT, Sharpe AH, Dana R. A novel function for programmed death ligand-1 regulation of angiogenesis. *Am J Pathol.* (2011) 178:1922–9. doi: 10.1016/j.ajpath.2010.12.027
44. Azuma T, Yao S, Zhu G, Flies AS, Flies SJ, Chen L. B7-H1 is a ubiquitous antiapoptotic receptor on cancer cells. *Blood* (2008) 111:3635–43. doi: 10.1182/blood-2007-11-123141
45. Ghebeh H, Lehe C, Barhoush E, Al-Romaih K, Tulbah A, Al-Alwan M, et al. Doxorubicin downregulatescell surface B7-H1 expression and upregulates its nuclear expression in breast cancer cells: role of B7-H1 as an anti-apoptotic molecule. *Breast Cancer Res.* (2010) 12:R48. doi: 10.1186/bcr2605
46. Gato-Canas M, Zuazo M, Arasanz H, Ibanez-Vea M, Lorenzo L, Fernandez-Hinojal G, et al. PDL1 Signals through conserved sequence motifs to overcome interferon-mediated cytotoxicity. *Cell Rep.* (2017) 20:1818–29. doi: 10.1016/j.celrep.2017.07.075
47. Cohen JN, Tewalt EF, Rouhani SJ, Buonomo EL, Bruce AN, Xu X, et al. Tolerogenic properties of lymphatic endothelial cells are controlled by the lymph node microenvironment. *PLoS ONE* (2014) 9:e87740. doi: 10.1371/journal.pone.0087740
48. Keir ME, Butte MJ, Freeman GJ, Sharpe AH. PD-1 and its ligands in tolerance and immunity. *Annu Rev Immunol.* (2008) 26:677–704. doi: 10.1146/annurev.immunol.26.021607.090331
49. Bardhan K, Anagnostou T, Boussiotis VA. The PD1:PD-L1/2 pathway from discovery to clinical implementation. *Front Immunol.* (2016) 7:550. doi: 10.3389/fimmu.2016.00550
50. Schutz F, Stefanovic S, Mayer L, Von Au A, Domschke C, Sohn C. PD-1/PD-L1 pathway in breast cancer. *Oncol Res Treat.* (2017) 40:294–7. doi: 10.1159/000464353
51. Kythreotou A, Siddique A, Mauri FA, Bower M, Pinato DJ. Pd-L1. *J Clin Pathol.* (2018) 71:189–94. doi: 10.1136/jclinpath-2017-204853
52. Dieterich LC, Ikenberg K, Cetintas T, Kapaklikaya K, Hutmacher C, Detmar M. Tumor-associated lymphatic vessels upregulate PDL1 to inhibit T-cell activation. *Front Immunol.* (2017) 8:66. doi: 10.3389/fimmu.2017.00066
53. Lane RS, Femel J, Breazeale AP, Loo CP, Thibault G, Kaempf A, et al. IFNγ-activated dermal lymphatic vessels inhibit cytotoxic T cells in melanoma and inflamed skin. *J Exp Med.* (2018) 215:3057–74. doi: 10.1084/jem.20180654
54. Hirosue S, Vokali E, Raghavan VR, Rincon-Restrepo M, Lund AW, Corthesy-Henrioud P, et al. Steady-state antigen scavenging, cross-presentation, and CD8+ T cell priming: a new role for lymphatic endothelial cells. *J Immunol.* (2014) 192:5002–11. doi: 10.4049/jimmunol.1302492

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REVIEW
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Modulation of Immunity by Lymphatic Dysfunction in Lymphedema

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The debilitating condition known as secondary lymphedema frequently occurs after lymphadenectomy and/or radiotherapy for the treatment of cancer. These therapies can damage lymphatic vessels leading to edema, fibrosis, inflammation and dysregulated adipogenesis, which result in profound swelling of an affected limb. Importantly, lymphedema patients often exhibit impaired immune function which predisposes them to a variety of infections. It is known that lymphadenectomy can compromise the acquisition of adaptive immune responses and antibody production; however the cellular mechanisms involved are poorly understood. Here we discuss recent progress in revealing the cellular and molecular mechanisms underlying poor immune function in secondary lymphedema, which has indicated a key role for regulatory T cells in immunosuppression in this disease. Furthermore, the interaction of $CD4^+$ T cells and macrophages has been shown to play a role in driving proliferation of lymphatic endothelial cells and aberrant lymphangiogenesis, which contribute to interstitial fluid accumulation in lymphedema. These new insights into the interplay between lymphatic vessels and the immune system in lymphedema will likely provide opportunities for novel therapeutic approaches designed to improve clinical outcomes in this problematic disease.

Keywords: immune function, inflammation, lymphedema, regulatory T cells, T-helper cells

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INTRODUCTION

The lymphatic system is a highly structured vascular network, important for interstitial fluid homeostasis, immune surveillance and lipid absorption, which consist of distinct types of lymphatic vessels. Interstitial fluid is absorbed by highly permeable initial lymphatics and transported by lymphatic pre-collectors to lymphatic collectors, which converge to form lymphatic trunks that ultimately transport lymph to the venous system via lymphatic ducts. Each type of lymphatic vessel is anatomically specialized for its function (1), but all lymphatic vessels share the feature of being lined by a single layer of lymphatic endothelial cells (LECs). Lymphatic vessels can undergo a variety of remodeling processes in development and disease, including lymphangiogenesis (the growth of new lymphatic vessels), which have important implications for lymphatic biology and immune function (2). Much progress has been made over recent years in defining the effects of lymphatic remodeling, lymphangiogenesis and LECs on immune function, particularly in the setting of cancer [for example see (3–5)]. More specifically, the establishment of tumor-associated immunity is thought to depend on lymphatic vessel remodeling and drainage. Further, there is emerging evidence that LECs are important for the maintenance of peripheral tolerance, modulating effector T cell responses and influencing leukocyte function (6). Here we review the role of lymphatic vessels in modulating immunity in secondary lymphedema, a prevalent condition caused by lymphatic dysfunction, which involves remodeling of lymphatic vessels and compromised immune function.

SECONDARY LYMPHEDEMA: CLINICAL ASPECTS AND PATHOPHYSIOLOGY

Secondary lymphedema is a chronic disease characterized by the accumulation of interstitial fluid in tissues due to damaged lymphatic vessels, leading to swelling, and dysfunction of limbs (7). It is an acquired condition that is etiologically distinct from primary, or hereditary, lymphedema, which is a rare disease caused by intrinsic abnormalities of lymphatic function due to defects in genes involved in the growth and development of the lymphatic vasculature (8). Secondary lymphedema is a slow but progressive condition which can be caused by trauma, infection and inflammation. Globally, the most common cause is lymphatic filariasis, due to lymphatic vascular invasion by filarial nematodes, which has been estimated to afflict 68 million people in 73 countries worldwide (9). However, disruption of the lymphatic vasculature due to surgical interventions (e.g., lymphadenectomy) and/or radiotherapy for breast cancer is the most common cause in the developed world (7), with an incidence of lymphedema of 21% among women who were diagnosed with breast cancer (10). The condition can occur not only in breast cancer patients, but in patients with any cancer types which require lymph node dissection or radiotherapy treatment, such as head and neck, genitourinary and gynecological cancers, and melanoma (11). The onset of secondary lymphedema can be highly variable and has been reported to occur immediately postoperatively or up to 30 years post-treatment in the context of breast cancer, and it is not clear what determines a patient's predisposition to develop the disease. Historically, secondary lymphedema has been considered underdiagnosed, and robust epidemiological data have been scant, however, it is clearly a relatively prevalent condition with between two and five million people estimated to suffer from it in the United States (12).

Secondary lymphedema can be highly debilitating both physically and psychologically for patients due to the reduced quality of life associated with limb discomfort, anxiety, depression, sexual dysfunction, and social isolation. Current treatment choices for lymphedema include massage, manual lymph drainage (13), remedial exercise (14), compression bandaging (15), electrophysical modalities (including low-level laser therapy and electrical stimulation) (16), elevation techniques, exercise programs, and dietary/weight loss interventions (17, 18). There are also a range of surgical options such as liposuction (19), various forms of vascular anastomosis (20), lymph node transplantation (21) and other regional tissue transfer procedures. Unfortunately these treatments have not proven to be reliably curative as they have limited efficacy in controlling the disease, and many do not address the cause. Notably, there are no molecular-based therapies for the condition although therapeutics targeting inflammation (ketoprofen) (22) or promoting lymphangiogenesis [Lymfactin and Ubenimex also known as Bestatin (23, 24)] are being tested for treatment of lymphedema in clinical trials programs-see ClinicalTrials.gov for further information about these trials (ClinicalTrials.gov identifiers: NCT02257970, NCT02700529, and NCT02994771). Given the absence of curative treatment options and prevalence of the condition, secondary lymphedema is considered an important unmet clinical need in medicine.

The pathological features of secondary lymphedema include edema, inflammation, dermal fibrosis and formation of fat tissue, and patients often exhibit impaired immune function predisposing them to a variety of infections (25). These features are thought to further restrict lymphatic function in lymphedematous tissue thereby establishing a vicious pathophysiological cycle (26). The types of infections observed in secondary lymphedema include cellulitis involving the deeper dermis and subcutaneous fat, erysipelas involving the superficial dermis and lymphangitis involving the superficial dermal lymphatics. Soft-tissue infections associated with secondary lymphedema can lead to sepsis and, on occasions, death (27). Therefore, patients can require lifelong prophylactic antibiotic therapy. Given the clinical management of lymphedema-associated infections can be highly problematic, it is important to understand how the immune response is impaired by the lymphatic injury which underlies secondary lymphedema. Such understanding could provide opportunities for prevention or improved treatment of secondary lymphedema.

IMMUNOLOGICAL VULNERABILITY OF SECONDARY LYMPHEDEMA

Extensive clinical literature and experience has made it clear that lymphedematous tissue is immunologically vulnerable. Not only infections, but also neoplasms and immune-related disorders, such as neutrophilic dermatosis and toxic epidermal necrolysis, occur more frequently than in normal tissue [for example see (28)]. Chronic secondary lymphedema is typically characterized by an altered abundance of immune cells. Clinical studies have demonstrated increased numbers of lymphocytes, plasma cells, macrophages, dendritic cells, and neutrophils in the affected skin and subdermal tissue of lymphedema patients (29, 30). Such immune cell populations can be important for development of lymphedema, for example a $CD4^+$ cell inflammatory response and T-helper 2 (Th2) cell differentiation can contribute to key pathological changes including fibrosis and lymphatic dysfunction (**Figure 1**) (31). The accumulation of macrophages and lymphocytes in lymphedematous tissue can be induced by lymphatic fluid stasis in animal models of lymphedema (32). Importantly, there is evidence from animal models that lymphatic vascular defects can be associated with inadequate humoral immunity (33) which is consistent with clinical studies in lymphedema patients which showed that vaccination in lymphedematous tissue was associated with significantly decreased antibody titres (34).

Lymphedematous tissue constitutes a highly abnormal environment from the perspective of immune function. It is to be expected that chronic lymph stasis in lymphedema would impair local immune surveillance by restricting the trafficking of immunocompetent cells in lymphedematous tissues. Further, the irregular accumulation of immune mediators (cytokines and chemokines) in lymphedematous tissue could be an initiating factor promoting activation of LECs and immune cells (35). It is

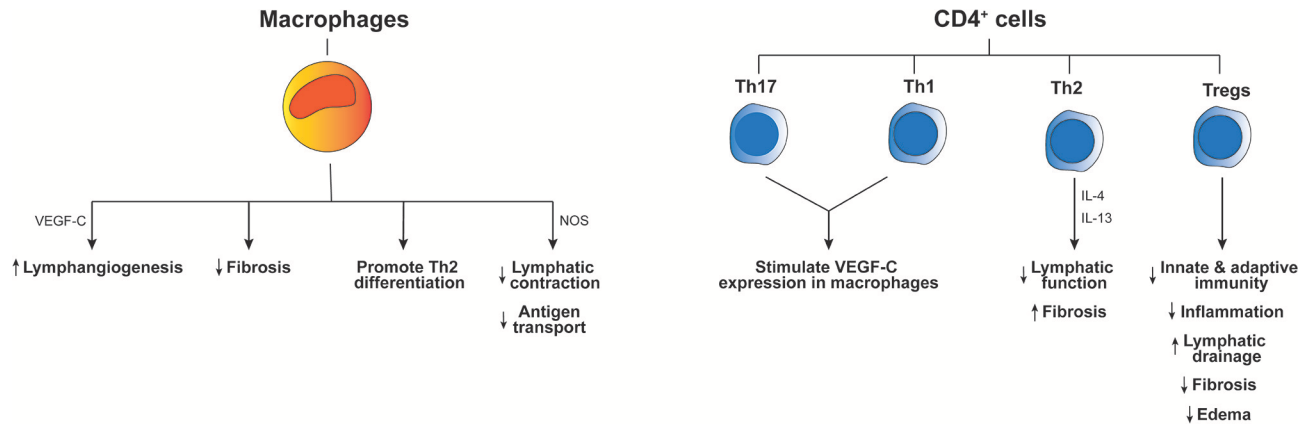

FIGURE 1 | Effects of immune cells on the pathogenesis of lymphedema. The diagram indicates how different immune cell types influence inflammation and other aspects of lymphedema pathology. Key molecular mediators produced by these cells types, which have been reported to drive the outcomes listed at the bottom of the figure, are indicated next to the long arrows. "Th" denotes T-helper cells, "Tregs" regulatory T cells, "IL" interleukin and "NOS" inducible nitric oxide synthase.

well-known that lymphatics can respond to immune mediators produced by macrophages, including TNF-α, IL-1β, and IFNγ (36), and are able to produce immune mediators which regulate macrophage function such as IL-6 and CCL21 (35, 37). It has also been shown that macrophages produce inducible nitric oxide synthase which causes a reduction of lymphatic vessel contraction (38) and thus may contribute to accumulation of tissue fluids and impairment of antigen transport to lymph nodes in lymphedema (**Figure 1**). In summary, it is likely that lymphatic activation perpetuates abnormal activation of macrophages, and visa-versa, which could contribute to immune dysfunction and abnormal inflammation in lymphedema. Nevertheless, the cellular mechanisms which cause immune deficits in lymphedema have begun to emerge only recently.

MECHANISMS OF IMMUNE DYSFUNCTION IN SECONDARY LYMPHEDEMA

A recent study exploring the function of T cells in lymphedema demonstrated a major increase in regulatory T cells (Tregs) in the lymphedematous extremity, compared to contralateral control tissue, in patients with breast cancer-related lymphedema (39). This finding was replicated in a mouse model of axillary lymph node dissection which showed increased infiltration of both $CD4^+$ T cells and Tregs. In this model, it was shown that Treg proliferation was localized to the tissue distal to the area of lymphatic injury caused by the surgery. Further analyses suggested that the Tregs downregulated local tissue inflammation post-lymphatic injury, and inhibited acquisition of T-cell-mediated immune responses (39). The loss of draining lymph nodes was also thought to diminish these responses. In addition, Tregs impaired bacterial phagocytosis, regulated humoral responses and compromised dendritic cell activation in this model after lymphatic injury (39). Overall, Tregs impaired both innate and adaptive immune responses, and depletion of these cells restored immune-mediated responses, indicating an important role for these cells in local immunosuppression in lymphedema (39) (**Figure 1**).

Immune function has been studied in transgenic mice expressing a soluble form of the lymphangiogenic receptor VEGFR-3 in skin (*K14-VEGFR-3-Ig* mice). These mice lack small dermal lymphatic vessels, develop lymphedema, and provide a model in which to monitor immune responses in the setting of lymphatic insufficiency (40). These mice produced lower antibody titres in response to dermal immunization, which was not due to compromised function of B cells, but was thought to be due to physiological differences in antigen transport to draining lymph nodes (33). T cell responses to dermal vaccination were delayed in these mice, although these responses were nevertheless robust. T-cell-mediated contact hypersensitivity (CHS) responses were strong, but the ability of these transgenic mice to induce CHS tolerance in the skin was impaired (33). The mice also exhibited hallmarks of autoimmunity, including antibody deposits in the skin, which supports the concept that lymphatic drainage to lymph nodes is important for maintaining immune tolerance against peripheral antigens. These findings provide mechanistic insight into how compromised lymphatic drainage in lymphedema plays a role in regulating humoral immunity and peripheral tolerance (33).

The effect of re-introducing lymph nodes, post-lymphatic damage, on immune responses and development of secondary lymphedema was monitored by Huang et al. in a mouse model of lymphatic ablation and popliteal lymph node dissection (41). Lymph node transplantation in this model led to a decreased accumulation of perilymphatic inflammatory cells, increased dendritic cell trafficking from the periphery to the inguinal node, and markedly improved adaptive immune responses. These changes were accompanied by decreases in hindlimb swelling and fibroadipose tissue deposition, as well as a pronounced lymphangiogenic response. The findings from this model may have clinical relevance for improving immune function post-lymphatic damage, given lymph node transfer is being used

in human patients and is being developed in animal models in combination with lymphangiogenic growth factor therapy (42–44).

EFFECTS OF IMMUNE CELLS ON LYMPHEDEMA PATHOPHYSIOLOGY

The involvement of $CD4^+$ T cells in lymphedema pathogenesis was studied by Ogata et al. who employed a mouse model of lymphedema based on ligating the major collecting lymphatic vessels in the skin of the abdomen and removing the associated axillary lymph node (30). This model exhibited excessive generation of immature lymphatic vessels that was essential for the early emergence of edema and the subsequent development of lymphedema pathology. $CD4^+$ T cells interacted with macrophages to promote lymphangiogenesis in this model, and both lymphangiogenesis and edema were reduced in macrophage-depleted or $CD4^+$ T-cell-deficient mice. From a mechanistic perspective, Th1 and Th17 cells activated macrophages to produce the lymphangiogenic growth factor VEGF-C, which likely drove the aberrant lymphangiogenesis. Inhibition of this mechanism suppressed both early lymphangiogenesis and development of lymphedema (30). Macrophages have also been reported to restrict fibrosis as depletion of these cells in a mouse model of secondary lymphedema significantly increased fibrosis, and impaired lymphatic transport, decreased VEGF-C expression and promoted Th2 differentiation (45). Th2 cells may also be involved in lymphedema pathogenesis as neutralization of two cytokines produced by these cells, IL-4 and IL-13, in a mouse model of secondary lymphedema promoted lymphatic function and restricted fibrosis (31). The role of $CD4^+$ T cells was also studied in a mouse model of secondary lymphedema by use of adoptive transfer techniques in CD4-deficient mice that underwent excision of skin and lymphatics in the tail or dissection of popliteal lymph nodes (46). This study revealed naïve $CD4^+$ T cells were activated in skin-draining lymph nodes and then migrated to lymphedematous skin. These activated cells promoted fibrosis and inflammation, and inhibited lymphangiogenesis and lymphatic function. Importantly, use of a sphingosine-1-phosphate receptor modulator to block release of T cells from lymph nodes prevented lymphedema in the mouse tail model employed in this study (46). It is now clear that $CD4^+$ T cells play a major role in the development of secondary lymphedema (**Figure 1**), at least in animal models, although the effect of these cells on lymphangiogenesis in lymphedema differed in different mouse models and therefore requires further clarification.

In a separate study, RNA sequencing of lymphedematous mouse skin suggested an upregulation of many T cell-related networks (47). More specifically, upregulation of Foxp3, a transcription factor specifically expressed by Tregs, indicated a potential role for these cells in lymphedema, consistent with findings discussed in the previous section. While global deletion of $CD4^+$ cells restricted lymphedema development in the mouse tail lymphedema model used in this study, targeted depletion of Tregs led to exacerbated edema associated with increased infiltration of immune cells and a mixed Th1/Th2 cytokine profile (47). Conversely, expansion of Tregs in the mouse model restricted lymphedema development. Therapeutic use of adoptively transferred Tregs upon lymphedema establishment reversed the major hallmarks of lymphedema such as edema, fibrosis and inflammation, and promoted lymphatic drainage (47). These findings on the role of Tregs are supported by the study of Garcia Nores et al. which showed that depletion of Tregs up-regulated local tissue inflammation after lymphatic injury (39). However, this study also showed that Tregs can locally impair adaptive immunity and clearance of bacteria after lymphatic injury. While it is clear that the number of Tregs in lymphedematous tissue is increased compared to normal tissue, the functional significance of these cells for development of lymphedema may differ depending on the relative importance of inflammation vs. adaptive immunity in the lymphedema model employed. This issue needs to be considered when assessing if Treg application could be a potential new therapeutic approach for treating lymphedema.

CONCLUDING REMARKS

Recent studies using animal models and clinical samples have established that immune function is significantly compromised in secondary lymphedema, and demonstrated that a variety of T-cell-related networks are up-regulated in this condition. Tregs, in particular, are increased in abundance in lymphedematous tissue and are thought to compromise immune function in this disease by promoting immunosuppression, although they can make a positive contribution by reducing the degree of inflammation. Further analysis of Treg function in secondary lymphedema is required to establish whether or not modulating the levels or function of these cells could be beneficial for prevention or treatment of this condition. This may need to be pursued in large animal models, as opposed to mice, to give a clearer picture of how targeting immune cells might be beneficial for lymphedema patients.

AUTHOR CONTRIBUTIONS

YY and MA conceived the topic and outlined the paper. YY, VA, SL, and MA wrote the paper.

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REFERENCES

1. Stacker SA, Williams SP, Karnezis T, Shayan R, Fox SB, Achen MG. Lymphangiogenesis and lymphatic vessel remodelling in cancer. *Nat Rev Cancer* (2014) 14:159–72. doi: 10.1038/nrc3677
2. Alitalo K. The lymphatic vasculature in disease. *Nature Med.* (2011) 17:1371–80. doi: 10.1038/nm.2545
3. Fankhauser M, Broggi MAS, Potin L, Bordry N, Jeanbart L, Lund AW, et al. Tumor lymphangiogenesis promotes T cell infiltration and potentiates immunotherapy in melanoma. *Sci Transl Med.* (2017) 9:eaal4712. doi: 10.1126/scitranslmed.aal4712
4. Hendry SA, Farnsworth RH, Solomon B, Achen MG, Stacker SA, Fox SB. The role of the tumor vasculature in the host immune response: implications for therapeutic strategies targeting the tumor microenvironment. *Front Immunol.* (2016) 7:621. doi: 10.3389/fimmu.2016.00621
5. Lund AW, Wagner M, Fankhauser M, Steinskog ES, Broggi MA, Spranger S, et al. Lymphatic vessels regulate immune microenvironments in human and murine melanoma. *J Clin Invest.* (2016) 126:3389–402. doi: 10.1172/JCI79434
6. Card CM, Yu SS, Swartz MA. Emerging roles of lymphatic endothelium in regulating adaptive immunity. *J Clin Invest.* (2014) 124:943–52. doi: 10.1172/JCI73316
7. Rockson SG. Lymphedema. *Am J Med.* (2001) 110:288–95. doi: 10.1016/S0002-9343(00)00727-0
8. Connell F, Brice G, Jeffery S, Keeley V, Mortimer P, Mansour S. A new classification system for primary lymphatic dysplasias based on phenotype. *Clin Genet.* (2010) 77:438–52. doi: 10.1111/j.1399-0004.2010.01394.x
9. Dickson BFR, Graves PM, McBride WJ. Lymphatic filariasis in mainland Southeast Asia: a systematic review and meta-analysis of prevalence and disease burden. *Trop Med Infect Dis.* (2017) 2:E32. doi: 10.3390/tropicalmed2030032
10. DiSipio T, Rye S, Newman B, Hayes S. Incidence of unilateral arm lymphoedema after breast cancer: a systematic review and meta-analysis. *Lancet Oncol.* (2013) 14:500–15. doi: 10.1016/S1470-2045(13)70076-7
11. Cormier JN, Askew RL, Mungovan KS, Xing Y, Ross MI, Armer JM. Lymphedema beyond breast cancer: a systematic review and meta-analysis of cancer-related secondary lymphedema. *Cancer* (2010) 116:5138–49. doi: 10.1002/cncr.25458
12. Rockson SG, Rivera KK. Estimating the population burden of lymphedema. *Ann N Y Acad Sci.* (2008) 1131:147–54. doi: 10.1196/annals.1413.014
13. Pinell XA, Kirkpatrick SH, Hawkins K, Mondry TE, Johnstone PA. Manipulative therapy of secondary lymphedema in the presence of locoregional tumors. *Cancer* (2008) 112:950–4. doi: 10.1002/cncr.23242
14. Singh B, Disipio T, Peake J, Hayes SC. Systematic review and meta-analysis of the effects of exercise for those with cancer-related lymphedema. *Arch Phys Med Rehabil.* (2016) 97:e313. doi: 10.1016/j.apmr.2015.09.012
15. Lawenda BD, Mondry TE, Johnstone PA. Lymphedema: a primer on the identification and management of a chronic condition in oncologic treatment. *CA Cancer J Clin.* (2009) 59:8–24. doi: 10.3322/caac.20001
16. Carati CJ, Anderson SN, Gannon BJ, Piller NB. Treatment of postmastectomy lymphedema with low-level laser therapy: a double blind, placebo-controlled trial. *Cancer* (2003) 98:1114–22. doi: 10.1002/cncr.11641
17. Murdaca G, Cagnati P, Gulli R, Spano F, Puppo F, Campisi C, et al. Current views on diagnostic approach and treatment of lymphedema. *Am J Med.* (2012) 125:134–40. doi: 10.1016/j.amjmed.2011.06.032
18. Shaw C, Mortimer P, Judd PA. A randomized controlled trial of weight reduction as a treatment for breast cancer-related lymphedema. *Cancer* (2007) 110:1868–74. doi: 10.1002/cncr.22994
19. Boyages J, Kastanias K, Koelmeyer LA, Winch CJ, Lam TC, Sherman KA, et al. Liposuction for advanced lymphedema: a multidisciplinary approach for complete reduction of arm and leg swelling. *Ann Surg Oncol.* (2015) 22 (Suppl 3):S1263–70. doi: 10.1245/s10434-015-4700-3
20. Chang DW. Lymphaticovenular bypass for lymphedema management in breast cancer patients: a prospective study. *Plast Reconstr Surg.* (2010) 126:752–8. doi: 10.1097/PRS.0b013e3181e5f6a9
21. Lin CH, Ali R, Chen SC, Wallace C, Chang YC, Chen HC, et al. Vascularized groin lymph node transfer using the wrist as a recipient site for management of postmastectomy upper extremity lymphedema. *Plast Reconstr Surg.* (2009) 123:1265–75. doi: 10.1097/PRS.0b013e31819e6529
22. Rockson SG, Tian W, Jiang X, Kuznetsova T, Haddad F, Zampell J, et al. Pilot studies demonstrate the potential benefits of antiinflammatory therapy in human lymphedema. *JCI Insight* (2018) 3:e123775. doi: 10.1172/jci.insight.123775
23. Scornik OA, Botbol V. Bestatin as an experimental tool in mammals. *Curr Drug Metab.* (2001) 2:67–85. doi: 10.2174/1389200013338748
24. Tian W, Rockson SG, Jiang X, Kim J, Begaye A, Shuffle EM, et al. Leukotriene B4 antagonism ameliorates experimental lymphedema. *Sci Transl Med.* (2017) 9:eaal3920. doi: 10.1126/scitranslmed.aal3920
25. Zampell JC, Aschen S, Weitman ES, Yan A, Elhadad S, De Brot M, et al. Regulation of adipogenesis by lymphatic fluid stasis: part I. Adipogenesis, fibrosis, and inflammation. *Plast Reconstr Surg.* (2012) 129:825–34. doi: 10.1097/PRS.0b013e3182450b2d
26. Cucchi F, Rossmeislova L, Simonsen L, Jensen MR, Bulow J. A vicious circle in chronic lymphoedema pathophysiology? An adipocentric view. *Obes Rev.* (2017) 18:1159–69. doi: 10.1111/obr.12565
27. Rockson SG. Addressing the unmet needs in lymphedema risk management. *Lymphat Res Biol.* (2006) 4:42–6. doi: 10.1089/lrb.2006.4.42
28. Wilkinson SM, Heagerty AH, Smith AG. Toxic epidermal necrolysis localized to an area of lymphoedema. *Clin Exp Dermatol.* (1992) 17:456–7. doi: 10.1111/j.1365-2230.1992.tb00260.x
29. Mortimer PS, Rockson SG. New developments in clinical aspects of lymphatic disease. *J Clin Invest.* (2014) 124:915–21. doi: 10.1172/JCI71608
30. Ogata F, Fujiu K, Matsumoto S, Nakayama Y, Shibata M, Oike Y, et al. Excess lymphangiogenesis cooperatively induced by macrophages and CD4(+) T cells drives the pathogenesis of lymphedema. *J Invest Dermatol.* (2016) 136:706–14. doi: 10.1016/j.jid.2015.12.001
31. Avraham T, Zampell JC, Yan A, Elhadad S, Weitman ES, Rockson SG, et al. Th2 differentiation is necessary for soft tissue fibrosis and lymphatic dysfunction resulting from lymphedema. *FASEB J.* (2013) 27:1114–26. doi: 10.1096/fj.12-222695
32. Zampell JC, Yan A, Elhadad S, Avraham T, Weitman E, Mehrara BJ. CD4(+) cells regulate fibrosis and lymphangiogenesis in response to lymphatic fluid stasis. *PLoS ONE* (2012) 7:e49940. doi: 10.1371/journal.pone.0049940
33. Thomas SN, Rutkowski JM, Pasquier M, Kuan EL, Alitalo K, Randolph GJ, et al. Impaired humoral immunity and tolerance in K14-VEGFR-3-Ig mice that lack dermal lymphatic drainage. *J Immunol.* (2012) 189:2181–90. doi: 10.4049/jimmunol.1103545
34. Sugaya M, Kuwano Y, Suga H, Miyagaki T, Ohmatsu H, Kadono T, et al. Lymphatic dysfunction impairs antigen-specific immunization, but augments tissue swelling following contact with allergens. *J Invest Dermatol.* (2012) 132:667–76. doi: 10.1038/jid.2011.349
35. Hancock DG, Potezny TM, White PM. Immune regulation by the peripheral lymphatics and its implications for wound healing and infection control in lymphoedema. *Wound Practice Res.* (2016) 24:76–83.
36. Chaitanya GV, Franks SE, Cromer W, Wells SR, Bienkowska M, Jennings MH, et al. Differential cytokine responses in human and mouse lymphatic endothelial cells to cytokines in vitro. *Lymphat Res Biol.* (2010) 8:155–64. doi: 10.1089/lrb.2010.0004
37. Sawa Y, Ueki T, Hata M, Iwasawa K, Tsuruga E, Kojima H, et al. LPS-induced IL-6, IL-8, VCAM-1, and ICAM-1 expression in human lymphatic endothelium. *J Histochem Cytochem.* (2008) 56:97–109. doi: 10.1369/jhc.7A7299.2007
38. Liao S, Cheng G, Conner DA, Huang Y, Kucherlapati RS, Munn LL, et al. Impaired lymphatic contraction associated with immunosuppression. *Proc Natl Acad Sci USA.* (2011) 108:18784–9. doi: 10.1073/pnas.1116152108
39. Garcia Nores GD, Ly CL, Savetsky IL, Kataru RP, Ghanta S, Hespe GE, et al. Regulatory T cells mediate local immunosuppression in lymphedema. *J Invest Dermatol.* (2018) 138:325–35. doi: 10.1016/j.jid.2017.09.011
40. Makinen T, Jussila L, Veikkola T, Karpanen T, Kettunen MI, Pulkkanen KJ, et al. Inhibition of lymphangiogenesis with resulting lymphedema in transgenic mice expressing soluble VEGF receptor-3. *Nature Med.* (2001) 7:199–205. doi: 10.1038/84651
41. Huang JJ, Gardenier JC, Hespe GE, Garcia Nores GD, Kataru RP, Ly CL, et al. Lymph node transplantation decreases swelling and restores immune responses in a transgenic model of lymphedema. *PLoS ONE* (2016) 11:e0168259. doi: 10.1371/journal.pone.0168259

42. Tervala TV, Hartiala P, Tammela T, Visuri MT, Yla-Herttuala S, Alitalo K, et al. Growth factor therapy and lymph node graft for lymphedema. *J Surg Res.* (2015) 196:200–7. doi: 10.1016/j.jss.2015.02.031
43. Viitanen TP, Visuri MT, Hartiala P, Maki MT, Seppanen MP, Suominen EA, et al. Lymphatic vessel function and lymphatic growth factor secretion after microvascular lymph node transfer in lymphedema patients. *Plast Reconstr Surg Glob Open* (2013) 1:1–9. doi: 10.1097/GOX.0b013e318293a532
44. Visuri MT, Honkonen KM, Hartiala P, Tervala TV, Halonen PJ, Junkkari H, et al. VEGF-C and VEGF-C156S in the pro-lymphangiogenic growth factor therapy of lymphedema: a large animal study. *Angiogenesis* (2015) 18:313–26. doi: 10.1007/s10456-015-9469-2
45. Ghanta S, Cuzzone DA, Torrisi JS, Albano NJ, Joseph WJ, Savetsky IL, et al. Regulation of inflammation and fibrosis by macrophages in lymphedema. *Am J Physiol Heart Circ Physiol.* (2015) 308:H1065–77. doi: 10.1152/ajpheart.00598.2014
46. Garcia Nores GD, Ly CL, Cuzzone DA, Kataru RP, Hespe GE, Torrisi JS, et al. CD4(+) T cells are activated in regional lymph nodes and migrate to skin to initiate lymphedema. *Nat Commun.* (2018) 9:1970. doi: 10.1038/s41467-018-04418-y
47. Gousopoulos E, Proulx ST, Bachmann SB, Scholl J, Dionyssiou D, Demiri E, et al. Regulatory T cell transfer ameliorates lymphedema and promotes lymphatic vessel function. *JCI Insight* (2016) 1:e89081. doi: 10.1172/jci.insight.89081

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REVIEW
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Inflammation and Lymphatic Function

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The lymphatic vasculature plays a crucial role in regulating the inflammatory response by influencing drainage of extravasated fluid, inflammatory mediators, and leukocytes. Lymphatic vessels undergo pronounced enlargement in inflamed tissue and display increased leakiness, indicating reduced functionality. Interfering with lymphatic expansion by blocking the vascular endothelial growth factor C (VEGF-C)/vascular endothelial growth factor receptor 3 (VEGFR-3) signaling axis exacerbates inflammation in a variety of disease models, including inflammatory bowel disease (IBD), rheumatoid arthritis and skin inflammation. In contrast, stimulation of the lymphatic vasculature, e.g., by transgenic or viral overexpression as well as local injections of VEGF-C, has been shown to reduce inflammation severity in models of rheumatoid arthritis, skin inflammation, and IBD. Strikingly, the induced expansion of the lymphatic vasculature improves lymphatic function as assessed by the drainage of dyes, fluorescent tracers or inflammatory cells and labeled antigens. The drainage performance of lymphatic vessels is influenced by vascular permeability and pumping activity, which are influenced by VEGF-C/VEGFR-3 signaling as well as several inflammatory mediators, including TNF-α, IL-1β, and nitric oxide. Considering the beneficial effects of lymphatic activation in inflammation, administration of pro-lymphangiogenic factors like VEGF-C, preferably in a targeted, inflammation site-specific fashion, represents a promising therapeutic approach in the setting of inflammatory pathologies.

Keywords: lymphatic vessels, lymphangiogenesis, inflammation, inflammatory bowel disease, arthritis, psoriasis, skin, inflammatory mediators

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INTRODUCTION

Inflammation is a defensive reaction of the organism against pathogens or irritants. It is characterized by the five cardinal symptoms of rubor (redness), calor (increased heat), tumor (swelling), dolor (pain), and functio laesa (impaired function), which are mostly mediated by the expansion and activation of blood vessels. Inflammation is commonly associated with the formation of new blood (angiogenesis) and lymphatic (lymphangiogenesis) vessels from the pre-existing vascular networks. Interestingly, while the activation of the blood vasculature has been reported to aggravate inflammation severity in a variety of disease models (1–3), lymphatic vessels generally appear to exert beneficial effects, possibly by improving the clearance of extravasated fluid, thus reducing edema formation and levels of pro-inflammatory mediators as well as numbers of immune cells.

This review provides an overview of studies investigating the role of lymphatic expansion and function in common inflammatory diseases such as skin inflammation, inflammatory bowel disease (IBD) and rheumatoid arthritis (RA). In addition, the known effects of inflammatory mediators on the lymphatic vasculature and commonly used mouse models are described.

The lymphatic vasculature is a hierarchically structured, one-way circuit composed of initial capillaries, which lack a continuous basement membrane and smooth muscle cell coverage, draining into larger, smooth muscle cell-covered collectors and ultimately lymph nodes. In the setting of inflammation, the lymphatic system is critically important, as it is needed to ensure tissue fluid homeostasis by draining the larger amounts of extravasated fluid originating from increasingly leaky, inflammatory blood vessels. Indeed, an increased interstitial fluid pressure has been found to lead to the dilation of initial lymphatic vessels, thus facilitating the entry of fluid and inflammatory cells into the lymphatic vasculature and thereby removal from the inflamed tissue (4). In addition, lymphatic vessels are crucial for immune surveillance, as they serve as main transport routes for cells and inflammatory mediators to lymph nodes, where immune responses are mounted.

The most-thoroughly characterized signaling axis involved in lymphatic expansion and development consists of the vascular endothelial growth factor receptor 3 (VEGFR-3) and its ligands VEGF-C and VEGF-D. VEGFR-3 is part of the receptor tyrosine kinase family and is expressed widely in vascular endothelial cells during embryonic development, but becomes strongly restricted to lymphatic endothelial cells (LECs) in the adult organism under physiological conditions (5).

VEGF-C is the main ligand of VEGFR-3 and induces proliferation and migration of endothelial cells (6, 7). It undergoes extensive post-translational proteolytic processing, which also regulates the molecule's binding properties. Fully processed VEGF-C binds VEGFR-3 and, albeit with a lower affinity, VEGFR-2 (8). A mutated form of VEGF-C in which the cysteine 156 is replaced with a serine (VEGF-C156Ser) selectively binds VEGFR-3 (9).

VEGF-D has been reported to induce proliferation of endothelial cells (10). In mice, VEGF-D exclusively binds VEGFR-3, while fully processed human VEGF-D may also bind VEGFR-2 (11).

In order to study the role of lymphatic vessels in different pathologies, various mouse lines with a modified VEGFR-3 signaling axis have been generated. In K14-VEGF-C mice, VEGF-C is overexpressed under the control of the keratin-14 promoter, resulting in elevated levels of the growth factor in the skin and an enlarged dermal lymphatic vascular network (12). A similar lymphatic hyperplasia has been observed in mice transgenic for VEGF-D (K14-VEGF-D) (13). In contrast, mice overexpressing a soluble form of VEGFR-3 in the skin (K14-VEGFR-3-Ig mice) lack dermal lymphatic vessels and develop edema in the feet and skin (14).

Apart from promoting or inhibiting lymphatic vascular expansion, the clearance capacity of lymphatic vessels is subject to regulation by various signals. Drainage performance is influenced by vascular permeability and pumping activity of lymphatic vessels. Mediators inducing increased lymphatic vessel permeability include TNF-α, IL-1β, histamine, and the VEGF-C/VEGFR-3 axis (15–17). Lymphatic contractions and thereby pumping are negatively regulated by various inflammatory mediators, including prostaglandins, histamine, and nitric oxide (NO), while VEGF-C has enhancing effects (18–21). NO regulates lymphatic vessel function via its effects on lymphatic smooth muscle cells leading to vasodilation. It is produced constitutively by the endothelial nitric oxide synthase (eNOS) under physiological conditions. In inflammation, however, its levels are elevated due to the higher expression of inducible nitric oxide synthase (iNOS) on immune cells and inflamed endothelium, which has been linked to reduced lymphatic contraction frequency (22).

THE LYMPHATIC VASCULATURE IN INFLAMMATORY DISEASES

Skin Inflammation

A wide range of skin pathologies including psoriasis, atopic dermatitis, rosacea, and UV damage are characterized by pronounced and often prolonged inflammation. The lymphatic vasculature is often aberrant in inflamed skin; in human psoriatic plaques for example, lymphatic vessels are dilated and tortuous (23–25). Nevertheless, lymphatic dysregulation in the human disease has attracted comparatively little attention.

Multiple mouse models have been established to facilitate the study of these diseases in general and the role of the lymphatic vasculature in particular. A common model are K14-VEGF-A transgenic mice which overexpress VEGF-A under the control of the keratin-14 promoter, resulting in chronically elevated levels of said growth factor in the skin and a concomitant expanded, leaky blood vasculature. Homozygous mice spontaneously develop a chronic skin inflammation at the age of 6 months (26). In hemizygous mice, a contact sensitizer (e.g., oxazolone) can be used to trigger a contact hypersensitivity reaction (CHS), leading to a similar chronic inflammatory skin disease (24).

In wild-type mice, skin inflammation may be elicited by inducing CHS, exposure to UVB radiation, injection of bacterial antigens like LPS or application of pro-inflammatory agents such as tetradecanoylphorbolacetate (TPA) or imiquimod (27).

Using these models, skin inflammation has been extensively studied in mice and the lymphatic vasculature has been demonstrated to be functionally impeded in UVB-irradiated, chronically inflamed ear skin. Evans blue injected into the inflamed skin stained strongly dilated lymphatic vessels that were extremely leaky, indicating reduced drainage capacity (1).

Stimulation of Lymphatic Vessels in Skin Inflammation

Activating the lymphatic vasculature in the setting of skin inflammation has been associated with reduced disease severity (summarized in **Table 1**). In K14-VEGF-A mice that had been crossed with K14-VEGF-C mice and were undergoing chronic CHS of the ear skin, the lymphatic vasculature was expanded and the inflammation, as assessed by edema formation,

TABLE 1 | Effects of lymphatic vessel stimulation in inflammatory diseases.

Animal model	Inflammatory stimulus	Method of lymphatic vasculature activation	Effects	References
SKIN INFLAMMATION				
K14-VEGF-A mice	Oxazolone	Transgenic VEGF-C delivery (crossed with K14-VEGF-C mice)	Reduced inflammatory edema and cell infiltration Expanded skin lymphatic vasculature Normalization of skin blood vasculature, epidermal differentiation and proliferation Improved lymphatic drainage function	(28)
		Local injection of recombinant VEGF-C156Ser	Reduced inflammatory edema Expanded skin lymphatic vasculature Normalization of skin blood vasculature Reduced inflammatory cell infiltration	
K14-VEGF-C mice	Injections of LPS or LTA and MDP	Transgenic VEGF-C delivery	Expanded lymphatic skin and LN vasculature Increased inflammatory cell migration to LNs Reduced inflammatory edema and erythema Faster antigen clearance	(29)
	TPA		Increased clearance of lymphatic-specific tracer	(30)
	UVB irradiation		Reduced inflammatory edema and epidermal thickening Expanded lymphatic vasculature Improved lymphatic drainage function	(31)
	Oxazolone		Reduced inflammatory edema and epidermal thickening Expanded lymphatic vasculature Lower levels of IL-1β and VEGF-A	
K14-VEGF-D mice	UVB irradiation	Transgenic VEGF-D delivery	Reduced inflammatory edema and epidermal thickening Expanded lymphatic vasculature Improved lymphatic drainage function	(31)
	Oxazolone		Reduced inflammatory edema and epidermal thickening Expanded lymphatic vasculature	
Wildtype mice	UVB irradiation	Local injection of recombinant VEGF-C156Ser	Reduced inflammatory edema and cell infiltration Expanded lymphatic vasculature	(32)
INFLAMMATORY BOWEL DISEASE				
Wildtype mice	DSS	Adenoviral delivery of VEGF-C	Reduced colitis severity and inflammatory cell infiltration Increased lymphatic vessel density and proliferation Improved lymphatic drainage function Increased inflammatory cell migration to LNs	(33)
IL-10 knockout mice	Lack of anti-inflammatory IL-10			
RHEUMATOID ARTHRITIS				
TNF-α transgenic mice	TNF-α overexpression	Adeno-associated viral delivery of VEGF-C	Expanded lymphatic vasculature Reduced synovial volume, bone and cartilage erosion and osteoclast numbers Improved joint movement and lymphatic clearance function	(34)
		iNOS inhibition	Improved lymphatic clearance function Restored lymphatic contractions	(35)

DSS, dextran sulfate sodium; LN, lymph node; LPS, lipopolysaccharide; LTA, lipoteichoic acid; LV, lymphatic vessel; MDP, muramyl dipeptide; TPA, tetradecanoylphorbolacetate.

inflammatory cell infiltrate, and altered epidermal proliferation or differentiation, was significantly reduced compared to control inflamed K14-VEGF-A mice. Strikingly, the vascular expansion was accompanied by an improved lymphatic clearance function. Local injections of VEGF-C156Ser had similar disease-alleviating effects, indicating that VEGFR-3- rather than VEGFR-2-mediated signaling is mainly responsible for the observed anti-inflammatory effects (28). In agreement with this observation, local injections of VEGF-C156Ser also triggered a strong lymphangiogenic response and reduced inflammatory ear

TABLE 2 | Effects of lymphatic vessel inhibition in inflammatory diseases.

Animal model	Inflammatory stimulus	Method of lymphatic vasculature inhibition	Effects	References
SKIN INFLAMMATION				
K14-VEGF-A mice	Oxazolone	Blocking antibody to VEGFR-3	Reduced lymphatic vasculature Increased inflammatory edema and epidermal thickening	(28)
Wild-type mice	Injections of LPS or LTA and MDP	Adenoviral VEGFR-3 overexpression	Delayed inflammation resolution Reduced lymphatic drainage and inflammatory cell migration	(29)
	UVB irradiation	Blocking antibody to VEGFR-3	Increased inflammatory edema and inflammatory cell invasion	(36)
INFLAMMATORY BOWEL DISEASE				
Wildtype mice	DSS	Blocking antibody to VEGFR-3	Increased colitis severity Reduced lymphatic vessel density, LV proliferation, lymphatic drainage function and cell migration to LN	(33)
IL-10 knockout mice	Lack of anti-inflammatory IL-10	Blocking antibody to VEGFR-3	Increased colitis severity Reduced lymphatic vessel density, LV proliferation, lymphatic drainage function and cell migration to LN	(33)
			Increased colitis severity and edema Enlarged lymphatic vessels	(37)
RHEUMATOID ARTHRITIS				
TNF-α transgenic mice	TNF-α overexpression	Blocking antibody to VEGFR-3	Reduced lymphatic vessel numbers and lymphatic drainage Smaller draining LNs Increased joint inflammation	(38)

DSS, dextran sulfate sodium; LN, lymph node; LPS, lipopolysaccharide; LTA, lipoteichoic acid, LV, lymphatic vessel; MDP, muramyl dipeptide.

swelling and CD11b-positive immune cell infiltration in UVB-irradiated ear skin inflammation (32).

These findings are in line with a different study investigating the role of macrophages and lymphatic vessels in cutaneous inflammation. K14-VEGF-C mice that were subjected to lipopolysaccharide (LPS)- or lipoteichoic acid (LTA)/muramyl dipeptide (MDP)-induced skin inflammation presented with an expanded dermal and lymph node lymphatic vasculature. In addition, inflammatory tissue swelling and skin reddening were reduced. While no difference in FITC-dextran clearance was found, inflammatory cell migration to the draining lymph nodes and the drainage of fluorescently labeled antigen was significantly accelerated in K14-VEGF-C mice. These effects appeared to be dependent on macrophages, as clodronate-mediated depletion of these cells reduced lymphangiogenesis and delayed inflammation resolution (29). An enhanced lymphatic drainage function due to lymphatic stimulation has also been reported in other studies, e.g., after repeated application of TPA to the back skin of K14-VEGF-C transgenic mice, in which a lymphatic-specific, near-infrared tracer was cleared more rapidly than in wild-type mice (30). Similarly, in a study of acute skin inflammation, both K14-VEGF-C and, to a lesser extent, K14-VEGF-D transgenic mice had improved clearance of Evans blue out of UVB-irradiated ear skin (31). Moreover, these mice also had less inflammatory edema and reduced epidermal thickening in oxazolone- and UVB-induced skin inflammation. The reduction in inflammation was generally more pronounced in VEGF-C transgenic mice than in VEGF-D transgenic animals, indicating stronger anti-inflammatory effects of VEGF-C (31).

Inhibition of Lymphatic Vessels in Skin Inflammation

In contrast to stimulation of the lymphatic vasculature, inhibiting lymphatic vessels has been shown to aggravate skin inflammation in several studies (summarized in **Table 2**). Antibody-mediated blocking of VEGFR-3 strikingly reduced the number of lymphatic vessels in the inflamed ear skin of K14-VEGF-A mice during a CHS reaction. At the same time, tissue swelling, epidermal thickening, keratinocyte proliferation and the numbers of CD8- and CD11b-positive cells were significantly increased, indicating a more severe inflammatory phenotype. Interestingly, blocking VEGFR-2 alone or in combination with VEGFR-3 alleviated inflammation, indicating that VEGFR-2-mediated inhibition of blood vessels is beneficial in skin inflammation and outweighs the detrimental effects of VEGFR-3 inhibition (28). Similarly, adenoviral overexpression of a soluble VEGFR-3 strongly reduced lymphangiogenesis in mice undergoing LPS- or LTA/MDP-induced skin inflammation, resulting in delayed inflammation-resolution, slower clearance of FITC-dextran as well as FITC-labeled LPS, and reduced migration of inflammatory cells from the skin to the draining lymph nodes (29). Systemic, antibody-mediated inhibition of VEGFR-3 also led to increased edema formation and CD11b-positive cell numbers in UVB-irradiated ear skin (36).

Inflammatory Bowel Disease

The term inflammatory bowel disease (IBD) comprises Crohn's disease (CD) and ulcerative colitis (UC), which are characterized by a chronic inflammation of the digestive tract. While UC generally affects the colon and presents with superficial ulcerations of the mucosa and submucosa, CD may occur at any location in the gastrointestinal tract and often causes transmural inflammation. As in the case of skin inflammation, research has long been focused on changes in the blood vasculature and VEGF-A has been suggested as an important mediator of IBD (39, 40).

In human patients suffering from IBD, lymphangiogenesis, lymphatic vessel obstruction, dilation, and submucosal edema are commonly observed (41–44) and abnormalities in the lymphatic vasculature had already been recognized during the original characterization of CD (45). In addition to morphological alterations, the functionality of IBD-associated lymphatic vessels is reduced. A study in patients with CD employed injections of the lymphatic-vessel-staining Patent Blue V dye in the inflamed colon and demonstrated morphological aberrations and functional impairment of the lymphatic vasculature, which could be correlated with disease severity. Strikingly, following surgical intervention and inflammation regression, lymphatic vessel appearance reverted back to normal, indicating that lymphatic vessel function may be involved in IBD pathogenesis in humans (46). In line with this, a lower density of lymphatic vessels could be linked to an increased risk of CD recurrence (47).

A multitude of studies have been performed in mouse models of IBD, the two most-commonly used being IL-10 knockout mice and dextran sulfate sodium (DSS)-induced colitis. IL-10-deficient mice spontaneously develop colitis at the age of 10–12 weeks, most likely due to the lacking anti-inflammatory and immunosuppressive activity of IL-10 (48, 49).

DSS-induced colitis relies on administration of the detergent DSS in drinking water, which damages the intestinal epithelium, most strongly in the distal colon, and compromises its barrier function, making the underlying tissue accessible to bacteria and associated substances. In order to model acute inflammation, mice are commonly given DSS for a certain amount of time (e.g., a week), for chronic inflammation, mice receive multiple cycles of DSS and intermittent regular drinking water (50, 51).

Stimulation of Lymphatic Vessels in Inflammatory Bowel Disease

Akin to skin inflammation, inducing the lymphatic vasculature is generally correlated with a reduction in inflammation severity (summarized in **Table 1**).

In IL-10 knockout mice as well as in animals undergoing DSS-induced colitis, adenoviral delivery of VEGF-C significantly increased lymphatic vessel density and was associated with a reduction in bodyweight loss and disease severity as assessed by stool consistency and presence or absence of fecal blood. Moreover, histological analyses revealed decreased submucosal tissue edema and inflammatory cell infiltration, while the proliferation of LECs was greatly increased. Quantification of Evans blue clearance out of inflamed distal colon tissue revealed an enhanced lymphatic drainage function, which was also reflected in an improved clearance of fluorescently labeled antigen-coated beads and an augmented inflammatory cell migration from the inflamed tissue to the draining lymph nodes. Similar to the observations in skin inflammation, depletion of macrophages by clodronate largely abolished the protective effects of VEGF-C (33). It has been suggested that VEGF-C may influence the cytokine balance in the inflamed colon. Indeed, *in vitro* experiments have shown VEGF-C to induce the upregulation of IL-10 by bone marrow-derived macrophages (33). In line with this, increased levels of IL-10 in combination with a reduction of IL-9, which is associated with intestinal barrier disruption, have been reported upon treatment with adenovirally delivered VEGF-C in mice undergoing DSS-induced colitis (52, 53).

Inhibition of Lymphatic Vessels in Inflammatory Bowel Disease

Blocking VEGFR-3 resulted in a worsened colitis in IL-10 knockout mice as well as DSS-treated animals in terms of the histological score (summarized in **Table 2**). Animals of both models presented with strongly reduced lymphatic vessel density and LEC proliferation upon VEGFR-3 inhibition. At the same time, lymphatic clearance of Evans blue and bacterial antigen as well as inflammatory cell mobilization to the draining lymph nodes were significantly reduced (33).

In a different, independent study, IL-10 knockout mice were treated with a blocking antibody to VEGFR-3. This resulted in enlarged and tortuous lymphatic vessels in the colon, increased submucosal edema and a higher leukocyte infiltration in the inflamed tissue as well as a higher disease severity score (37).

Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a chronic inflammatory disease affecting the joints and characterized by episodic flares (54). In its chronic stage, RA is commonly associated with lymphadenopathy and a decrease in lymphatic drainage function, as shown for example by tracking the drainage of intradermally-injected, radioactively labeled albumin from the forearm (55). Lymphangiogenesis is also commonly observed in the joints of human RA patients and has been reproduced in mouse models of the disease (56, 57).

Commonly used mouse models of rheumatoid arthritis include TNF-α transgenic mice and K/B × N mice. The former overexpress human TNF-α and spontaneously develop chronic progressive joint inflammation at the age of ∼4 weeks (58). K/B × N mice model the autoimmunity aspect of RA and are based on a mouse line transgenic for a T cell receptor specific for bovine ribonuclease. After breeding onto the NOD background, accidental recognition of a NOD-derived antigen triggers the onset of joint inflammation at 4 weeks after birth (59).

Lymphatic function has mainly been studied in these animals and a two-phase model has been proposed [reviewed in (60)]. In an initial "expansion" phase during joint inflammation, lymphangiogenesis and popliteal lymph node expansion with or without increased lymphatic vessel contractions limit the inflammatory response (57, 61, 62). During the following "collapse" phase, popliteal lymph nodes shrink and lymphatic

vessel contractions as well as lymphatic drainage function decrease significantly. At the same time, the joint inflammation increases in severity (61, 63–65). Blocking TNF-α signaling resulted in increased lymphatic contractions and reduced joint inflammation (66).

Similar changes in lymph node characteristics have also been reported in human patients, where lymph node hypertrophy could be observed in the vast majority of patients suffering from active RA, while healthy individuals and patients in remission showed no lymph node alterations (67).

Stimulation of Lymphatic Vessels in Rheumatoid Arthritis

Stimulating the lymphatic vasculature has been associated with reduced disease severity in animal models of RA (summarized in **Table 1**). Adeno-associated viral (AAV) delivery of VEGF-C in the inflamed ankle joints of 6-week-old TNF-α transgenic mice partially reversed the inflammation-associated increase in synovial volume and significantly improved leg mobility. Histological analyses revealed that mice treated with VEGF-C had less cartilage and bone destruction than animals injected with a control vector. In chronic arthritis (mice at 5 months of age), lymphatic drainage of indocyanine green (ICG) out of the footpad was strongly decreased in TNF-α transgenic compared to wild-type mice. AAV-mediated delivery of VEGF-C significantly improved the clearance of ICG out of the paws and increased the number of lymphatic vessels in the pannus of the inflamed joint (34).

In an alternative approach, based on the observation that increased levels of NO in inflammation reduce lymphatic pumping, lymphatic vessel function was studied using inhibition of NOS. Local application of L-N6-(1-iminoethyl)lysine 5-tetrazole-amide (L-NIL), a moderately selective inhibitor of iNOS (68), in TNF-α transgenic mice with collapsed lymph nodes restored lymphatic contractions and strongly improved ICG transport from the footpad to popliteal lymph nodes, while Nω-nitro-l-arginine methyl ester (L-NAME), an unspecific inhibitor of both eNOS and iNOS was not associated with beneficial effects (35). Although the impact on disease severity in these mice was not assessed in the study, it provides evidence that selective inhibition of iNOS might offer an alternative and clinically relevant approach for RA therapy.

Inhibition on Lymphatic Vessels in Rheumatoid Arthritis

Inhibiting the lymphatic vasculature led to worsened inflammation in mouse models of arthritis (summarized in **Table 2**). Injecting TNF-α transgenic mice that had developed joint inflammation with a VEGFR-3-blocking antibody for 2 months significantly reduced the number of lymphatic capillaries in the draining popliteal lymph nodes and inflamed ankles. Blocking VEGFR-3 also aggravated inflammation of the knee and ankle joints, as the increase in synovial volume over time as well as its absolute size were elevated in these animals compared to IgG-treated controls. Similarly, histological analyses of hematoxylin-eosin-stained sections revealed exacerbated inflammation after VEGFR-3 inhibition. Akin to the effects observed in chronic skin inflammation, blocking VEGFR-2 was associated with a reduced inflammatory reaction, as assessed by synovial volume and histological scoring. Lymphatic drainage function, as assessed by tracking the ICG signal in paws and draining popliteal lymph nodes following injection into the footpad, was dramatically reduced upon blocking VEGFR-3 (38).

The Effect of Inflammatory Mediators on the Lymphatic Vasculature

Inflammatory lymphangiogenesis is mostly mediated by VEGF-A and VEGF-C which are produced by keratinocytes and stromal cells like fibroblasts as well as immune cells, most importantly macrophages (69–71). Indeed, several inflammatory mediators have been found to induce VEGF-C transcription (72–74).

Macrophages are of critical importance, as demonstrated in a model of IBD and LPS-induced skin inflammation, where depletion of macrophages aggravated the inflammation (29, 33). While VEGFs are important for inflammation-induced lymphangiogenesis, there are many additional factors at play. IL-17, a crucial cytokine in the pathogenesis of psoriasis for example, has been shown to induce lymphangiogenesis *in vitro* and in cornea micropocket assays (75), and IL-8 promoted lymphangiogenesis in cell culture experiments and in an animal model of lymphedema (76). Similarly, inhibition of TGF-β, which mediates anti-inflammatory effects, supported lymphangiogenesis in a mouse model of peritonitis and in lymphedema (77, 78). In line with this, cytokines characteristic for T_H2 cells like IL-4 and IL-13, which are often linked to inflammation resolution, inhibited lymphangiogenesis (79). Interestingly, several inflammatory mediators have anti-lymphangiogenic activity. Interferon-γ (IFN-γ), which is produced by activated T cells, decreased lymphatic vessel formation of both human and murine lymphatic endothelial cells *in vitro* as well as in mouse lymph nodes (15, 80). Likewise, TNF-α inhibited capillary formation and proliferation of mouse LECs, while IL-1β had no consistent effects on proliferation, but reduced barrier function of LECs (15). Indeed, inflammatory mediators not only influence lymphangiogenesis, but also impact lymphatic function more directly. Prostaglandins, IL-1β, IL-6, and TNF-α reduced lymphatic pumping frequency (81, 82). Similarly, inflammatory mediators affect lymphatic vessel permeability, as demonstrated *in vitro* by assessing the effect of a wide array of inflammatory mediators on rat lymphatic endothelial cell monolayers, where IL-6, TNF-α, and IFN-γ strikingly increased the permeability, probably by reducing vascular endothelial (VE)-cadherin expression (83). Few studies have addressed lymphatic vessel permeability *in vivo*, but results of those that have showed impaired barrier function as well as pronounced leakiness and reported VEGF-A as important mediator of these effects, possibly by signaling via VEGFR-2 (1, 84).

It is important to consider that cytokines and growth factors often have pleiotropic effects, making it challenging to distinguish between direct and indirect mechanisms. IL-17 for example has been reported to induce VEGF-D expression, thereby triggering lymphangiogenesis indirectly (75). The wide array of signaling

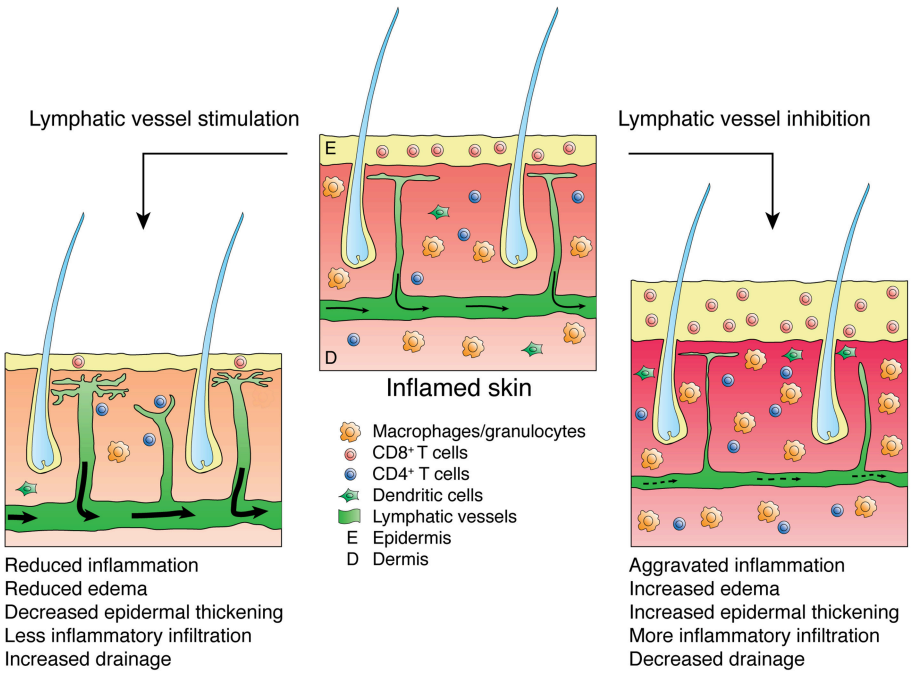

FIGURE 1 | Effects of lymphatic vessel stimulation or inhibition on skin inflammation. Inflamed skin presents with epidermal thickening, edema and infiltration by inflammatory leukocytes (e.g., CD8-positive cells or macrophages and granulocytes). Stimulation of the lymphatic vasculature alleviates inflammation, reducing edema, epidermal thickening and inflammatory infiltration while improving lymphatic drainage, thus lowering the numbers of inflammatory cells in the inflamed skin. Inhibition of the lymphatic vasculature aggravates inflammation and reduces lymphatic clearance.

molecules involved in inflammation as well as their different and often pleiotropic effects on the lymphatic vasculature result in a highly complex network of signals which is still incompletely understood.

CONCLUSIONS

The lymphatic vasculature represents a crucial, although often under-appreciated, player in inflammation. Lymphatic vessels serve as the main transport route for inflammatory mediators, fluid, antigen and immune cells, thus playing a pivotal role in inflammation initiation and resolution. Indeed, it has been controversial whether expansion of the lymphatic endothelium contributes to inflammation by facilitating transport of leukocytes to lymph nodes and mounting of immune responses, or whether lymphatic vessels support inflammation resolution by draining inflammatory mediators and cells from the site of inflammation. However, in recent years, a number of studies detailed above have reported alleviated inflammation severity following activation and/or expansion of the lymphatic vasculature (depicted for skin inflammation in **Figure 1**), thus indicating that promoting the lymphatic vasculature supports inflammation resolution and may represent a valid therapeutic approach. It should be considered, however, that VEGF-C/VEGFR-3 signaling itself might also account for some of the anti-inflammatory effects observed in VEGF-C transgenic mice, as it has been shown to reduce the production of pro-inflammatory cytokines and protect mice from septic shock (85).

Interestingly, the lymphatic vasculature is also affected by established standard therapies used for the treatment of inflammatory diseases, e.g., in RA, where blocking TNF-α resulted in an increased lymphangiogenic response and increased lymphatic contractions in the inflamed tissue (66, 86). Other therapies aimed at blocking certain cytokines (e.g., IL-17 in psoriasis) may also exert parts of their anti-inflammatory effects by modulating the lymphatic vasculature. Curiously, some anti-inflammatory agents have been associated with anti-lymphangiogenic activity. Glucocorticoids reduced lymphangiogenesis in cornea inflammation and chronic airway inflammation mediated by *M. pulmonis* infection (87, 88). In addition, prostaglandin E2, whose biosynthesis is inhibited by cyclooxygenase (COX)-blocking non-steroidal anti-inflammatory drugs (NSAIDs), has been reported to induce VEGF-C expression and lymphangiogenesis in the setting of lung cancer (73). Coherently, inhibition of COX-2 reduced tumor-induced lymphangiogenesis (89). A possible explanation for these findings could be that potent therapeutic agents inhibit inflammation strongly enough to also reduce the concomitant inflammation-induced lymphangiogenesis. Moreover, while prostaglandin E2 interferes with lymphatic expansion, it has also been reported to inhibit lymphatic function (81). Therefore, glucocorticoids and NSAIDs may improve lymphatic clearance despite reducing lymphangiogenesis.

However, further studies are needed to thoroughly investigate these possibilities.

It is important to consider that immunomodulatory properties of the lymphatic endothelium, which have received increasing attention over the last decade, may explain the observed anti-inflammatory effects of lymphatic vessel induction at least partially. A good example is the receptor D6, which is highly expressed by lymphatic endothelial cells and scavenges inflammatory cytokines. Mice deficient for D6 suffered from more severe skin inflammation and colitis compared to wild-type animals (90, 91), hence, lymphatic expansion may increase the levels of D6 and accordingly lower the levels of inflammatory mediators in the inflamed tissue, resulting in reduced disease severity. However, the immunomodulatory roles of the lymphatic vasculature are outside the scope of this review.

Although VEGF-C has been associated with anti-inflammatory effects in a variety of diseases as described above, its biological roles are highly complex and may be organ- and disease-dependent. In the setting of experimental obesity for example, transgenically overexpressed VEGF-C induced pro-inflammatory macrophage chemotaxis, increased weight gain and worsened metabolic parameters such as insulin resistance (92). In contrast, blockade of VEGF-C and VEGF-D by overexpression of a soluble form of VEGFR-3 reduced macrophage infiltration and improved insulin sensitivity in diet-induced obesity (93). Similarly, in tumor studies, VEGF-C has been reported to induce tumor lymphangiogenesis and stimulate the migration of macrophages (94), which may explain the observed increase in tumor metastasis in VEGF-C transgenic mice (95).

Applying VEGF-C in these diseases might be counter-productive and these findings therefore highlight the complexity of VEGF-C biology and emphasize the necessity of thoroughly evaluating possible beneficial and detrimental effects of VEGF-C in individual pathologies.

Considering all available data, the induction of lymphangiogenesis and activation of the lymphatic vasculature in the setting of inflammation appears to represent a potent therapeutic approach. It is therefore striking that this strategy has not been explored more thoroughly, let alone exploited clinically. A major obstacle has been the lack of clinically feasible delivery systems of lymphangiogenic factors. In a recent study, however, a targeted F8-VEGF-C fusion protein that specifically accumulates in the inflamed tissue was characterized and shown to reduce inflammation in two mouse models of skin inflammation, possibly filling this therapeutic gap (96).

AUTHOR CONTRIBUTIONS

SS designed and wrote the manuscript. MD designed and revised the manuscript.

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REFERENCES

1. Kajiya K, Hirakawa S, Detmar M. Vascular endothelial growth factor-A mediates ultraviolet B-induced impairment of lymphatic vessel function. *Am J Pathol*. (2006) 169:1496–503. doi: 10.2353/ajpath.2006.060197
2. Chidlow JH, Langston W, Greer JJM, Ostanin D, Abdelbaqi M, Houghton J, et al. Differential angiogenic regulation of experimental colitis. *Am J Pathol*. (2006) 169:2014–30. doi: 10.2353/ajpath.2006.051021
3. Elshabrawy HA, Chen Z, Volin MV, Ravella S, Virupannavar S, Shahrara S. The pathogenic role of angiogenesis in rheumatoid arthritis. *Angiogenesis* (2015) 18:433–48. doi: 10.1007/s10456-015-9477-2
4. Skobe M, Detmar M. Structure, function, and molecular control of the skin lymphatic system. *J Investig Dermatol Symp Proc*. (2000) 5:14–9. doi: 10.1046/j.1087-0024.2000.00001.x
5. Kaipainen A, Korhonen J, Mustonen T, van Hinsbergh VW, Fang GH, Dumont D, et al. Expression of the fms-like tyrosine kinase 4 gene becomes restricted to lymphatic endothelium during development. *Proc Natl Acad Sci USA*. (1995) 92:3566–70. doi: 10.1073/pnas.92.8.3566
6. Joukov V, Pajusola K, Kaipainen A, Chilov D, Lahtinen I, Kukk E, et al. A novel vascular endothelial growth factor, VEGF-C, is a ligand for the Flt4 (VEGFR-3) and KDR (VEGFR-2) receptor tyrosine kinases. *EMBO J*. (1996) 15:1751. doi: 10.1002/j.1460-2075.1996.tb00359.x
7. Makinen T, Veikkola T, Mustjoki S, Karpanen T, Catimel B, Nice EC, et al. Isolated lymphatic endothelial cells transduce growth, survival and migratory signals via the VEGF-C/D receptor VEGFR-3. *EMBO J*. (2001) 20:4762–73. doi: 10.1093/emboj/20.17.4762
8. Joukov V, Sorsa T, Kumar V, Jeltsch M, Claesson-Welsh L, Cao Y, et al. Proteolytic processing regulates receptor specificity and activity of VEGF-C. *Embo J*. (1997) 16:3898–911. doi: 10.1093/emboj/16.13.3898
9. Joukov V, Kumar V, Sorsa T, Arighi E, Weich H, Saksela O, et al. A recombinant mutant vascular endothelial growth factor-C that has lost vascular endothelial growth factor receptor-2 binding, activation, and vascular permeability activities. *J Biol Chem*. (1998) 273:6599–602. doi: 10.1074/jbc.273.12.6599
10. Achen MG, Jeltsch M, Kukk E, Makinen T, Vitali A, Wilks AF, et al. Vascular endothelial growth factor D (VEGF-D) is a ligand for the tyrosine kinases VEGF receptor 2 (Flk1) and VEGF receptor 3 (Flt4). *Proc Natl Acad Sci USA*. (1998) 95:548–53. doi: 10.1073/pnas.95.2.548
11. Baldwin ME, Catimel B, Nice EC, Roufail S, Hall NE, Stenvers KL, et al. The specificity of receptor binding by vascular endothelial growth factor-d is different in mouse and man. *J Biol Chem*. (2001) 276:19166–71. doi: 10.1074/jbc.M100097200
12. Jeltsch M, Kaipainen A, Joukov V, Meng X, Lakso M, Rauvala H, et al. Hyperplasia of lymphatic vessels in VEGF-C transgenic mice. *Science* (1997) 276:1423–5. doi: 10.1126/science.276.5317.1423
13. Veikkola T, Jussila L, Makinen T, Karpanen T, Jeltsch M, Petrova TV, et al. Signalling via vascular endothelial growth factor receptor-3 is sufficient for lymphangiogenesis in transgenic mice. *Embo J*. (2001) 20:1223–31. doi: 10.1093/emboj/20.6.1223
14. Makinen T, Jussila L, Veikkola T, Karpanen T, Kettunen MI, Pulkkanen KJ, et al. Inhibition of lymphangiogenesis with resulting lymphedema in transgenic mice expressing soluble VEGF receptor-3. *Nat Med*. (2001) 7:199–205. doi: 10.1038/84651
15. Chaitanya GV, Franks SE, Cromer W, Wells SR, Bienkowska M, Jennings MH, et al. Differential cytokine responses in human and mouse lymphatic endothelial cells to cytokines in vitro. *Lymphat Res Biol*. (2010) 8:155–64. doi: 10.1089/lrb.2010.0004

16. Breslin JW, Yuan SY, Wu MH. VEGF-C alters barrier function of cultured lymphatic endothelial cells through a VEGFR-3-dependent mechanism. *Lymphat Res Biol*. (2007) 5:105–13. doi: 10.1089/lrb.2007.1004
17. Breslin JW. ROCK and cAMP promote lymphatic endothelial cell barrier integrity and modulate histamine and thrombin-induced barrier dysfunction. *Lymphat Res Biol*. (2011) 9:3–11. doi: 10.1089/lrb.2010.0016
18. Kurtz KH, Moor AN, Souza-Smith FM, Breslin JW. Involvement of H1 and H2 receptors and soluble guanylate cyclase in histamine-induced relaxation of rat mesenteric collecting lymphatics. *Microcirculation* (2014) 21:593–605. doi: 10.1111/micc.12138
19. Nizamutdinova IT, Maejima D, Nagai T, Bridenbaugh E, Thangaswamy S, Chatterjee V, et al. Involvement of histamine in endothelium-dependent relaxation of mesenteric lymphatic vessels. *Microcirculation* (2014) 21:640–8. doi: 10.1111/micc.12143
20. Gasheva OY, Zawieja DC, Gashev AA. Contraction-initiated NO-dependent lymphatic relaxation: a self-regulatory mechanism in rat thoracic duct. *J Physiol*. (2006) 575:821–32. doi: 10.1113/jphysiol.2006.115212
21. Breslin JW, Gaudreault N, Watson KD, Reynoso R, Yuan SY, Wu MH. Vascular endothelial growth factor-C stimulates the lymphatic pump by a VEGF receptor-3-dependent mechanism. *Am J Physiol Heart Circ Physiol*. (2007) 293:H709–H718. doi: 10.1152/ajpheart.00102.2007
22. Liao S, Cheng G, Conner DA, Huang Y, Kucherlapati RS, Munn LL, et al. Impaired lymphatic contraction associated with immunosuppression. *Proc Natl Acad Sci USA*. (2011) 108:18784–9. doi: 10.1073/pnas.1116152108
23. Braverman IM. Electron microscopic studies of the microcirculation in psoriasis. *J Invest Dermatol*. (1972) 59:91–8. doi: 10.1111/1523-1747.ep12625852
24. Kunstfeld R. Induction of cutaneous delayed-type hypersensitivity reactions in VEGF-A transgenic mice results in chronic skin inflammation associated with persistent lymphatic hyperplasia. *Blood* (2004) 104:1048–57. doi: 10.1182/blood-2003-08-2964
25. Henno A, Blacher S, Lambert C, Colige A, Seidel L, Noël A, et al. Altered expression of angiogenesis and lymphangiogenesis markers in the uninvolved skin of plaque-type psoriasis. *Br J Dermatol*. (2009) 160:581–90. doi: 10.1111/j.1365-2133.2008.08889.x
26. Xia Y-P, Li B, Hylton D, Detmar M, Yancopoulos GD, Rudge JS. Transgenic delivery of VEGF to mouse skin leads to an inflammatory condition resembling human psoriasis. *Blood* (2003) 102:161–8. doi: 10.1182/blood-2002-12-3793
27. van der Fits L, Mourits S, Voerman JSA, Kant M, Boon L, Laman JD, et al. Imiquimod-induced psoriasis-like skin inflammation in mice is mediated via the IL-23/IL-17 axis. *J Immunol*. (2009) 182:5836–45. doi: 10.4049/jimmunol.0802999
28. Huggenberger R, Ullmann S, Proulx ST, Pytowski B, Alitalo K, Detmar M. Stimulation of lymphangiogenesis via VEGFR-3 inhibits chronic skin inflammation. *J Exp Med*. (2010) 207:2255–69. doi: 10.1084/jem.20100559
29. Kataru RP, Jung K, Jang C, Yang H, Schwendener RA, Baik JE, et al. Critical role of CD11b+ macrophages and VEGF in inflammatory lymphangiogenesis, antigen clearance, and inflammation resolution. *Blood* (2009) 113:5650–9. doi: 10.1182/blood-2008-09-176776
30. Christiansen AJ, Dieterich LC, Ohs I, Bachmann SB, Bianchi R, Proulx ST, et al. Lymphatic endothelial cells attenuate inflammation via suppression of dendritic cell maturation. *Oncotarget* (2016) 7:39421–35. doi: 10.18632/oncotarget.9820
31. Huggenberger R, Siddiqui SS, Brander D, Ullmann S, Zimmermann K, Antsiferova M, et al. An important role of lymphatic vessel activation in limiting acute inflammation. *Blood* (2011) 117:4667–78. doi: 10.1182/blood-2010-10-316356
32. Kajiya K, Sawane M, Huggenberger R, Detmar M. Activation of the VEGFR-3 pathway by VEGF-C attenuates UVB-induced edema formation and skin inflammation by promoting lymphangiogenesis. *J Invest Dermatol*. (2009) 129:1292–8. doi: 10.1038/jid.2008.351
33. D'Alessio S, Correale C, Tacconi C, Gandelli A, Pietrogrande G, Vetrano S, et al. VEGF-C–dependent stimulation of lymphatic function ameliorates experimental inflammatory bowel disease. *J Clin Invest*. (2014) 124:3863–78. doi: 10.1172/JCI72189
34. Zhou Q, Guo R, Wood R, Boyce BF, Liang Q, Wang Y-J, et al. Vascular endothelial growth factor C attenuates joint damage in chronic inflammatory arthritis by accelerating local lymphatic drainage in mice. *Arthritis Rheum*. (2011) 63:2318–28. doi: 10.1002/art.30421
35. Liang Q, Ju Y, Chen Y, Wang W, Li J, Zhang L, et al. Lymphatic endothelial cells efferent to inflamed joints produce iNOS and inhibit lymphatic vessel contraction and drainage in TNF-induced arthritis in mice. *Arthritis Res Ther*. (2016) 18:62. doi: 10.1186/s13075-016-0963-8
36. Kajiya K, Detmar M. An important role of lymphatic vessels in the control of UVB-induced edema formation and inflammation. *J Invest Dermatol*. (2006) 126:919–21. doi: 10.1038/sj.jid.5700126
37. Jurisic G, Sundberg JP, Detmar M. Blockade of VEGF receptor-3 aggravates inflammatory bowel disease and lymphatic vessel enlargement. *Inflamm Bowel Dis*. (2013) 19:1983–9. doi: 10.1097/MIB.0b013e31829292f7
38. Guo R, Zhou Q, Proulx ST, Wood R, Ji R-C, Ritchlin CT, et al. Inhibition of lymphangiogenesis and lymphatic drainage via vascular endothelial growth factor receptor 3 blockade increases the severity of inflammation in a mouse model of chronic inflammatory arthritis. *Arthritis Rheum*. (2009) 60:2666–76. doi: 10.1002/art.24764
39. Scaldaferri F, Vetrano S, Sans M, Arena V, Straface G, Stigliano E, et al. VEGF-A links angiogenesis and inflammation in inflammatory bowel disease pathogenesis. *Gastroenterology* (2009) 136:585–95.e585. doi: 10.1053/j.gastro.2008.09.064
40. Chidlow JH, Glawe JD, Pattillo CB, Pardue S, Zhang S, Kevil CG. $VEGF_{164}$ isoform specific regulation of T-cell-dependent experimental colitis in mice. *Inflamm Bowel Dis*. (2011) 17:1501–12. doi: 10.1002/ibd.21525
41. Geleff S, Schoppmann SF, Oberhuber G. Increase in podoplanin-expressing intestinal lymphatic vessels in inflammatory bowel disease. *Virchows Arch*. (2003) 442:231–7. doi: 10.1007/s00428-002-0744-4
42. Pedica F, Ligorio C, Tonelli P, Bartolini S, Baccarini P. Lymphangiogenesis in Crohn's disease: an immunohistochemical study using monoclonal antibody D2-40. *Virchows Arch*. (2008) 452:57–63. doi: 10.1007/s00428-007-0540-2
43. Heatley RV, Bolton PM, Hughes LE, Owen EW. Mesenteric lymphatic obstruction in Crohn's disease. *Digestion* (1980) 20:307–13. doi: 10.1159/000198452
44. Van Kruiningen HJ, Hayes AW, Colombel JF. Granulomas obstruct lymphatics in all layers of the intestine in Crohn's disease. *Apmis* (2014) 122:1125–9. doi: 10.1111/apm.12268
45. Crohn BB, Ginzburg L, Oppenheimer GD. Regional ileitis: a pathologic and clinical entity. *JAMA* (1932) 99:1323–9. doi: 10.1001/jama.1932.02740680019005
46. Tonelli F, Giudici F, Liscia G. Is lymphatic status related to regression of inflammation in Crohn's disease? *World J Gastrointest Surg*. (2012) 4:228–33. doi: 10.4240/wjgs.v4.i10.228
47. Rahier J-F, Dubuquoy L, Colombel J-F, Jouret-Mourin A, Delos M, Ferrante M, et al. Decreased lymphatic vessel density is associated with postoperative endoscopic recurrence in Crohn's disease. *Inflamm Bowel Dis*. (2013) 19:2084–90. doi: 10.1097/MIB.0b013e3182971cec
48. Kühn R, Löhler J, Rennick D, Rajewsky K, Müller W. Interleukin-10-deficient mice develop chronic enterocolitis. *Cell* (1993) 75:263–74.
49. Spencer DM, Veldman GM, Banerjee S, Willis J, Levine AD. Distinct inflammatory mechanisms mediate early versus late colitis in mice. *Gastroenterology* (2002) 122:94–105. doi: 10.1053/gast.2002.30308
50. Okayasu I, Hatakeyama S, Yamada M, Ohkusa T, Inagaki Y, Nakaya R. A novel method in the induction of reliable experimental acute and chronic ulcerative colitis in mice. *Gastroenterology* (1990) 98:694–702. doi: 10.1016/0016-5085(90)90290-H
51. Chassaing B, Aitken JD, Malleshappa M, Vijay-Kumar M. Dextran sulfate sodium (DSS)-induced colitis in mice. *Curr Protoc Immunol*. (2014) 104:Unit15.25.−15.25.14. doi: 10.1002/0471142735.im1525s104
52. Vyas SP, Goswami R. A Decade of Th9 Cells: Role of Th9 Cells in Inflammatory Bowel Disease. *Front Immunol* (2018) 9:1139. doi: 10.3389/fimmu.2018.01139
53. Wang X, Zhao J, Qin L. VEGF-C mediated enhancement of lymphatic drainage reduces intestinal inflammation by regulating IL-9/IL-17 balance and improving gut microbiota in experimental chronic colitis. *Am J Transl Res*. (2017) 9:4772–84. Available online at: http://www.ajtr.org/files/ajtr0064922.pdf
54. Firestein GS. Evolving concepts of rheumatoid arthritis. *Nature* (2003) 423:356–61. doi: 10.1038/nature01661

55. Jayson MI, Cavill I, Barks JS. Lymphatic clearance rates in rheumatoid arthritis. *Ann Rheum Dis*. (1971) 30:638–9. doi: 10.1136/ard.30.6.638
56. Xu H, Edwards J, Banerji S, Prevo R, Jackson DG, Athanasou NA. Distribution of lymphatic vessels in normal and arthritic human synovial tissues. *Ann Rheum Dis*. (2003) 62:1227–9. doi: 10.1136/ard.2003.005876
57. Zhang Q, Lu Y, Proulx ST, Guo R, Yao Z, Schwarz EM, et al. Increased lymphangiogenesis in joints of mice with inflammatory arthritis. *Arthritis Res Ther*. (2007) 9:R118. doi: 10.1186/ar2326
58. Keffer J, Probert L, Cazlaris H, Georgopoulos S, Kaslaris E, Kioussis D, et al. Transgenic mice expressing human tumour necrosis factor: a predictive genetic model of arthritis. *Embo J*. (1991) 10:4025–31. doi: 10.1002/j.1460-2075.1991.tb04978.x
59. Kouskoff V, Korganow AS, Duchatelle V, Degott C, Benoist C, Mathis D. Organ-specific disease provoked by systemic autoimmunity. *Cell* (1996) 87:811–22. doi: 10.1016/S0092-8674(00)81989-3
60. Bouta EM, Bell RD, Rahimi H, Xing L, Wood RW, Bingham CO, et al. Targeting lymphatic function as a novel therapeutic intervention for rheumatoid arthritis. *Nat Rev Rheumatol*. (2018) 14:94–106. doi: 10.1038/nrrheum.2017.205
61. Proulx ST, Kwok E, You Z, Beck CA, Shealy DJ, Ritchlin CT, et al. MRI and quantification of draining lymph node function in inflammatory arthritis. *Ann NY Acad Sci USA*. (2007) 1117:106–23. doi: 10.1196/annals.1402.016
62. Zhou Q, Wood R, Schwarz EM, Wang Y-J, Xing L. Near-infrared lymphatic imaging demonstrates the dynamics of lymph flow and lymphangiogenesis during the acute versus chronic phases of arthritis in mice. *Arthritis Rheum*. (2010) 62:1881–9. doi: 10.1002/art.27464
63. Proulx ST, Kwok E, You Z, Papuga MO, Beck CA, Shealy DJ, et al. Longitudinal assessment of synovial, lymph node, and bone volumes in inflammatory arthritis in mice by in vivo magnetic resonance imaging and microfocal computed tomography. *Arthritis Rheum*. (2007b) 56:4024–37. doi: 10.1002/art.23128
64. Bouta EM, Ju Y, Rahimi H, de Mesy-Bentley KL, Wood RW, Xing L, et al. Power Doppler ultrasound phenotyping of expanding versus collapsed popliteal lymph nodes in murine inflammatory arthritis. *PLoS ONE* (2013) 8:e73766. doi: 10.1371/journal.pone.0073766
65. Bouta EM, Wood RW, Brown EB, Rahimi H, Ritchlin CT, Schwarz EM. *In vivo* quantification of lymph viscosity and pressure in lymphatic vessels and draining lymph nodes of arthritic joints in mice. *J Physiol*. (2014) 592:1213–23. doi: 10.1113/jphysiol.2013.266700
66. Bouta EM, Kuzin I, de Mesy Bentley K, Wood RW, Rahimi H, Ji R-C, et al. Brief report: treatment of tumor necrosis factor-transgenic mice with anti-tumor necrosis factor restores lymphatic contractions, repairs lymphatic vessels, and may increase monocyte/macrophage egress. *Arthr Rheumatol*. (2017) 69:1187–93. doi: 10.1002/art.40047
67. Manzo A, Caporali R, Vitolo B, Alessi S, Benaglio F, Todoerti M, et al. Subclinical remodelling of draining lymph node structure in early and established rheumatoid arthritis assessed by power Doppler ultrasonography. *Rheumatology* (2011) 50:1395–400. doi: 10.1093/rheumatology/ker076
68. Víteček J, Lojek A, Valacchi G, Kubala L. Arginine-based inhibitors of nitric oxide synthase: therapeutic potential and challenges. *Mediators Inflamm*. (2012) 2012:318087–22. doi: 10.1155/2012/318087
69. Detmar M, Brown LF, Claffey KP, Yeo KT, Kocher O, Jackman RW, et al. Overexpression of vascular permeability factor/vascular endothelial growth factor and its receptors in psoriasis. *J Exp Med*. (1994) 180:1141–6. doi: 10.1084/jem.180.3.1141
70. Kim KE, Koh Y-J, Jeon B-H, Jang C, Han J, Kataru RP, et al. Role of CD11b+ macrophages in intraperitoneal lipopolysaccharide-induced aberrant lymphangiogenesis and lymphatic function in the diaphragm. *Am J Pathol*. (2009) 175:1733–45. doi: 10.2353/ajpath.2009.090133
71. Cursiefen C, Chen L, Borges LP, Jackson D, Cao J, Radziejewski C, et al. VEGF-A stimulates lymphangiogenesis and hemangiogenesis in inflammatory neovascularization via macrophage recruitment. *J Clin Invest* (2004) 113:1040–50. doi: 10.1172/JCI20465
72. Ristimäki A, Narko K, Enholm B, Joukov V, Alitalo K. Proinflammatory cytokines regulate expression of the lymphatic endothelial mitogen vascular endothelial growth factor-C. *J Biol Chem*. (1998) 273:8413–8.
73. Su J-L, Shih J-Y, Yen M-L, Jeng Y-M, Chang C-C, Hsieh C-Y, et al. Cyclooxygenase-2 induces EP1- and HER-2/Neu-dependent vascular endothelial growth factor-C up-regulation: a novel mechanism of lymphangiogenesis in lung adenocarcinoma. *Cancer Res*. (2004) 64:554–64. doi: 10.1158/0008-5472.CAN-03-1301
74. Cha H-S, Bae E-K, Koh J-H, Chai J-Y, Jeon CH, Ahn K-S, et al. Tumor necrosis factor-alpha induces vascular endothelial growth factor-C expression in rheumatoid synoviocytes. *J Rheumatol*. (2007) 34:16–9. Available online at: http://www.jrheum.org/content/34/1/16.long
75. Chauhan SK, Jin Y, Goyal S, Lee HS, Fuchsluger TA, Lee HK, et al. A novel pro-lymphangiogenic function for Th17/IL-17. *Blood* (2011) 118:4630–4. doi: 10.1182/blood-2011-01-332049
76. Choi I, Lee YS, Chung HK, Choi D, Ecoiffier T, Lee HN, et al. Interleukin-8 reduces post-surgical lymphedema formation by promoting lymphatic vessel regeneration. *Angiogenesis* (2013) 16:29–44. doi: 10.1007/s10456-012-9297-6
77. Oka M, Iwata C, Suzuki HI, Kiyono K, Morishita Y, Watabe T, et al. Inhibition of endogenous TGF-beta signaling enhances lymphangiogenesis. *Blood* (2008) 111:4571–9. doi: 10.1182/blood-2007-10-120337
78. Avraham T, Daluvoy S, Zampell J, Yan A, Haviv YS, Rockson SG, et al. Blockade of transforming growth factor-beta1 accelerates lymphatic regeneration during wound repair. *Am J Pathol*. (2010) 177:3202–14. doi: 10.2353/ajpath.2010.100594
79. Savetsky IL, Ghanta S, Gardenier JC, Torrisi JS, García Nores GD, Hespe GE, et al. Th2 cytokines inhibit lymphangiogenesis. *PLoS ONE* (2015) 10:e0126908. doi: 10.1371/journal.pone.0126908
80. Kataru RP, Kim H, Jang C, Choi DK, Koh BI, Kim M, et al. T lymphocytes negatively regulate lymph node lymphatic vessel formation. *Immunity* (2011) 34:96–107. doi: 10.1016/j.immuni.2010.12.016
81. Rehal S, Blanckaert P, Roizes S, von der Weid, PY. (2009). Characterization of biosynthesis and modes of action of prostaglandin E2 and prostacyclin in guinea pig mesenteric lymphatic vessels. *Br. J. Pharmacol*. 158, 1961–1970. doi: 10.1111/j.1476-5381.2009.00493.x
82. Aldrich MB, Sevick-Muraca EM. Cytokines are systemic effectors of lymphatic function in acute inflammation. *Cytokine* (2013) 64:362–9. doi: 10.1016/j.cyto.2013.05.015
83. Cromer WE, Zawieja SD, Tharakan B, Childs EW, Newell MK, Zawieja DC. The effects of inflammatory cytokines on lymphatic endothelial barrier function. *Angiogenesis* (2014) 17:395–406. doi: 10.1007/s10456-013-9393-2
84. Nagy JA, Vasile E, Feng D, Sundberg C, Brown LF, Detmar MJ, et al. Vascular permeability factor/vascular endothelial growth factor induces lymphangiogenesis as well as angiogenesis. *J Exp Med*. (2002) 196:1497–506. doi: 10.1084/jem.20021244
85. Zhang Y, Lu Y, Ma L, Cao X, Xiao J, Chen J, et al. Activation of vascular endothelial growth factor receptor-3 in macrophages restrains TLR4-NF-κB signaling and protects against endotoxin shock. *Immunity* (2014) 40:501–14. doi: 10.1016/j.immuni.2014.01.013
86. Polzer K, Baeten D, Soleiman A, Distler J, Gerlag DM, Tak PP, et al. Tumour necrosis factor blockade increases lymphangiogenesis in murine and human arthritic joints. *Ann Rheum Dis*. (2008) 67:1610–6. doi: 10.1136/ard.2007.083394
87. Hos D, Saban DR, Bock F, Regenfuss B, Onderka J, Masli S, et al. Suppression of inflammatory corneal lymphangiogenesis by application of topical corticosteroids. *Arch Ophthalmol*. (2011) 129:445–52. doi: 10.1001/archophthalmol.2011.42
88. Yao L-C, Baluk P, Feng J, McDonald DM. Steroid-resistant lymphatic remodeling in chronically inflamed mouse airways. *Am J Pathol*. (2010) 176:1525–41. doi: 10.2353/ajpath.2010.090909
89. Iwata C, Kano MR, Komuro A, Oka M, Kiyono K, Johansson E, et al. Inhibition of cyclooxygenase-2 suppresses lymph node metastasis via reduction of lymphangiogenesis. *Cancer Res*. (2007) 67:10181–9. doi: 10.1158/0008-5472.CAN-07-2366
90. Jamieson T, Cook DN, Nibbs RJB, Rot A, Nixon C, McLean P, et al. The chemokine receptor D6 limits the inflammatory response *in vivo*. *Nat Immunol*. (2005) 6:403–11. doi: 10.1038/ni1182
91. Vetrano S, Borroni EM, Sarukhan A, Savino B, Bonecchi R, Correale C, et al. The lymphatic system controls intestinal inflammation and inflammation-associated Colon Cancer through the chemokine decoy receptor D6. *Gut* (2010) 59:197–206. doi: 10.1136/gut.2009.183772

92. Karaman S, Hollmén M, Yoon SY, Alkan HF, Alitalo K, Wolfrum C, et al. Transgenic overexpression of VEGF-C induces weight gain and insulin resistance in mice. *Sci Rep*. (2016) 6:31566. doi: 10.1038/srep31566
93. Karaman S, Hollmén M, Robciuc MR, Alitalo A, Nurmi H, Morf B, et al. Blockade of VEGF-C and VEGF-D modulates adipose tissue inflammation and improves metabolic parameters under high-fat diet. *Mol Metab*. (2015) 4:93–105. doi: 10.1016/j.molmet.2014.11.006
94. Skobe M, Hamberg LM, Hawighorst T, Schirner M, Wolf GL, Alitalo K, et al. Concurrent induction of lymphangiogenesis, angiogenesis, and macrophage recruitment by vascular endothelial growth factor-C in melanoma. *Am J Pathol*. (2001) 159:893–903. doi: 10.1016/S0002-9440(10)61765-8
95. Hirakawa S, Brown LF, Kodama S, Paavonen K, Alitalo K, Detmar M. VEGF-C-induced lymphangiogenesis in sentinel lymph nodes promotes tumor metastasis to distant sites. *Blood* (2007) 109:1010–7. doi: 10.1182/blood-2006-05-021758
96. Schwager S, Renner S, Hemmerle T, Karaman S, Proulx ST, Fetz R, et al. Antibody-mediated delivery of VEGF-C potently reduces chronic skin inflammation. *JCI Insight* (2018) 3:1983. doi: 10.1172/jci.insight.124850

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REVIEW
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Lymph Node Subcapsular Sinus Macrophages as the Frontline of Lymphatic Immune Defense

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Lymphatic vessels collect and transport lymph and pathogens to the draining lymph node (LN) to generate proper immune protection. A layer of macrophages that strategically line the LN subcapsular sinus (SCS) is directly exposed to the afferent lymph and are denoted as SCS macrophages. These macrophages are the frontline of immune defense that interact with lymph-borne antigens. The importance of these macrophages in limiting the spread of pathogens has been demonstrated in both viral and bacterial infection. In anti-microbial responses, these macrophages can directly or indirectly activate other LN innate immune cells to fight against pathogens, as well as activate T cells or B cells for adaptive immunity. As the first layer of immune cells embracing the tumor-derived antigens, SCS macrophages also actively participate in cancer immune regulation. Recent studies have shown that the LNs' SCS macrophage layer is interrupted in disease models. Despite their importance in fighting the spread of pathogens and in activating anti-tumor immunity, the mechanism and the immunological functional consequences for their disruption are not well-understood. Understanding the mechanism of these macrophages will enhance their capability for therapeutic targeting.

Keywords: subcapsular sinus macrophage, CD169, lymph node (LN), free-floating antigens, virus, bacteria, cancer

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INTRODUCTION

The lymphatic system consists of two major parts: lymphatic vessels and lymph nodes (LNs). Lymphatic vessels are present throughout the body, acting as a road map for immune surveillance. These vessels are responsible for collecting interstitial fluid, soluble proteins, peptides, metabolites, invading pathogens, and immune cells in the tissue, then transporting the collected contents to the draining LNs via afferent lymphatic vessels (1–3). Initial lymphatic vessels (also named lymphatic capillaries) have discontinuous junction molecules which are highly permeable, and permit easy access of fluid and other content from peripheral tissues (4). Initial lymphatic vessels congregate to contractile lymphatic vessels, also known as collecting lymphatic vessels. Collecting lymphatic vessels direct lymph to the LN. Once in the LN, free-floating antigens, migrating antigen-presenting cells, and resident LN immune cells meet to initiate immune activation. After immune surveillance in the LN, efferent lymphatic vessels return lymph and activated immune cells to the circulation in order to enter the site of pathogen invasion for immune protection.

The transport of tissue-originated antigen-loaded antigen-presenting cells via lymphatic vessels has been largely studied. Migrating dendritic cells enter lymphatic vessels through the portals formed by the discontinuous basement membrane between adjacent endothelial cells, and is

dependent on CCR7 expression on dendritic cells (DC) and chemokines CCL19 and CC21 expressed on lymphatic endothelial cells (5–8). However, not all antigens transported in lymphatics are loaded on dendritic cells. Some free-floating lymph-borne antigens can travel with lymph to the LN. The importance of how LN-resident antigen-presenting cells react to free-floating antigens in lymph has been gaining more interest in the past decade. As lymph enters the LN, fluid fills the sinus lumen. Lining the floor of the sinuses are sinus macrophages that directly embrace the lymph coming from the afferent lymphatic vessels (**Figure 1**). These macrophages sample the free-floating antigens in the afferent lymph within several minutes after administration of model antigen tracers or pathogens (9, 10). Larger molecules and particles, such as viruses and bacteria, are captured by sinus macrophages (11–15) and sinus DCs (16). Smaller antigens, such as ovalbumin (OVA), can be captured by sinus macrophages and DCs. Additionally, smaller antigens can enter the LN conduits and are sampled by the LN conduit-associated DCs (17, 18). The first wave of DC activation occurs several hours before tissue-originated antigen-bearing DCs enter the LN, acting as another layer of protection in the event pathogens evade detection at the site of invasion (18–21). In fact, even in the absence of tissue-migrating antigen-presenting cells, the LN-resident antigen-presenting cells are capable of generating a protective immune response against invading pathogens (16, 22, 23). Therefore, LN sinus resident macrophages function as a frontline of immune protection to lymph-borne pathogens.

During cancer lymphatic metastasis, metastatic tumor cells and tumor-derived antigens travel through lymphatic vessels to the tumor draining lymph node. Metastatic tumor cells were observed to first accumulate at the subcapsular sinus (24). LN metastatic tumor cells can invade the LN blood vessels as early as 2 days post-injection and spread to distant organs from the tumor draining LN (25, 26). Subcapsular sinus macrophages are the first layer of immune cells that are exposed to the metastatic tumor cells and tumor-derived antigens coming from the afferent lymphatic vessels. Studies in this field can reveal exciting new prospects when it comes to developing cancer immunotherapy. We reviewed the literature on how these macrophages are responsible for activating an immune response to the invading pathogens or tumor-derived antigens, as well as how the interruption of these macrophages in the LN is associated with disease.

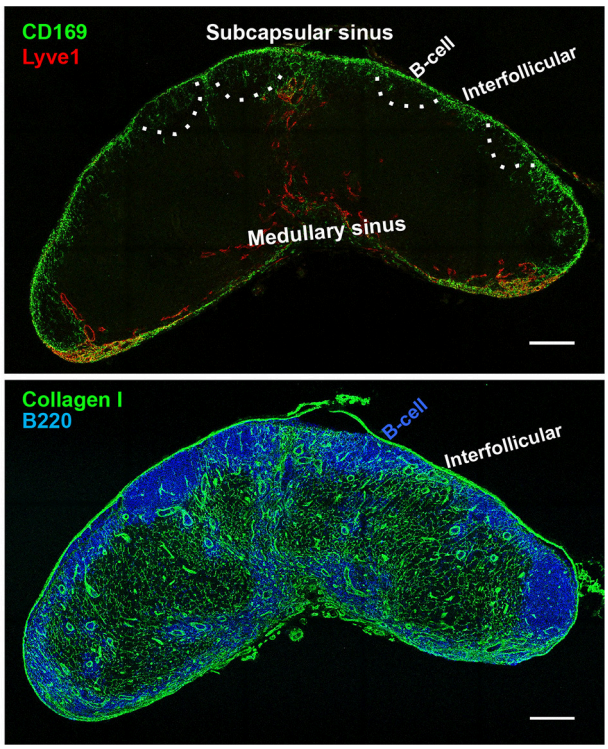

FIGURE 1 | Lymph node sinus macrophages. Confocal microscope image of a wild-type inguinal lymph node at 20× magnification. **(A)** Lymph node subcapsular sinus (SCS) and the medullary sinus (MS) are distinguished by the morphology of lymphatic endothelial cells (Lyve-1, red). $CD169^+$ macrophages are concentrated in the SCS, with much sparser distribution in the MS (CD169, green). B-cell zones are indicated by dashed lines according to the staining using serial section in **(B)**. **(B)** Underneath the SCS macrophages are the B cell follicles (B220). Between the B cell follicles are the interfollicular zones which contain collagen I^+ conduits. SCS macrophages are restricted in the SCS, but invade slightly deeper into the LN parenchyma at the interfollicular zone (Collagen I, green). Scale bars, 200 μm.

LN SINUS MACROPHAGES

Sinus macrophages are not uniform across the entire LN; they can be subdivided into two major populations: the subcapsular sinus (SCS) macrophages and the medullary sinus macrophages according to their anatomical location in the LN (**Figure 1**). There are also sinus dendritic cells that sparsely populate the subcapsular sinus. Functionally, both sinus macrophages and DCs can acquire pathogen or particles from the passing lymph in the SCS. The sinus macrophages differ phenotypically, as SCS macrophages express Mac1 (CD11b/CD18), Siglec-1 (CD169), but lack the expression of F4/80, a murine macrophage marker (27). On top of that, a small proportion of the SCS $CD169^+$ cells are $CD169^+CD11c^+$, indicating their DC phenotype (12, 16). Yet researchers still title these cells as macrophages, because, despite lacking the common F4/80 murine macrophage marker, SCS macrophage differentiation depends on the "macrophage colony-stimulating factor" cytokine, also known as CSF-1 (27–29). On the other hand, the phenotype of medullary sinus macrophages is more indicative of their macrophage characterization as they express F4/80 and Mac1. Some of the medullary sinus macrophages also express CD169 at a relatively lower level, therefore SCS macrophages are specifically distinguished as $CD169^+F4/80^-$, while medullary sinus macrophages are $CD169^+F4/80^+$ or $CD169^{low/-}F4/80^+$ (9).

Sinus macrophages also differ from each other functionally. Classically activated macrophages, known as M1 macrophages, typically produce pro-inflammatory cytokines, mediate pathogen resistance, and contribute to tissue destruction (30). This largely

describes the medullary sinus macrophages, given their high lysozyme content and ability to process antigens, but no evidence has been shown for their capability to produce pro-inflammatory cytokines (31, 32). In contrast, SCS macrophages show relatively low phagocytic activity, but have demonstrated the ability to produce pro-inflammatory cytokines, namely type I interferon's (27, 33, 34). Therefore, while both sinus macrophages exhibit components of M1 macrophage function, a consensus on their categorization has yet to be reached in the field.

The origin and development of SCS macrophages has been studied to better understand their function. As stated earlier, the CSF-1/CSF-1 receptor signaling interaction is pivotal for the presence of SCS macrophages. Transgenic mice with a recessive osteopetrotic mutation (op/op) demonstrate a CSF-1 deficiency and show a significant reduction in SCS macrophages. Similarly, anti-CSF-1 receptor treatment to block the CSF-1 ligand from binding to CSF-1 receptor significantly depleted SCS macrophages, while medullary sinus macrophages remained intact (28). However, while medullary sinus macrophages are unaffected by blocking CSF-1/CSF-1 receptor interaction, CSF-1 receptor deficient mice show a significant depletion of $F4/80^+$ macrophages, indicating the requirement of CSF-1 receptor activation for $F4/80^+$ macrophage development (29). In addition to CSF-1, SCS macrophages appear to need the lymphotoxin signal for their development. Lymphotoxin receptor LTβR is shown to be present on the surface of both SCS macrophages and medullary sinus macrophages, however chimeric mice lacking the LTβR (*ltbr*$^{-/-}$) only show a deficiency in SCS macrophages (27). The activation of LTβR on SCS macrophages largely depends on $LT\alpha_1\beta_2$, the ligand for LTβR, present on LN B cells that are located just underneath the SCS in the LN. μMT mice, which lack mature B cells in the LN, show significantly fewer macrophages with the SCS phenotype ($CD169^+F4/80^-$) and an abundance of the medullary sinus phenotype ($CD169^+F4/80^+$) (34). Furthermore, by ablating lymphotoxin signaling with LTβR-Ig, a soluble lymphotoxin receptor that blocks downstream signaling, a similar deficiency in the SCS macrophage phenotype can be found in wild-type mice as the μMT mice. Medullary sinus macrophages appeared unaffected by lymphotoxin signaling blockade (34). Based on these observations, while medullary sinus macrophages rely on CSF-1 receptor signaling for their development, SCS macrophages require CSF-1 receptor and LTβR for their development and the maintenance of their phenotype.

SCS MACROPHAGES PREVENT LYMPH-BORNE PATHOGEN SYSTEMIC SPREADING

Because SCS macrophages directly embrace pathogenic particles arriving from afferent lymphatic vessels, SCS macrophages have been widely studied in antimicrobial immunity, including anti-viral and anti-bacterial responses (**Figure 2A**). Studies on the function of SCS macrophages has first been demonstrated in preventing virus from spreading from the LN to the blood circulation or other organs after subcutaneous infection. Multiphoton intravital microscopy showed $CD11b^+CD169^+MHCII^+$ macrophages located on the floor of the popliteal SCS functioning as a "flypaper" to capture fluorescently labeled vesicular stomatitis virus (VSV) particles after a subcutaneous injection at the footpad (11). This observation extends to different viruses, such adenovirus, vaccinia virus and murine cytomegalovirus (MCMV), as luciferase-labeled MCMV is limited to the LN for several days before spreading systemically (11, 35). Artificially depleting the SCS macrophages prior to VSV challenge led to a significant reduction in animal survival and a marked increase in viral titers found in the brain and spinal cord (33).

The "flypaper" function of SCS macrophages is also applicable to lymph-borne bacteria. Fluorescently labeled *Pseudomonas aeruginosa*, an extracellular bacterium, was found in the LN parenchyma and blood 8 h post-injection when the macrophages were depleted, while bacteria were limited to the SCS when the macrophage layer was intact (36). More specifically, lipid antigens, such as lipopolysaccharide found on bacteria, has also been shown to localize with the SCS macrophages (37). This further defines the "flypaper" function of the SCS macrophages as it is not only preventing a systemic spread, but specifically limits pathogens to the SCS in the LN. Restricting pathogens to the SCS is at least partially achieved by the expression of CD169 on the macrophages, as CD169 interacts with α2,3-linked sialic acids expressed on the surface of cells or microbes. Biotinylated exosomes specifically bound to SCS macrophages on tissue sections while biotinylated bovine serum did not, suggesting the $CD169^+$ macrophages retain extracellular vesicles and microbes rather than free flow proteins at the sinus (38).

However, evident by their minimal phagocytosis function and failure to adequately process the self-quenching DQ Green probe, SCS macrophages are poorly phagocytic and cannot clear the microbes directly (27). Instead, these macrophages ensure enough immune stimulation by supporting replication of captured pathogens. Fluorescently labeled VSV was robustly replicated in wild-type LNs, while mice lacking the SCS macrophages showed no virus replication (33, 34). Without the immune protection generated by this layer of macrophages, viruses may invade deeper into the parenchyma and infect LN neurons or LN fibroblasts, and eventually disseminate into other organs (33, 35). The mechanism of SCS restricting virus spreading does not apply to all types of viral infection. Capture of the influenza virus alternatively depends on medullary sinus DCs to generate durable B cell responses (39). Like the subcapsular sinus macrophages, medullary sinus macrophages recruit additional immune cells to clear their targeted pathogen. While both SCS macrophages and medullary sinus macrophages demonstrate early activation marker CD69 to UV-inactivated influenza virus, medullary sinus macrophages have been shown to be preferentially activated through the secretion of IFN-β, which further induces IL-1α expression, leading to the expression of dendritic cell and monocyte chemoattractant, MCP-1 (40). Understanding the mechanism of how influenza virus escape SCS macrophages and alternatively activate medullary sinus macrophages may help influenza vaccine design.

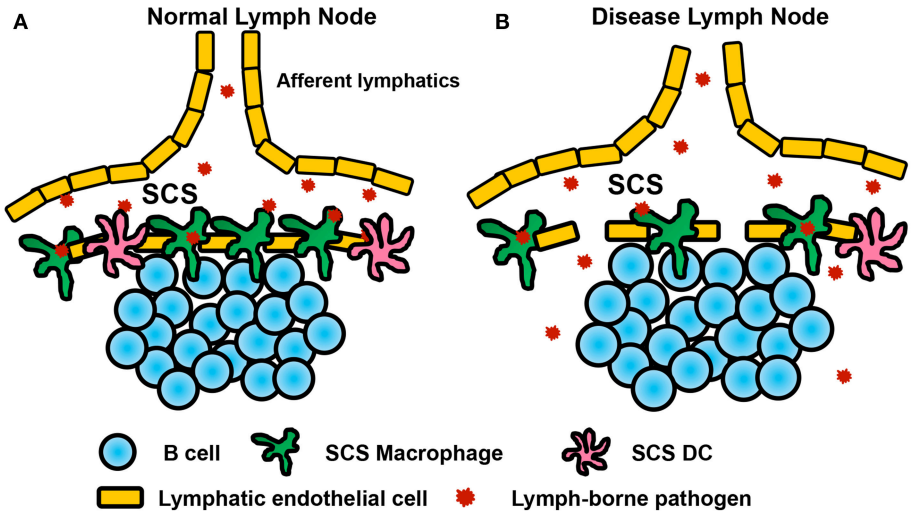

FIGURE 2 | Function of the subcapsular sinus macrophage layer in normal and inflamed lymph nodes. **(A)** Lymph-borne free floating particles and pathogens travel with lymph and enter the lymph node subcapsular sinus via the afferent lymphatics. Subcapsular sinus macrophages are the first layer of cells in the draining lymph node that capture and retain lymph-borne pathogens from entering the lymph node parenchyma likely via the interaction between CD169 and its ligand, α2,3-linked sialic acids, expressed on the surface of cells or microbes. After pathogen capture, SCS macrophages can relay the antigen to B cells just underneath the SCS to prime B cell and humoral responses. SCS macrophage activation produces different types of cytokines to recruit and communicate with other immune cells, such as NK cells, γδ T cells, non-classical $CD8^+$ T cells, neutrophils, monocytes, T cells etc. to combat the invading pathogens. The SCS macrophage layer prevents pathogen from invading the lymph node parenchyma or systemic spreading. **(B)** In an inflamed LN during diseased condition, the SCS macrophage layer is interrupted, allowing pathogen to invade the lymph node parenchyma or systemic spreading. The immunological consequence of disrupting SCS macrophage appears contraversial in different types of infection or in cancer progression. The reason behind SCS macrophage layer disruption remains unclear as well.

Once activated, SCS macrophages function by communicating with other LN resident lymphocytes or recruiting other cells to the SCS to provide rapid and robust anti-microbial responses to lymph-borne antigens. As B cells reside directly underneath the SCS macrophage layer, the early studies exploring the function of SCS macrophages identified that activating these cells attract B cells from the follicles to the SCS. SCS macrophages then relay captured antigens to B cells using a complement-dependent and -independent pathway (41). Multiphoton intravital microscopy visualized the accumulation of virus serotype-specific B cells at the SCS depending on the virus challenge, indicating their migration is highly selective (11, 42). Co-stimulatory molecule CD86 was upregulated and B cell receptors were internalized within 6 h after virus challenge, indicative of the activation of B cells. B cell activation after viral challenge failed in the LN when SCS macrophages were depleted with clodronate liposome (CLL). After the early activation, B cells migrate to the boundary between the B and T cell zones of the LN (43). Here, an interaction occurs between the primed B cell and the helper T cells, causing a proliferation of B cells and germinal center formation. Upon macrophage depletion, the antiviral B cells remained spread in the LN and take a much longer time to migrate to T-B cell border. However, depleting the macrophage layer only delayed rather than completely prevented B cell activation or humoral responses, raising the question about the exact function of these cells (11).

Antigen challenge also recruits innate immune cells to the LN SCS. In the case of lipid antigens, α-galactosylceramide was used to coat 200 nm silica particles to stimulate immune cell activation. iNKT cells migrate toward the SCS and are arrested within a few hours. Three days post-injection, the LNs had been inflamed and the number of iNKT cells present in the LN were 10-fold higher than normal (37). Modified vaccinia virus Ankara, a viral vector, was shown to induce NK cell motility and transition from the interfollicular zone and outer T cell zone to the SCS. Depleting the SCS macrophages with CLL reduced the NK cell accumulation and activation normally triggered by virus challenge (44). Similar to the viral challenge, parasite infection with *Toxoplasma gondii* causes NK cell accumulation in the SCS. However, depletion of SCS macrophage with CLL did not reduce the proportion of NK cells, only suppressed NK cell activation during *T. gondii* infection (45). Parasitic challenges such as QS-21, an adjuvant component of malaria, colocalized with SCS macrophages. Depleting these macrophages using CLL reduced monocyte, neutrophil, and dendritic cell recruitment to the draining LN (46). However, while neutrophil recruitment to the LN occurred in response to *Staphylococcus aureus* infection, SCS macrophage depletion via CLL did not change neutrophil movement to SCS (47). Based on these results, it is apparent that SCS macrophage activation recruits and activates different types of immune cells to the SCS when responding to different types of lymph-borne microbes.

SCS macrophages appear to produce different types of cytokines to aid in their function, which potentially explains the different immune cell recruitment against lymph-borne pathogens. In response to lymph-borne virus pathogens, infected SCS macrophages produce interferon-α. Activated SCS macrophages additionally recruit plasmacytoid DCs to the SCS

to express type I interferon to initiate anti-viral immunity (33). Mice lacking the SCS macrophages show lower levels of both interferon-α and interferon-β mRNA levels in their LNs, which is correlated with a lower survival rate after viral challenge (34). In the case of bacterial infection, the number of interferon-γ producing lymphoid cells increased 4 h after subcutaneous *P. aeruginosa* infection (36). SCS macrophages are not necessarily the only cell population to express interferon-γ, as the NLR dependent inflammasome activation in these cells enhances other lymphoid cells, such as NK cells, γδ T cells, and non-classical $CD8^+$ T cells, to produce interferon-γ. Absence of the SCS macrophages significantly reduces cytokine production and limits the recruitment of important innate immune cells to restrict bacteria spread (36). This could also adversely affect the initiation of the adaptive immunity as inflammasome activation induces an influx of innate immune cells and T cells (48).

INTERRUPTION OF SCS MACROPHAGES IN DISEASES

While the early activation of SCS macrophages has been studied extensively, disease models pose a different perspective. It appears that pathogen-induced inflammation can disrupt the SCS macrophage layer (**Figure 2B**). Recruitment of neutrophils or NK cells appear to interrupt the SCS macrophage layer, but the mechanism and the function of SCS macrophage dissociation from the SCS remains unclear (45, 49). Using either CpG or LPS, the SCS macrophages were able to dissociate from the SCS, leading to disrupted protective layer. The observed macrophage dissociation appears to be CCR7 dependent, as CCR7-deficient animals seem resistant to inflammation-induced SCS macrophage disruption. Transferring activated bone marrow derived DCs to the LN is sufficient to disrupt SCS macrophage layer, indicating DC activation may cause SCS macrophages to dissociate from the SCS. However, since a subpopulation of $CD169^+$ macrophages are $CD11c^+$, whether the CCR7-dependent SCS macrophage dissociation is only restricted to $CD169^+CD11c^+$ cell or all SCS $CD169^+$ cells remains unclear. The mechanism of SCS macrophage dissociation from the SCS remains to be clarified. When SCS macrophages were dissociated during inflammation, B cells were incapable of receiving the antigen and showed diminished activation as measured by germinal center formation and immunoglobulin production (50). These results appear to contradict experimental SCS macrophage depletion, where only early cognate B cell migration to SCS or to the border of T cell and B cell zone is affected, but does not prevent total B cell activation (11, 15). Influenza vaccination is capable of inducing lymph node subcapsular sinus and medullary sinus macrophage necrosis 12 h post-injection. However, the necrosis was independent of neutrophil or NK cell recruitment. Virus challenge activated TLR7 and Myd88, causing necrosis of the subcapsular sinus, but not the medullary sinus, macrophages (40).

Recent interest has sparked over these macrophages in the context of anti-tumor immunity. LN metastases are a key component in patient prognosis. Metastatic tumor cells present in the sentinel lymph node were able to spread systemically via the lymph node blood vessels or the efferent lymphatic vessels (25, 26). SCS macrophages in the tumor draining LN directly interacts with metastatic tumor cells or tumor-derived antigens coming from the afferent lymphatic vessels. The idea that SCS macrophages can limit the spread of cancer, similar to how they limit the spread of lymph-borne microbes, has developed into a relatively new field of study. Clinical studies have determined a correlation between $CD169^+$ macrophage density in human sentinel LNs and a favorable tumor prognosis. Consistent between multiple different types of tumors, indicators for the favorable prognosis often include a lower number of LN metastases and increased $CD8^+$ T cell tumor infiltration, reflecting the SCS macrophages' functions of limiting cancer spread and immune activation (51–53). To activate the antitumor $CD8^+$ T cell response, SCS macrophages are capable of capturing irradiated tumor cells. Like microbes, subcutaneously injected apoptotic tumor cells travel to the LN and are captured mainly by $CD169^+$ macrophages. Then, activated SCS macrophages recruit and prime anti-tumor $CD8^+$ T cells at the SCS. Mice with their $CD169^+$ cells depleted in a CD169-DTR model were incapable of activating $CD8^+$ T cells or rejecting tumor cells after a vaccination with irradiated tumor cells (12).

However, a growing tumor and its complex tumor microenvironment significantly changes the function of SCS macrophages. Instead of capturing tumor cells as seen with injected irradiated tumor cells, growing melanoma tumors deposit tumor-derived antigens into B cell follicles in patients (54). The accumulation of fluorescent tumor-derived antigen in the follicular dendritic cells in the germinal centers was observed using B16F10 melanoma. Depletion of the SCS macrophages ablated tumor-derived antigen accumulation in the follicular dendritic cells, demonstrating the necessity for SCS macrophages in depositing tumor-derived antigens into the B cell follicle (54). However, a recent publication has shown a contrasting observation; depletion of SCS macrophages increases tumor-derived exosome penetration deep into the B cell follicles and enhances B cell activation as measured by plasma immunoglobulin levels (15). In this study, growing tumors appear to disrupt the SCS macrophages in the tumor-draining LN and permits tumor-derived exosome entry into the B cell follicle. The increase in B cell response was correlated with a larger tumor size, suggesting the SCS macrophages are necessary to limit a pro-tumor B cell response (15). Because of these contradicting studies, further studies are required to reveal the function of SCS macrophage in anti-tumor immunity in the tumor-draining LN.

In contrast to the disruption of the macrophage layer seen in microbial infection, inflammation, or melanoma mentioned above, a recent study showed that inflammatory bowel disease increases the $CD11b^+CD169^+$ macrophages in the draining mesenteric LN. Depletion of the $CD169^+$ macrophages in a CD169-DTR model showed reduced symptoms of inflammation, indicating that these macrophages promoted inflammation in the inflammatory bowel disease model (55). Whether these changes depend on the anatomical location of the LN or the disease models remains to be investigated.

CONCLUSION AND PROSPECTIVE

The lymphatic system collects invading bacterial and viral pathogens and drains them to the LN for efficient processing and clearance. In this process, the LN sinus macrophages are among the first immune cells that interact with lymph-borne pathogens. With the evidence from different models, it is clear that SCS macrophages are essential for the response against lymph-borne pathogens. Unlike typical macrophages, the SCS macrophages are incapable of breaking down pathogens. The SCS macrophages appear to diversify its ability to target and initiate specific immune responses to a variety of lymph-borne pathogens by relaying antigens to B cells, producing cytokine signaling cascades to cause influx of dendritic cells, neutrophils,

TABLE 1 | Summary of SCS macrophages in different studies.

Model	Cytokines	Recruited cells	SCS macrophages	Depletion method	References
VIRUSES					
VSV	IFN-α, IFN-I	B cells, Plasmacytoid dendritic cells	–	CLL CD11c-DTR	(11, 33, 34)
Adenovirus	–	B cells	–	CLL	(11)
MCMV	–	–	–	CLL CD169-DTR	(35)
Influenza virus	IL-1α IFN-β	B cells NK cells Neutrophils	*MS DCs,* *MS macrophages* Necrosis	CLL CD169-DTR	(39, 40)
CpG	–	Dendritic Cells B cells	Dissociation, CCR7 dependent migration	CLL	(50)
BACTERIA					
Pseudomonas aeruginosa	IFN-γ IL-18 IL-1β	NK cells γδ T cells NKT cells αβ TCR CD8+ T cells Neutrophils	–	CLL	(36)
Staphylococcus aureus	C5aR	Neutrophils	Dissociation	CLL	(47, 50)
Lipid antigens (α-galactosylceramide coated on silica particles)	CD1d IL-2 IFN-γ	*i*NKT cells	Dissociation	CLL	(37, 50)
Glycolipids (α-linked galacturonic glycosphingolipid on silica particles)	–	*i*NKT cells	–	CLL	(37)
LPS	–	Dendritic Cells B cells	Dissociation, CCR7 dependent migration	CLL	(50)
PARASITES					
Toxoplasma gondii	IFN-γ	NK cells	Dissociation	CLL	(45, 49)
QS-21 (Malaria component)	IL-1β	Monocytes Neutrophils Eosinophils Dendritic cells		CLL	(46)
CANCER					
Exosomes	–	CD4+ T cells CD8+ T cells	–	$CD169^{-/-}$	(38, 56)
Irradiated tumor cells	IFN-γ	CD8+ T cells		CD169-DTR	(12)
Melanoma and melanoma-derived exosomes	–	Follicular DCs B cells	Dissociation	CLL CD169-DTR	(15, 54)
OTHER DISEASES					
Colitis	IL-17, IL-21, IL-23, IL-6, IL-1β, TNFα, IL-12, IL-18, CCL8, CCL3	Th17 cells	Increase	CD169-DTR	(55)

NK cells, or in some conditions, presenting antigens to T cells. Using cytokine production and immune cell recruitment, SCS macrophages can mount an early immune response against free-floating pathogens and prevent their LN invasion or systemic spreading. While there is a consensus that SCS macrophages limit the systemic dissemination of pathogens, there does not appear to be a universal mechanism for their action (**Table 1**). The requirement of SCS macrophages appear to be more critical for innate immunity, since depletion of SCS macrophages allow pathogens to escape the draining LN and spread systemically, adversely affecting survival rate. Surprisingly, although SCS macrophages appears critical to relay antigens to B cells, artificial depletion of SCS macrophages did not substantially interrupt the overall anti-microbial adaptive immune responses, except several hours of delay in the induction of adaptive immunity.

Several studies have shown infection induces SCS macrophage dissociation from the SCS (**Figure 2**). CpG or LPS induces SCS macrophage migration deeper into the LN parenchyma, which impairs B cell responses to a secondary infection (50). Tumor progression induces SCS macrophage dissociation from the SCS in the tumor draining lymph node and results in B cell activation and tumor growth (15). Why the effect of dissociated SCS macrophages on subsequent immune protection appears contradictory between infectious diseases and cancer progression remains unclear. As it is now clear that inactivated influenza virus causes macrophage necrosis, one interpretation could be that challenge of microbes or materials mimicking microbial products causes more severe damage to SCS macrophages when compared to tumor derived antigens. Another possibility is that microbial product challenge lasts several hours, while infection or tumors may continuously deliver antigens for several days. Additionally, disease induced SCS macrophage dissociation also differs from the artificial SCS macrophage depletion as the former did not complete abrogate the SCS macrophage layer and some of these macrophages are relocated deeper into the LN parenchyma. Most studies use CLL and/or diphtheria toxin (DT) in a CD169-DTR to deplete SCS macrophages (**Table 1**) and both may cause off-target cell death. Additionally, the induced cell death may impact the function of immune cells in the LN. Thus, the mechanisms that cause SCS macrophage dissociation would substantially impact the immune protection to a subsequent challenge, such as secondary infection or continuous tumor-derived antigen delivery. More studies are required to understand why the SCS macrophages leave their position after stimulation and what is the immunological consequence of SCS macrophage dissociation from the SCS.

Currently, the mechanisms of how SCS macrophages participate in fighting against lymph-borne pathogens are better studied. The role of SCS macrophages in anti-tumor immunity in the tumor draining LN is still young. The collective literature in anti-microbial studies suggest future studies center around how SCS macrophage communication with other immune cells at different stages of tumor progression could provide pivotal insights into the development of immunotherapy.

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All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REFERENCES

1. Drayton DL, Liao S, Mounzer RH, Ruddle NH. Lymphoid organ development: from ontogeny to neogenesis. *Nat Immunol.* (2006) 7:344–53. doi: 10.1038/ni1330
2. Liao S, von der Weid PY. Lymphatic system: an active pathway for immune protection. *Semin Cell Dev Biol.* (2014) 38:83–9. doi: 10.1016/j.semcdb.2014.11.012
3. Randolph GJ, Ivanov S, Zinselmeyer BH, Scallan JP. The lymphatic system: integral roles in immunity. *Annu Rev Immunol.* (2017) 35:31–52. doi: 10.1146/annurev-immunol-041015-055354
4. Baluk P, Fuxe J, Hashizume H, Romano T, Lashnits E, Butz S, et al. Functionally specialized junctions between endothelial cells of lymphatic vessels. *J Exp Med.* (2007) 204:2349–62. doi: 10.1084/jem.20062596
5. Pflicke H, Sixt M. Preformed portals facilitate dendritic cell entry into afferent lymphatic vessels. *J Exp Med.* (2009) 206:2925–35. doi: 10.1084/jem.20091739
6. Nitschke M, Aebischer D, Abadier M, Haener S, Lucic M, Vigl B, et al. Differential requirement for ROCK in dendritic cell migration within lymphatic capillaries in steady-state and inflammation. *Blood.* (2012) 120:2249–58. doi: 10.1182/blood-2012-03-417923
7. Forster R, Schubel A, Breitfeld D, Kremmer E, Renner-Muller I, Wolf E, et al. CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs. *Cell.* (1999) 99:23–33. doi: 10.1016/S0092-8674(00)80059-8
8. Ohl L, Mohaupt M, Czeloth N, Hintzen G, Kiafard Z, Zwirner J, et al. CCR7 governs skin dendritic cell migration under inflammatory and steady-state conditions. *Immunity.* (2004) 21:279–88. doi: 10.1016/j.immuni.2004.06.014
9. Gray EE, Cyster JG. Lymph node macrophages. *J Innate Immun.* (2012) 4:424–36. doi: 10.1159/000337007
10. Kuka M, Iannacone M. The role of lymph node sinus macrophages in host defense. *Ann N Y Acad Sci.* (2014) 1319:38–46. doi: 10.1111/nyas.12387
11. Junt T, Moseman EA, Iannacone M, Massberg S, Lang PA, Boes M, et al. Subcapsular sinus macrophages in lymph nodes clear lymph-borne viruses and present them to antiviral B cells. *Nature.* (2007) 450:110–14. doi: 10.1038/nature06287
12. Asano K, Nabeyama A, Miyake Y, Qiu CH, Kurita A, Tomura M, et al. CD169-positive macrophages dominate antitumor immunity by crosspresenting dead cell-associated antigens. *Immunity.* (2011) 34:85–95. doi: 10.1016/j.immuni.2010.12.011
13. Martinez-Pomares L, Gordon S. CD169+ macrophages at the crossroads of antigen presentation. *Trends Immunol.* (2012) 33:66–70. doi: 10.1016/j.it.2011.11.001
14. Ravishankar B, Shinde R, Liu H, Chaudhary K, Bradley J, Lemos HP, et al. Marginal zone CD169+ macrophages coordinate apoptotic cell-driven cellular recruitment and tolerance. *Proc Natl Acad Sci USA.* (2014) 111:4215–20. doi: 10.1073/pnas.1320924111
15. Pucci F, Garris C, Lai CP, Newton A, Pfirschke C, Engblom C, et al. SCS macrophages suppress melanoma by restricting tumor-derived

vesicle-B cell interactions. *Science.* (2016) 352:242–6. doi: 10.1126/science.aaf1328
16. Gerner MY, Torabi-Parizi P, Germain RN. Strategically localized dendritic cells promote rapid T cell responses to lymph-borne particulate antigens. *Immunity.* (2015) 42:172–85. doi: 10.1016/j.immuni.2014.12.024
17. Gretz JE, Norbury CC, Anderson AO, Proudfoot AE, Shaw S. Lymph-borne chemokines and other low molecular weight molecules reach high endothelial venules via specialized conduits while a functional barrier limits access to the lymphocyte microenvironments in lymph node cortex. *J Exp Med.* (2000) 192:1425–40. doi: 10.1084/jem.192.10.1425
18. Sixt M, Kanazawa N, Selg M, Samson T, Roos G, Reinhardt DP, et al. The conduit system transports soluble antigens from the afferent lymph to resident dendritic cells in the T cell area of the lymph node. *Immunity.* (2005) 22:19–29. doi: 10.1016/j.immuni.2004.11.013
19. Mempel TR, Henrickson SE, Von Andrian UH. T-cell priming by dendritic cells in lymph nodes occurs in three distinct phases. *Nature.* (2004) 427:154–9. doi: 10.1038/nature02238
20. Bajenoff M, Germain RN. B-cell follicle development remodels the conduit system and allows soluble antigen delivery to follicular dendritic cells. *Blood.* (2009) 114:4989–97. doi: 10.1182/blood-2009-06-229567
21. Roozendaal R, Mempel TR, Pitcher LA, Gonzalez SF, Verschoor A, Mebius RE, et al. Conduits mediate transport of low-molecular-weight antigen to lymph node follicles. *Immunity.* (2009) 30:264–76. doi: 10.1016/j.immuni.2008.12.014
22. Radtke AJ, Kastenmuller W, Espinosa DA, Gerner MY, Tse SW, Sinnis P, et al. Lymph-node resident CD8alpha+ dendritic cells capture antigens from migratory malaria sporozoites and induce CD8+ T cell responses. *PLoS Pathog.* (2015) 11:e1004637. doi: 10.1371/journal.ppat.1004637
23. Iezzi G, Frohlich A, Ernst B, Ampenberger F, Saeland S, Glaichenhaus N, et al. Lymph node resident rather than skin-derived dendritic cells initiate specific T cell responses after Leishmania major infection. *J Immunol.* (2006) 177:1250–6. doi: 10.4049/jimmunol.177.2.1250
24. Jeong HS, Jones D, Liao S, Wattson DA, Cui CH, Duda DG, et al. Investigation of the lack of angiogenesis in the formation of lymph node metastases. *J Natl Cancer Inst.* (2015) 107:djv155. doi: 10.1093/jnci/djv155
25. Brown M, Assen FP, Leithner A, Abe J, Schachner H, Asfour G, et al. Lymph node blood vessels provide exit routes for metastatic tumor cell dissemination in mice. *Science.* (2018) 359:1408–11. doi: 10.1126/science.aal3662
26. Pereira ER, Kedrin D, Seano G, Gautier O, Meijer EFJ, Jones D, et al. Lymph node metastases can invade local blood vessels, exit the node, and colonize distant organs in mice. *Science.* (2018) 359:1403–7. doi: 10.1126/science.aal3622
27. Phan TG, Green JA, Gray EE, Xu Y, Cyster JG. Immune complex relay by subcapsular sinus macrophages and noncognate B cells drives antibody affinity maturation. *Nat Immunol.* (2009) 10:786–93. doi: 10.1038/ni.1745
28. Witmer-Pack MD, Hughes DA, Schuler G, Lawson L, McWilliam A, Inaba K, et al. Identification of macrophages and dendritic cells in the osteopetrotic (op/op) mouse. *J Cell Sci.* (1993) 104(Pt 4):1021–9.
29. MacDonald KP, Palmer JS, Cronau S, Seppanen E, Olver S, Raffelt NC, et al. An antibody against the colony-stimulating factor 1 receptor depletes the resident subset of monocytes and tissue- and tumor-associated macrophages but does not inhibit inflammation. *Blood.* (2010) 116:3955–63. doi: 10.1182/blood-2010-02-266296
30. Chávez-Galán L, Olleros ML, Vesin D, Garcia I. Much more than M1 and M2 macrophages, there are also CD169(+) and TCR(+) macrophages. *Front Immunol.* (2015) 6:263. doi: 10.3389/fimmu.2015.00263
31. Steer HW, Foot RA. Changes in the medulla of the parathymic lymph nodes of the rat during acute gastro-intestinal inflammation. *J Anat.* (1987) 152:23–36.
32. Nossal GJ, Abbot A, Mitchell J. Antigens in immunity. XIV. Electron microscopic radioautographic studies of antigen capture in the lymph node medulla. *J Exp Med.* (1968) 127:263–76. doi: 10.1084/jem.127.2.263
33. Iannacone M, Moseman EA, Tonti E, Bosurgi L, Junt T, Henrickson SE, et al. Subcapsular sinus macrophages prevent CNS invasion on peripheral infection with a neurotropic virus. *Nature.* (2010) 465:1079–83. doi: 10.1038/nature09118
34. Moseman EA, Iannacone M, Bosurgi L, Tonti E, Chevrier N, Tumanov A, et al. B cell maintenance of subcapsular sinus macrophages protects against a fatal viral infection independent of adaptive immunity. *Immunity.* (2012) 36:415–26. doi: 10.1016/j.immuni.2012.01.013
35. Farrell HE, Davis-Poynter N, Bruce K, Lawler C, Dolken L, Mach M, et al. Lymph node macrophages restrict murine cytomegalovirus dissemination. *J Virol.* (2015) 89:7147–58. doi: 10.1128/JVI.00480-15
36. Kastenmüller W, Torabi-Parizi P, Subramanian N, Lämmermann T, Germain RN. A spatially-organized multicellular innate immune response in lymph nodes limits systemic pathogen spread. *Cell.* (2012) 150:1235–48. doi: 10.1016/j.cell.2012.07.021
37. Barral P, Polzella P, Bruckbauer A, van Rooijen N, Besra GS, Cerundolo V, et al. CD169(+) macrophages present lipid antigens to mediate early activation of iNKT cells in lymph nodes. *Nat Immunol.* (2010) 11:303–12. doi: 10.1038/ni.1853
38. Saunderson SC, Dunn AC, Crocker PR, McLellan AD. CD169 mediates the capture of exosomes in spleen and lymph node. *Blood.* (2014) 123:208–16. doi: 10.1182/blood-2013-03-489732
39. Gonzalez SF, Lukacs-Kornek V, Kuligowski MP, Pitcher LA, Degn SE, Kim YA, et al. Capture of influenza by medullary dendritic cells via SIGN-R1 is essential for humoral immunity in draining lymph nodes. *Nat Immunol.* (2010) 11:427–34. doi: 10.1038/ni.1856
40. Chatziandreou N, Farsakoglu Y, Palomino-Segura M, D'Antuono R, Pizzagalli DU, Sallusto F, et al. Macrophage death following influenza vaccination initiates the inflammatory response that promotes dendritic cell function in the draining lymph node. *Cell Rep.* (2017) 18:2427–40. doi: 10.1016/j.celrep.2017.02.026
41. Heesters BA, van der Poel CE, Das A, Carroll MC. Antigen presentation to B cells. *Trends Immunol.* (2016) 37:844–54. doi: 10.1016/j.it.2016.10.003
42. Carrasco YR, Batista FD. B cells acquire particulate antigen in a macrophage-rich area at the boundary between the follicle and the subcapsular sinus of the lymph node. *Immunity.* (2007) 27:160–71. doi: 10.1016/j.immuni.2007.06.007
43. Okada T, Miller MJ, Parker I, Krummel MF, Neighbors M, Hartley SB, et al. Antigen-engaged B cells undergo chemotaxis toward the T zone and form motile conjugates with helper T cells. *PLoS Biol.* (2005) 3:e150. doi: 10.1371/journal.pbio.0030150
44. Garcia Z, Lemaître F, van Rooijen N, Albert ML, Levy Y, Schwartz O, et al. Subcapsular sinus macrophages promote NK cell accumulation and activation in response to lymph-borne viral particles. *Blood.* (2012) 120:4744–50. doi: 10.1182/blood-2012-02-408179
45. Coombes JL, Han SJ, van Rooijen N, Raulet DH, Robey EA. Infection-induced regulation of natural killer cells by macrophages and collagen at the lymph node subcapsular sinus. *Cell Rep.* (2012) 2:124–35. doi: 10.1016/j.celrep.2012.06.001
46. Detienne S, Welsby I, Collignon C, Wouters S, Coccia M, Delhaye S, et al. Central role of CD169+ lymph node resident macrophages in the adjuvanticity of the QS-21 component of AS01. *Sci Rep.* (2016) 6:39475. doi: 10.1038/srep39475
47. Bogoslowski A, Butcher EC, Kubes P. Neutrophils recruited through high endothelial venules of the lymph nodes via PNAd intercept disseminating. *Proc Natl Acad Sci USA.* (2018) 115:2449–54. doi: 10.1073/pnas.1715756115
48. Sagoo P, Garcia Z, Breart B, Lemaître F, Michonneau D, Albert ML, et al. *In vivo* imaging of inflammasome activation reveals a subcapsular macrophage burst response that mobilizes innate and adaptive immunity. *Nat Med.* (2016) 22:64–71. doi: 10.1038/nm.4016
49. Chtanova T, Schaeffer M, Han SJ, van Dooren GG, Nollmann M, Herzmark P, et al. Dynamics of neutrophil migration in lymph nodes during infection. *Immunity.* (2008) 29:487–96. doi: 10.1016/j.immuni.2008.07.012
50. Gaya M, Castello A, Montaner B, Rogers N, Reis e Sousa C, Bruckbauer A, et al. Host response. Inflammation-induced disruption of SCS macrophages impairs B cell responses to secondary infection. *Science.* (2015) 347:667–72. doi: 10.1126/science.aaa1300
51. Shiota T, Miyasato Y, Ohnishi K, Yamamoto-Ibusuki M, Yamamoto Y, Iwase H, et al. The clinical significance of CD169-positive lymph node macrophage in patients with breast cancer. *PLoS ONE.* (2016) 11:e0166680. doi: 10.1371/journal.pone.0166680

52. Asano T, Ohnishi K, Shiota T, Motoshima T, Sugiyama Y, Yatsuda J, et al. CD169-positive sinus macrophages in the lymph nodes determine bladder cancer prognosis. *Cancer Sci.* (2018) 109:1723–30. doi: 10.1111/cas.13565
53. Ohnishi K, Komohara Y, Saito Y, Miyamoto Y, Watanabe M, Baba H, et al. CD169-positive macrophages in regional lymph nodes are associated with a favorable prognosis in patients with colorectal carcinoma. *Cancer Sci.* (2013) 104:1237–44. doi: 10.1111/cas.12212
54. Moalli F, Proulx ST, Schwendener R, Detmar M, Schlapbach C, Stein JV. Intravital and whole-organ imaging reveals capture of melanoma-derived antigen by lymph node subcapsular macrophages leading to widespread deposition on follicular dendritic cells. *Front Immunol.* (2015) 6:114. doi: 10.3389/fimmu.2015.00114
55. Li Q, Wang D, Hao S, Han X, Xia Y, Li X, et al. CD169 Expressing macrophage, a key subset in mesenteric lymph nodes promotes mucosal inflammation in dextran sulfate sodium-induced colitis. *Front Immunol.* (2017) 8:669. doi: 10.3389/fimmu.2017.00669
56. Black LV, Saunderson SC, Coutinho FP, Muhsin-Sharafaldine MR, Damani TT, Dunn AC, et al. The CD169 sialoadhesin molecule mediates cytotoxic T-cell responses to tumour apoptotic vesicles. *Immunol Cell Biol.* (2016) 94:430–8. doi: 10.1038/icb.2015.111

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Leucocyte Trafficking via the Lymphatic Vasculature—Mechanisms and Consequences

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The lymphatics fulfill a vital physiological function as the conduits through which leucocytes traffic between the tissues and draining lymph nodes for the initiation and modulation of immune responses. However, until recently many of the molecular mechanisms controlling such migration have been unclear. As a result of careful research, it is now apparent that the process is regulated at multiple stages from initial leucocyte entry and intraluminal crawling in peripheral tissue lymphatics, through to leucocyte exit in draining lymph nodes where the migrating cells either participate in immune responses or return to the circulation *via* efferent lymph. Furthermore, it is increasingly evident that most if not all leucocyte populations migrate in lymph and that such migration is not only important for immune modulation, but also for the timely repair and resolution of tissue inflammation. In this article, I review the latest research findings in these areas, arising from new insights into the distinctive ultrastructure of lymphatic capillaries and lymph node sinuses. Accordingly, I highlight the emerging importance of the leucocyte glycocalyx and its novel interactions with the endothelial receptor LYVE-1, the intricacies of endothelial chemokine secretion and sequestration that direct leucocyte trafficking and the significance of the process for normal immune function and pathology.

Keywords: lymphatic, trafficking, transmigration, dendritic cell, endothelium, chemokine, hyaluronan, LYVE-1

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INTRODUCTION

The lymphatics form an extensive network that facilitates the drainage of plasma leaked from the peripheral vasculature and its re-uptake by the venous circulation for maintenance of fluid homeostasis (1, 2). Moreover, they constitute an essential compartment of the immune system, providing conduits for the trafficking of antigen loaded dendritic cells (DCs), memory and regulatory T cells (T_{MEM} and T_{REG}) and neutrophils to draining lymph nodes (dLNs) in the process of immune activation, modulation and peripheral tolerance (3–8). In addition, they mediate the clearance of macrophages that remove pathogens and tissue debris during resolution of tissue inflammation and infection, and are exploited by microbial pathogens such as Group A *streptococci*, *Salmonella, Brucella* and *M. tuberculosis*, and parasitic nematodes that use the lymphatics for host colonization and systemic dissemination (9–13). Understanding the mechanisms by which cells enter and exit lymphatics in tissues and lymph nodes and their detailed choreography will therefore be essential to understanding how such processes help regulate immunity, and how they might be manipulated for therapeutic intervention.

The lymphatic network is quite distinct from the blood vasculature in terms of both structure and physiology. Notably, the lymphatics start as blind-ended capillaries that are freely permeable to fluids, and have discontinuous overlapping junctions pre-adapted to cell transit, unlike the conventional tight junctions that seal most blood vessels (8, 14, 15). In addition, unlike the blood circulation, cell trafficking in most afferent lymphatics involves intravasation rather than extravasation, in keeping with their role in accommodating the passage to dLNs of tissue resident leucocytes and transient immune cell populations recruited from the circulation (3–5, 8, 16–18). Moreover, during entry to lymphatic capillaries, leucocytes are exposed to the very low shear rates associated with interstitial fluid flow, as distinct from the high shear rates experienced during extravasation from blood capillaries (19). Reflecting such different environments, some of the molecular mechanisms for leucocyte entry and trafficking in the lymphatics are quite different to those in blood vessels. Nevertheless, as will be apparent from this present review, some are broadly similar. Just as extravasation of leucocytes from blood is triggered by inflammation and the induced expression of dedicated chemokines and adhesion molecules in the vascular endothelium, so too is the entry of most leucocytes to afferent lymph vessels. Indeed, as discussed later, certain key adhesion molecules are shared by both vasculatures.

In the following sections, I describe the latest findings on how leucocytes exploit chemotactic and adhesive mechanisms to enter and migrate within lymphatic vessels, as well as exit the lymphatic sinuses in dLNs to fulfill their various immune functions. I begin with an outline of the major leucocyte populations that migrate in lymph, and the characteristic architecture of lymphatic endothelial junctions. Based largely on knowledge gained from studies on DCs, I go on to provide a detailed account of the key steps in lymphatic trafficking from interstitial migration, lymphatic entry, and intraluminal crawling, to transit within downstream dLNs **(Figure 1)**. In my discourse, I highlight the newly discovered role of the lymphatic endothelial HA receptor LYVE-1 in lymphatic entry and its functional relationship with other more ubiquitous adhesion receptors in endothelial transit, and the possibility of their co-operation in a "lymphatic synapse." I also describe the co-ordinated triggering of chemokine release by transmigrating DCs, and some of the additional mechanisms employed by neutrophils and certain T cell populations during lymphatic transit. Lastly, I speculate on how knowledge of lymphatic trafficking mechanisms might be exploited in the future to develop new therapies for immune and inflammatory disorders.

LEUCOCYTE POPULATIONS THAT TRAFFIC *VIA* LYMPH

Classic cannulation studies carried out in domestic animals and applicable also to mice and humans showed the major cell populations migrating in normal afferent lymph are T cells (80–90%), followed by antigen presenting DCs and very small numbers of B cells which together account for most of the remaining 10–15%. Most of the T cells are antigen-experienced $CD4^+$ $CD45RO^+$ effector memory (T_{EM}) cells, recently re-defined as the recirculating memory (T_{RCM}) subset (20, 21), which, having entered the extra-lymphoid tissues from blood, engage in immune surveillance for cognate antigens before exiting *via* the afferent lymphatics to dLNs where they modulate recall immune responses (22–25). Notably, lymphocytes of the CD4 subset in afferent lymph outnumber those of the cytotoxic CD8 subset by some 5× fold (26–28), which mostly remain immotile as tissue-resident (T_{RM}) cells. Furthermore, more recent cell tracking studies using photoconvertible Kaede mice have revealed that a significant proportion (25%) of the CD4 population are $FOXP3^+$ T_{REGs}, thus uncovering a previously unrecognized role for the lymphatics in conveying these important immunoregulatory cells. By comparison, only low numbers of naïve T cells are usually present in afferent lymph, and despite the fact these can be shown to enter lymphatic capillaries after adoptive transfer in mice, their normally low frequency in tissue means they rarely do so *in vivo*. The likely functional significance of T_{EM} cell migration in afferent lymph may be to allow the amplification and polarization of immune responses in the dLNs and maintenance of the T cell memory pool, as well as enabling the re-entry of these lymphocytes to the circulation to target pathogen dissemination in further tissue sites (7, 21).

The second most numerous leucocyte population in afferent lymph are DCs, which ferry endocytosed antigens from the tissues, primarily for immune priming in dLNs. Although small numbers of DCs migrate in lymph under steady state conditions to maintain peripheral tolerance to self-antigens (29–33), the majority are mobilized by inflammation, which induces a program of differentiation and the expression of appropriate chemokine receptors for vessel entry (34–36) (see below). DCs, more than any other cell type, have been the subject of studies into the mechanisms of lymphatic trafficking, not least because they are normal tissue residents whose migration can be readily monitored by dye uptake in experimental mice (37). Moreover, in laboratory animals the lymph migrating DCs are almost as numerous as T cells, owing to the fact the pathogen-low environment in which they are bred and maintained generates a smaller pool of circulating memory cells (38).

During inflammation, the numbers of T cells and DCs in afferent lymph increase several fold along with an increase in lymph vessel permeability and the rate of lymph flow (8, 34). Furthermore, the afferent lymph can contain type I and II macrophages which are recruited to inflamed tissues for the clearance of debris and remodeling of the extracellular matrix (ECM) and which utilize the lymphatics for subsequent exit in a process that is becoming increasingly appreciated as critical for resolution and the return to normal homeostasis (39–41). In addition, this lymph contains subsets of neutrophils that are rapidly recruited to the tissues during sepsis and trauma and subsequently exit *via* the inflamed lymphatics to dLNs (42, 43). Although the majority of neutrophils in tissues are short-lived ($T_{1/2}$ 6–12 h) and undergo early apoptosis before removal by macrophage efferocytosis (44–46), the lymph-migrating cells have an extended lifespan (47). Most notably they can transport phagocytosed pathogens such as Leishmania, *H. pylori* and *M.*

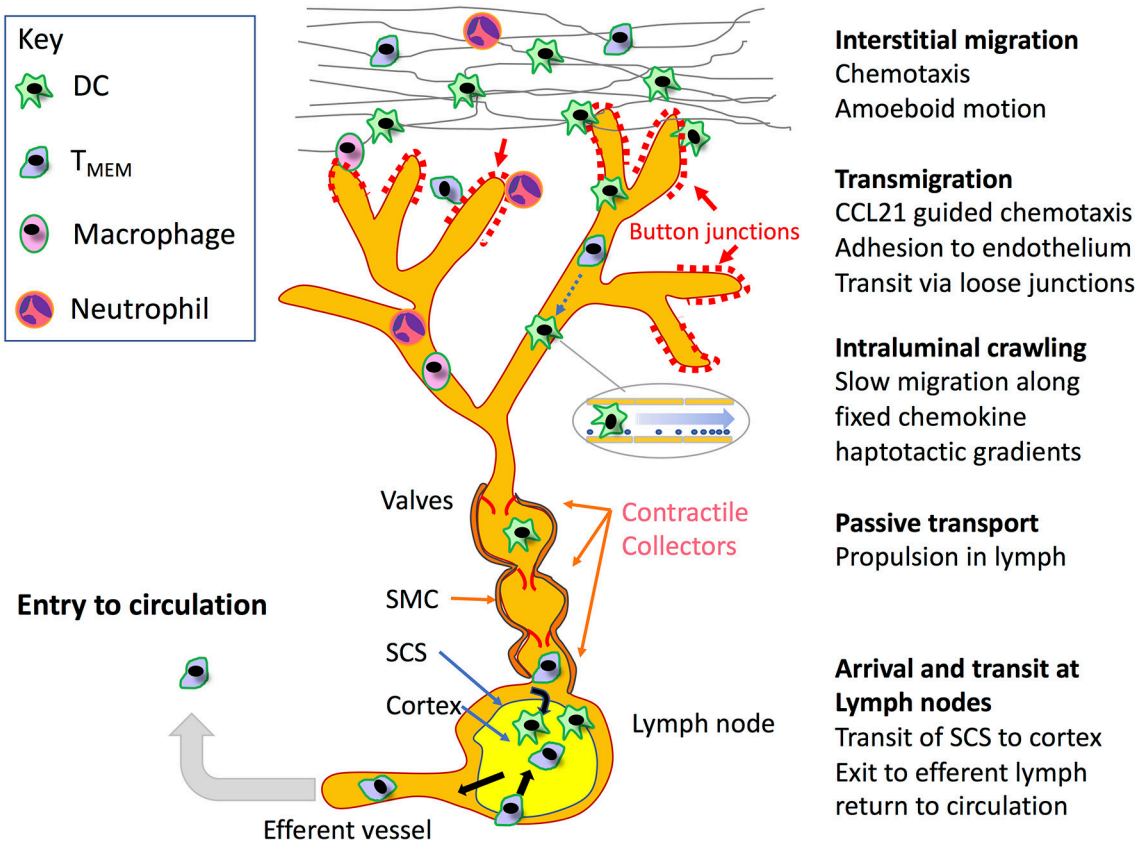

FIGURE 1 | Key steps in leucocyte migration through the lymphatic system. Beginning in the interstitium, motile leucocytes including recirculating (T_{RCM}) cells and neutrophils recruited from the blood, in addition to antigen loaded tissue resident DCs and macrophages are mobilized through inflammation to migrate toward initial lymphatic capillaries for access to dLNs by means of chemokine-directed amoeboid movement and in some cases integrin-mediated adhesion to fibronectin. The leucocytes then transit the blind-ended lymphatic capillaries at discrete sites within their discontinuous button-like endothelial junctions through receptor-mediated adhesion and chemokine directed squeezing, or in the case of neutrophils *via* lipoxin mediated chemorepulsion (detailed in **Figures 2**,**4**, respectively). Although not depicted, it is also possible that leucocytes can enter downstream vessels through conventional zipper-like junctions, and exit at intermediate stages to the surrounding tissues. On entering the capillary lumen, the transmigrated cells crawl in a semi-directional manner along the endothelial surface, guided by gradients of immobilized CCL21 established under low shear flow, using adhesion to ICAM-1 for traction. On entering downstream valved collectors, the leucocytes are now conveyed by passive transport, propelled by lymph flow generated by smooth muscle contraction. Ultimately, the migrating cells arrive within the SCS of dLNs, where they immediately transmigrate to the underlying cortex to initiate or modify immune responses in the case of DCs and likely neutrophils and macrophages, or continue to the medullary sinuses where they transit to the cortex to either remain there or pass to the efferent lymphatics to re-enter the blood circulation in the case of T_{MEMs}. Although not shown in the Figure, naïve T and B cells also recirculate directly from the blood through lymph nodes at specialized high-endothelial venules to enter the cortex and re-exit through efferent lymph.

bovis BCG to dLNs where they can influence the polarity of protective T cell responses through cytokine release and crosstalk with DCs, thus bridging the gap between innate and adaptive immunity (48–51). Indeed, neutrophils can migrate *via* lymph more rapidly than any other leucocyte populations, reportedly arriving in the ipsilateral dLNs some 12–72 h earlier than either DCs or macrophages (52–56).

In contrast to afferent lymph, the leucocyte population present in the efferent lymphatics that exit from the lymph node hilum are mostly naïve T and B cells. Having entered the lymph nodes through high endothelial blood venules in a separate circuit to probe for antigens presented by DCs in the cortex and paracortex, these are ultimately returned to the circulation through the subclavian vein (36). Notably, during the onset of infection or inflammation, the efflux of this recirculating population from the lymph node is halted transiently (3–4 days) so as to prolong their residence time and thus increase the efficiency of immune recognition (57).

THE DISTINCTIVE ARCHITECTURE OF INITIAL LYMPHATIC VESSELS

As already mentioned, the afferent lymphatics initiate as blind-ended capillaries that branch and merge with larger collecting vessels, emptying their contents into dLNs before exiting as efferent vessels that reconnect either directly, or *via* other intervening nodes to the venous blood **(Figure 1)**. In keeping with their fluid draining function, the blind-ended capillaries have only a rudimentary basement membrane (BM), and lack any

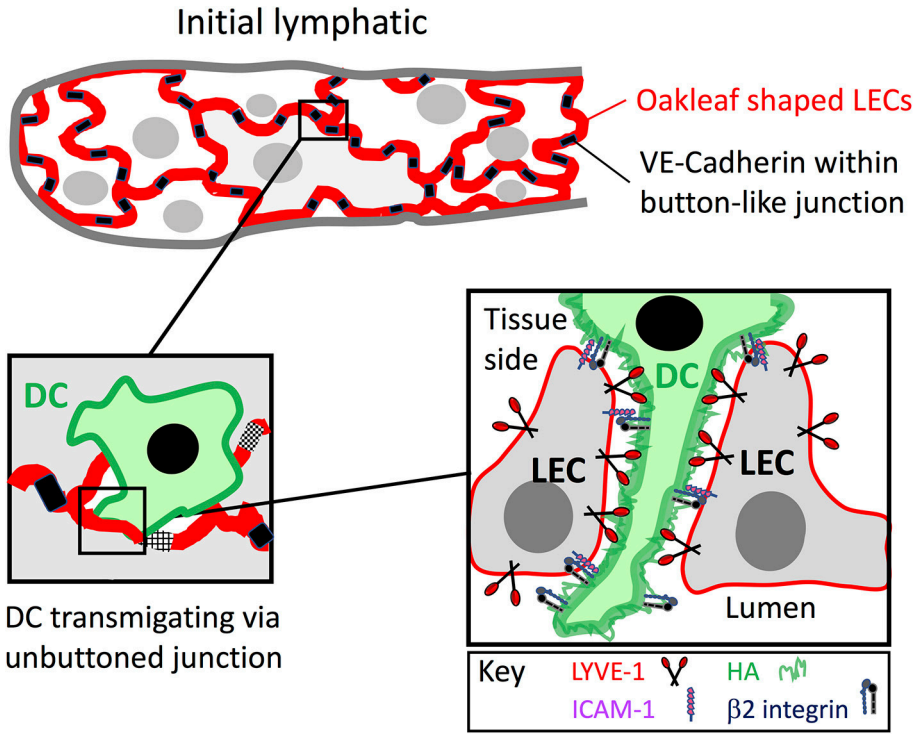

FIGURE 2 | Leucocyte entry to initial lymphatic capillaries through formation of LYVE-1 endothelial transmigratory cups. Entry to the afferent lymphatics proceeds within the discontinuous junctions between the oakleaf shaped lymphatic endothelial cells of initial capillaries that are tightly buttoned at their sides by VE-cadherin and lined at their tips by the HA receptor LYVE-1 (red). As depicted in the Figure, migrating leucocytes such as DCs, macrophages and likely T cells that assemble a surface HA glycocalyx engage with LYVE-1 and transduce signals for VE-cadherin disassembly and junctional unbuttoning that lead to formation of a continuous LYVE-1 lined interface at portals termed transmigratory cups (see text). The weak avidity dependent nature of HA interactions with the LYVE-1 homodimers, combined with the multivalent nature of the long HA polymers are thought to enable low friction migration of the DCs through the endothelial openings. In addition, ICAM-1 and VCAM-1 expressed within the cups bind to integrin ligands on the DC surface activated by local secretion of CCL21 and likely provide the necessary traction for diapedesis. The precise details of the interplay between LYVE-1 and these receptors are not yet known.

investment by actin-containing smooth muscle cells. Moreover, the endothelial cells that make up the first few millimeters of these initial capillaries have a distinctive oakleaf shape that allows them to interdigitate and form loose discontinuous junctions **(Figure 2)**, quite unlike the continuous junctions of endothelia in most blood vessels (14, 58–61). As revealed by electron microscopy (EM) and confocal imaging studies of such capillaries in mice, the alternating membrane flaps making up these structures are pinned at their sides by discrete assemblages some 3 μm wide and spaced 3 μm apart, that contain the adherens-junction protein VE-cadherin and the tight junction proteins Claudin-5, ZO-1 (zonula occludens-1), ESAM (endothelial selective adhesion molecule) and JAM-A (14). In contrast, the flaps remain free at their tips, where they guard openings of ∼0.5–1 μm, that are decorated by CD31 and the lymphatic endothelial HA receptor LYVE-1 as detailed below (14). It is through these openings that DCs appear to enter the lymphatic capillaries. Importantly, the process is far from passive, as the dimensions of the DCs are many times greater than the gaps through which they must enter, and hence transit requires pushing and squeezing *via* intimate contact with the endothelium (14, 62–65). While the rationale for such an elaborate arrangement of buttons and flaps is not fully clear, it likely represents a compromise between the conflicting requirements of high vessel permeability for fluid uptake and a more restrictive barrier for regulating leucocyte entry. Indeed, for the experimenter, such architecture poses problems for establishing *in vitro* models to study vessel entry, as primary cultured lymphatic endothelial cell (LEC) monolayers form only partial surrogates of discontinuous junctions (66), and full authentication requires the application of whole animal models or crawl-in assays with tissue explants.

In contrast to the initial capillaries, downstream pre-collector and valved collector vessels lack buttons and instead adopt conventional tight or "zipper" like junctions that allow the formation of a more fully sealed vasculature (14, 15). The collectors are also covered by smooth muscle cells that promote contractile pumping and which convey migrating leucocytes to the dLNs with minimal leakage. Of note, these zippered junctions constitute the default state of capillaries in the developing embryo and early neonate, and button-like junctions emerge only during the later neonatal period, co-incident with the establishment of full immune function (67). Curiously, the nascent lymphatics generated by lymphangiogenesis in chronically inflamed tissues

also have conventional zippers, raising the possibility that vessel entry *via* zippers rather than buttons may be permissible in certain contexts (15).

MIGRATION THROUGH INTERSTITIAL MATRIX AND THE PERI-LYMPHATIC BASEMENT MEMBRANE

In order to access the initial lymphatic capillaries for exit from the tissues and onward trafficking to lymph nodes, leucocytes must first migrate distances of several hundred microns through the surrounding interstitial matrix, a variably dense complex of type I collagen fibrils, fibronectin, hyaluronan (HA), and heparan sulfate proteoglycans (HSPGs) (68). The rate of such interstitial migration is thought to be comparable with that of subsequent migration through afferent lymph capillaries [4.4 vs. 5.7 μm/min in the case of DCs (69) and see below] and the time taken for DCs to navigate to a lymphatic capillary from the point of initial mobilization has been estimated as ∼1 h (70). In mice, under conditions of normal homeostasis, the numbers of migrating cells in sites such as skin are small and the populations consist mostly of immature dermal DCs and effector T_{MEM} cells *en route* to lymph nodes during immune surveillance. However, in response to inflammation, as deduced from *in vivo* studies using skin contact sensitizing agents and complete Freund's adjuvant (CFA), this traffic increases significantly. For practical reasons, most studies of leucocyte interstitial migration in the context of lymphatic trafficking have focused on DCs in the mouse dermis and epidermis. Although sessile in the steady state, these cells are subsequently induced to differentiate and crawl toward LVs, in response to inflammatory signals received from cytokines, prostaglandins and leukotrienes released from keratinocytes activated by microbial products and other Toll-like receptor (TLR) ligands (71). The movement of activated DCs is further stimulated by the increase in interstitial fluid flow that is characteristic of tissue inflammation. Nevertheless, the major driving force for leucocyte interstitial migration under resting and inflamed conditions is chemotaxis, directed primarily by the chemokine CCL21 secreted radially from lymphatic endothelium and its G-protein coupled receptor CCR7 on both migrating DCs and T cells (32, 36, 57, 72). Elegant studies by Weber and Sixt using single cell tracking in mouse ear skin revealed DC migration begins in a random fashion until the cells reach a distance of ∼90 μm from an initial capillary, at which point their migration becomes directional and persistent (73). This directionality of motion is imposed by fixed, haptotactic gradients of CCL21 secreted constitutively from lymphatic vessel endothelial cells and sequestered by collagen and HSPGs in the surrounding interstitial matrix *via* the positively charged chemokine N-terminal domain (73). Moreover, the gradients fall away with distance from the vessel periphery in such a way as to provide a polarity that can be sensed by DCs in the typical size range of 15–50 μm. Importantly, DC migration under both resting and inflamed conditions is independent of β2 integrin/ICAM-1 adhesion, as demonstrated by seminal studies in mice that compared intact and pan-integrin knockout cells (74). Migration proceeds instead by "amoeboid movement" whereby CCL21 directs the pushing and squeezing of DCs through the 3D collagen matrix, primarily by triggering actin polymerization and actomyosin contraction at the leading and trailing edges *via* the small GTPases Rac1 and Rac2 and nuclear contraction *via* the Rho associated kinase ROCK (69, 74, 75). To date few studies have addressed the mechanisms of interstitial migration employed by leucocyte populations other than DCs. Although in the case of $CD4^+$ effector T_{MEM}, such migration in CFA inflamed ear dermis was also found to be independent of β2 integrin/ICAM-1 adhesion (76), it was nevertheless reliant on guidance *via* β1and β3 integrin-mediated adhesion to matrix fibronectin, laid down in association with parallel oriented collagen fibers (77). In the looser fibronectin-rich ECM network that is characteristic of inflamed tissues, such adhesion may be a greater requirement for the chemokine driven migration of T cells due to their smaller size and inability to extend dendritic processes.

Lastly, before engaging with the external surface of initial lymphatic capillaries, migrating leucocytes must traverse the surrounding BM, a rudimentary structure comprised mainly of type IV collagen and the non-network forming laminin isoform α4, before they can transmigrate the endothelium to enter the vessel lumen. In contrast to the more close-knit BM of blood vessels (78), the BM of lymphatic capillaries is sparse and highly perforated by gaps of ∼1 μm diameter that are completely devoid of any ECM components. Making up 30% of the vessel surface, these gaps offer points through which migrating cells can access the underlying vessel endothelium with the aid of physical expansion (to ∼2 μm). Studies of inflamed mouse ear skin indicate that those gaps which overlie sites of vessel entry are marked by discrete deposits of sequestered CCL21 which direct the transit of DCs by chemotaxis/haptotaxis rather than simply chemokinesis. Indeed, migrating DCs have been observed to extend cellular processes toward these CCL21 puncta and apparently make physical contact with them (71). Furthermore, detailed imaging of such events in *ex vivo* crawl-in assays with dermal tissue explants (79) suggested these invadopodia-like protrusions enable DCs to transiently expand the BM portals by physical squeezing without the parallel requirement for proteolytic re-modeling observed during leucocyte transit of blood vascular BMs. On traversing the BM the migrating leucocytes then encounter the lymphatic endothelium next to their interdigitating flaps, which bend inwards to accommodate entry to the vessel lumen (79).

TRANSMIGRATION OF THE VESSEL ENDOTHELIUM AND ENTRY TO THE LYMPHATICS

Integrin-Mediated Adhesion *via* Endothelial Microvilli

Similar to leucocyte transmigration of blood vessel endothelium, transmigration of lymphatic endothelium involves prior adhesive interactions between β2 integrins and their Ig superfamily counter receptors. Of note, early *in vivo* studies using mouse

models of skin inflammation and contact hypersensitivity showed that the endothelial leucocyte adhesion receptor ICAM-1 is involved in the migration of epidermal Langerhans cells to skin dLNs (80, 81). Nevertheless, the requirement for adhesion receptors in lymphatic transmigration has been disputed, and one particularly prominent study using pan-integrin ($Int^{-/-}$) deficient mice reported that under normal resting conditions, DCs can enter lymphatic vessels and migrate to dLNs independently of all integrins, just as documented for interstitial migration, by means of chemokine-directed amoeboid motion (74). Importantly however, these studies tracked the migration of exogenous DCs that had been adoptively transferred to a non-inflamed dermis and hence reported a mode of trafficking that proceeds only quite rarely in normal tissues (74). Indeed, other studies that tracked endogenous DC trafficking in the uninflamed skin of CD11c YFP^{+}/VE-Cadherin Cre/Rosa26 Fl RFP^{+} chimeric mice detected few if any cells entering dermal lymphatic capillaries, and such entry was observed only after adoptive transfer into tissue inflamed by either contact hypersensitization, exposure to adjuvants (CFA) or treatment with bacterial LPS (71). Hence, an integrin-independent mode of lymphatic vessel transmigration likely applies only to the relatively small minority of immature DCs that traffic constitutively in immune surveillance.

In contrast, in inflamed tissues where DCs migrate more extensively *via* lymph, transmigration is indeed dependent on integrin-mediated adhesion mechanisms. As evidenced by studies with cultured primary LECs, and confirmed by transcriptional profiling of lymph vessel endothelium isolated from inflamed mouse skin, exposure to inflammatory cytokines and contact sensitizing agents results in rapid ($T_{1/2}$ ~3 h) upregulation of the key integrin counter-receptors ICAM-1 and VCAM-1 together with various other adhesion molecules associated with leucocyte endothelial transit such as E-selectin, and a range of different chemokines that are attractants for DCs, monocytes, lymphocytes and neutrophils (82, 83). Furthermore, ICAM-1 and VCAM-1 blocking mAbs have been demonstrated to impair adhesion and transmigration of bone marrow DCs (BMDC) across inflamed LEC monolayers *in vitro*, as well as entry and trafficking of endogenous DCs to dLNs *in vivo*, both in murine models of skin hypersensitivity and dermal vaccine-induced T-cell immunity (82, 84). Likewise, in CD45.2 mice, LFA-1 blocking mAbs were shown to impair trafficking of injected CD45.1 DCs to dLNs from TNFα treated footpads (85). Most notably, it has been observed using *in vitro* confocal imaging of murine BMDCs engaged in transit across inflamed lymphatic endothelium, that ICAM-1 and VCAM-1 are concentrated within finger-like projections somewhat analogous to the transmigratory cups described originally in blood vascular endothelium (86–89) (**Figure 2** and see below). These extend around the transmigrating cells, facilitating their adhesion *via* activated (mAb 24^{+ve}) forms of β2 integrin (LFA-1) present in complementary projections on the DC surface (85). Reportedly, similar ICAM-1 lined membrane protrusions were also observed around transmigrating DCs *in vivo* both in mouse ear and human dermal tissues.

The transmigration of T cells across lymphatic endothelium also involves integrin-mediated adhesion. Accordingly, in studies employing antibody blockade, ICAM-1 and its β2 integrin ligand LFA-1 were shown to be functionally required for T cell adhesion and transmigration of TNFα treated murine LECs *in vitro*, as well as entry to lymphatic vessels and trafficking to dLNs in the inflamed skin of oxazolone and adjuvant treated mice *in vivo* (76). Indeed, in an analogous manner to DCs, ICAM-1 was observed to be distributed in microvillar projections around $CD4^{+}$ T cells and ionomycin/PMA-activated peripheral blood mononuclear cells adhering to a lymphatic endothelium (85). Hence it appears that similar mechanisms are employed by both CD4 T cells and DCs for lymphatic vessel entry through extension of ICAM/VCAM enriched endothelial microvilli (19, 90). Furthermore, as detailed later in this review, the initial stages of neutrophil entry involves adhesion through the β2 integrins LFA-1 and Mac-I and most likely their main lymphatic endothelial counter-receptor ICAM-1, as evidenced from experiments using adoptive transfer of $Int^{-/-}$ neutrophils and receptor blocking mAbs (42, 43, 56, 91). Indeed, it was clearly shown that administration of either β2 integrin or ICAM-1 blocking mAbs impaired the entry of GFP*lysM* labeled neutrophils to the initial lymphatic capillaries in mice, causing the cells to logjam at the vessel periphery (56), in a manner reminiscent of DCs given similar blockade in oxazolone-treated mouse skin (17, 82). Such harnessing of integrins, ICAM-1 and VCAM-1 for leucocyte adhesion to the basolateral surface of lymphatic vessel endothelium and subsequent transendothelial migration is in many ways analogous to their involvement in the abluminal crawling of newly extravasated leucocytes on the pericyte surface of blood vessels (92) and stands as an example of how comparable mechanisms can operate in the two vasculatures albeit in reverse orientation.

Transit *via* Hyaluronan and LYVE-1 Transmigratory Cups

In addition to integrins and their counter-receptors, it has recently been demonstrated that transmigration of DCs requires critical involvement of LYVE-1 (93) within the button-like junctions of initial lymphatic capillaries (14, 15) **(Figure 2)**, and its engagement with the large mucopolysaccharide ligand HA present on the DC surface (19, 90). Closely related to the leucocyte receptor CD44 (94, 95), LYVE-1 contains a conserved lectin-like HA-binding domain, termed the Link module, at its N-terminus (19, 96). Although HA is a ubiquitous component of perivascular ECMs (97), it can also be synthesized by DCs and other leucocytes including macrophages and T cells as a surface glycocalyx (98–100), and it is the selective interaction of LYVE-1 with this latter structure in preference to ambient HA that facilitates DC transmigration (90, 101). Such specificity is possible because of the strict avidity-dependent nature of LYVE-1: HA interactions. Because the receptor binds only a short 8–20 saccharide region of HA with low affinity (K_D 125 μM), it therefore relies on homo-dimerization and clustering, as well as a high ligand density to achieve the multiplicity of co-ordinate binding interactions required for tethering of the

polymer chains (96, 101, 102), a phenomenon that has been termed superselectivity (103). Intriguingly, *in vitro* confocal imaging studies using primary murine LEC monolayers have shown that LYVE-1 is recruited along with ICAM-1 and VCAM-1 to the transmigratory cups that form upon initial DC contact with endothelium, and furthermore that engagement of LYVE-1 with the HA glycocalyx is actually critical for their formation (90). Consistent with a functional role for the receptor in DC transmigration, LYVE-1 HA blocking mAbs also impaired both the adhesion and transit of DCs across LEC monolayers *in vitro*. Similar features of LYVE-1 transmigratory cups have been observed *in vivo*, during DC transit of lymphatic capillaries in oxazolone sensitized mouse skin. Moreover, interference with LYVE-1 mediated DC interactions in such studies by LYVE-1 gene deletion, antibody blockade or DC HA glycocalyx depletion resulted in the characteristic logjamming of endogenous and adoptively transferred DCs on the basolateral surface of dermal lymphatics, and impaired their capacity to prime antigen specific T cells in dLNs, mirroring the effects seen with β2 integrin blockade (90). Notably, besides DCs, LYVE-1 also mediates adhesion and transmigration of macrophages across lymphatic endothelium through similar mechanisms (102). Indeed, in a murine model of myocardial infarction where damage-inducing M1 macrophages infiltrate the ischaemic myocardium and are subsequently cleared *via* cardiac lymphatics (104, 105), the process is blocked by *Lyve1* deletion, which delays resolution and leads to fibrotic scarring (41). Whether or not T cells also engage LYVE-1 in such structures has yet to be determined. However, in common with DCs and macrophages these also have a capacity for HA biosynthesis, and hence may well assemble a similar HA surface glycocalyx (98). Curiously, another receptor, CLEVER-1, containing an HA-binding "Link" domain related to LYVE-1 has been reported to mediate CD4 and CD8 T cell trafficking through afferent murine skin lymphatics (see **Table 1**) as well as transmigration across monolayers of lymphatic endothelium *in vitro* (115–117). However, the mode of action of CLEVER-1 is distinctly different to LYVE-1, as the Link module was shown to be non-functional and the site mediating transmigration instead found to reside within a distant EGF repeat region (116, 118).

Importantly, the role of LYVE-1 in lymphatic transmigration extends beyond merely supporting cell adhesion. For example, it has been demonstrated that engagement of the receptor can transduce signals for endothelial junctional relaxation, in particular the phosphorylation and detachment of VE-cadherin located within the button-like foci of initial capillaries from which LYVE-1 is selectively excluded (14, 106). Hence, engagement with the DC glycocalyx and un-buttoning of the VE-cadherin lined junctions at vessel entry sites may well promote coalescence of the alternating endothelial flaps and redistribution of LYVE-1 to form a single continuous interface for leucocyte diapedesis (2, 19). In addition, the large contour lengths of HA polymers, which can extend to several microns, likely mask access to the more compact integrins [extracellular domains ∼20 nm (107)] on the underlying DC surface, and allow the polysaccharide to make primary contact with the capillary endothelium. It has also been postulated that the low affinity of LYVE-1 HA-binding supports crawling of DCs along the vessel surface toward junctional portals through the inherently low friction of the interaction (19). This role as lubricant is supported by physicochemical studies of LYVE-1 HA binding mechanics at the single molecule level using atomic force microscopy, which indicate the individual interactions are weak and that they rupture collectively under the low forces experienced in interstitial flow [see (19)]. It contrasts markedly with the behavior of CD44, a receptor tuned for leucocyte capture in post-capillary venules, which forms bonds that are stronger and detach sequentially in a Velcro (hook and loop) like fashion in response to the higher forces experienced in blood flow (108, 109). Nevertheless, the transit of cells through lymphatic endothelium must involve traction, and if this is not provided by LYVE-1, then it is likely that DCs use both HA and integrin-based adhesion either on different faces of the cell, or in sequential fashion during diapedesis.

Undoubtedly, many other adhesion molecules located in and around the buttoned junctions of lymphatic endothelium contribute to leucocyte transmigration, and may even specify the entry of discrete leucocyte populations. A number of such receptors including Mannose receptor (110–114), ALCAM (CD166, Activated Leucocyte Cell Adhesion Molecule) (119), L1CAM (CD171) (120), 4-1BB (CD137) (121), CD99, and CD31 (PECAM-1) (122) have already been implicated in the process from various *in vitro* and *in vivo* studies (**Table 1**). However, the precise functional roles played by each of these receptors, and how they are individually choreographed during lymphatic trafficking have yet to be elucidated.

DIRECTIONAL GUIDANCE OF LEUCOCYTE TRANSMIGRATION BY CHEMOKINES

The Key Roles of CCL21 and CCR7

In concert with adhesion receptors, critical cues for the guidance of leucocytes during the process of lymphatic vessel entry are provided by chemokines synthesized and secreted in the main by underlying LECs. However, the emerging view is that these may operate as much by inducing the transient arrest of migrating leucocytes at endothelial junctions as by guiding their migration along conventional chemotactic gradients. CCL21 released from the endothelium has been identified as the primary chemokine controlling the entry of DCs to afferent lymph, based initially on the findings from elegant studies in mice showing that CCL21 neutralizing mAbs or CCR7 gene deletion decreased or delayed DC migration from the dermis to dLNs (32, 72, 123, 124). Curiously, mice express two separate genes for CCL21 that encode a lymph node isoform $CCL21^{ser}$ and an afferent vessel isoform $CCL21^{leu}$ (125, 126), and it has logically been assumed (though not formally proven) that the latter ($CCL21^{leu}$) controls the lymphatic entry step (32, 72, 123, 124, 126). Although a naturally occurring genetic deletion of $CCL21^{ser}$ in the *plt/plt* mouse line compromises DC trafficking *via* lymph (125–127), this likely reflects a more distal defect in either entry or retention in downstream dLNs.

In addition to DCs, CCL21 is also the primary chemokine driving the entry of T cells to afferent lymph vessels in the

TABLE 1 | Adhesion receptors in lymphatic endothelium involved in regulating leucocyte entry and trafficking.

Receptor	Comment	Key references
ICAM-1	Immunoglobulin superfamily receptor for leucocyte β2 integrin ligands LFA-1 and Mac-1, upregulated in inflammation	See main text
VCAM-1	Immunoglobulin superfamily receptor for leucocyte β1 integrin ligands, upregulated in inflammation	See main text
LYVE-1	Avidity dependent Link superfamily HA receptor binds selectively to migrating leucocyte glycocalyx	See main text
CLEVER-1	Multidomain scavenger receptor in afferent LVs and LN HEVs. Supports adhesion of lymphocytes, monocytes, and granulocytes. mAbs impair migration to dLNs. Ligands yet to be identified	See main text (103–105)
Mannose receptor (CD206)	C-type lectin receptor supports lymphocyte adhesion and migration to dLNs by binding E-selectin and sulphated glycans	(106–110)
ALCAM (CD166)	Mediates DC adhesion to LECs *in vitro* and migration from lung to dLN *in vivo.* Binds CD6, L1CAM, Galectins	(111)
L1CAM (CD171)	Homotypic adhesion molecule expressed in inflamed lymphatics and DCs. Disruption impaired DC endothelial transmigration *in vitro* and trafficking to dLNs *in vivo*	(112)
4-1BB (CD137)	Induced in LEC by TNFα, IL-1, LPS. Ligation potentiates DC transmigration by upregulating ICAM-1, VCAM-1, CCL21	(113)
CD34 (PECAM-1) and CD99	Homophilic adhesion molecules at LEC:LEC junctions and luminal surfaces. Both support DC adhesion/transmigration	(114)

periphery, in particular the antigen-experienced $CD4^+$ and $CD8^+$ T_{EM} population that exits from the circulation to patrol the inflamed tissues. Approximately 50% of this population in the skin of humans and mice are $CCR7^+$, and as confirmed from cannulation studies in sheep, almost all the T cells that migrate in afferent lymph express CCR7 and respond chemotactically to CCL21 (23, 128). Furthermore, parallel studies with CFSE labeled T cells in gene deficient mice have indicated their entry to the afferent lymphatics in both dermis and lung and trafficking to dLNs is almost entirely dependent on expression of CCR7 (20, 23). These migratory $CCR7^+$ T cells which can ultimately re-enter the blood *via* the thoracic duct are clearly distinct from the $CCR7^-$ T effector (T_{EM}) population that remains resident within the tissues as sentinels, and have since been defined phenotypically as a ($CCR7^{int/+}$ $CD62L^{int}$ $CD69^-$ $CD103^{+/-}$ E-selectin ligand$^+$) recirculating memory (T_{RCM}) cell subset, based on their tracking in photoconvertible fluorescent *Kaede* mice (21). Indeed, they also include the important immunosuppressive $CD4^+$ T regulatory cell (T_{REG}) as well as inflammation-associated Th1 and Th17 cell subsets (129, 130). Curiously however, the importance of CCL21/CCR7 for T cell entry and trafficking is diminished in chronic as compared to acute inflammation (130), and hence it is likely that other inflammation-induced chemokines become involved at later time points (see below).

It has also been reported that CCL21 chemotaxis helps direct the entry of neutrophils to afferent lymphatics. Accordingly, in a study of skin inflammation evoked by topical CFA administration in mice, the lymph migrating neutrophil population was identified exclusively as $CCR7^+$ and trafficking to dLNs was decreased almost 4× fold in $CCR7^{-/-}$ animals (55). Likewise, the entry of neutrophils to the cremaster muscle lymphatics following TNFα treatment, which induces CCL21 release, was reported to be almost completely (>97%) inhibited in $CCR7^{-/-}$ mice (43). Whether CCL21 is the primary chemokine in every context is however open to question, as another study of neutrophil migration in *S. aureus* treated mice found that neutrophil entry to the dermal lymphatics was directed by CXCL12, on the basis of inhibition by the CXCR4 receptor antagonist AMD3100 (91).

Leucocyte-Induced Chemokine Release

Synthesis of CCL21 is markedly upregulated in murine and human lymphatic endothelium in response to inflammation (35, 66, 131) whereby the chemokine accumulates in intracellular storage vesicles in readiness for secretion, notably at the basolateral surface of the endothelium where leucocytes transmigrate (132). Intriguingly, in a recent seminal study of DCs by Vaahtomeri *et al* it was reported that leucocytes themselves can trigger such secretion from lymphatic vessels through a contact dependent mechanism in which the transmigrating cells extend filopodia toward the endothelium, provoking a Ca^{2+} flux that triggers disassembly of cortical actin and the exocytosis of pre-stored CCL21 from trans Golgi vesicles along linear microtubule tracks, for fusion with the plasma membrane (**Figure 3**) (65). As visualized using EM, the exocytosed CCL21 is then retained focally in the form of minute puncta at the basolateral surface of the endothelium close to intercellular junctions where they are thought to induce local arrest of DCs through β2 integrin activation. Such confined release may well avoid the desensitization of CCR7 on the migrating cells that might otherwise be evoked by formation of a conventional transendothelial CCL21 gradient (133). Diapedesis—the actual transit process, then proceeds by CCL21-driven re-arrangement of the DC actomyosin cytoskeleton that allows the cell to push and squeeze through the flap-like protrusions between adjacent oakleaf shaped endothelial cells in the initial capillary junctions (65), aided by Semaphorin 3A induced signaling *via* RhoA and ROCK for contraction of the uropod (134). The CCL21 secreted focally in response to DC contact is thus functionally distinct from the CCL21 that is secreted homeostatically for interstitial migration. Interestingly, the DC adhesion-induced CCL21

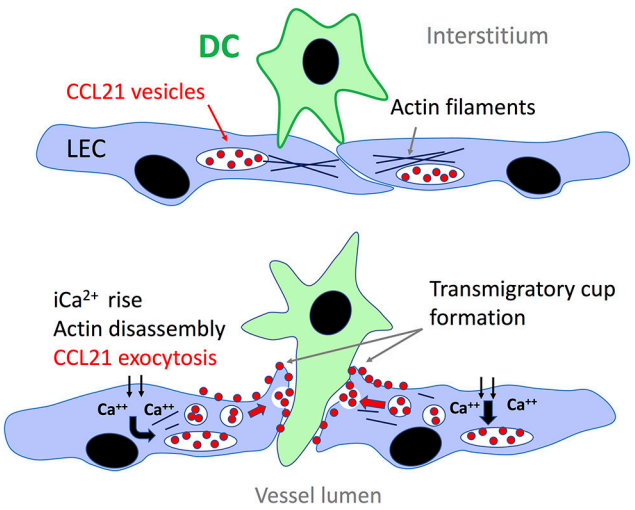

FIGURE 3 | Local secretion of CCL21 from lymphatic endothelium triggered by DC contact. Contacts with the basolateral surface of lymphatic endothelium *via* filopodia extended from the migrating DCs trigger an influx of Ca^{2+} that leads to disassembly of the adjacent cortical actin network and enables translocation of endothelial trans-Golgi vesicles containing pre-stored CCL21 to the plasma membrane along parallel microtubule tracks. Fusion of the vesicles with the plasma membrane, most likely orchestrated by Ca^{2+} binding synaptotagmins, leads to the local release of CCL21 which is retained on the plasma membrane surface close to the endothelium:DC interface, where it directs DC transit through β2 integrin activation and actomyosin-mediated pushing and squeezing, upon the formation of LYVE-1 and ICAM-1/VCAM-1 lined transmigratory cups (see text and **Figure 2**). The identity of the adhesion molecules involved in making initial contact between DC and lymphatic endothelium are not currently known.

exocytotic mechanism described by Vaahtomeri et al. does not appear to be initiated by integrins. Indeed, the identities of the receptors on DCs and endothelium responsible have yet to be determined. Moreover, it is likely that the process of DC-induced CCL21 secretion orchestrates the assembly of LYVE-1, ICAM-1, and VCAM-1 enriched endothelial transmigratory cups, as independent studies reported that corresponding structures consistently formed close to CCL21 puncta in LEC monolayers *in vitro*, and their assembly was blocked by CCL21 neutralizing antibody (76, 85, 90, 135). It remains to be determined whether other leucocyte populations such as $CD4^+$ T cells can trigger local CCL21 secretion in a similar manner to DCs and whether the coupling of chemokine release and transmigratory cup formation is a general phenomenon for vessel entry.

Additional Inflammatory Chemokines and Exosome—Mediated Secretion

Besides CCL21, LECs synthesize a variety of other chemokines including CCL1, CCL2, CCL5, CCL20, CXCL12, and CX3CL1 that are chemotactic for T cells, DCs and monocytes expressing the G-protein coupled receptors CCR8, CCR2, CCR5, CCR4, and CX_3CR, respectively, and CXCL1, CXCL2, CXCL5, and CXCL8 that are chemotactic for neutrophils that express CXCR1 and CXCR2. In common with CCL21 these are all upregulated by exposure of the endothelium to inflammatory cytokines or other inflammatory stimuli (82, 83). In most cases however, the mechanisms underlying their release and extracellular localization in relation to junctional entry sites are not so well-understood. Amongst those chemokines that have been studied in any significant detail, CXCL12 (SDF-1) has been reported to direct the entry of DCs and epidermal Langerhans cells to lymphatic vessels in mice and migration to dLNs as assessed by FITC skin painting (136, 137). More specifically, its receptor CXCR4 was shown to be highly expressed by lymph-migrating MHC class II^+ DC in skin and co-administration of a synthetic CXCR4 antagonist (4-F-benzoyl-TN14003) impaired their migration to dLNs and capacity to promote T cell proliferation after contact hypersensitization with the hapten DNBS (136). Nevertheless, the authors concluded that the role of CXCL12/CXCR4 axis, although operating in parallel for DC migration was subordinate to that of CCL21 and CCR7. Furthermore, CXCL12 does not appear to control the exit of T cells to afferent lymphatic vessels in inflamed skin, despite the fact they express CXCR4 and exhibit responsiveness to the chemokine *in vitro* (138). Additionally, CCL1, which regulates DC and tumor cell transit across the LN SCS *via* its primary receptor CCR8, has also been implicated in directing transmigration of monocytes and monocyte derived DCs across inflamed peripheral lymphatics (139–141).

More recently, CX3CL1 was shown to promote both *in vitro* transmigration and *in vivo* lymphatic entry of $CX3CR^+$ monocyte-derived DCs in the skin of oxazolone-hypersensitised mice in parallel with CCL21 (142). Unique amongst chemokines, CX3CL1 is synthesized as a membrane-anchored molecule that is subsequently cleaved by proteases including the disintegrin and metalloproteases ADAM10 and ADAM17 to generate a conventional soluble chemoattractant, and it is this form that is released basolaterally from cytokine-activated endothelium (142). Moreover, as reported within the last few months (143), CX3CL1 is also secreted from lymphatic vessels in $CD9^+$ and $CD63^+$ exosomes that form halos around the periphery of lymphatic vessels in inflamed mouse and human tissues. Intriguingly, these exosomes which carry the membrane-anchored form of CX3CL1 on their surface can elicit cellular protrusions in monocyte-derived DCs and promote their transmigration across human dermal LEC (HDLEC) monolayers, in co-operation with CCL21, as well as entry to intact lymphatic vessels in *ex vivo* exposed skin (143). Why lymphatic vessels should employ two such different modes of CX3CL1 release is unclear. However, as exosomes act as vehicles for the release of chemokines other than CX3CL1 it is possible they direct the entry of multiple different inflammatory leucocyte populations.

Interestingly, most of the remaining chemokines such as CCL2, CCL5, CCL20, and the neutrophil chemokines CXCL2, CXCL5, and CXCL8 are preferentially secreted from the luminal rather than the basolateral face of lymphatic endothelium (17, 56, 66, 132, 142), unlike CCL21 and CX3CL1, and hence it is currently unclear how they might regulate leucocyte entry to afferent lymphatic vessels. Indeed, the reduced trafficking of epidermal Langerhans cells and $CD8^+$ dermal DCs to dLNs observed in $CCR2^{-/-}$ and to a lesser extent in $CCR5^{-/-}$ mice

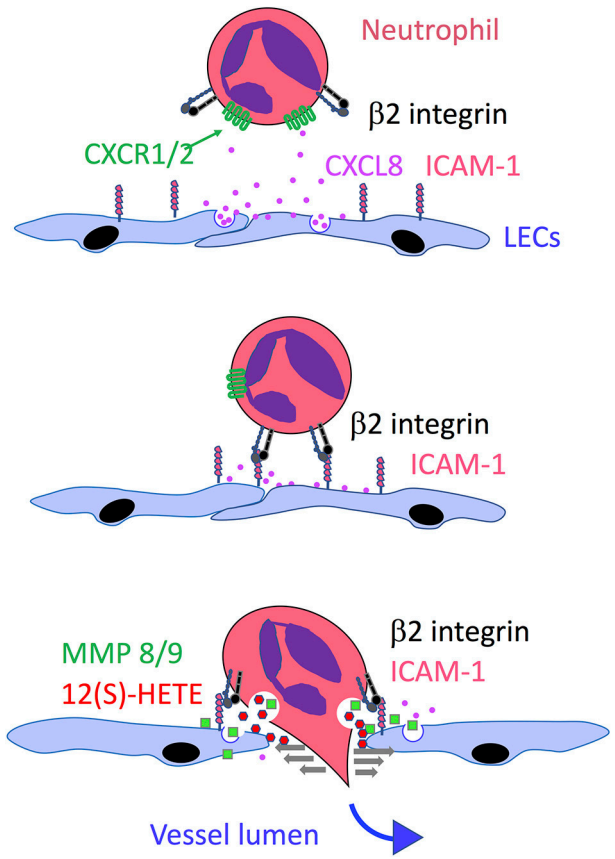

FIGURE 4 | Model of neutrophil transmigration across lymphatic endothelium, Based on a combination of *in vitro* studies with HDLECs and *in vivo* studies in mice, the model depicts the guidance of neutrophils toward the basolateral surface of endothelium following inflammation-induced release of the chemokine CXCL8 (IL-8) which transmits signals through its G protein coupled receptors CXCR1 and CXCR2 to activate the neutrophil β2 integrin LFA-1 and engage its endothelial counter-receptor ICAM-1. This in turn triggers local secretion of the matrix metalloproteinases MMP8 and MMP9 and neutrophil elastase (which also bind to the endothelial surface) and exocytosis of the chemorepellent 12(S)-HETE that together promote transient junctional retraction and neutrophil transmigration.

appeared to result from their accumulation *inside* rather than *outside* dermal lymphatics and hence it is more likely that such chemokines direct intraluminal crawling rather than initial vessel entry (144).

Finally, the steady-state levels of secreted chemokines on and around lymphatic capillaries are regulated by a group of "atypical" chemokine receptors present in lymphatic endothelium that lack signaling capacity and act primarily as chemokine scavengers. This group which includes ACKR1 (Duffy antigen), ACKR2, formerly known as D6, ACKR3 (CXCR7), and ACKR4 (CCRL1) bind and internalize inflammatory CC chemokines and prevent their inappropriate accumulation on the surface of lymphatic capillaries while also helping to establish the polarity of their gradients (145). Accordingly, the action of ACKR2 which scavenges the inflammatory CC chemokines CCL2, CCL3, CCL4, and CCL5 is thought to aid in the preferential entry of mature activated $CCR7^+$ DCs *via* CCL21, largely by preventing an accumulation of CCR2/CCR5 macrophages at the vessel surface that might otherwise block their access (146, 147). It has also been posited that ACKR4 helps preserve the responsiveness of DCs to CCL21 during entry to dermal lymphatics in inflamed tissues by scavenging CCL19 released from stromal cells and preventing a build-up of the chemokine that might otherwise de-sensitize CCR7 and lead to DC stasis (148).

Chemoattractive Guidance for T Cells *via* Sphingosine-1-Phosphate and Lymphotoxins

Besides conventional chemokines, T cells also engage two other chemoattractive pathways for vessel entry, that function co-operatively with CCL21 and may impinge at least partly on the integrin: ICAM/VCAM mediated transmigratory mechanism described above. The first involves the chemotactic lipid sphingosine 1 phosphate (S1P), best known as a regulator of lymphocyte exit from lymph nodes but which appears also to be a negative regulator of $CD4^+$ T cell entry to initial lymphatics in inflamed peripheral tissues. Normally present at high concentrations in lymph and low concentrations in lymph nodes, the resulting gradients of S1P direct transit of T cells bearing the G-protein coupled receptor S1PR to efferent lymph (149, 150). However, in a murine alloantigen-induced model of inflammation, increased synthesis of S1P in peripheral tissue or administration of the S1PR1 functional antagonist FTY720 (fingolimod) signals retention and arrest of $CD4^+$ T cells, which logjam around the basolateral surface of initial lymphatic capillaries (151). Moreover, as shown using *in vitro* transmigration assays with monolayers of the lymphatic endothelial like cell line SVEC4-10, S1P treatment of T cells blocked their transit and caused their arrest through β2 integrin-mediated adhesion to ICAM-1 and VCAM-1 (151). Whether this S1P/S1PR driven chemotactic mechanism also operates in conjunction with CCL21/CCR7 to positively regulate T cell transmigration and whether ICAM-1 and VCAM-1 are recruited to structures similar to or distinct from DC transmigratory cups during S1P mediated transmigration/arrest remains uncertain.

Secondly, the main immunoregulatory T_{REG} population that migrates between tissues and lymph nodes to maintain peripheral tolerance and immune suppression have been shown to employ lymphotoxin (LT), a lymphokine member of the TNF superfamily, to transit inflamed lymphatic vessels by engaging its signal transducing receptor LTβR in lymphatic endothelium (152, 153). Best known for its role in inducing lymph node neogenesis *via* lymphoid tissue inducer (LTi) cells, the trimeric lymphotoxin molecule is expressed on the surface of T_{REG} as a membrane-bound $LT\alpha_1\beta_2$ heterotrimer. Moreover, genetic deletion of LTα in murine T_{REGs} or treatment with soluble LTβR Ig fusion protein was shown to disrupt their entry to lymphatic capillaries in mouse skin, while leaving the transit of naïve CD4 and CD8 cells unaffected (153). More specifically, the interaction between LT and LTβR *in vitro* induces the rapid extension of VCAM-1 enriched lamellipodia-like protrusions akin to

transmigratory cups in lymphatic endothelium, that appear to facilitate T_{REG} transmigration (153). Curiously however, the VCAM counter-receptor involved in LT-mediated T_{REG} transit has not been identified. While RNA array data suggest that T_{REGs} express much higher levels of LTα than other T cell types (154), it remains unclear whether this mechanism is used by other T or B cell populations for lymphatic transmigration, or indeed whether the LTβR is redistributed to T_{REG} transmigratory structures in lymphatic endothelium together with LYVE-1 and VCAM-1 must await further investigation.

TRANSMIGRATION *VIA* CHEMOREPULSION—THE UNUSUAL MECHANISM USED BY NEUTROPHILS

In comparison to other leucocyte populations, neutrophils deploy a particularly unique and complex mechanism to enter lymphatic capillaries. While studies in mice harboring bacterial infections have shown that entry involves adhesion *via* β2 integrins, like that of DCs and T cells, more detailed *in vitro* studies using inflamed human LEC monolayers and mouse tissue explants revealed that such adhesion is just the first in a co-ordinated series of events that induce release of neutrophil elastase and the matrix metalloproteinases MMP8 and MMP9 and focal secretion of the arachidonate-derived chemorepellant lipid 12-hydroxyeicosatetraenoate (12(S)HETE), that together evoke local endothelial junctional retraction (**Figure 4**) (56, 155). These studies indicated the openings created by such retraction were transient and resolved spontaneously without any attendant cell death. Moreover, they served as portals for enhanced transit of successive waves of neutrophils which formed loosely attached swarms over the LEC monolayers resembling those previously described in lymph nodes of infected mice. Notably, the rate of *in vitro* neutrophil transmigration was some 10× fold higher than that of DCs, even in neutrophil pre-exposed LEC monolayers, suggesting they exclusively target such portals for entry without inducing significant endothelial damage (56). The contact dependent nature of this neutrophil-induced activation process is curiously reminiscent of the contact-induced secretion of CCL21 by transmigrating DCs. Indeed, neutrophil adhesion to human LEC monolayers triggered the secretion of several chemokines including CXCL2, CXCL5, and CXCL8 from the basolateral surface that could potentially direct *in vitro* neutrophil transit, although experiments with neutralizing mAbs revealed only CXCL8 (IL-8) fulfilled this role (56). Whether a murine IL-8 ortholog guides neutrophil transmigration similarly in mice along with the recently reported actions of CCL21 and CXCL12 (43, 55, 91, 136, 137) remains to be determined. Importantly, neutrophils as distinct from other lymph migrating leucocyte populations do not synthesize an HA glycocalyx, and hence cannot engage LYVE-1 transmigratory cups for vessel entry. The likely significance of the alternative transmigration mechanism is that it offers a far more rapid mode of lymphatic entry than through the button junctions of initial capillaries, in keeping with the primary function of these cells in the rapid response to tissue injury. It is also noteworthy that an analogous lipoxin-mediated process of endothelial retraction has also been described for lymphatic metastasis of tumors in mice, and certain human cancers (156).

INTRALUMINAL CRAWLING

Having transited the lymphatic endothelium, migrating leucocytes enter the vessel lumen and begin their onward journey to the dLNs. In initial capillaries, which are non-contractile and lack smooth muscle investment, the rate of lymph flow has been variably estimated as up to 200–300 μm/min (157, 158), some 2–3 orders of magnitude slower than in blood capillaries and sinusoids, and only marginally exceeding that in the interstitium (69). It is not until these capillaries merge into downstream contractile collectors that the flow rates even approach those of blood vessels. Surprisingly, intravital imaging studies revealed that DCs migrate within initial capillaries (6–8 μm/min) at an even slower rate than lymph itself, and that the majority of newly transmigrated cells are not conveyed by passive flow, but rather crawl along the luminal surface of the lymphatic capillaries until they enter downstream collectors (69–71, 159). Moreover, the crawling leucocytes exhibit semi-random patterns of migration, frequently changing direction before moving downstream (160). Indeed, using time-lapsed microscopy of YFP tagged DCs in the ear tissue of lymphatic reporter mice, it was confirmed that the rate and directionality of intraluminal crawling is almost completely unaffected by changes in lymph flow and even proceeds in its absence (160). Instead, crawling was shown to be driven by chemotaxis/haptotaxis, guided by physical gradients of CCL21 sequestered on the luminal surface of the vessel, as evidenced by confocal and immune EM imaging of mouse dermis, and by the demonstration that its downstream directionality in mouse dermal lymphatics was abrogated by CCL21 blocking mAbs or CCR7 gene deletion in the case of both endogenous and adoptively transferred DCs (160). The spontaneous establishment of such intraluminal gradients was elegantly demonstrated *in vitro* by flow chamber experiments with transfected LECs which affirmed that under levels of shear close to those of afferent lymph (0.015 dynes/cm^2), fluorescent CCL21 secreted from the luminal surface of the endothelium underwent re-binding downstream to form authentic, directionally oriented gradients for DC migration (160) (see also **Figure 1**). The macromolecules responsible for sequestering the CCL21 gradient on the endothelium likely include the lymphatic marker podoplanin (161) that reportedly binds the chemokine with high affinity (K_D 70 nM) in Biacore analyses (162), and HSPGs, whose digestion or specific knockdown has been shown to disrupt CCL21-dependent DC adhesion to LEC monolayers under flow (163–165). Thus, lymph flow generates the chemotactic gradients which drive intraluminal crawling, rather than conveying cells through physical propulsion. The necessary traction for crawling is provided by β2 integrin-mediated adhesion to ICAM-1 on the luminal surface of the endothelium, whose expression is upregulated in inflammation and whose blockade by mAbs was shown to reduce DC crawling velocity *in vitro* (69). Like DC

migration in the interstitium, efficient intraluminal crawling of DCs in inflamed lymphatics also depends on signaling *via* the Rho-associated protein kinase ROCK for dissociation of β2 integrin from ICAM-1 and uropod retraction in a continual process of adhesion and detachment from the endothelium (69).

As regards other leucocyte populations, semi-directional crawling behavior has also been observed for $CD4^+$ T cells migrating in the lumen of initial dermal lymphatic capillaries, as evidenced in a recent study that used time-lapsed imaging to track CD2 DsRed fluorescent lymphocytes in *Prox1* GFP mice reporter mice (76). Furthermore, the speed of T cell intraluminal crawling and the degree of motility were both markedly increased in the inflamed dermal lymphatics of skin contact hypersensitised mice, supported again by integrin-mediated adhesive interactions with ICAM-1 on the inner surface of the endothelium (76). Just as described for DCs, these T cells became detached from the luminal endothelium as the initial capillaries merged with downstream collectors where they were passively drawn into lymph flow by vessel pumping. Neutrophils also crawl within the lumen of initial lymphatic capillaries in a mostly downstream direction toward lymphatic collectors, and at broadly comparable velocities (mean 6–13 μm/min) to DCs and T cells. In common with these other leucocyte populations, migration was shown to be directed by haptotactic gradients of CCL21 sequestered on the capillary floor in the direction of lymph flow, with traction provided by β2 integrin mediated ICAM-1 adhesion (91). The faster recruitment of neutrophils to dLNs compared to DCs and T cells *via* afferent lymph may therefore be the product of more rapid mobilization in the tissues, the ability to translocate stored CCR7 from intracellular vesicles rather than relying on *de novo* synthesis (43), and a more efficient mode of endothelial transmigration.

On passing from initial capillaries to the smooth muscle invested pre-collectors and collectors, migrating leucocytes encounter a large increase in lymph flow rate (>1 mm/min) that likely renders intraluminal crawling redundant, and hence it is thought the cells are conveyed toward downstream lymph nodes by passive lymph flow.

ARRIVAL AND TRANSIT AT LNS

The major destination for leucocytes migrating through tissue lymphatics is the lymph node, an organ the size of a small bean in most peripheral tissues. With the exception of recirculating T_{RCM} cells which reside transiently before returning to the blood circulation *via* efferent lymphatics, most leucocytes reaching the nodes proceed no further and having fulfilled their immune function, ultimately die there. In either case the cells arrive into the subcapsular sinus (SCS), a labyrinthine compartment continuous with the afferent lymphatics that is situated just beneath the outer capsule of the node. From there, they transit across the SCS endothelium to access the T and B cell-rich cortex, in the case of DCs and neutrophils to prime or re-activate T cell immune responses, and in the case of T_{MEM} and T_{REGs} to influence or downregulate such responses, before they egress and circulate back to the tissues (see **Figure 1**). While exit from the nodes is known to be directed by the sphingosine 1-phosphate (S1P) receptor S1PR on recirculating cells and S1P in efferent lymph (150, 166, 167), it has often been assumed that cell entry from afferent lymph is a more passive process. However, it is becoming increasingly clear that the SCS endothelium represents a checkpoint for nodal entry that is regulated both by chemokines and adhesion receptors.

In one particularly informative study using mouse eGFP tagged leucocytes microinjected into pre-nodal (popliteal) lymphatics, it was found that DCs and $CD4^+$ T cells used separate routes to transit across the SCS (168). Whereas, DCs invariably crossed directly through the floor of the SCS to the cortical zones, T cells instead continued to the adjoining medullary sinuses, previously considered as exit routes from the nodes, before transmigrating to the underlying parenchyma (168). Moreover, DC transmigration across the SCS was shown to be directed by CCR7/CCL21 dependent chemotaxis, whereas $CD4^+$ T cells required this chemokine receptor pair only for subsequent haptotactic crawling within the underlying parenchyma (168). Curiously however, when $CD4^+$ T cells were co-injected into mice, the former then switched to the SCS route for transmigration, suggesting that DCs in some way remodel the endothelium during transit. More recent work has revealed that the role of CCL21 in directing DC transit can also be aided or augmented by other chemokines. Notably CCL1 released from the floor of the SCS was reported to induce the entry of monocyte-derived DCs to the LN parenchyma *via* its cognate receptor CCR8 (139). More recently, in Th2 immunized mice, the alternative CCR8 ligand CCL8 released from $CD169^+$ SCS macrophages was shown to potentiate transit of DCs across the SCS by enhancing CCR7 signal transduction (141). Significantly, polarized expression of the alternative CCL19/CCL21 scavenging receptor CCRL1 (ACKR4) in the ceiling of the SCS was reported to maintain the gradient of CCL21 inside the SCS that drives DC transit, as its deletion led to trapping of DCs within the SCS lumen (169).

Knowledge of the contribution made by adhesion receptors in leucocyte transmigration across the SCS floor is still rather sketchy in comparison with peripheral lymphatics. Curiously, a key role has been identified for the transmembrane protein PLVAP (*aka* Pal-E, MECA 32) that forms diaphragm-like structures in the SCS and certain blood vessels and which primarily regulates the size selective entry of macromolecules to the underlying cortical fibrillar conduit network for presentation by LN HEVs (170). Notably, deletion of PLVAP was found to result not only in the uncontrolled entry of small macromolecules to the cortex, but also the enhanced transmigration of injected splenic T cells. Based on *in vitro* studies with isolated SCS endothelium, it was concluded that PLVAP diaphragms guard entry portals remote from VE-cadherin buttoned junctions, through which T cells transmigrate by extension of their leading edges (170). Whether PLVAP plays a direct or indirect role in the process and also mediates transit of DCs and other leucocytes remains unknown. More recently, transcriptional profiling has identified further candidate receptors that might regulate the differential transit of leucocytes across SCS and medullary sinuses. In particular, the macrophage scavenger

receptor MSR1 (CD203) was found to be selectively expressed in SCS, where it was shown to mediate T cell adhesion in *ex vivo* assays with frozen sections. Like PLVAP however, the receptor appears to act as a regulator of SCS transit rather than a gatekeeper, as the process was enhanced not retarded in $MSR^{-/-}$ mice (171). Studies have yet to determine whether transit through SCS or other LN sinuses involves leucocyte adhesion *via* β2 integrins in resting or inflammatory conditions, or interactions between the leucocyte HA glycocalyx and LYVE-1 as in the case of initial lymphatic capillaries. Nevertheless, the observation that transit of DCs is accompanied by marked morphological changes in the floor of the SCS endothelium, including modulation of LYVE-1 and realignment of the SCS-lining $CD169^+$ macrophages is indicative that the process is complex and that these and other adhesion receptors may well play contributory roles (36). Finally, it should be stressed that dLN sinuses and the surrounding afferent lymphatic network undergo considerable expansion following antigen challenge or inflammation in peripheral tissues, through a process of lymphangiogenesis driven by VEGF-A released primarily by B cells and macrophages arriving through afferent lymph (172–174). This is accompanied by a transient increase in LN size, cellularity and lymph flow that induces DC mobilization and migration, and markedly enhances DC transit into the deep underlying LN cortex and paracortex for lymphocyte activation (174–176). It is highly likely that such changes also enhance the entry of other migrant leucocyte populations to dLNs and it is hoped that future research in this area will yield much needed insights into the underlying mechanisms.

SUMMARY AND CONCLUSIONS

Over the past decade, research using new techniques and animal models for tracking and imaging cell migration, combined with the efforts of a wide interdisciplinary community of interested scientists, has led to huge advances in our understanding of leucocyte trafficking in the lymphatic system and its immune significance. As this pace of advance seems set to continue in the immediate future, we can anticipate that the resulting mechanistic insights will translate into new targets and therapies for immune disorders and even the treatment of lymph metastasising cancers.

As outlined in this text, we now have detailed insight into the first key step in such trafficking, the entry of cells to the lymphatic vessels. In the case of DCs, this has revealed an intricate and closely co-ordinated mechanism in which physical contact of migrating leucocytes with lymphatic endothelium triggers the local exocytosis of CCL21 and formation of LYVE-1^+ transmigratory cups which envelop the migrating cells and promote their transit into the vessel lumen. Moreover, parallel observations that transmigrating T cells and macrophages elicit the formation of similar endothelial protrusions containing ICAM-1, VCAM-1 and/or LYVE-1, and reliance on an HA glycocalyx or β2 integrin adhesion, raise the possibility that lymph-migrating leucocytes exploit a common mechanism for vessel entry (76, 85, 101). Indeed, this could be considered as a form of "lymphatic synapse," through which appropriate input from other chemokine receptors or signaling components such as lymphotoxin/LTβR and S1P/S1PR1 might direct selective entry of T_{REGs} or other lymph migrating cell populations including Natural Killer (NK) cells and innate lymphoid cells (ILCs). However, the notion of a synapse may not apply to neutrophils which use a unique mechanism of integrin-dependent proteolysis and lipoxin-mediated endothelial retraction to "invade" lymphatic vessels. This unusually specialized process may have evolved to enable these professional phagocytes to exit almost instantaneously from sites of infection and reach the dLNs well-ahead of slower migrating DCs. In comparison, we know little about how the docking and adhesion of transmigrating leucocytes is choreographed and how the many "accessory" adhesion molecules including the Mannose receptor, ALCAM, CLEVER-1, CD31, CD99, and others (**Table 1**) integrate with key cup-forming components such as ICAM-1, VCAM-1, and LYVE-1. Though most leucocytes that employ such cups have been observed to enter the afferent lymphatics at button junctions in initial vessels, it seems unlikely that these are the only junctional types to allow entry. During inflammation-induced lymphangiogenesis for example, newly sprouting vessels assemble zippered rather than buttoned junctions, and in chronic inflammation, when leucocyte traffic *via* lymph is markedly increased, zippers replace buttons. It will be interesting to determine whether leucocytes have the ability to enter through zipper junctions and whether the process involves different molecular mechanisms to buttons and transit *via* a transcellular or paracellular route. A further priority will be to ascertain whether these mechanisms of lymphatic entry are universally applicable or vary between tissue beds such as the intestines, brain and central nervous system (177), given that most of our current insight has been gained from studies on mouse dermis, due to its greater accessibility.

Recent research has also provided surprising insight into how leucocytes, having entered the initial lymphatics, migrate within the vessel lumen toward downstream lymph nodes. Rather than being conveyed by passive lymph flow, it is now apparent that DCs, $CD4^+$ T cells and neutrophils actively crawl along the internal surface of initial vessels using guidance from CCL21 and transient integrin-mediated adhesion. Yet why such mechanisms should have evolved to deliberately slow the downstream progress of antigen presenting and immune effector cells is unclear. Might the intimate contact with endothelium imposed by intraluminal crawling enable for example *en route* uptake of foreign antigens or maturation signals by DCs, or MHC-mediated antigen presentation to recirculating T cells or T_{REGs} for immune tolerance? Could it provide a platform for interactions between different leucocyte populations themselves? Or might intraluminal crawling provide DCs or T cells with the option to exit and re-enter lymphatic vessels prior to reaching dLNs in order to sample or respond to antigen in the surrounding tissues? Though not yet reported in initial lymphatics vessels, there is evidence that migrating leucocytes exit collectors in adipose tissue during bacterial infection, and

that DCs which interdigitate the vessel endothelium to regulate vessel permeability and sample the surrounding tissue can subsequently detach and migrate to dLNs (178).

With the many insights into lymphatic trafficking and its immune consequences that have been gained from basic research, there is increasing scope for clinical translation and the development of new immune based therapies based on migration blockade. Judicious targeting of integrins, ICAM-1, chemokine receptors, S1P and particularly lymph-specific adhesion molecules such as LYVE-1 by appropriate blocking mAbs may be envisaged as therapeutic strategies for transplant rejection, to prevent DC migration from engrafted tissues and consequently activation of alloimmune responses in host dLNs. This is particularly applicable in the case of corneal allografts, where such therapies could be applied locally, thus avoiding off-target effects associated with systemic antibody administration. In the case of T_{REGs}, the inclusion of lymphotoxin blockade to impair their migration to lymph nodes for immune suppression might also provide an adjunct to checkpoint inhibitors for tumor immunotherapy. As a corollary, our understanding of the main factors regulating DC migration from inflamed tissues could be exploited to optimize vaccine delivery. For many years the notion of using DCs as adjuvants for adoptive cancer immunotherapy has fuelled efforts to enhance their maturation *ex vivo* using TLR ligands and inflammatory cytokines and to optimize antigen loading and presentation. Combining these approaches with pre-conditioning of vaccination sites to boost lymphatic vessel density, the efficiency of DCs for vessel entry and nodal transport should lead to much greater clinical efficacy. Likewise, boosting the exit of macrophages *via* lymphatics could aid in the resolution of inflammation and tissue recovery in conditions such as myocardial infarction where their delayed removal results in a failure of cardiomyocyte replenishment, and an increase in tissue scarring and fibrosis (41, 179–181).

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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REFERENCES

1. Swartz MA, Hubbell JA, Reddy ST. Lymphatic drainage function and its immunological implications: from dendritic cell homing to vaccine design. *Semin Immunol.* (2008) 20:147–56. doi: 10.1016/j.smim.2007.11.007
2. Jackson DG. Lymphatic regulation of cell trafficking. *J Clin Cell Immunol.* (2014) 5:258–67. doi: 10.4172/2155-9899.1000258
3. Randolph GJ, Angeli V, Swartz MA. Dendritic-cell trafficking to lymph nodes through lymphatic vessels. *Nat Rev Immunol.* (2005) 5:617–28. doi: 10.1038/nri1670
4. Platt AM, Randolph GJ. Dendritic cell migration through the lymphatic vasculature to lymph nodes. *Adv Immunol.* (2013) 120:51–68. doi: 10.1016/B978-0-12-417028-5.00002-8
5. Teijeira A, Russo E, Halin C. Taking the lymphatic route: dendritic cell migration to draining lymph nodes. *Semin Immunopathol.* (2014). doi: 10.1007/s00281-013-0410-8
6. Tewalt EF, Cohen JN, Rouhani SJ, Engelhard VH. Lymphatic endothelial cells - key players in regulation of tolerance and immunity. *Front Immunol.* (2012) 3:305. doi: 10.3389/fimmu.2012.00305
7. Hunter MC, Teijeira A, Halin C. T cell trafficking through lymphatic vessels. *Front Immunol.* (2016) 7:613. doi: 10.3389/fimmu.2016.00613
8. Randolph GJ, Ivanov S, Zinselmeyer BH, Scallan JP. The Lymphatic system: integral roles in immunity. *Annu Rev Immunol.* (2017) 35:31–52. doi: 10.1146/annurev-immunol-041015-055354
9. Lynskey NN, Banerji S, Johnson LA, Holder KA, Reglinski M, Wing PA, et al. Rapid lymphatic dissemination of encapsulated group A streptococci via lymphatic vessel endothelial receptor-1 interaction. *PLoS Pathog.* (2015) 11:e1005137. doi: 10.1371/journal.ppat.1005137
10. Lerner TR, de Souza Carvalho-Wodarz C, Repnik U, Russell MR, Borel S, Diedrich CR, et al. Lymphatic endothelial cells are a replicative niche for *Mycobacterium tuberculosis*. *J Clin Invest.* (2016) 126:1093–108. doi: 10.1172/JCI83379
11. Babu S, Nutman TB. Immunology of lymphatic filariasis. *Parasite Immunol.* (2014) 36:338–46. doi: 10.1111/pim.12081
12. Corthesy-Theulaz IE, Hopkins S, Bachmann D, Saldinger PF, Porta N, Haas R, et al. Mice are protected from Helicobacter pylori infection by nasal immunization with attenuated *Salmonella typhimurium* phoPc expressing urease A and B subunits. *Infect Immun.* (1998) 66:581–6.
13. von Bargen K, Gagnaire A, Arce-Gorvel V, de Bovis B, Baudimont F, Chasson L, et al. Cervical lymph nodes as a selective niche for brucella during oral infections. *PLoS ONE.* (2014) 10:e0121790. doi: 10.1371/journal.pone.0121790
14. Baluk P, Fuxe J, Hashizume H, Romano T, Lashnits E, Butz S, et al. Functionally specialized junctions between endothelial cells of lymphatic vessels. *J Exp Med.* (2007) 204:2349–62. doi: 10.1084/jem.20062596
15. Yao LC, Baluk P, Srinivasan RS, Oliver G, McDonald DM. Plasticity of button-like junctions in the endothelium of airway lymphatics in development and inflammation. *Am J Pathol.* (2012) 180:2561–75. doi: 10.1016/j.ajpath.2012.02.019
16. Schineis P, Runge P, Halin C. Cellular traffic through afferent lymphatic vessels. *Vascul Pharmacol.* (2018) 112:31–41. doi: 10.1016/j.vph.2018.08.001
17. Johnson LA, Jackson DG. Cell traffic and the lymphatic endothelium. *Ann N Y Acad Sci.* (2008) 1131:119–33. doi: 10.1196/annals.1413.011
18. Johnson LA, Jackson DG. Control of dendritic cell trafficking in lymphatics by chemokines. *Angiogenesis.* (2014) 17:335–45. doi: 10.1007/s10456-013-9407-0
19. Jackson DG. Hyaluronan in the lymphatics: the key role of the hyaluronan receptor LYVE-1 in leucocyte trafficking. *Matrix Biol.* (in press). doi: 10.1016/j.matbio.2018.02.001
20. Bromley SK, Thomas SY, Luster AD. Chemokine receptor CCR7 guides T cell exit from peripheral tissues and entry into afferent lymphatics. *Nat Immunol.* (2005) 6:895–901. doi: 10.1038/ni1240
21. Bromley SK, Yan S, Tomura M, Kanagawa O, Luster AD. Recirculating memory T cells are a unique subset of $CD4^+$ T cells with a distinct

phenotype and migratory pattern. *J Immunol*. (2013) 190:970–6. doi: 10.4049/jimmunol.1202805
22. Mackay CR, Kimpton WG, Brandon MR, Cahill RN. Lymphocyte subsets show marked differences in their distribution between blood and the afferent and efferent lymph of peripheral lymph nodes. *J Exp Med*. (1988) 167:1755–65. doi: 10.1084/jem.167.6.1755
23. Debes GF, Arnold CN, Young AJ, Krautwald S, Lipp M, Hay JB, et al. Chemokine receptor CCR7 required for T lymphocyte exit from peripheral tissues. *Nat Immunol*. (2005) 6:889–94. doi: 10.1038/ni1238
24. Cahill RN, Frost H, Trnka Z. The effects of antigen on the migration of recirculating lymphocytes through single lymph nodes. *J Exp Med*. (1976) 143:870–88. doi: 10.1084/jem.143.4.870
25. Cahill RN, Poskitt DC, Frost DC, Trnka Z. Two distinct pools of recirculating T lymphocytes: migratory characteristics of nodal and intestinal T lymphocytes. *J Exp Med*. (1977) 145:420–8. doi: 10.1084/jem.145.2.420
26. Mackay CR. T-cell memory: the connection between function, phenotype and migration pathways. *Immunol Today*. (1991) 12:189–92. doi: 10.1016/0167-5699(91)90051-T
27. Mackay CR, Marston WL, Dudler L. Naive and memory T cells show distinct pathways of lymphocyte recirculation. *J Exp Med*. (1990) 171:801–17. doi: 10.1084/jem.171.3.801
28. Yawalkar N, Hunger RE, Pichler WJ, Braathen LR, Brand CU. Human afferent lymph from normal skin contains an increased number of mainly memory/effector $CD4^+$ T cells expressing activation, adhesion and co-stimulatory molecules. *Eur J Immunol*. (2000) 30:491–7. doi: 10.1002/1521-4141(200002)30:2<491::AID-IMMU491>3.0.CO;2-H
29. Huang FP, Platt N, Wykes M, Major JR, Powell TJ, Jenkins CD, et al. A discrete subpopulation of dendritic cells transports apoptotic intestinal epithelial cells to T cell areas of mesenteric lymph nodes. *J Exp Med*. (2000) 191:435–44. doi: 10.1084/jem.191.3.435
30. Scheinecker C, McHugh R, Shevach EM, Germain RN. Constitutive presentation of a natural tissue autoantigen exclusively by dendritic cells in the draining lymph node. *J Exp Med*. (2002) 196:1079–90. doi: 10.1084/jem.20020991
31. Steinman RM, Hawiger D, Nussenzweig MC. Tolerogenic dendritic cells. *Annu Rev Immunol*. (2003) 21:685–711. doi: 10.1146/annurev.immunol.21.120601.141040
32. Ohl L, Mohaupt M, Czeloth N, Hintzen G, Kiafard Z, Zwirner J, et al. CCR7 governs skin dendritic cell migration under inflammatory and steady-state conditions. *Immunity*. (2004) 21:279–88. doi: 10.1016/j.immuni.2004.06.014
33. Jakubzick C, Bogunovic M, Bonito AJ, Kuan EL, Merad M, Randolph GJ. Lymph-migrating, tissue-derived dendritic cells are minor constituents within steady-state lymph nodes. *J Exp Med*. (2008) 205:2839–50. doi: 10.1084/jem.20081430
34. MacPherson GG, Jenkins CD, Stein MJ, Edwards C. Endotoxin-mediated dendritic cell release from the intestine. Characterization of released dendritic cells and TNF dependence. *J Immunol*. (1995) 154:1317–22.
35. MartIn-Fontecha A, Sebastiani S, Hopken UE, Uguccioni M, Lipp M, Lanzavecchia A, et al. Regulation of dendritic cell migration to the draining lymph node: impact on T lymphocyte traffic and priming. *J Exp Med*. (2003) 198:615–21. doi: 10.1084/jem.20030448
36. Forster R, Braun A, Worbs T. Lymph node homing of T cells and dendritic cells via afferent lymphatics. *Trends Immunol*. (2012) 33:271–80. doi: 10.1016/j.it.2012.02.007
37. Tomura M, Yoshida N, Tanaka J, Karasawa S, Miwa Y, Miyawaki A, et al. Monitoring cellular movement *in vivo* with photoconvertible fluorescence protein "Kaede" transgenic mice. *Proc Natl Acad Sci USA*. (2008) 105:10871–6. doi: 10.1073/pnas.0802278105
38. Beura LK, Hamilton SE, Bi K, Schenkel JM, Odumade OA, Casey KA, et al. Normalizing the environment recapitulates adult human immune traits in laboratory mice. *Nature*. (2016) 532:512–6. doi: 10.1038/nature17655
39. Bellingan GJ, Caldwell H, Howie SEM, Dransfield I, Haslett C. *In vivo* fate of the inflammatory macrophage during the resolution of inflammation. *J Immunol*. (1996) 157:2577–85.
40. Bellingan GJ, Laurent GJ. Fate of macrophages once having ingested apoptotic cells: lymphatic clearance or *in situ* apoptosis? In: Rossi AG, Sawatzky DA, editors. *The resolution of inflammation*. Basel: Birkhauser Verlag (2008) 75–91. doi: 10.1007/978-3-7643-7506-5_5
41. Vieira JM, Norman S, Villa Del Campo C, Cahill TJ, Barnette DN, Gunadasa-Rohling M, et al. The cardiac lymphatic system stimulates resolution of inflammation following myocardial infarction. *J Clin Invest*. (2018) 128:3402–12. doi: 10.1172/JCI97192
42. Gorlino CV, Ranocchia RP, Harman MF, Garcia IA, Crespo MI, Moron G, et al. Neutrophils exhibit differential requirements for homing molecules in their lymphatic and blood trafficking into draining lymph nodes. *J Immunol*. (2014) 193:1966–74. doi: 10.4049/jimmunol.1301791
43. Arokiasamy S, Zakian C, Dilliway J, Wang W, Nourshargh S, Voisin MB. Endogenous TNFalpha orchestrates the trafficking of neutrophils into and within lymphatic vessels during acute inflammation. *Sci Rep*. (2017) 7:44189. doi: 10.1038/srep44189
44. Nathan C. Neutrophils and immunity: challenges and opportunities. *Nat Rev Immunol*. (2006) 6:173–82. doi: 10.1038/nri1785
45. Borregaard N. Neutrophils, from marrow to microbes. *Immunity*. (2010) 33:657–70. doi: 10.1016/j.immuni.2010.11.011
46. Kolaczkowska E, Kubes P. Neutrophil recruitment and function in health and inflammation. *Nat Rev Immunol*. (2013) 13:159–75. doi: 10.1038/nri3399
47. Kim MH, Granick JL, Kwok C, Walker NJ, Borjesson DL, Curry FR, et al. Neutrophil survival and c-kit^+-progenitor proliferation in *Staphylococcus aureus*-infected skin wounds promote resolution. *Blood*. (2011) 117:3343–52. doi: 10.1182/blood-2010-07-296970
48. Bennouna S, Bliss SK, Curiel TJ, Denkers EY. Crosstalk in the innate immune system: neutrophils instruct recruitment and activation of dendritic cells during microbial infection. *J Immunol*. (2003) 171:6052–8. doi: 10.4049/jimmunol.171.11.6052
49. Megiovanni AM, Sanchez F, Robledo-Sarmiento M, Morel C, Gluckman JC, Boudaly S. Polymorphonuclear neutrophils deliver activation signals and antigenic molecules to dendritic cells: a new link between leukocytes upstream of T lymphocytes. *J Leukoc Biol*. (2006) 79:977–88. doi: 10.1189/jlb.0905526
50. Beauvillain C, Delneste Y, Scotet M, Peres A, Gascan H, Guermonprez P, et al. Neutrophils efficiently cross-prime naive T cells *in vivo*. *Blood*. (2007) 110:2965–73. doi: 10.1182/blood-2006-12-063826
51. Culshaw S, Millington OR, Brewer JM, McInnes IB. Murine neutrophils present Class II restricted antigen. *Immunol Lett*. (2008) 118:49–54. doi: 10.1016/j.imlet.2008.02.008
52. Abadie V, Badell E, Douillard P, Ensergueix D, Leenen PJ, Tanguy M, et al. Neutrophils rapidly migrate via lymphatics after *Mycobacterium bovis* BCG intradermal vaccination and shuttle live bacilli to the draining lymph nodes. *Blood*. (2005) 106:1843–50. doi: 10.1182/blood-2005-03-1281
53. Maletto BA, Ropolo AS, Alignani DO, Liscovsky MV, Ranocchia RP, Moron VG, et al. Presence of neutrophil-bearing antigen in lymphoid organs of immune mice. *Blood*. (2006) 108:3094–102. doi: 10.1182/blood-2006-04-016659
54. Chtanova T, Schaeffer M, Han SJ, van Dooren GG, Nollmann M, Herzmark P, et al. Dynamics of neutrophil migration in lymph nodes during infection. *Immunity*. (2008) 29:487–96. doi: 10.1016/j.immuni.2008.07.012
55. Beauvillain C, Cunin P, Doni A, Scotet M, Jaillon S, Loiry ML, et al. CCR7 is involved in the migration of neutrophils to lymph nodes. *Blood*. (2011) 117:1196–204. doi: 10.1182/blood-2009-11-254490
56. Rigby DA, Ferguson DJ, Johnson LA, Jackson DG. Neutrophils rapidly transit inflamed lymphatic vessel endothelium via integrin-dependent proteolysis and lipoxin-induced junctional retraction. *J Leukoc Biol*. (2015) 98:897–912. doi: 10.1189/jlb.1HI0415-149R
57. Permanyer M, Bosnjak B, Forster R. Dendritic cells, T cells and lymphatics: dialogues in migration and beyond. *Curr Opin Immunol*. (2018) 53:173–9. doi: 10.1016/j.coi.2018.05.004
58. Leak LV. The structure of lymphatic capillaries in lymph formation. *Fed Proc*. (1976) 35:1863–71.
59. Leak LV, Burke JF. Fine structure of the lymphatic capillary and the adjoining connective tissue area. *Am J Anat*. (1966) 118:785–809. doi: 10.1002/aja.1001180308
60. Leak LV, Jamuar MP. Ultrastructure of pulmonary lymphatic vessels. *Am Rev Respir Dis*. (1983) 128(2 Pt 2):S59–65.
61. Zoltzer H. Initial lymphatics–morphology and function of the endothelial cells. *Lymphology*. (2003) 36:7–25.

62. Romani N, Ratzinger G, Pfaller K, Salvenmoser W, Stossel H, Koch F, et al. Migration of dendritic cells into lymphatics-the Langerhans cell example: routes, regulation, and relevance. *Int Rev Cytol*. (2001) 207:237–70. doi: 10.1016/S0074-7696(01)07007-3
63. Stoitzner P, Holzmann S, McLellan AD, Ivarsson L, Stossel H, Kapp M, et al. Visualization and characterization of migratory Langerhans cells in murine skin and lymph nodes by antibodies against Langerin/CD207. *J Invest Dermatol*. (2003) 120:266–74. doi: 10.1046/j.1523-1747.2003.12042.x
64. Stoitzner P, Pfaller K, Stossel H, Romani N. A close-up view of migrating Langerhans cells in the skin. *J Invest Dermatol*. (2002) 118:117–25. doi: 10.1046/j.0022-202x.2001.01631.x
65. Vaahtomeri K, Brown M, Hauschild R, De Vries I, Leithner AF, Mehling M, et al. Locally triggered release of the chemokine CCL21 promotes dendritic cell transmigration across lymphatic endothelia. *Cell Rep*. (2017) 19:902–9. doi: 10.1016/j.celrep.2017.04.027
66. Kriehuber E, Breiteneder-Geleff S, Groeger M, Soleiman A, Schoppmann SF, Stingl G, et al. Isolation and characterization of dermal lymphatic and blood endothelial cells reveal stable and functionally specialized cell lineages. *J Exp Med*. (2001) 194:797–808. doi: 10.1084/jem.194.6.797
67. Ni A, Lashnits E, Yao LC, Baluk P, McDonald DM. Rapid remodeling of airway vascular architecture at birth. *Dev Dyn*. (2010) 239:2354–66. doi: 10.1002/dvdy.22379
68. Lammermann T, Germain RN. The multiple faces of leukocyte interstitial migration. *Semin Immunopathol*. (2014) 36:227–51. doi: 10.1007/s00281-014-0418-8
69. Nitschke M, Aebischer D, Abadier M, Haener S, Lucic M, Vigl B, et al. Differential requirement for ROCK in dendritic cell migration within lymphatic capillaries in steady-state and inflammation. *Blood*. (2012) 120:2249–58. doi: 10.1182/blood-2012-03-417923
70. Sen D, Forrest L, Kepler TB, Parker I, Cahalan MD. Selective and site-specific mobilization of dermal dendritic cells and Langerhans cells by Th1- and Th2-polarizing adjuvants. *Proc Natl Acad Sci USA*. (2010) 107:8334–9. doi: 10.1073/pnas.0912817107
71. Tal O, Lim HY, Gurevich I, Milo I, Shipony Z, Ng LG, et al. DC mobilization from the skin requires docking to immobilized CCL21 on lymphatic endothelium and intralymphatic crawling. *J Exp Med*. (2011) 208:2141–53. doi: 10.1084/jem.20102392
72. Saeki H, Moore AM, Brown MJ, Hwang ST. Cutting edge: secondary lymphoid-tissue chemokine (SLC) and CC chemokine receptor 7 (CCR7) participate in the emigration pathway of mature dendritic cells from the skin to regional lymph nodes. *J Immunol*. (1999) 162:2472–5.
73. Weber M, Hauschild R, Schwarz J, Moussion C, de Vries I, Legler DF, et al. Interstitial dendritic cell guidance by haptotactic chemokine gradients. *Science*. (2013) 339:328–32. doi: 10.1126/science.1228456
74. Lammermann T, Bader BL, Monkley SJ, Worbs T, Wedlich-Soldner R, Hirsch K, et al. Rapid leukocyte migration by integrin-independent flowing and squeezing. *Nature*. (2008) 453:51–5. doi: 10.1038/nature06887
75. Benvenuti F, Hugues S, Walmsley M, Ruf S, Fetler L, Popoff M, et al. Requirement of Rac1 and Rac2 expression by mature dendritic cells for T cell priming. *Science*. (2004) 305:1150–3. doi: 10.1126/science.1099159
76. Teijeira A, Hunter MC, Russo E, Proulx ST, Frei T, Debes GF, et al. T cell migration from inflamed skin to draining lymph nodes requires intralymphatic crawling supported by ICAM-1/LFA-1 interactions. *Cell Rep*. (2017) 18:857–65. doi: 10.1016/j.celrep.2016.12.078
77. Overstreet MG, Gaylo A, Angermann BR, Hughson A, Hyun YM, Lambert K, et al. Inflammation-induced interstitial migration of effector $CD4^+$ T cells is dependent on integrin alphaV. *Nat Immunol*. (2013) 14:949–58. doi: 10.1038/ni.2682
78. Wang S, Voisin MB, Larbi KY, Dangerfield J, Scheiermann C, Tran M, et al. Venular basement membranes contain specific matrix protein low expression regions that act as exit points for emigrating neutrophils. *J Exp Med*. (2006) 203:1519–32. doi: 10.1084/jem.20051210
79. Pflicke H, Sixt M. Preformed portals facilitate dendritic cell entry into afferent lymphatic vessels. *J Exp Med*. (2009) 206:2925–35. doi: 10.1084/jem.20091739
80. Ma J, Wang JH, Guo YJ, Sy MS, Bigby M. *In vivo* treatment with anti-ICAM-1 and anti-LFA-1 antibodies inhibits contact sensitization-induced migration of epidermal Langerhans cells to regional lymph nodes. *Cell Immunol*. (1994) 158:389–99. doi: 10.1006/cimm.1994.1285
81. Xu H, Guan H, Zu G, Bullard D, Hanson J, Slater M, et al. The role of ICAM-1 molecule in the migration of Langerhans cells in the skin and regional lymph node. *Eur J Immunol*. (2001) 31:3085–93. doi: 10.1002/1521-4141(2001010)31:10<3085::AID-IMMU3085>3.0.CO;2-B
82. Johnson LA, Clasper S, Holt AP, Lalor PF, Baban D, Jackson DG. An inflammation-induced mechanism for leukocyte transmigration across lymphatic vessel endothelium. *J Exp Med*. (2006) 203:2763–77. doi: 10.1084/jem.20051759
83. Vigl B, Aebischer D, Nitschke M, Iolyeva M, Rothlin T, Antsiferova O, et al. Tissue inflammation modulates gene expression of lymphatic endothelial cells and dendritic cell migration in a stimulus-dependent manner. *Blood*. (2011) 118:205–15. doi: 10.1182/blood-2010-12-326447
84. Teoh D, Johnson LA, Hanke T, McMichael AJ, Jackson DG. Blocking development of a $CD8^+$ T cell response by targeting lymphatic recruitment of APC. *J Immunol*. (2009) 182:2425–31. doi: 10.4049/jimmunol.0803661
85. Teijeira A, Garasa S, Pelaez R, Azpilikueta A, Ochoa C, Marre D, et al. Lymphatic endothelium forms integrin-engaging 3D structures during DC transit across inflamed lymphatic vessels. *J Invest Dermatol*. (2013) 133:2276–85. doi: 10.1038/jid.2013.152
86. Carman CV, Springer TA. A transmigratory cup in leukocyte diapedesis both through individual vascular endothelial cells and between them. *J Cell Biol*. (2004) 167:377–88. doi: 10.1083/jcb.200404129
87. Nieminen M, Henttinen T, Merinen M, Marttila-Ichihara F, Eriksson JE, Jalkanen S. Vimentin function in lymphocyte adhesion and transcellular migration. *Nat Cell Biol*. (2006) 8:156–62. doi: 10.1038/ncb1355
88. Millan J, Hewlett L, Glyn M, Toomre D, Clark P, Ridley AJ. Lymphocyte transcellular migration occurs through recruitment of endothelial ICAM-1 to caveola- and F-actin-rich domains. *Nat Cell Biol*. (2006) 8:113–23. doi: 10.1038/ncb1356
89. Shulman Z, Shinder V, Klein E, Grabovsky V, Yeger O, Geron E, et al. Lymphocyte crawling and transendothelial migration require chemokine triggering of high-affinity LFA-1 integrin. *Immunity*. (2009) 30:384–96. doi: 10.1016/j.immuni.2008.12.020
90. Johnson LA, Lawrance W, Roshorn YM, Hanke T, Banerji S, Jackson DG. The lymphatic vessel endothelial receptor LYVE-1 mediates dendritic cell entry to the afferent lymphatics via transmigratory cups that engage the leukocyte hyaluronan glycocalyx. *Nat Immunol*. (2017) 18:762–70. doi: 10.1038/ni.3750
91. Hampton HR, Bailey J, Tomura M, Brink R, Chtanova T. Microbe-dependent lymphatic migration of neutrophils modulates lymphocyte proliferation in lymph nodes. *Nat Commun*. (2015) 6:7139. doi: 10.1038/ncomms8139
92. Weninger W, Biro M, Jain R. Leukocyte migration in the interstitial space of non-lymphoid organs. *Nat Rev Immunol*. (2014) 14:232–46. doi: 10.1038/nri3641
93. Banerji S, Ni J, Wang SX, Clasper S, Su J, Tammi R, et al. LYVE-1, a new homologue of the CD44 glycoprotein, is a lymph-specific receptor for hyaluronan. *J Cell Biol*. (1999) 144:789–801. doi: 10.1083/jcb.144.4.789
94. Teriete P, Banerji S, Noble M, Blundell CD, Wright AJ, Pickford AR, et al. Structure of the regulatory hyaluronan binding domain in the inflammatory leukocyte homing receptor CD44. *Mol Cell*. (2004) 13:483–96. doi: 10.1016/S1097-2765(04)00080-2
95. Banerji S, Wright AJ, Noble M, Mahoney DJ, Campbell ID, Day AJ, et al. Structures of the Cd44-hyaluronan complex provide insight into a fundamental carbohydrate-protein interaction. *Nat Struct Mol Biol*. (2007) 14:234–9. doi: 10.1038/nsmb1201
96. Banerji S, Hide BR, James JR, Noble ME, Jackson DG. Distinctive properties of the hyaluronan-binding domain in the lymphatic endothelial receptor Lyve-1 and their implications for receptor function. *J Biol Chem*. (2010) 285:10724–35. doi: 10.1074/jbc.M109.047647
97. Jackson DG. Immunological functions of hyaluronan and its receptors in the lymphatics. *Immunol Rev*. (2009) 230:216–31. doi: 10.1111/j.1600-065X.2009.00803.x
98. Mahaffey CL, Mummert ME. Hyaluronan synthesis is required for IL-2-mediated T cell proliferation. *J Immunol*. (2007) 179:8191–9. doi: 10.4049/jimmunol.179.12.8191

99. Mummert ME. Immunologic roles of hyaluronan. *Immunol Res*. (2005) 31:189–206. doi: 10.1385/IR:31:3:189
100. Mummert ME, Mummert D, Edelbaum D, Hui F, Matsue H, Takashima A. Synthesis and surface expression of hyaluronan by dendritic cells and its potential role in antigen presentation. *J Immunol*. (2002) 169:4322–31. doi: 10.4049/jimmunol.169.8.4322
101. Lawrance W, Banerji S, Day AJ, Bhattacharjee S, Jackson DG. Binding of hyaluronan to the native lymphatic vessel endothelial receptor LYVE-1 is critically dependent on Receptor Clustering and Hyaluronan Organization. *J Biol Chem*. (2016) 291:8014–30. doi: 10.1074/jbc.M115.708305
102. Banerji S, Lawrance W, Metcalfe C, Briggs DC, Yamauchi A, Dushek O, et al. Homodimerization of the lymph vessel endothelial receptor LYVE-1 through a redox-labile disulfide is critical for hyaluronan binding in lymphatic endothelium. *J Biol Chem*. (2016) 291:25004–18. doi: 10.1074/jbc.M116.736926
103. Dubacheva GV, Curk T, Mognetti BM, Auzely-Velty R, Frenkel D, Richter RP. Superselective targeting using multivalent polymers. *J Am Chem Soc*. (2014) 136:1722–5. doi: 10.1021/ja411138s
104. Randolph GJ. Emigration of monocyte-derived cells to lymph nodes during resolution of inflammation and its failure in atherosclerosis. *Curr Opin Lipidol*. (2008) 19:462–8. doi: 10.1097/MOL.0b013e32830d5f09
105. Randolph GJ. Proliferating macrophages prevail in atherosclerosis. *Nat Med*. (2013) 19:1094–5. doi: 10.1038/nm.3316
106. Hou WH, Liu IH, Tsai CC, Johnson FE, Huang SS, Huang JS. CRSBP-1/LYVE-1 ligands disrupt lymphatic intercellular adhesion by inducing tyrosine phosphorylation and internalization of VE-cadherin. *J Cell Sci*. (2011) 124(Pt 8):1231–44. doi: 10.1242/jcs.078154
107. Nermut MV, Green NM, Eason P, Yamada SS, Yamada KM. Electron microscopy and structural model of human fibronectin receptor. *EMBO J*. (1988) 7:4093–9. doi: 10.1002/j.1460-2075.1988.tb03303.x
108. Bano F, Banerji S, Howarth M, Jackson D, Richter R. A Single molecule assay to probe monovalent and multivalent bonds between hyaluronan and its key leukocyte receptor CD44 under force. *Sci Rep*. (2016) 6:34176. doi: 10.1038/srep34176
109. Bano F, Tammi MI, Kang DW, Harris EN, Richter RP. Single-molecule unbinding forces between the polysaccharide hyaluronan and its binding proteins. *Biophys J*. (2018) 114:2910–22. doi: 10.1016/j.bpj.2018.05.014
110. Linehan SA, Martinez-Pomares L, da Silva RP, Gordon S. Endogenous ligands of carbohydrate recognition domains of the mannose receptor in murine macrophages, endothelial cells and secretory cells; potential relevance to inflammation and immunity. *Eur J Immunol*. (2001) 31:1857–66. doi: 10.1002/1521-4141(200106)31:6<1857::AID-IMMU1857>3.0.CO;2-D
111. Linehan SA, Martinez-Pomares L, Stahl PD, Gordon S. Mannose receptor and its putative ligands in normal murine lymphoid and nonlymphoid organs: *in situ* expression of mannose receptor by selected macrophages, endothelial cells, perivascular microglia, and mesangial cells, but not dendritic cells. *J Exp Med*. (1999) 189:1961–72. doi: 10.1084/jem.189.12.1961
112. Irjala H, Alanen K, Grenman R, Heikkila P, Joensuu H, Jalkanen S. Mannose receptor (MR) and common lymphatic endothelial and vascular endothelial receptor (CLEVER)-1 direct the binding of cancer cells to the lymph vessel endothelium. *Cancer Res*. (2003) 63:4671–6.
113. Marttila-Ichihara F, Turja R, Miiluniemi M, Karikoski M, Maksimow M, Niemela J, et al. Macrophage mannose receptor on lymphatics controls cell trafficking. *Blood*. (2008) 112:64–72. doi: 10.1182/blood-2007-10-118984
114. Salmi M, Karikoski M, Elima K, Rantakari P, Jalkanen S. CD44 binds to macrophage mannose receptor on lymphatic endothelium and supports lymphocyte migration via afferent lymphatics. *Circ Res*. (2013) 112:1577–82. doi: 10.1161/CIRCRESAHA.111.300476
115. Irjala H, Elima K, Johansson EL, Merinen M, Kontula K, Alanen K, et al. The same endothelial receptor controls lymphocyte traffic both in vascular and lymphatic vessels. *Eur J Immunol*. (2003) 33:815–24. doi: 10.1002/eji.200323859
116. Salmi M, Koskinen K, Henttinen T, Elima K, Jalkanen S. CLEVER-1 mediates lymphocyte transmigration through vascular and lymphatic endothelium. *Blood*. (2004) 104:3849–57. doi: 10.1182/blood-2004-01-0222
117. Karikoski M, Irjala H, Maksimow M, Miiluniemi M, Granfors K, Hernesniemi S, et al. Clever-1/Stabilin-1 regulates lymphocyte migration within lymphatics and leukocyte entrance to sites of inflammation. *Eur J Immunol*. (2009) 39:3477–87. doi: 10.1002/eji.200939896
118. Prevo R, Banerji S, Ni J, Jackson DG. Rapid plasma membrane-endosomal trafficking of the lymph node sinus and high endothelial venule scavenger receptor/homing receptor stabilin-1 (FEEL-1/CLEVER-1). *J Biol Chem*. (2004) 279:52580–92. doi: 10.1074/jbc.M406897200
119. Iolyeva M, Karaman S, Willrodt AH, Weingartner S, Vigl B, Halin C. Novel role for ALCAM in lymphatic network formation and function. *FASEB J*. (2013) 27:978–90. doi: 10.1096/fj.12-217844
120. Maddaluno L, Verbrugge SE, Martinoli C, Matteoli G, Chiavelli A, Zeng Y, et al. The adhesion molecule L1 regulates transendothelial migration and trafficking of dendritic cells. *J Exp Med*. (2009) 206:623–35. doi: 10.1084/jem.20081211
121. Teijeira A, Palazon A, Garasa S, Marre D, Auba C, Rogel A, et al. CD137 on inflamed lymphatic endothelial cells enhances CCL21-guided migration of dendritic cells. *FASEB J*. (2012) 26:3380–92. doi: 10.1096/fj.11-201061
122. Torzicky M, Viznerova P, Richter S, Strobl H, Scheinecker C, Foedinger D, et al. Platelet endothelial cell adhesion molecule-1 (PECAM-1/CD31) and CD99 are critical in lymphatic transmigration of human dendritic cells. *J Invest Dermatol*. (2012) 132:1149–57. doi: 10.1038/jid.2011.420
123. Gunn MD, Kyuwa S, Tam C, Kakiuchi T, Matsuzawa A, Williams LT, et al. Mice lacking expression of secondary lymphoid organ chemokine have defects in lymphocyte homing and dendritic cell localization. *J Exp Med*. (1999) 189:451–60. doi: 10.1084/jem.189.3.451
124. Forster R, Schubel A, Breitfeld D, Kremmer E, Renner-Muller I, Wolf E, et al. CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs. *Cell*. (1999) 99:23–33. doi: 10.1016/S0092-8674(00)80059-8
125. Vassileva G, Soto H, Zlotnik A, Nakano H, Kakiuchi T, Hedrick JA, et al. The reduced expression of 6Ckine in the plt mouse results from the deletion of one of two 6Ckine genes. *J Exp Med*. (1999) 190:1183–8. doi: 10.1084/jem.190.8.1183
126. Mori S, Nakano H, Aritomi K, Wang CR, Gunn MD, Kakiuchi T. Mice lacking expression of the chemokines CCL21-ser and CCL19 (plt mice) demonstrate delayed but enhanced T cell immune responses. *J Exp Med*. (2001) 193:207–18. doi: 10.1084/jem.193.2.207
127. Nakano H, Gunn MD. Gene duplications at the chemokine locus on mouse chromosome 4: multiple strain-specific haplotypes and the deletion of secondary lymphoid-organ chemokine and EBI-1 ligand chemokine genes in the plt mutation. *J Immunol*. (2001) 166:361–9. doi: 10.4049/jimmunol.166.1.361
128. Clark RA, Chong B, Mirchandani N, Brinster NK, Yamanaka K, Dowgiert RK, et al. The vast majority of CLA^+ T cells are resident in normal skin. *J Immunol*. (2006) 176:4431–9. doi: 10.4049/jimmunol.176.7.4431
129. Menning A, Hopken UE, Siegmund K, Lipp M, Hamann A, Huehn J. Distinctive role of CCR7 in migration and functional activity of naive- and effector/memory-like Treg subsets. *Eur J Immunol*. (2007) 37:1575–83. doi: 10.1002/eji.200737201
130. Brown MN, Fintushel SR, Lee MH, Jennrich S, Geherin SA, Hay JB, et al. Chemoattractant receptors and lymphocyte egress from extralymphoid tissue: changing requirements during the course of inflammation. *J Immunol*. (2010) 185:4873–82. doi: 10.4049/jimmunol.1000676
131. Eberhard Y, Ortiz S, Ruiz Lascano A, Kuznitzky R, Serra HM. Up-regulation of the chemokine CCL21 in the skin of subjects exposed to irritants. *BMC Immunol*. (2004) 5:7. doi: 10.1186/1471-2172-5-7
132. Johnson L, Jackson D. Inflammation-induced secretion of CCL21 in lymphatic endothelium is a key regulator of integrin-mediated dendritic cell transmigration. *Int Immunol*. (2010) 22:639–49. doi: 10.1093/intimm/dxq435
133. Shulman Z, Cohen SJ, Roediger B, Kalchenko V, Jain R, Grabovsky V, et al. Transendothelial migration of lymphocytes mediated by intraendothelial vesicle stores rather than by extracellular chemokine depots. *Nat Immunol*. (2011) 13:67–76. doi: 10.1038/ni.2173
134. Takamatsu H, Takegahara N, Nakagawa Y, Tomura M, Taniguchi M, Friedel RH, et al. Semaphorins guide the entry of dendritic cells into

the lymphatics by activating myosin II. *Nat Immunol*. (2010) 11:594–600. doi: 10.1038/ni.1885
135. Rouzaut A, Garasa S, Teijeira A, Gonzalez I, Martinez-Forero I, Suarez N, et al. Dendritic cells adhere to and transmigrate across lymphatic endothelium in response to IFN-alpha. *Eur J Immunol*. (2010) 40:3054–63. doi: 10.1002/eji.201040523
136. Kabashima K, Shiraishi N, Sugita K, Mori T, Onoue A, Kobayashi M, et al. CXCL12-CXCR4 engagement is required for migration of cutaneous dendritic cells. *Am J Pathol*. (2007) 171:1249–57. doi: 10.2353/ajpath.2007.070225
137. Kabashima K, Sugita K, Shiraishi N, Tamamura H, Fujii N, Tokura Y. CXCR4 engagement promotes dendritic cell survival and maturation. *Biochem Biophys Res Commun*. (2007) 361:1012–6. doi: 10.1016/j.bbrc.2007.07.128
138. Geherin SA, Wilson RP, Jennrich S, Debes GF. CXCR4 is dispensable for T cell egress from chronically inflamed skin via the afferent lymph. *PLoS ONE*. (2014) 9:e95626. doi: 10.1371/journal.pone.0095626
139. Qu C, Edwards EW, Tacke F, Angeli V, Llodra J, Sanchez-Schmitz G, et al. Role of CCR8 and other chemokine pathways in the migration of monocyte-derived dendritic cells to lymph nodes. *J Exp Med*. (2004) 200:1231–41. doi: 10.1084/jem.20032152
140. Das S, Sarrou E, Podgrabinska S, Cassella M, Mungamuri SK, Feirt N, et al. Tumor cell entry into the lymph node is controlled by CCL1 chemokine expressed by lymph node lymphatic sinuses. *J Exp Med*. (2013) 210:1509–28. doi: 10.1084/jem.20111627
141. Sokol CL, Camire RB, Jones MC, Luster AD. The chemokine receptor CCR8 promotes the migration of dendritic cells into the lymph node parenchyma to initiate the allergic immune response. *Immunity*. (2018) 49:449–63 e6. doi: 10.1016/j.immuni.2018.07.012
142. Johnson LA, Jackson DG. The chemokine CX3CL1 promotes trafficking of dendritic cells through inflamed lymphatics. *J Cell Sci*. (2013) 126(Pt 22):5259–70. doi: 10.1242/jcs.135343
143. Brown M, Johnson LA, Leone DA, Majek P, Vaahtomeri K, Senfter D, et al. Lymphatic exosomes promote dendritic cell migration along guidance cues. *J Cell Biol*. (2018) 217:2205–21. doi: 10.1083/jcb.201612051
144. Sato N, Ahuja SK, Quinones M, Kostecki V, Reddick RL, Melby PC, et al. CC chemokine receptor (CCR)2 is required for langerhans cell migration and localization of T helper cell type 1 (Th1)-inducing dendritic cells. Absence of CCR2 shifts the Leishmania major-resistant phenotype to a susceptible state dominated by Th2 cytokines, b cell outgrowth, and sustained neutrophilic inflammation. *J Exp Med*. (2000) 192:205–18. doi: 10.1084/jem.192.2.205
145. Graham GJ. D6 and the atypical chemokine receptor family: novel regulators of immune and inflammatory processes. *Eur J Immunol*. (2009) 39:342–51. doi: 10.1002/eji.200838858
146. Lee KM, McKimmie CS, Gilchrist DS, Pallas KJ, Nibbs RJ, Garside P, et al. D6 facilitates cellular migration and fluid flow to lymph nodes by suppressing lymphatic congestion. *Blood*. (2011) 118:6220–9. doi: 10.1182/blood-2011-03-344044
147. McKimmie CS, Singh MD, Hewit K, Lopez-Franco O, Le Brocq M, Rose-John S, et al. An analysis of the function and expression of D6 on lymphatic endothelial cells. *Blood*. (2013) 121:3768–77. doi: 10.1182/blood-2012-04-425314
148. Bryce SA, Wilson RA, Tiplady EM, Asquith DL, Bromley SK, Luster AD, et al. ACKR4 on stromal cells scavenges CCL19 to enable CCR7-dependent trafficking of APCs from inflamed skin to lymph nodes. *J Immunol*. (2016) 196:3341–53. doi: 10.4049/jimmunol.1501542
149. Chiba K, Matsuyuki H, Maeda Y, Sugahara K. Role of sphingosine 1-phosphate receptor type 1 in lymphocyte egress from secondary lymphoid tissues and thymus. *Cell Mol Immunol*. (2006) 3:11–9.
150. Pham TH, Baluk P, Xu Y, Grigorova I, Bankovich AJ, Pappu R, et al. Lymphatic endothelial cell sphingosine kinase activity is required for lymphocyte egress and lymphatic patterning. *J Exp Med*. (2010) 207:17–27. doi: 10.1084/jem.20091619
151. Ledgerwood LG, Lal G, Zhang N, Garin A, Esses SJ, Ginhoux F, et al. The sphingosine 1-phosphate receptor 1 causes tissue retention by inhibiting the entry of peripheral tissue T lymphocytes into afferent lymphatics. *Nat Immunol*. (2008) 9:42–53. doi: 10.1038/ni1534
152. Zhang N, Schroppel B, Lal G, Jakubzick C, Mao X, Chen D, et al. Regulatory T cells sequentially migrate from inflamed tissues to draining lymph nodes to suppress the alloimmune response. *Immunity*. (2009) 30:458–69. doi: 10.1016/j.immuni.2008.12.022
153. Brinkman CC, Iwami D, Hritzo MK, Xiong Y, Ahmad S, Simon T, et al. Treg engage lymphotoxin beta receptor for afferent lymphatic transendothelial migration. *Nat Commun*. (2016) 7:12021. doi: 10.1038/ncomms12021
154. Heng TS, Painter MW, Immunological Genome Project C. The immunological genome project: networks of gene expression in immune cells. *Nat Immunol*. (2008) 9:1091–4. doi: 10.1038/ni1008-1091
155. Teijeira A, Halin C. Editorial: breaching their way through: neutrophils destroy intercellular junctions to transmigrate rapidly across lymphatic endothelium. *J Leukoc Biol*. (2015) 98:880–2. doi: 10.1189/jlb.3CE0615-273
156. Kerjaschki D, Bago-Horvath Z, Rudas M, Sexl V, Schneckenleithner C, Wolbank S, et al. Lipoxygenase mediates invasion of intrametastatic lymphatic vessels and propagates lymph node metastasis of human mammary carcinoma xenografts in mouse. *J Clin Invest*. (2011) 121:2000–12. doi: 10.1172/JCI44751
157. Berk DA, Swartz MA, Leu AJ, Jain RK. Transport in lymphatic capillaries. II. Microscopic velocity measurement with fluorescence photobleaching. *Am J Physiol*. (1996) 270(1 Pt 2):H330–7.
158. Swartz MA, Berk DA, Jain RK. Transport in lymphatic capillaries. I. Macroscopic measurements using residence time distribution theory. *Am J Physiol*. (1996) 270(1 Pt 2):H324–9.
159. Russo E, Nitschke M, Halin C. Dendritic cell interactions with lymphatic endothelium. *Lymphat Res Biol*. (2013) 11:172–82. doi: 10.1089/lrb.2013.0008
160. Russo E, Teijeira A, Vaahtomeri K, Willrodt AH, Bloch JS, Nitschke M, et al. Intralymphatic CCL21 promotes tissue egress of dendritic cells through afferent lymphatic vessels. *Cell Rep*. (2016) 14:1723–34. doi: 10.1016/j.celrep.2016.01.048
161. Breiteneder-Geleff S, Matsui K, Soleiman A, Meraner P, Poczewski H, Kalt R, et al. Podoplanin, novel 43-kd membrane protein of glomerular epithelial cells, is down-regulated in puromycin nephrosis. *Am J Pathol*. (1997) 151:1141–52.
162. Kerjaschki D, Regele HM, Moosberger I, Nagy-Bojarski K, Watschinger B, Soleiman A, et al. Lymphatic neoangiogenesis in human kidney transplants is associated with immunologically active lymphocytic infiltrates. *J Am Soc Nephrol*. (2004) 15:603–12. doi: 10.1097/01.ASN.0000113316.52371.2E
163. Yin X, Johns SC, Kim D, Mikulski Z, Salanga CL, Handel TM, et al. Lymphatic specific disruption in the fine structure of heparan sulfate inhibits dendritic cell traffic and functional T cell responses in the lymph node. *J Immunol*. (2014) 192:2133–42. doi: 10.4049/jimmunol.1301286
164. Yin X, Johns SC, Lawrence R, Xu D, Reddi K, Bishop JR, et al. Lymphatic endothelial heparan sulfate deficiency results in altered growth responses to vascular endothelial growth factor-C (VEGF-C). *J Biol Chem*. (2011) 286:14952–62. doi: 10.1074/jbc.M110.206664
165. Yin X, Truty J, Lawrence R, Johns SC, Srinivasan RS, Handel TM, et al. A critical role for lymphatic endothelial heparan sulfate in lymph node metastasis. *Mol Cancer*. (2010) 9:316. doi: 10.1186/1476-4598-9-316
166. Matloubian M, Lo CG, Cinamon G, Lesneski MJ, Xu Y, Brinkmann V, et al. Lymphocyte egress from thymus and peripheral lymphoid organs is dependent on S1P receptor 1. *Nature*. (2004) 427:355–60. doi: 10.1038/nature02284
167. Cyster JG, Schwab SR. Sphingosine-1-phosphate and lymphocyte egress from lymphoid organs. *Annu Rev Immunol*. (2012) 30:69–94. doi: 10.1146/annurev-immunol-020711-075011
168. Braun A, Worbs T, Moschovakis GL, Halle S, Hoffmann K, Bolter J, et al. Afferent lymph-derived T cells and DCs use different chemokine receptor CCR7-dependent routes for entry into the lymph node and intranodal migration. *Nat Immunol*. (2011) 12:879–87. doi: 10.1038/ni.2085
169. Ulvmar MH, Werth K, Braun A, Kelay P, Hub E, Eller K, et al. The atypical chemokine receptor CCRL1 shapes functional CCL21 gradients in lymph nodes. *Nat Immunol*. (2014) 15:623–30. doi: 10.1038/ni.2889
170. Rantakari P, Auvinen K, Jappinen N, Kapraali M, Valtonen J, Karikoski M, et al. The endothelial protein PLVAP in lymphatics controls the entry of lymphocytes and antigens into lymph nodes. *Nat Immunol*. (2015) 16:386–96. doi: 10.1038/ni.3101
171. Iftakhar EKI, Fair-Makela R, Kukkonen-Macchi A, Elima K, Karikoski M, Rantakari P, et al. Gene-expression profiling of different arms of lymphatic

vasculature identifies candidates for manipulation of cell traffic. *Proc Natl Acad Sci USA*. (2016) 113:10643–8. doi: 10.1073/pnas.1602357113
172. Angeli V, Ginhoux F, Llodra J, Quemeneur L, Frenette PS, Skobe M, et al. B cell-driven lymphangiogenesis in inflamed lymph nodes enhances dendritic cell mobilization. *Immunity*. (2006) 24:203–15. doi: 10.1016/j.immuni.2006.01.003
173. Halin C, Tobler NE, Vigl B, Brown LF, Detmar M. VEGF-A produced by chronically inflamed tissue induces lymphangiogenesis in draining lymph nodes. *Blood*. (2007) 110:3158–67. doi: 10.1182/blood-2007-01-066811
174. Kataru RP, Jung K, Jang C, Yang H, Schwendener RA, Baik JE, et al. Critical role of $CD11b^{+}$ macrophages and VEGF in inflammatory lymphangiogenesis, antigen clearance, and inflammation resolution. *Blood*. (2009) 113:5650–9. doi: 10.1182/blood-2008-09-176776
175. Drayton DL, Liao S, Mounzer RH, Ruddle NH. Lymphoid organ development: from ontogeny to neogenesis. *Nat Immunol*. (2006) 7:344–53. doi: 10.1038/ni1330
176. Kataru RP, Kim H, Jang C, Choi DK, Koh BI, Kim M, et al. T lymphocytes negatively regulate lymph node lymphatic vessel formation. *Immunity*. (2011) 34:96–107. doi: 10.1016/j.immuni.2010.12.016
177. Aspelund A, Antila S, Proulx ST, Karlsen TV, Karaman S, Detmar M, et al. A dural lymphatic vascular system that drains brain interstitial fluid and macromolecules. *J Exp Med*. (2015) 212:991–9. doi: 10.1084/jem.20142290
178. Ivanov S, Scallan JP, Kim KW, Werth K, Johnson MW, Saunders BT, et al. CCR7 and IRF4-dependent dendritic cells regulate lymphatic collecting vessel permeability. *J Clin Invest*. (2016) 126:1581–91. doi: 10.1172/JCI84518
179. Nahrendorf M, Pittet MJ, Swirski FK. Monocytes: protagonists of infarct inflammation and repair after myocardial infarction. *Circulation*. (2010) 121:2437–45. doi: 10.1161/CIRCULATIONAHA.109.916346
180. Aspelund A, Robciuc MR, Karaman S, Makinen T, Alitalo K. Lymphatic system in cardiovascular medicine. *Circ Res*. (2016) 118:515–30. doi: 10.1161/CIRCRESAHA.115.306544
181. Henri O, Pouehe C, Houssari M, Galas L, Nicol L, Edwards-Levy F, et al. Selective stimulation of cardiac lymphangiogenesis reduces myocardial edema and fibrosis leading to improved cardiac function following myocardial infarction. *Circulation*. (2016) 133:1484–97. Discussion 97. doi: 10.1161/CIRCULATIONAHA.115.020143

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REVIEW
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Regulation of Immune Function by the Lymphatic System in Lymphedema

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The lymphatic vasculature has traditionally been thought to play a passive role in the regulation of immune responses by transporting antigen presenting cells and soluble antigens to regional lymph nodes. However, more recent studies have shown that lymphatic endothelial cells regulate immune responses more directly by modulating entry of immune cells into lymphatic capillaries, presenting antigens on major histocompatibility complex proteins, and modulating antigen presenting cells. Secondary lymphedema is a disease that develops when the lymphatic system is injured during surgical treatment of cancers or is damaged by infections. We have used mouse models of lymphedema in order to understand the effects of chronic lymphatic injury on immune responses and have shown that lymphedema results in a mixed T helper cell and T regulatory cell (Treg) inflammatory response. Prolonged T helper 2 biased immune responses in lymphedema regulate the pathology of this disease by promoting tissue fibrosis, inhibiting formation of collateral lymphatics, decreasing lymphatic vessel pumping capacity, and increasing lymphatic leakiness. Treg infiltration following lymphatic injury results from proliferation of natural Tregs and suppresses innate and adaptive immune responses. These studies have broad clinical relevance since understanding how lymphatic injury in lymphedema can modulate immune responses may provide a template with which we can study more subtle forms of lymphatic injury that may occur in physiologic conditions such as aging, obesity, metabolic tumors, and in the tumor microenvironment.

Keywords: lymphatic vessels, immune function, Th2 type T cells, inflammation, fibrosis

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THE LYMPHATIC SYSTEM DIRECTLY AND INDIRECTLY REGULATES IMMUNE RESPONSES

The lymphatic system is comprised of a series of blind ended, single cell thick initial lymphatic vessels that drain progressively into successively larger vessels and eventually return interstitial fluid back to the systemic circulation. Lymphatic flow is regulated by coordinated pumping of smooth muscle cells that partially envelop collecting lymphatics and compressive forces from surrounding skeletal muscles. One-way valves in collecting lymphatics ensure forward flow of interstitial fluid and prevent reflux when a segment of the collecting vessel located between two valves contracts (1).

In addition to draining interstitial fluid, the lymphatics system is responsible for lipid and fatty acid absorption and is an important regulator of cholesterol metabolism (2). The lymphatic system

also regulates immune responses by transporting bacteria, foreign antigens, particulate matter, exosomes, and immune cells to regional lymph nodes and lymphoid structures (3). Regulation of immune responses occurs at multiple levels and is both active and passive in nature. Active mechanisms of immune response regulation by the lymphatics includes regulation of immune cell entry and migration through the lymphatic system by LEC cytokine, chemokine, and adhesion molecule expression. In addition, LECs modulate immune responses and regulate autoimmunity by transferring self-antigen to DCs (4), or by directly inducing T cells tolerance using their PD-L1 molecule or MHC II-self antigen peptide complex that acquired from DCs (5–7). The lymphatic system can also control immune responses indirectly by modulating the rate at which antigens and cells are delivered to regional lymph nodes by regulating lymphatic vessel tone and pumping (8–11).

Given the important role of the lymphatic system in a wide range of physiologic processes, it is not surprising therefore that abnormalities in lymphatic function have been implicated in inflammatory disorders (12, 13), immune tolerance (14), metabolic abnormalities such as obesity and metabolic syndrome (2), cardiovascular disease including hypertension and atherosclerosis (15), cancer growth and metastasis (16–18), infectious diseases (19, 20), and septic shock (21). Genetic, iatrogenic, traumatic, or infectious abnormalities of the lymphatic system cause severe complications including lymphedema, chylous ascites, chylothorax, and lymphatic vascular anomalies. Recent findings suggest that many of these abnormalities are related not only to changes in lymphatic fluid transport function, but also lymphatic regulation of immune responses.

LYMPHATIC FUNCTION IS VARIABLE AND CAN REGULATE IMMUNE RESPONSES

Lymphatic function is highly variable clinically and modulated by numerous factors including chronic inflammation, tumors, external stimuli such as radiation, age, obesity, and metabolic dysfunction. For example, reports published in the late 1990s showed that aging results in structural changes in the lymphatic system including loss of elasticity, reduced smooth muscle coverage, decreased number of mesenteric collecting vessels, and decreased mesenteric lymphatic flow (22, 23). More recent studies have shown that aging results in ultrastructural changes in collecting lymphatics resulting in tissue degeneration and loss of extracellular matrix components, decreased expression of contractile and regulatory proteins, and increased lymphatic vascular permeability (24, 25). These structural changes, together with changes in gradients of eNOS, iNOS, and histamine significantly decrease aging lymphatic vessel contraction, interstitial fluid transport function, transport of pathogens to regional lymph nodes, and clearance of macromolecules from the central nervous system (26, 27). Similar changes in lymphatic function have been reported in obesity. For example, obesity results in structural and physiologic changes in the lymphatic system including increased lymphatic leakiness, decreased collecting vessel contractility, and decreased lymph node size and changes in lymph node architecture (2). Obese patients have decreased clearance of interstitial fluid as compared to lean individuals (28), obesity increases the risk of developing lymphedema after surgery (29), and severe obesity can lead to the spontaneous development of lymphedema (30). Interestingly, obesity induced lymphatic abnormalities decrease adaptive immune responses and are reversible with treatments that promote lymphangiogenesis and increase lymphatic transport (31). These findings are important because they suggest that common comorbid conditions have significant effects on the lymphatic system and these changes in turn significantly modulate immune responses.

Variability in lymphatic function resulting from aging, obesity, or metabolic syndrome may play a key role in immune responses to solid tumors and provide a rationale for the fact that these comorbid conditions increase the risk of tumor development and metastasis. Solid tumors such as melanoma and breast cancer are surrounded by abnormal, leaky lymphatics with impaired lymphatic transport function. Tumor, draining lymph node lymphangiogenesis and increased VEGF-C expression by inflammatory cells increase tumor growth and metastasis. Lymphatic vessel density and VEGF-C expression correlates with cytotoxic T cell infiltration and expression of immunosuppressive factors (iNOS, IDO, Arg-1) in patients with melanoma indicating a possibility of LECs playing a dual role in promoting and hindering anti-tumor responses (32). These changes are associated with increased risk of local/regional tumor recurrence and decreased survival. Intradermal implantation of melanoma in mice that lack dermal lymphatics due to transgenic expression of K-14 VEGFR3-Ig results in more rapid tumor growth locally, decreased distant metastasis, and decreased inflammatory cell infiltration, and impaired dendritic cell migration to regional lymph node (33). Interestingly, K-14-VEGFR3-Ig mice had impaired tumor specific immune responses after vaccination. Lymphatic endothelial cell (LEC) presentation of tumor antigens on major histocompatibility complex proteins (MHCI or MHCII) in the context of PD-L1 (checkpoint molecule programmed death ligand 1) and the absence of co-stimulatory molecules results in suppression of T cell mediated immune responses by decreasing T cell activation and proliferation and increasing apoptosis (4, 34). Taken together, these findings suggest that tumor lymphatics regulate tumor immune response and modulate the tumor microenvironment (35).

LYMPHEDEMA RESULTS IN CHRONIC INFLAMMATION

Lymph node dissection for cancer treatment is the most common cause of lymphedema development in Western Countries. Because lymphedema in this scenario develops secondary to surgical injury, this type of lymphedema is referred to as secondary lymphedema. Patients with secondary lymphedema develop progressive fibroadipose deposition in the affected limb and have an increased risk of developing infections and secondary malignancies. These pathologic changes cause

significant morbidity and decrease quality of life (36). It is estimated that 20–40% of patients who undergo treatment for solid malignancies such as breast cancer, melanoma, gynecological or urologic tumors, or sarcomas go on to develop lymphedema (37). Because these cancers are common, there is a large number of patients who are diagnosed with lymphedema annually. This fact, together with the fact that lymphedema is a life-long disease and survival following cancer treatment has significantly improved, is responsible for the increasing number of patients who suffer from lymphedema. Although estimates of the number of patients who suffer from secondary lymphedema are variable and range between 5 and 10 million individuals, it is important to note that even the most conservative estimates make lymphedema among the most common chronic disorders and the most common long-term complication of cancer treatment.

Historically, development of lymphedema has been thought to be secondary to impaired development of collateral lymphatics that bypass the zone of injury. Indeed, this concept led to the multiple preclinical studies reporting on the use of exogenous lymphangiogenic growth factors as a therapeutic treatment for lymphedema (38–40). However, more recent studies have shown that although abnormal collateral lymphatic formation is a pathologic finding in patients with lymphedema, the clinical development of lymphedema may not be due to impaired production of lymphangiogenic cytokines such as VEGF-C (41, 42). In fact, patients with lymphedema have increased serum levels of VEGF-C (43) and transgenic mice that over-express VEGF-C have more severe pathologic changes of lymphedema in a tail model (44). These findings suggest that abnormalities in lymphangiogenesis alone are not enough to cause lymphedema. Rather, lymphatic injury appears to serve as an initiating factor setting into motion other pathologic changes that in some patients results in the development of lymphedema. This hypothesis is supported by the fact that lymph node dissection does not always cause lymphedema; instead only a subset of patients (about 1 in 3) who undergo this treatment go on to develop the disease. Further, the hypothesis that lymphatic injury is simply an initiating event that is necessary but alone insufficient to cause lymphedema is supported by the fact that the development of lymphedema in most cases occurs in a delayed fashion. Typically, patients who undergo lymph node dissection have minor swelling that resolves spontaneously 2–6 weeks after the initial surgery. In some patients however, this swelling recurs permanently 8–24 months later.

Recent studies from our lab and others have shown that lymphatic injury results in chronic inflammatory changes in the skin distal to the zone of injury and that this response, in turn regulates development of lymphedema by causing lymphatic leakiness, decreasing lymphatic pumping, increasing tissue fibrosis, and impairing development of collateral lymphatics. These inflammatory changes illustrate the important coordination of immune responses by the lymphatic system. The changes in inflammatory responses after significant lymphatic injury during surgery enable us to study the effects of more subtle forms of lymphatic injury as may occur in aging, obesity, metabolic dysfunction, or the tumor microenvironment. Thus, studying lymphedema is broadly relevant and may provide important insight into the role of the lymphatic system in regulating immune responses in other physiologic or pathologic events.

LYMPHATIC INJURY RESULTS IN UPREGULATION OF ENDOGENOUS DANGER SIGNAL MOLECULES

Danger-associated molecular patterns (DAMPs) are the endogenous cellular products released by stressed, damaged or cells undergoing necrosis that alarm and activate the innate immune system components. By activating innate immune system, DAMPs create a pro-inflammatory state in the damaged tissues with an intention of host defense. However, in excess DAMPs can be harmful due to continuous activation of innate immune reactions (45, 46). Earlier studies by our group revealed the spatial and temporal expression patterns of High mobility group box 1 (HMGB1) and Heat shock protein 70 (HSP-70), two of the well-studied DAMPs (47). Using a mouse tail model of lymphedema tissues and human lymphedema biopsy samples, these studies have shown that DAMP expression occurred along a spatial gradient relative to the site of injury with the highest expression occurring closest to the zone of lymphatic injury and decreasing more distally. DAMP expression was localized to virtually all tissue cells including LECs, blood endothelial cells, adipocytes, and other stromal cells. More importantly, the expression of DAMPs persisted chronically even 6 weeks post-surgery, a time period that is far longer than wound healing related to the initial surgery. Other studies have shown that HMGB1 promotes lymphangiogenesis *in vivo* and *in vitro* (48, 49). In support of this, we found that blockade of HMGB1 in the mouse tail lymphedema model inhibited inflammatory lymphangiogenesis.

DAMPs initiate innate immune responses by interacting with pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs). To understand the role of DAMPs interaction with PRRs in lymphedema development, we previously studied lymphedema development in different TLR knockout mice (TLR 2, 4, and 9 KO) using a mouse tail model of the disease (47). Consistent with our findings with HMGB1 blockade resulting in impaired lymphangiogenesis, we found that TLR knockout mice had more severe lymphedema, decreased lymphatic transport, abnormal lymphatic structures, decreased number of lymphatic capillaries, increased collagen deposition and dermal fibrosis, and increased infiltration of T cells as compared with wild-type controls. Taken together, these studies indicate that lymphatic injury chronically activates DAMPs, that eventually activates TLRs. The net result of this DAMP-TLR cascade activation during lymphatic injury is regulation of inflammatory lymphangiogenesis and chronic inflammatory reactions.

ROLE OF MACROPHAGES DURING LYMPHATIC INJURY AND LYMPHEDEMA PROGRESSION

In similar lines with several other inflammation pathologies, macrophage recruitment and accumulation is significantly observed during lymphedema both in human biopsy (50, 51)

and animal lymphedema models (50, 52). It is reported that macrophage recruitment is significantly high immediately after lymphatic injury compared to later stages of lymphedema (53). Macrophages seem to play multiple roles in lymphedema pathology based on results from several groups. Studies from our group shows that depletion of macrophages promotes impaired lymphatic function, infiltration of $CD4^+$ cells and aggravates fibrosis (50). In addition we have demonstrated that during lymphatic injury, M2-differentiated macrophages are responsible for initiation of superficial dermal lymphangiogenesis by secreting lymphangiogenic growth factors like VEGF-C (54). Furthermore, it is being reported that, macrophage induced VEGF-C production is positively influenced by prostaglandin E2 (55) or $CD4^+$ T cells and blocking of COX2, IFN-γ or IL-17 abrogates VEGF-C expression by macrophages (52) indicating interplay between inflammatory mediators and T cells with macrophages during lymphedema. Macrophages play a double-edged sword role in lymphedema pathology, because it plays important role in initial lymphangiogenesis post-lymphatic surgery transiently alleviating fluid accumulation (54). However, macrophages strongly express iNOS and are potential source of nitric oxide (NO) which attenuates collecting lymphatic vessels contraction and pumping significantly decreasing lymphatic function and eventually accumulation of lymph fluid and immunosuppression (9). Macrophages are also a major source of IL-6, a cytokine implicated in mediating chronic inflammation and adipose metabolism in lymphedema and found abundantly in lymphedema tissues (56, 57). Furthermore, macrophages are important source of TGF-β a major anti-lymphangiogenic cytokine that inhibits lymphangiogenesis and is copiously present in lymphedema tissues (58, 59). Taken together, these studies suggest that macrophages play a complex paracrine role in pathology of lymphedema regulating lymphangiogenesis, fibrosis and lymphatic function mostly through varied kinds of growth factor and cytokine secretion that have a dual impact on lymphatic endothelial cells.

LYMPHATIC INJURY RESULTS IN ACTIVATION OF DENDRITIC CELLS IN THE SKIN AND MIGRATION TO REGIONAL LYMPH NODES

How do chronic inflammatory responses in lymphedema get initiated? We have studied this question using mouse models of lymphedema and adoptive transfer of labeled cells to track the homing, activation, and migration of inflammatory cells (60). Adoptive transfer using intravenous injection is useful tool since this approach can provide insight into the behavior of circulating and skin resident inflammatory cells. To study activation of chronic T cell responses, we injected labeled dendritic cells (DCs) since these leukocytes are powerful antigen presenting cells that regulate adaptive T cell inflammatory responses. Wild-type or CD4 knockout mice (CD4KO) underwent popliteal lymph node dissection (PLND) and allowed to recover. Two weeks following surgery, we adoptively transferred bone marrow DCs using intravenous injection and analyzed DCs in the skin and the inguinal lymph node (the next draining basin) using flow cytometry. Interestingly, we found that adoptively transferred DCs rapidly migrated to the lymphedematous skin (within 6 h of injection) where they expressed activation markers. Over the next 24 h, activated DCs migrated to the inguinal lymph node. Importantly, DC activation or migration was identical in wild-type and CD4KO mice suggesting that DC activation precedes chronic $CD4^+$ cell inflammatory reactions in lymphedema. These findings are supported by previous studies demonstrating that the lymphatics play a key role in regulating DC migration. Activated DCs upregulate cell surface expression of the chemokine receptor CCR7 (C-C chemokine receptor 7) whose ligands [CCL21 (C-C motif ligand 21) and CCL19] are expressed by LECs. Gradients of CCL21 guide DCs to initial lymphatics (61) and docking to CCL21 (62), and adhesion molecules such as intracellular adhesion molecule 1 (ICAM1), vascular cell adhesion molecule (VCAM1) expressed by LEC is required for entry into the vessel lumen (63). DCs enter the lymphatic vessel through gaps between LECs (64) and are guided to lymph nodes by gradients of CCL21 in lymphatic fluid (65) as well as passive lymphatic fluid flow (**Figure 1**).

$CD4^+$ CELL INFLAMMATORY RESPONSES ARE NECESSARY FOR THE DEVELOPMENT OF LYMPHEDEMA

DCs activate naïve T cell in lymph nodes by presenting antigens in the context of co-stimulatory molecules. This process is also important for chronic T cell inflammatory reactions in lymphedema. Histological and flow cytometry analysis of tissue biopsies from patients with lymphedema as well as mouse models of lymphedema demonstrate that the predominant inflammatory cell infiltrate is comprised of $CD4^+$ cells. In fact, the severity of lymphedema correlates significantly with the degree of $CD4^+$ cell inflammatory response. T cell inflammatory responses are necessary for the development of lymphedema since nude mice (lack all T cells) or mice lacking $CD4^+$ cells (CD4KO) do not develop lymphedema after skin and lymphatic excision. Similarly, depletion of T cells using antibodies or topical treatment with tacrolimus, a medication that prevents T cell proliferation/differentiation prevents development of lymphedema in preclinical models and can be used to treat the disease once it has developed. In contrast, depletion of other inflammatory cell types such as cytotoxic T cells, B cells, and macrophages either has no significant effect or worsens the severity of lymphedema (66).

T CELL ACTIVATION IN LYMPHEDEMA REQUIRES T CELL RECEPTOR ACTIVATION AND CO-STIMULATORY MOLECULE EXPRESSION

T cell activation and differentiation in lymphedema requires T cell receptor activation in the context of co-stimulatory

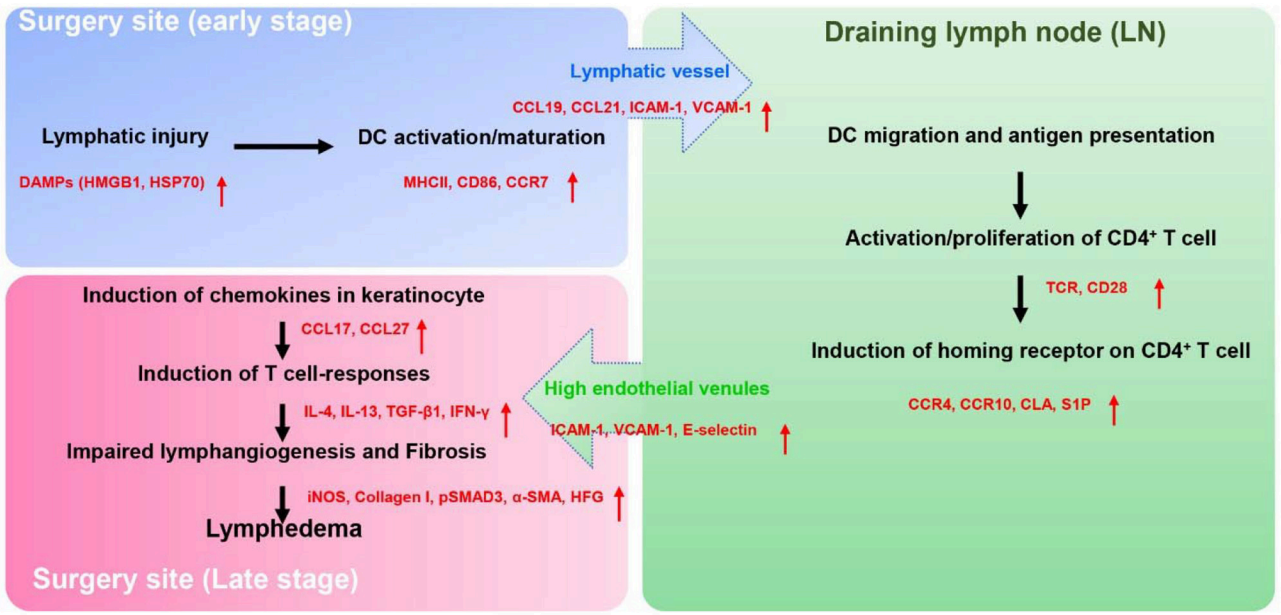

FIGURE 1 | The proposed pathophysiology of secondary lymphedema. Lymphatic injury results in activation of DCs and maturation of DCs. Activated DCs migrate to skin draining lymph node(s) through the interaction of their chemokine receptors (CCR7) and chemokine (CCL19, CCL21) produced by LECs. Within the lymph node, activated DCs interact via T cell receptors and co-stimulatory molecules (CD28) with naïve $CD4^+$ T cells resulting in T cell activation and Th1/Th2 differentiation. Activated $CD4^+$ T cells express skin homing receptors (CCR4, CCR10, and CLA), are actively released from the lymph node into the systemic circulation, and home preferentially migrate to the skin in the area of lymphatic injury following gradients of CCL17, CCL27 which are mainly produced by keratinocyte. Upon the arrival in the skin, activated $CD4^+$ T cells produce the Th1 (IFN-γ) or Th2 inflammatory mediators (IL-4, IL-13, TGF-β1), which promote lymphedema development by causing fibroadipose tissue deposition, impair lymphangiogenesis, decreased lymphatic pumping, increased lymphatic leakiness, and chronic inflammation.

molecule expression by antigen presenting cells in regional lymph nodes (67). To demonstrate this concept, we used the adoptive transfer experimental approach to study T cell activation in lymphedema. In these experiments, CD4KO mice underwent PLND and 2 weeks later were intravenously injected with naïve $CD4^+$ cells harvested from wild-type mice or RAG2/OTII mice since these transgenic mice have clonal T cells that only express the ovalbumin T cell receptor (68). This experiment was therefore designed to determine if T cell receptor activation is necessary for skin homing of activated T cells in lymphedema. In other experiments, we tested the hypothesis that T cell receptor activation requires co-stimulatory molecule expression by antigen presenting cells by adoptively transferring naïve wild-type $CD4^+$ cells into either wild-type or transgenic mice lacking CD28 (a costimulatory molecule necessary for full T cell activation) (69). In these experiments, in contrast to our findings with adoptive transfer of DCs, we found that naïve T cells initially migrated to the ipsilateral inguinal lymph node (i.e., the next draining lymph node basin downstream from where the initial lymph node dissection was performed). Within the lymph node, both T cell receptor activation and co-stimulatory molecule activation were necessary for activation of $CD4^+$ cells following lymphatic injury. Thus, adoptively transferred $CD4^+$ cells harvested from RAG2/OTII mice were not activated in the inguinal lymph node and did not migrate to the lymphedematous skin. Similarly, adoptive transfer of wild-type $CD4^+$ cells to CD28 knockout mice failed to result in T cell activation or T cell homing. Taken together, these findings suggest that T cells in lymphedema are activated in regional lymph nodes by antigen presenting cells in response to antigenic stimuli (60). Identification of T cell activating antigens in lymphedema is an active topic of study in our lab (**Figure 1**).

LYMPHEDEMA RESULTS IN A MIXED T HELPER CELL DIFFERENTIATION RESPONSE

Lymphatic injury results in a mixed T cell inflammatory reaction consisting of T helper 1 (Th1), T helper 2 (Th2), and Tregs (66). Lymphedematous skin from clinical biopsy specimens and mouse models of lymphedema are infiltrated with large numbers of $CD4^+$ cells that co-express interferon gamma (IFN-γ; putative Th1 cells) and $CD4^+$ cells that co-express interleukin 4 (IL4) or IL13 (putative Th2 cells). T cells in lymphedematous tissues tend to cluster around initial lymphatics and lymphatic collectors (60, 70). Using adoptive transfer experiments, we found that naïve $CD4^+$ cells are activated in regional lymph nodes and characterized by cell surface expression of Th1 ($CD45^+/CD4^+/CCR5^+/CXCR3^+$) and Th2 ($CD45^+/CD4^+/CCR4^+/CCR8^+$) cells. More importantly, we found that release of activated T cells from the lymph node via sphingosine 1 phosphate (S1P) signaling into the systemic

circulation is necessary for the development of lymphedema. Treatment with an S1P inhibitor (FTY720) prevented release of activated T cells from the lymph node and prevented development of lymphedema in a mouse tail model of the disease.

Once activated T cells are released from the lymph node and actively home to lymphedematous skin by expressing skin homing cell surface receptors (71). $CD4^+$ cell migration to the skin in other inflammatory conditions is regulated by cell surface expression of chemokine receptors including cutaneous leukocyte antigen (CLA), cc chemokine receptor 4 (CCR4), CCR8, and CCR10 (72, 73). This fact, together with the finding that T cell inflammatory reactions are important regulators of lymphedema, suggests that the expression of skin homing receptors may also play an important role in the development of lymphedema. This hypothesis is supported by the finding that the expression of CLA ligand E-selectin (74) as well as other leukocyte adhesion molecules (ICAM1, VCAM1) is significantly increased in lymphedematous skin (60). Similarly, we have found that the expression of ligands for CCR4 [chemokine c-c motif ligand 17 (CCL17)] and CCR10 (CCL27) (75) is markedly increased in keratinocytes of lymphedematous skin. Thus, migration of activated T cells to lymphedematous skin is not random but rather a tightly coordinated active process that may enable us to design rational treatment options that may be useful for the treatment of this morbid disease (**Figure 1**).

Th2 DIFFERENTIATION IS NECESSARY FOR PATHOLOGIC CHANGES IN LYMPHEDEMA

Lymphedema is characterized by fibro-adipose tissue deposition, impaired lymphatic pumping, lymphatic leak, and decreased formation of collateral lymphatics. Previous studies in our lab and others have shown that T cells in general, and Th2 cells in particular play a key role in these pathologic processes (52, 60, 66, 70, 76). In fact, we have hypothesized that lymphedema is simply fibrotic organ failure of the lymphatic system. This hypothesis is supported by the histological characteristics of lymphedema demonstrating progressive collagen deposition and encasement of initial lymphatics by thick collagen bundles (66, 77). In addition, clinical studies have shown that late stage lymphedema results in fibrosis of collecting lymphatics with resultant luminal obliteration and failure of the pump mechanism (78, 79). The fibrotic hypothesis of lymphedema also provides a rationale for the delayed onset of symptoms following surgery since the critical threshold of fibrosis necessary to become symptomatic takes time to occur. In addition, fibrosis is a common cause of organ failure affecting virtually every other organ system in one form or another. Similar to lymphedema, these diseases are progressive and eventually become irreversible with severe end organ injury.

Previous studies have shown that T helper cells play a key role in organ fibrosis in a variety of pathologic conditions including liver fibrosis, pulmonary fibrosis, and scleroderma (80–82). Although the inciting events causing fibrosis in these conditions is highly variable and the parenchyma in these organ systems is distinct, the cellular mechanisms that regulate fibrosis in these conditions appears to be conserved and dependent on chronic Th2 biased immune responses. Ordinarily, Th2 cells play an important role in responses to parasites, however, chronic Th2 biased inflammatory responses promote tissue fibrosis by increasing collagen deposition, decreasing collagen breakdown, and increasing expression of profibrotic growth factors such as IL4, IL13, and TGF-b1 (83–85). Because Th1 immune responses often balance and oppose Th2 responses, in general Th1 biased responses are anti-fibrotic.

The regulation of organ fibrosis by chronic mixed Th1/Th2 inflammatory responses is referred to as the Th1/Th2 paradigm (86) and also appears to play a key role in the development of lymphedema. This hypothesis is supported by the fact that inhibition of Th2 differentiation with antibodies that neutralize IL4 or IL13, cytokines necessary for naïve T helper cell differentiation along the Th2 lineage, is highly effective in preventing the development of lymphedema in mouse models (70). Similarly, this treatment strategy is effective in reversing lymphedema once it has become established. Mice with impaired Th2 differentiation capacity do not develop lymphedema following lymphatic injury; in contrast, mice with impaired Th1 differentiation have a phenotype that is indistinguishable from wild-type littermates (71). Inhibition of Th2 differentiation markedly decreases accumulation of inflammatory cells in the skin, decreases collagen deposition and lymphatic fibrosis, reduces lymphatic leakiness, and preserves collecting lymphatic pumping capacity. Inhibition of Th2 responses decreases accumulation of perilymphatic inflammatory cells and markedly decreases expression of induced nitric oxide (iNOS) by perilymphatic inflammatory cells. This is important since increased iNOS expression in inflammatory conditions decreases lymphatic pumping capacity by decreasing gradients of endothelial derived nitric oxide expression by lymphatic cells (9). Finally, we have shown that T cell derived cytokines including IFN-γ, IL4, IL13, and TGF-β1 have potent anti-lymphangiogenic activity and impair LEC proliferation, differentiation, and migration (59, 87–89). Thus, Th2 mediated inflammatory responses impair lymphatic function by multiple mechanisms and play a central role in the pathology of lymphedema. More importantly, we have shown that other causes of lymphatic injury such as high fat diet induced obesity have a similar phenotype including peri-lymphatic accumulation of inflammatory cells, decreased lymphangiogenesis, lymphatic leaking, and impaired lymphatic pumping suggesting that our findings in lymphedema have broader physiologic relevance (90, 91).

Taken together, our findings in lymphedema suggest that lymphatic injury results in a mixed Th1/Th2 immune response secondary to T cell receptor mediated interactions with dendritic cells in regional lymph nodes and that these activated T cells migrate specifically to lymphedematous skin due to expression of cell surface receptors. Within the skin,

TABLE 1 | The cellular and molecular factors in lymphedema development.

Role in lymphedema development	Cell type	Mediator	Physiological function in lymphedema	References
Promotion of lymphedema development	Dendritic cell	CCR7	- Migration of DC into lymph node	(61, 97, 98)
		MHCII, CD86	- T cell activation via antigen presentation	(60)
	Helper T cell	TCR, CD28	- T cell activation	(67–69)
		CCR4, CCR8, CCR10, CLA, S1P	- T cell homing to lymphedematous tissue	(72, 73, 75, 98)
	Th1 cell	IFN-γ	- Inhibition of lymphangiogenesis	(89)
	Th2 cell	IL-4, IL-13	- Th2 cells differentiation - Inhibition of lymphangiogenesis - Promotion of fibrosis	(59, 70, 88)
		TGF-β1	- Promotion of fibrosis	(59, 87, 88)
	Th17 cell	IL-17A	- Inhibition of lymphatic vessel formation	(52, 99)
	Lymphatic endothelial cell	CCL21, CCL19, ICAM-1, VCAM-1	- Migration of DC into lymph node	(61–63)
		eNOS	- Promotion of lymphatic vessel contraction	(9)
	Blood endothelial cell	ICAM-1, V-CAM1, E-selectin	- T cell homing to lymphedematous tissue	(60, 74)
	Macrophage	iNOS	- Inhibition of lymphatic collector contraction	(9, 90)
	Keratinocyte	CCL17, CCL27	- T cell homing to lymphedematous tissue	(75)
Inhibition of lymphedema development	Macrophage	VEGF-C, VEGF-A	- Promotion of lymphangiogenesis	(47, 50)
		IL-6	- Regulation of chronic inflammation and adipose metabolism	(56, 57, 100)
	Regulatory T cell	N.D.	- Inhibition of infiltration and activation of immune cell (Th1/Th2 cell, macrophage, neutrophils, activated DC)	(66, 95, 96)
Not involved in lymphedema development	Natural killer cell	N.D.	- Depletion of NK cells does not reverse lymphedema	(66)
	Cytotoxic T cell	N.D.	- Depletion of $CD8^+$ cells depletion does not reverse lymphedema	(66)
	B cell	N.D.	- No significant differences in the percentage of B cells in mice model of lymphedema	(66)

N.D. stands for not determined.

Th2 cells proliferate and regulate pathologic changes including fibrosis, lymphatic leakiness, impaired pumping, and decreased formation of collateral lymphatics that eventually result in lymphedema.

HOW DOES LYMPHATIC INJURY REGULATE T REGULATORY CELL PROLIFERATION AND DIFFERENTIATION?

Tregs are immune cells that play a central role in regulating inflammatory responses, autoimmunity, and immune tolerance in a wide variety of physiologic settings. Tregs inhibit immune responses by a myriad of mechanisms including regulation of immune cell proliferation, apoptosis, and activation, production of cytokines, prevention of co-stimulation, and uptake of interleukin 2 (92, 93). These responses provide a homeostatic mechanism that prevents excessive inflammatory reactions. Tregs can be broadly divided into induced Tregs or natural Tregs; natural Tregs develop in the thymus from bone marrow derived T cell precursors (94). Induced Tregs, in contrast, develop from mature conventions T helper cells outside of the thymus, play an important role in the regulation of autoimmunity.

In addition to a mixed Th1/Th2 immune response, our lab and others have shown that lymphedema results in the accumulation of Tregs in lymphedematous tissues (66, 95, 96). Biopsy specimens of patients with unilateral upper extremity lymphedema demonstrated a nearly 6-fold increase in the number of Tregs in the lymphedematous skin (95). Using a mouse model of axillary lymph node dissection (ALND), we showed that the majority of Tregs present in the forelimb skin distal to the zone of lymphatic injury are proliferating, natural Tregs ($CD4^+$/$FoxP3^+$/$Nrp\text{-}1^+$). In contrast, we found no changes in the number of induced Tregs in the skin and no changes in any Treg population in the blood or the spleen suggesting that Treg activation and proliferation was localized to the forelimb skin rather than systemic changes. Depletion of Tregs using diphtheria toxin treatment in Fox-P3-diphtheria toxin receptor (FoxP3-DTR) transgenic mice significantly

increased the number of Th1 and Th2 cells in the forelimb tissues. In addition, this treatment increased the number of infiltrating macrophages ($CD11b^+F4/80^+$), neutrophils ($Ly\text{-}6G^+$), and activated DCs ($CD11c^+MHCII^+CD86^+$). Consistent with the immunosuppressive effects of Tregs in general, we found that Treg depletion improved T cell and B cell mediated immune responses after sensitization of the forelimb skin distal to the zone of lymphatic injury. Moreover, we found that Treg depletion increased bacterial phagocytosis and removal as compared with control mice after injection of heat inactivated bacterial particles in the forelimb skin. Other recent studies have shown that depletion of Tregs in mouse models of lymphedema results in increased severity of lymphedema while adoptive transfer of Tregs ameliorates the phenotype (96). Taken together, these findings suggest that Treg infiltration following lymphatic injury acts to suppress chronic inflammatory responses and may be homeostatic in nature. In addition, chronic infiltration of Tregs in lymphedematous tissues and subsequent suppression of immune responses may provide a rationale for the increased risk of infections and developing secondary malignancies in patients with lymphedema. These two papers in combination reveal the duality in Tregs function in lymphedema pathology by modulating one common factor namely inflammation. More importantly, these findings clearly show that lymphatic function can regulate Treg migration, proliferation, and differentiation.

CONCLUSIONS

The lymphatic system, acting via direct and indirect mechanisms, is an important regulator of immune responses (**Table 1**). Lymphatic injury occurring either as a result of iatrogenic causes or secondary to physiologic changes such as obesity, tumor formation, metabolic syndrome, or infection can modulate immune response by regulating trafficking of antigen presenting cells, decreasing transport of particulate matter or antigens, regulating T cell differentiation, and modulating immunosuppressive immune responses. These changes may modulate the severity of the underlying condition and, in some cases, may promote the development of a vicious cycle of events. Thus, understanding the mechanisms regulating immune modulation by the lymphatic system is an important goal and has broad biologic relevance.

AUTHOR CONTRIBUTIONS

BM, RK, JB, HP, and IW did the literature search, complied, and wrote the manuscript. SR and JS proof read the manuscript. RK and JB prepared the figures and tables.

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REFERENCES

1. Tammela T, Alitalo K. Lymphangiogenesis: molecular mechanisms and future promise. *Cell.* (2010) 140:460–76. doi: 10.1016/j.cell.2010.01.045
2. Escobedo N, Oliver G. The lymphatic vasculature: its role in adipose metabolism and obesity. *Cell Metab.* (2017) 26:598–609. doi: 10.1016/j.cmet.2017.07.020
3. Trevaskis NL, Kaminskas LM, Porter CJ. From sewer to saviour - targeting the lymphatic system to promote drug exposure and activity. *Nat Rev Drug Discov.* (2015) 14:781–803. doi: 10.1038/nrd4608
4. Hirosue S, Vokali E, Raghavan VR, Rincon-Restrepo M, Lund AW, Corthesy-Henrioud P, et al. Steady-state antigen scavenging, cross-presentation, and CD8+ T cell priming: a new role for lymphatic endothelial cells. *J Immunol.* (2014) 192:5002–11. doi: 10.4049/jimmunol.1302492
5. Cohen JN, Tewalt EF, Rouhani SJ, Buonomo EL, Bruce AN, Xu X, et al. Tolerogenic properties of lymphatic endothelial cells are controlled by the lymph node microenvironment. *PLoS ONE.* (2014) 9:e87740. doi: 10.1371/journal.pone.0087740
6. Cohen JN, Guidi CJ, Tewalt EF, Qiao H, Rouhani SJ, Ruddell A, et al. Lymph node-resident lymphatic endothelial cells mediate peripheral tolerance via Aire-independent direct antigen presentation. *J Exp Med.* (2010) 207:681–8. doi: 10.1084/jem.20092465
7. Dubrot J, Duraes FV, Potin L, Capotosti F, Brighouse D, Suter T, et al. Lymph node stromal cells acquire peptide-MHCII complexes from dendritic cells and induce antigen-specific CD4(+) T cell tolerance. *J Exp Med.* (2014) 211:1153–66. doi: 10.1084/jem.20132000
8. Randolph GJ, Ivanov S, Zinselmeyer BH, Scallan JP. The lymphatic system: integral roles in immunity. *Annu Rev Immunol.* (2017) 35:31–52. doi: 10.1146/annurev-immunol-041015-055354
9. Liao S, Cheng G, Conner DA, Huang Y, Kucherlapati RS, Munn LL, et al. Impaired lymphatic contraction associated with immunosuppression. *Proc Natl Acad Sci USA.* (2011) 108:18784–9. doi: 10.1073/pnas.1116152108
10. Lee KM, McKimmie CS, Gilchrist DS, Pallas KJ, Nibbs RJ, Garside P, et al. D6 facilitates cellular migration and fluid flow to lymph nodes by suppressing lymphatic congestion. *Blood.* (2011) 118:6220–9. doi: 10.1182/blood-2011-03-344044
11. Kuan EL, Ivanov S, Bridenbaugh EA, Victora G, Wang W, Childs EW, et al. Collecting lymphatic vessel permeability facilitates adipose tissue inflammation and distribution of antigen to lymph node-homing adipose tissue dendritic cells. *J Immunol.* (2015) 194:5200–10. doi: 10.4049/jimmunol.1500221
12. Zhang Q, Lu Y, Proulx ST, Guo R, Yao Z, Schwarz EM, et al. Increased lymphangiogenesis in joints of mice with inflammatory arthritis. *Arthritis Res Ther.* (2007) 9:R118. doi: 10.1186/ar2326
13. D'Alessio S, Correale C, Tacconi C, Gandelli A, Pietrogrande G, Vetrano S, et al. VEGF-C-dependent stimulation of lymphatic function ameliorates experimental inflammatory bowel disease. *J Clin Invest.* (2014) 124:3863–78. doi: 10.1172/JCI72189
14. Liao S, von der Weid PY. Lymphatic system: an active pathway for immune protection. *Sem Cell Dev Biol.* (2015) 38:83–9. doi: 10.1016/j.semcdb.2014.11.012
15. Jones D, Min W. An overview of lymphatic vessels and their emerging role in cardiovascular disease. *J Cardiovasc Dis Res.* (2011) 2:141–52. doi: 10.4103/0975-3583.85260
16. Padera TP, Meijer EF, Munn LL. The lymphatic system in disease processes and cancer progression. *Annu Rev Biomed Eng.* (2016) 18:125–58. doi: 10.1146/annurev-bioeng-112315-031200
17. Stacker SA, Williams SP, Karnezis T, Shayan R, Fox SB, Achen MG. Lymphangiogenesis and lymphatic vessel remodelling in cancer. *Nat Rev Cancer.* (2014) 14:159–72. doi: 10.1038/nrc3677
18. Lund AW, Medler TR, Leachman SA, Coussens LM. Lymphatic vessels, inflammation, and immunity in skin cancer. *Cancer Discov.* (2016) 6:22–35. doi: 10.1158/2159-8290.CD-15-0023

19. Mzingwane ML, Tiemessen CT. Mechanisms of HIV persistence in HIV reservoirs. *Rev Med Virol.* (2017) 27:e1924. doi: 10.1002/rmv.1924
20. Pantaleo G, Graziosi C, Butini L, Pizzo PA, Schnittman SM, Kotler DP, et al. Lymphoid organs function as major reservoirs for human immunodeficiency virus. *Proc Natl Acad Sci USA.* (1991) 88:9838–42. doi: 10.1073/pnas.88.21.9838
21. Zhang Y, Lu Y, Ma L, Cao X, Xiao J, Chen J, et al. Activation of vascular endothelial growth factor receptor-3 in macrophages restrains TLR4-NF-kappaB signaling and protects against endotoxin shock. *Immunity.* (2014) 40:501–14. doi: 10.1016/j.immuni.2014.01.013
22. Pal S, Meininger CJ, Gashev AA. Aged lymphatic vessels and mast cells in perilymphatic tissues. *Int J Mol Sci.* (2017) 18:E965. doi: 10.3390/ijms18050965
23. Bridenbaugh EA, Nizamutdinova IT, Jupiter D, Nagai T, Thangaswamy S, Chatterjee V, et al. Lymphatic muscle cells in rat mesenteric lymphatic vessels of various ages. *Lymphat Res Biol.* (2013) 11:35–42. doi: 10.1089/lrb.2012.0025
24. Chevalier S, Ferland G, Tuchweber B. Lymphatic absorption of retinol in young, mature, and old rats: influence of dietary restriction. *FASEB J.* (1996) 10:1085–90. doi: 10.1096/fasebj.10.9.8801171
25. Zolla V, Nizamutdinova IT, Scharf B, Clement CC, Maejima D, Akl T, et al. Aging-related anatomical and biochemical changes in lymphatic collectors impair lymph transport, fluid homeostasis, and pathogen clearance. *Aging Cell.* (2015) 14:582–94. doi: 10.1111/acel.12330
26. Ma Q, Ineichen BV, Detmar M, Proulx ST. Outflow of cerebrospinal fluid is predominantly through lymphatic vessels and is reduced in aged mice. *Nat Commun.* (2017) 8:1434. doi: 10.1038/s41467-017-01484-6
27. Gasheva OY, Knippa K, Nepiushchikh ZV, Muthuchamy M, Gashev AA. Age-related alterations of active pumping mechanisms in rat thoracic duct. *Microcirculation.* (2007) 14:827–39. doi: 10.1080/10739680701444065
28. Arngrim N, Simonsen L, Holst JJ, Bulow J. Reduced adipose tissue lymphatic drainage of macromolecules in obese subjects: a possible link between obesity and local tissue inflammation? *Int J Obesity*. (2013) 37:748–50. doi: 10.1038/ijo.2012.98
29. Helyer LK, Varnic M, Le LW, Leong W, McCready D. Obesity is a risk factor for developing postoperative lymphedema in breast cancer patients. *Breast J.* (2010) 16:48–54. doi: 10.1111/j.1524-4741.2009.00855.x
30. Greene AK, Grant FD, Slavin SA. Lower-extremity lymphedema and elevated body-mass index. *N Engl J Med.* (2012) 366:2136–7. doi: 10.1056/NEJMc1201684
31. Savetsky IL, Albano NJ, Cuzzone DA, Gardenier JC, Torrisi JS, Garcia Nores GD, et al. Lymphatic function regulates contact hypersensitivity dermatitis in obesity. *J Invest Dermatol.* (2015) 135:2742–52. doi: 10.1038/jid.2015.283
32. Bordry N, Broggi MAS, de Jonge K, Schaeuble K, Gannon PO, Foukas PG, et al. Lymphatic vessel density is associated with CD8(+) T cell infiltration and immunosuppressive factors in human melanoma. *Oncoimmunology.* (2018) 7:e1462878. doi: 10.1080/2162402X.2018.1462878
33. Lund AW, Wagner M, Fankhauser M, Steinskog ES, Broggi MA, Spranger S, et al. Lymphatic vessels regulate immune microenvironments in human and murine melanoma. *J Clin Invest.* (2016) 126:3389–402. doi: 10.1172/JCI79434
34. Tewalt EF, Cohen JN, Rouhani SJ, Guidi CJ, Qiao H, Fahl SP, et al. Lymphatic endothelial cells induce tolerance via PD-L1 and lack of costimulation leading to high-level PD-1 expression on CD8 T cells. *Blood.* (2012) 120:4772–82. doi: 10.1182/blood-2012-04-427013
35. Swartz MA, Iida N, Roberts EW, Sangaletti S, Wong MH, Yull FE, et al. Tumor microenvironment complexity: emerging roles in cancer therapy. *Cancer Res.* (2012) 72:2473–80. doi: 10.1158/0008-5472.CAN-12-0122
36. Gjorup CA, Groenvold M, Hendel HW, Dahlstroem K, Drzewiecki KT, Klausen TW, et al. Health-related quality of life in melanoma patients: Impact of melanoma-related limb lymphoedema. *Eur J Cancer.* (2017) 85:122–32. doi: 10.1016/j.ejca.2017.07.052
37. Rockson SG, Rivera KK. Estimating the population burden of lymphedema. *Ann N Y Acad Sci.* (2008) 1131:147–54. doi: 10.1196/annals.1413.014
38. Szuba A, Skobe M, Karkkainen MJ, Shin WS, Beynet DP, Rockson NB, et al. Therapeutic lymphangiogenesis with human recombinant VEGF-C. *FASEB J.* (2002) 16:1985–7. doi: 10.1096/fj.02-0401fje
39. Hwang JH, Kim IG, Lee JY, Piao S, Lee DS, Lee TS, et al. Therapeutic lymphangiogenesis using stem cell and VEGF-C hydrogel. *Biomaterials.* (2011) 32:4415–23. doi: 10.1016/j.biomaterials.2011.02.051
40. Yoon YS, Murayama T, Gravereaux E, Tkebuchava T, Silver M, Curry C, et al. VEGF-C gene therapy augments postnatal lymphangiogenesis and ameliorates secondary lymphedema. *J Clin Invest.* (2003) 111:717–25. doi: 10.1172/JCI15830
41. Ongstad EL, Bouta EM, Roberts JE, Uzarski JS, Gibbs SE, Sabel MS, et al. Lymphangiogenesis-independent resolution of experimental edema. *Am J Physiol Heart Circ Physiol.* (2010) 299:H46–54. doi: 10.1152/ajpheart.00008.2010
42. Goldman J, Le TX, Skobe M, Swartz MA. Overexpression of VEGF-C causes transient lymphatic hyperplasia but not increased lymphangiogenesis in regenerating skin. *Circ Res.* (2005) 96:1193–9. doi: 10.1161/01.RES.0000168918.27576.78
43. Jensen MR, Simonsen L, Karlsmark T, Lanng C, Bulow J. Higher vascular endothelial growth factor-C concentration in plasma is associated with increased forearm capillary filtration capacity in breast cancer-related lymphedema. *Physiol Rep.* (2015) 3:e12403. doi: 10.14814/phy2.12403
44. Gousopoulos E, Proulx ST, Bachmann SB, Dieterich LC, Scholl J, Karaman S, et al. An important role of VEGF-C in promoting lymphedema development. *J Invest Dermatol.* (2017) 137:1995–2004. doi: 10.1016/j.jid.2017.04.033
45. Bianchi ME. DAMPs, PAMPs and alarmins: all we need to know about danger. *J Leukoc Biol.* (2007) 81:1–5. doi: 10.1189/jlb.0306164
46. Patel S. Danger-Associated Molecular Patterns (DAMPs): the derivatives and triggers of inflammation. *Curr Allergy Asthma Rep.* (2018) 18:63. doi: 10.1007/s11882-018-0817-3
47. Zampell JC, Elhadad S, Avraham T, Weitman E, Aschen S, Yan A, et al. Toll-like receptor deficiency worsens inflammation and lymphedema after lymphatic injury. *Am J Physiol Cell Physiol.* (2012) 302:C709–19. doi: 10.1152/ajpcell.00284.2011
48. Li Y, He J, Zhong D, Li J, Liang H. High-mobility group box 1 protein activating nuclear factor-kappaB to upregulate vascular endothelial growth factor C is involved in lymphangiogenesis and lymphatic node metastasis in colon cancer. *J Int Med Res.* (2015) 43:494–505. doi: 10.1177/0300060515581671
49. Qiu Y, Chen Y, Fu X, Zhang L, Tian J, Hao Q. HMGB1 promotes lymphangiogenesis of human lymphatic endothelial cells *in vitro*. *Med Oncol.* (2012) 29:358–63. doi: 10.1007/s12032-010-9778-7
50. Ghanta S, Cuzzone DA, Torrisi JS, Albano NJ, Joseph WJ, Savetsky IL, et al. Regulation of inflammation and fibrosis by macrophages in lymphedema. *Am J Physiol Heart Circ Physiol.* (2015) 308:H1065–77. doi: 10.1152/ajpheart.00598.2014
51. Fujimoto N, Nakanishi G, Manabe T, Fujimura T, Tanaka T. Intralymphatic histiocytosis comprises M2 macrophages in superficial dermal lymphatics with or without smooth muscles. *J Cutan Pathol.* (2016) 43:898–902. doi: 10.1111/cup.12754
52. Ogata F, Fujiu K, Matsumoto S, Nakayama Y, Shibata M, Oike Y, et al. Excess lymphangiogenesis cooperatively induced by macrophages and CD4(+) T cells drives the pathogenesis of lymphedema. *J Invest Dermatol.* (2016) 136:706–14. doi: 10.1016/j.jid.2015.12.001
53. Rutkowski JM, Moya M, Johannes J, Goldman J, Swartz MA. Secondary lymphedema in the mouse tail: lymphatic hyperplasia, VEGF-C upregulation, and the protective role of MMP-9. *Microvasc Res.* (2006) 72:161–71. doi: 10.1016/j.mvr.2006.05.009
54. Gardenier JC, Hespe GE, Kataru RP, Savetsky IL, Torrisi JS, Nores GD, et al. Diphtheria toxin-mediated ablation of lymphatic endothelial cells results in progressive lymphedema. *JCI Insight.* (2016) 1:e84095. doi: 10.1172/jci.insight.84095
55. Kashiwagi S, Hosono K, Suzuki T, Takeda A, Uchinuma E, Majima M. Role of COX-2 in lymphangiogenesis and restoration of lymphatic flow in secondary lymphedema. *Lab Invest.* (2011) 91:1314–25. doi: 10.1038/labinvest.2011.84
56. Karlsen TV, Karkkainen MJ, Alitalo K, Wiig H. Transcapillary fluid balance consequences of missing initial lymphatics studied in a mouse model of primary lymphoedema. *J Physiol.* (2006) 574:583–96. doi: 10.1113/jphysiol.2006.108308
57. Cuzzone DA, Weitman ES, Albano NJ, Ghanta S, Savetsky IL, Gardenier JC, et al. IL-6 regulates adipose deposition and homeostasis in

lymphedema. *Am J Physiol Heart Circ Physiol.* (2014) 306:H1426–34. doi: 10.1152/ajpheart.01019.2013
58. Oka M, Iwata C, Suzuki HI, Kiyono K, Morishita Y, Watabe T, et al. Inhibition of endogenous TGF-beta signaling enhances lymphangiogenesis. *Blood.* (2008) 111:4571–9. doi: 10.1182/blood-2007-10-120337
59. Avraham T, Daluvoy S, Zampell J, Yan A, Haviv YS, Rockson SG, et al. Blockade of transforming growth factor-beta1 accelerates lymphatic regeneration during wound repair. *Am J Pathol.* (2010) 177:3202–14. doi: 10.2353/ajpath.2010.100594
60. Garcia Nores GD, Ly CL, Cuzzone DA, Kataru RP, Hespe GE, Torrisi JS, et al. CD4(+) T cells are activated in regional lymph nodes and migrate to skin to initiate lymphedema. *Nat Commun.* (2018) 9:1970. doi: 10.1038/s41467-018-04418-y
61. Weber M, Hauschild R, Schwarz J, Moussion C, de Vries I, Legler DF, et al. Interstitial dendritic cell guidance by haptotactic chemokine gradients. *Science.* (2013) 339:328–32. doi: 10.1126/science.1228456
62. Tal O, Lim HY, Gurevich I, Milo I, Shipony Z, Ng LG, et al. DC mobilization from the skin requires docking to immobilized CCL21 on lymphatic endothelium and intralymphatic crawling. *J Exp Med.* (2011) 208:2141–53. doi: 10.1084/jem.20102392
63. Teijeira A, Rouzaut A, Melero I. Initial afferent lymphatic vessels controlling outbound leukocyte traffic from skin to lymph nodes. *Front Immunol.* (2013) 4:433. doi: 10.3389/fimmu.2013.00433
64. Baluk P, Fuxe J, Hashizume H, Romano T, Lashnits E, Butz S, et al. Functionally specialized junctions between endothelial cells of lymphatic vessels. *J Exp Med.* (2007) 204:2349–62. doi: 10.1084/jem.20062596
65. Russo E, Teijeira A, Vaahtomeri K, Willrodt AH, Bloch JS, Nitschke M, et al. Intralymphatic CCL21 promotes tissue egress of dendritic cells through afferent lymphatic vessels. *Cell Rep.* (2016) 14:1723–34. doi: 10.1016/j.celrep.2016.01.048
66. Zampell JC, Yan A, Elhadad S, Avraham T, Weitman E, Mehrara BJ. CD4(+) cells regulate fibrosis and lymphangiogenesis in response to lymphatic fluid stasis. *PLoS ONE.* (2012) 7:e49940. doi: 10.1371/journal.pone.0049940
67. Esensten JH, Helou YA, Chopra G, Weiss A, Bluestone JA. CD28 costimulation: from mechanism to therapy. *Immunity.* (2016) 44:973–88. doi: 10.1016/j.immuni.2016.04.020
68. Shinkai Y, Rathbun G, Lam KP, Oltz EM, Stewart V, Mendelsohn M, et al. RAG-2-deficient mice lack mature lymphocytes owing to inability to initiate V(D)J rearrangement. *Cell.* (1992) 68:855–67. doi: 10.1016/0092-8674(92)90029-C
69. Shahinian A, Pfeffer K, Lee KP, Kundig TM, Kishihara K, Wakeham A, et al. Differential T cell costimulatory requirements in CD28-deficient mice. *Science.* (1993) 261:609–12. doi: 10.1126/science.7688139
70. Avraham T, Zampell JC, Yan A, Elhadad S, Weitman ES, Rockson SG, et al. Th2 differentiation is necessary for soft tissue fibrosis and lymphatic dysfunction resulting from lymphedema. *FASEB J.* (2013) 27:1114–26. doi: 10.1096/fj.12-222695
71. Ly CL, Nores GDG, Kataru RP, Mehrara BJ. T helper 2 differentiation is necessary for development of lymphedema. *Transl Res.* (2018). doi: 10.1016/j.trsl.2018.12.003. [Epub ahead of print].
72. Agace WW. Tissue-tropic effector T cells: generation and targeting opportunities. *Nat Rev Immunol.* (2006) 6:682–92. doi: 10.1038/nri1869
73. Syrbe U, Siveke J, Hamann A. Th1/Th2 subsets: distinct differences in homing and chemokine receptor expression? *Springer Sem Immunopathol.* (1999) 21:263–85. doi: 10.1007/BF00812257
74. Ni Z, Walcheck B. Cutaneous lymphocyte-associated antigen (CLA) T cells up-regulate P-selectin ligand expression upon their activation. *Clin Immunol.* (2009) 133:257–64. doi: 10.1016/j.clim.2009.07.010
75. Reiss Y, Proudfoot AE, Power CA, Campbell JJ, Butcher EC. CC chemokine receptor (CCR)4 and the CCR10 ligand cutaneous T cell-attracting chemokine (CTACK) in lymphocyte trafficking to inflamed skin. *J Exp Med.* (2001) 194:1541–7. doi: 10.1084/jem.194.10.1541
76. Gousopoulos E, Proulx ST, Scholl J, Uecker M, Detmar M. Prominent lymphatic vessel hyperplasia with progressive dysfunction and distinct immune cell infiltration in lymphedema. *Am J Pathol.* (2016) 186:2193–203. doi: 10.1016/j.ajpath.2016.04.006
77. Suami H, Pan WR, Taylor GI. Changes in the lymph structure of the upper limb after axillary dissection: radiographic and anatomical study in a human cadaver. *Plast Reconstr Surg.* (2007) 120:982–91. doi: 10.1097/01.prs.0000277995.25009.3e
78. Mihara M, Hara H, Hayashi Y, Narushima M, Yamamoto T, Todokoro T, et al. Pathological steps of cancer-related lymphedema: histological changes in the collecting lymphatic vessels after lymphadenectomy. *PLoS ONE.* (2012) 7:e41126. doi: 10.1371/journal.pone.0041126
79. Modi S, Stanton AW, Svensson WE, Peters AM, Mortimer PS, Levick JR. Human lymphatic pumping measured in healthy and lymphoedematous arms by lymphatic congestion lymphoscintigraphy. *J Physiol.* (2007) 583:271–85. doi: 10.1113/jphysiol.2007.130401
80. Wynn TA. Common and unique mechanisms regulate fibrosis in various fibroproliferative diseases. *J Clin Invest.* (2007) 117:524–9. doi: 10.1172/JCI31487
81. Vasakova M, Striz I, Slavcev A, Jandova S, Kolesar L, Sulc J. Th1/Th2 cytokine gene polymorphisms in patients with idiopathic pulmonary fibrosis. *Tissue Antigens.* (2006) 67:229–32. doi: 10.1111/j.1399-0039.2006.00560.x
82. Buttner C, Skupin A, Reimann T, Rieber EP, Unteregger G, Geyer P, et al. Local production of interleukin-4 during radiation-induced pneumonitis and pulmonary fibrosis in rats: macrophages as a prominent source of interleukin-4. *Am J Respir Cell Mol Biol.* (1997) 17:315–25. doi: 10.1165/ajrcmb.17.3.2279
83. Massague J, Cheifetz S, Laiho M, Ralph DA, Weis FM, Zentella A. Transforming growth factor-beta. *Cancer Surv.* (1992) 12:81–103.
84. Cheever AW, Williams ME, Wynn TA, Finkelman FD, Seder RA, Cox TM, et al. Anti-IL-4 treatment of Schistosoma mansoni-infected mice inhibits development of T cells and non-B, non-T cells expressing Th2 cytokines while decreasing egg-induced hepatic fibrosis. *J Immunol.* (1994) 153:753–9.
85. Chiaramonte MG, Cheever AW, Malley JD, Donaldson DD, Wynn TA. Studies of murine schistosomiasis reveal interleukin-13 blockade as a treatment for established and progressive liver fibrosis. *Hepatology.* (2001) 34:273–82. doi: 10.1053/jhep.2001.26376
86. Wynn TA. Fibrotic disease and the T(H)1/T(H)2 paradigm. *Nat Rev Immunol.* (2004) 4:583–94. doi: 10.1038/nri1412
87. Shin K, Kataru RP, Park HJ, Kwon BI, Kim TW, Hong YK, et al. TH2 cells and their cytokines regulate formation and function of lymphatic vessels. *Nat Commun.* (2015) 6:6196. doi: 10.1038/ncomms7196
88. Savetsky IL, Ghanta S, Gardenier JC, Torrisi JS, Garcia Nores GD, et al. Th2 cytokines inhibit lymphangiogenesis. *PLoS ONE.* (2015) 10:e0126908. doi: 10.1371/journal.pone.0126908
89. Kataru RP, Kim H, Jang C, Choi DK, Koh BI, Kim M, et al. T lymphocytes negatively regulate lymph node lymphatic vessel formation. *Immunity.* (2011) 34:96–107. doi: 10.1016/j.immuni.2010.12.016
90. Torrisi JS, Hespe GE, Cuzzone DA, Savetsky IL, Nitti MD, Gardenier JC, et al. Inhibition of inflammation and iNOS improves lymphatic function in obesity. *Sci Rep.* (2016) 6:19817. doi: 10.1038/srep19817
91. Garcia Nores GD, Cuzzone DA, Albano NJ, Hespe GE, Kataru RP, Torrisi JS, et al. Obesity but not high-fat diet impairs lymphatic function. *Int J Obesity.* (2016) 40:1582–90. doi: 10.1038/ijo.2016.96
92. Josefowicz SZ, Lu LF, Rudensky AY. Regulatory T cells: mechanisms of differentiation and function. *Annu Rev Immunol.* (2012) 30:531–64. doi: 10.1146/annurev.immunol.25.022106.141623
93. Kretschmer K, Apostolou I, Hawiger D, Khazaie K, Nussenzweig MC, von Boehmer H. Inducing and expanding regulatory T cell populations by foreign antigen. *Nat Immunol.* (2005) 6:1219–27. doi: 10.1038/ni1265
94. Adeegbe DO, Nishikawa H. Natural and induced T regulatory cells in cancer. *Front Immunol.* (2013) 4:190. doi: 10.3389/fimmu.2013.00190
95. Garcia Nores GD, Ly CL, Savetsky IL, Kataru RP, Ghanta S, Hespe GE, et al. Regulatory T cells mediate local immunosuppression in lymphedema. *J Invest Dermatol.* (2018) 138:325–35. doi: 10.1016/j.jid.2017.09.011
96. Gousopoulos E, Proulx ST, Bachmann SB, Scholl J, Dionyssiou D, Demiri E, et al. Regulatory T cell transfer ameliorates lymphedema and promotes lymphatic vessel function. *JCI Insight.* (2016) 1:e89081. doi: 10.1172/jci.insight.89081
97. Ohl L, Mohaupt M, Czeloth N, Hintzen G, Kiafard Z, Zwirner J, et al. CCR7 governs skin dendritic cell migration under inflammatory and steady-state conditions. *Immunity.* (2004) 21:279–88. doi: 10.1016/j.immuni.2004.06.014

98. Saeki H, Moore AM, Brown MJ, Hwang ST. Cutting edge: secondary lymphoid-tissue chemokine (SLC) and CC chemokine receptor 7 (CCR7) participate in the emigration pathway of mature dendritic cells from the skin to regional lymph nodes. *J Immunol.* (1999) 162:2472–5.
99. Park HJ, Yuk CM, Shin K, Lee SH. Interleukin-17A negatively regulates lymphangiogenesis in T helper 17 cell-mediated inflammation. *Mucosal Immunol.* (2018) 11:590–600. doi: 10.1038/mi.2017.76
100. Scheller J, Chalaris A, Schmidt-Arras D, Rose-John S. The pro- and anti-inflammatory properties of the cytokine interleukin-6. *Biochim Biophys Acta.* (2011) 1813:878–88. doi: 10.1016/j.bbamcr.2011.01.034

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Lymphatic Function in Autoimmune Diseases

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Lymphatic vessels are critical for clearing fluid and inflammatory cells from inflamed tissues and also have roles in immune tolerance. Given the functional association of the lymphatics with the immune system, lymphatic dysfunction may contribute to the pathophysiology of rheumatic autoimmune diseases. Here we review the current understanding of the role of lymphatics in the autoimmune diseases rheumatoid arthritis, scleroderma, lupus, and dermatomyositis and consider the possibility that manual therapies such as massage and acupuncture may be useful in improving lymphatic function in autoimmune diseases.

Keywords: lymphatics, autoimmune disease, rheumatoid arthritis, scleroderma, systemic lupus erythematosus, lymphatic massage

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INTRODUCTION

As early as the Fourth century, Aristotle described lymphatic vessels as fibers positioned between blood vessels and nerves, containing colorless liquid (1). While our understanding of the lymphatic system has advanced since Aristotle's time, the functional significance of the lymphatic network to health and disease is still being unraveled. The lymphatic system is a network of vessels that drains protein-rich lymph from the extracellular fluid, transports it through a series of lymph nodes (LNs), and finally returns it to the bloodstream. Beyond their role in maintaining tissue fluid homeostasis, lymphatic vessels are an important part of the immune system: they allow transport of antigens from the periphery to LNs, where immune cells are primed, expanded, and eventually transported to the site of inflammation (2, 3). In addition to ferrying lymph and immune cells, the lymphatic system itself is directly involved in immune modulation and induction of tolerance to self-antigens (4, 5). Given the function of the lymphatic system in immunity, lymphatic dysfunction may also contribute to the pathophysiology of autoimmune diseases. Here, we review the current understanding of the lymphatic function within autoimmune disease. We begin with a brief overview of the lymphatic system, discuss what is known about lymphatic function in a number of rheumatic diseases, starting from the best studied to the least studied in terms of lymphatic function, and conclude with a consideration of manual therapies as potential approaches to improve lymphatic function in disease. Our goal is to bring more attention to this under-explored, yet promising, area of study.

OVERVIEW OF THE LYMPHATIC SYSTEM

Lymphatic vessels form an extensive network throughout the body with the exception of only a few tissues including bone, heart myocardium and skeletal muscles, as well as the parenchyma of kidney, liver, and adrenal and thyroid glands. These exceptions either have little interstitial fluid or have an alternative drainage system, such as fenestrated blood vessels (6).

The lymphatic system is composed of initial lymphatic capillaries that merge to form collecting lymphatic vessels. The collecting vessels transport the lymph to and from a series of LNs and eventually drain into the thoracic duct that connects to the blood circulation by draining into the subclavian veins (6). Lymphatic capillaries are blind-ended and are composed of a thin layer of lymphatic endothelial cells (LECs) with discontinuous basement membrane and "button-like" cell junctions, allowing unidirectional flow of cells and fluid into the vessel (7). In contrast to the capillaries, collecting lymphatic vessels (LVs) have intraluminal valves that prevent backflow of lymphatic fluid (8) and several perivascular layers of lymphatic muscle cells, with characteristics of both smooth muscle cells and cardiac striated muscle cells, that provide vascular tone and rhythmic contractions of the vessels, enabling anti-gravity, active fluid transportation (9, 10). Previously, distinguishing blood endothelial cells from LECs was difficult. But recent research identified several LEC-specific markers, including Lyve-1, vascular endothelial growth factor receptor-3 (VEGFR-3), podoplanin (PDPN), and Prox-1, among others, that enabled tremendous advancements in the study of lymphatic vessel development and function (11, 12).

Previous studies have shown that the systemic vasculature can reabsorb up to 90% of extravasated water, while the remaining 10% is absorbed by the lymphatic vessels (13). However, congenital or acquired dysfunction of the lymphatic system result not only in lymphedema and its sequalae such as skin thickening, fibrosis, and adipose degeneration, but also in poor immune function, susceptibility to infections, and impaired wound healing, among other deleterious health effects (8). These observations point at the greater role lymphatic vessels have than simple fluid transportation. Literature has shown that LECs directly affect immune cell activity in many different ways, including secretion of transforming growth factor-β (TGFβ) leading to suppression of dendritic cell (DC) maturation (14); production of IL-7 to increase IL-2 sensitivity in regulatory T cells to increase their immune-regulatory function (15) and to sustain inflammation-induced lymphoid follicles in disease (16); secretion of colony stimulating factor-1 (CSF-1) promoting differentiation, proliferation and survival of macrophages that contribute to tumor growth (17). LECs also present peripheral tissue antigens together with programmed death-ligand-1 (PD-L1) leading to CD8+ T-cell response inhibition (18) and modulate CD4+ T-cell response via low level antigen presentation with MHC-II during inflammation (19). That lymphatic dysfunction exacerbates autoimmune disease is supported by the development of autoantibodies in mice lacking dermal lymphatics (5). Understanding the lymphatic system in the context of autoimmune diseases has the potential to provide insight into disease mechanisms and new approaches to treatment.

LYMPHATICS AND RHEUMATOID ARTHRITIS

Rheumatoid arthritis (RA) is one of the most studied autoimmune conditions, with regards to the role of lymphatics in the context of disease. RA is an autoimmune systemic disease, affecting 0.5–1% of the population, with its hallmark being symmetric polyarthritis, usually with small-joint distribution (20). Local lymph node enlargement was first described in RA in 1896 (21), but it wasn't until more specific markers of the lymphatic system were discovered that its role could be specifically investigated.

It is thought that together with the joint inflammation occurring in RA, the local lymphatics undergo two stages of alterations. As a response to the initial, pre-arthritic, synovial inflammation, the lymphatics undergo an "expansion phase," whereby they increase their capacity to remove excess cellular debris and inflammatory cells from the site of inflammation; whether by lymphangiogenesis (22), or by increased lymphatic vessel contraction frequency (23). This process is important to allow for the resolution of the inflammatory process; if the expansion process is stunted, by inhibition of lymphangiogenesis for example, the joint inflammation becomes more severe and clinical synovitis develops (24). Beyond the lymphatic vessel changes, the draining lymph nodes themselves increase in size during the expansion phase (23, 25), likely due to increased volume and pressure of fluid within the afferent vessels (25), intra-nodal lymphangiogenesis (23), and infiltration of a unique subtype of $IgM^{+}CD23^{+}CD21^{hi}CD1d^{hi}$ B cells found in inflamed lymph nodes, known as Bin cells (26, 27). Nevertheless, while the removal of the excess debris is important to allow for inflammation resolution in the acute setting, the inflammatory cells, and catabolic factors that are being removed have been shown to directly damage the LECs and lymphatic muscle cells, both in the afferent lymphatic vessels and the draining lymph nodes (28). As a result of this ongoing stress on the lymphatic system, the lymphatics progress to the "collapsed phase," in which the local lymphatic conduit system breaks down, and the lymph node is no longer able to efficiently drain the fluid from the inflamed synovium (24, 25). The lymphatic vessels are damaged, with increased leakiness and reduced contractions, leading to poor lymphatic clearance, and stasis of the inflammatory fluid within the joint and the afferent lymphatic vessels (23, 28–31). The process is thought to be mediated by several factors, including inflammatory cytokines in the vessels triggering LEC expression of inducible nitric oxide synthase (iNOS), as well as iNOS-producing activated myeloid cells, now static within the lymphatic vessels. The increased local NO production abrogates the constitutive endothelial NOS (eNOS) activity that is an important mediator of lymphatic vessel contraction (32, 33). Reduced vessel contraction is likely also due to increased fluid flow and pressure inside the vessels, beyond the vessels' ability to compensate (34, 35). At the same time, Bin cells in the draining lymph node migrate from the lymph node follicles to the sinuses, as extensively reviewed by Bouta et al. (31), leading to clogging of lymph node sinuses and blocking passive lymphatic drainage. The resulting impairment in lymphatic drainage contributes to increased joint inflammation and synovial hyperplasia, eventually leading to joint destruction (36). Importantly, known and effective RA treatments, such as tumor necrosis factor (TNF) inhibition and anti-CD20 therapy, have both shown to also have a beneficial effect on lymphatic flow. Inhibition of TNF has been shown to restore lymphatic vessel contractility (28),

and anti-CD20 therapy, i.e., Rituximab, depletes Bin cells from the lymphatic sinuses, thereby promoting the restoration of lymphatic flow (37).

Preliminary work with indocyanine green near-infrared (ICG-NIR) fluorescence imaging has been promising in providing sensitive, real-time non-invasive means to evaluate the layout and function of the lymphatic vasculature (31, 38, 39), and the first study of lymphatic flow in RA patients with this modality is currently being performed (ClinicalTrials.gov NCT02680067). Meanwhile, the change in size of the local draining lymph nodes has been shown to reflect joint inflammatory activity, as well as response to therapy (40). Even prior to clinical lymphadenopathy, evaluation of draining lymph nodes of inflamed joints by power Doppler ultrasound (PDUS), demonstrated hypertrophy of the lymph node cortex, in addition to power Doppler signal amplification in cortical and hilar regions likely indicating increased flow. Importantly, these findings reversed with treatment (41). At the same time, low PDUS signal at baseline despite active arthritis, likely representing a collapsed lymph node, predicts poor clinical response to therapy (40). Similarly, in a pilot study using contrast enhanced MRI (CE-MRI) to monitor LN size before and after treatment with Certolizumab in RA patients, there was an inverse correlation between the extent of treatment-related pain relief and decrease in LN size. The LNs with the more notable reduction in size are the ones that are more likely to have undergone collapse, leading to inadequate inflammation resolution and reduced pain relief (42). Thus, in addition to providing an important, non-invasive means by which to monitor disease activity, response to therapy, and even predict prognosis; these findings also support the bi-phasic lymphatic response model, namely the "expansion phase" and "collapse phase," seen in murine models of inflammatory arthritis.

LYMPHATICS AND SYSTEMIC SCLEROSIS

Scleroderma is an autoimmune connective tissue disease characterized by abnormalities in vasculature, immune function, and extracellular matrix that ultimately manifest as fibrosis of the cutaneous, vascular, musculoskeletal, gastrointestinal, pulmonary, cardiac, and renal systems. Although the etiology of the disease is poorly understood, vascular injury and abnormal endothelial cell function are hypothesized to be among the primary defects responsible for disease pathogenesis (43, 44).

Studies into vascular abnormalities leading to fibrosis have generally been more focused on blood endothelial cells (BECs), while the roles of LECs and lymphatic dysfunction have been less well-studied. In 1999, Leu et al. first demonstrated using lymphangiography that scleroderma lesional skin had signs of lymphatic microangiopathy, with absence or fragmentation of visualizable lymphatic networks and evidence of vessel leakiness and backflow (45). Similar findings using immunohistochemical staining of scleroderma skin recently showed a decrease in lymphatic vessels and increased cross-sectional area of the remaining vessels, suggesting vessel dilatation and a block in lymphatic flow downstream (44). The changes were most significant in the reticular dermis, with a similar trend found in the papillary dermis. Lymphatic changes have been found in other fibrosing conditions as well (**Table 1**), further supporting the idea that lymphatic dysfunction may be a therapeutic target in scleroderma.

LYMPHATICS AND SYSTEMIC LUPUS ERYTHEMATOSUS AND DERMATOMYOSITIS

Systemic lupus erythematosus (SLE) is the prototypical systemic autoimmune disease, affecting between in 6.5 and 187 per 100,000 people worldwide, with a 9:1 female predominance and mortality rate that is three times that of the general population (57, 58). There have been no systematic studies of lymphatic function in SLE to date. There are, however, hints that there might be lymphatic dysfunction in SLE, as there are case reports of chylous ascites or pleural effusions, lymph fluid found in the abdomen or thoracic cavity, respectively, that can result from lymphatic obstruction in the mesentery (59–62). Lymphedema from peripheral lymphatic obstruction has also been described (63). These occurrences are rare, however, and whether more subtle problems with lymphatic flow that could perhaps result from the lymphadenopathy commonly found in SLE (64, 65) are not known.

Similarly, lymphatic function in dermatomyositis, a group of autoimmune diseases primarily directed against the muscle and skin (66), has not been systematically studied. Dermatomyositis patients can rarely present with generalized edema, which may reflect poor lymphatic function and lymphedema (67). Gottron's papules are characteristic red, raised lesions on the knuckles of dermatomyositis patients, and one study examining the histopathology of these papules noted dilated $PDPN^{+}$ lymphatic vessels (68). Interestingly, benign lymphadenopathy is less frequent in dermatomyositis than in SLE or RA, and, because of the association of cancer with dermatomyositis in adults, lymphadenopathy in dermatomyositis has to be evaluated carefully for metastatic cancer or lymphomas (69).

POTENTIAL APPROACHES TO IMPROVING LYMPHATIC FUNCTION IN RHEUMATIC DISEASES: NEW AND ANCIENT

As we begin to understand the role of lymphatic dysfunction in autoimmune diseases, it is also time to consider how we might improve lymphatic function as part of disease treatment. Schwarz and colleagues have recently outlined molecularly targeted therapies that are currently being investigated (31). In contrast to pharmacologic approaches, manual therapies have been used since ancient times and are currently the mainstay in improving lymphatic flow in diseases. Below, we briefly discuss some of these approaches to consider their potential utility in improving lymphatic function in autoimmune diseases.

Lymphatic-directed massage techniques are used in the treatment of primary and secondary forms of lymphedema, such as that which occurs in the arms of 20% of breast cancer surgery patients when axillary lymph nodes have been removed (70).

TABLE 1 | Summary of lymphatic dysfunction in fibrosis of different organs.

Disease model	Findings	Mechanism/molecules involved	References
LUNG FIBROSIS			
Human lung	Enlarged mediastinal lymph nodes (32% in SSc vs. 2% controls)		(46)
	• Increased alveolar lymphangiogenesis early in the disease • Lymphatic area directly proportional to the severity of the disease	CD11b+ macrophages form LECs in alveolar spaces of IPF patients but not controls	(47)
Radiation-exposed mouse lung	Progressive loss of pulmonary lymphatic vessels	Increase in VEGF-C and D expressing alveolar macrophages	(48)
SKIN FIBROSIS			
Human Skin	• Decreased lymphatic vessel counts in SSc patients • Inverse correlation between low vessel counts with fingertip ulcers		(49)
	Decreased density of reticular dermis lymphatic vessels		(44)
	Lymphatic microangiopathy		(45, 50)
Mouse tail skin radiation-induced fibrosis	Decrease in dermal capillary lymphatic vessels and LEC	TGF-β signaling inhibition protects from radiation-induced soft tissue fibrosis and lymphatic dysfunction.	(51)
LIVER FIBROSIS			
Sprague Dawley rat model	Increased lymphatic diameter in CCl4 induced fibrosis mice compared to control mice		(52)
Human liver	• Increase in area of each lymphatic vessel • Increase in number of lymphatic vessels per section and directly proportional to the fibrosis severity		(53)
RENAL AND PERITONEAL FIBROSIS			
Human kidney	• Presence of LEC in the tubulointerstitial fibrotic lesions and not in control sample • Lymphatic vessel proliferation in tubulointerstitial fibrosis and inflammatory interstitial areas, filled with mononuclear cells in the lymphatic lumen		(54)
Unilateral Ureteral Obstruction rat model	Increased lymphangiogenesis	Increased TGF-β and VEGF-C expression	(55)
Rat remnant kidney model	• Massive proliferation of lymphatic vessels in fibrotic tubulointerstitial regions. • Mononuclear clusters in lymphatic vessels		(56)

SSc, systemic sclerosis; LEC, lymphatic endothelial cells; IPF, idiopathic pulmonary fibrosis; VEGF, vascular endothelial growth factor; TGF-β, transforming growth factor-beta.

Manual lymphatic drainage (MLD) is a specific light pressure massage technique that moves from the trunk to the distal portion of the affected limb to stimulate lymph flow away from the peripheral tissue (71). Indeed, hand edema is observed in systemic sclerosis patients in the early edematous phase, and MLD has been shown to significantly reduce the swelling and improve hand function in these patients (72). Similarly, a dry brushing massage technique used in Ayurvedic medicine that originated in India 5,000 years ago is meant to relieve lymphatic congestion that is thought to contribute to stress and disease. It has also been used to reduce lymphedema and inflammation from lymphatic filariasis (73). Interestingly, the sports industry, which has been interested in promoting post-training recovery and reducing edema, is investigating the utility of peristaltic pulse dynamic compression (PPDC) devices that simulate manual lymphatic therapies. Recently, PPDC was shown to increase the pressure-to-pain threshold in elite athletes (74) and also to induce expression of anti-inflammatory genes (75), although it is yet unclear whether these effects are attributable to improving lymphatic flow. Potentially, then, lymphatic massage techniques could be used to improve lymphatic function to help reduce tissue inflammation in autoimmune diseases.

Interestingly, acupuncture, a component of traditional Chinese medicine, may potentially have a lymphatic basis. Acupoints are specific points on the body that practitioners target in an attempt to mobilize stagnant *qi*, thought to be a form of energy that flows through the meridian system and enhance well-being (76). Recent analysis of acupoints demonstrates that they co-localize with tissue planes rich in nerves, blood and lymphatic vessels, and mast cells. Acupuncture involves insertion

of metal needles into the skin and spinning the needle between the acupuncturist's fingers. It has been proposed that this process disturbs local tissues and transmits a biomechanical signal to surrounding cells and structures (77) that can stimulate lymphatic vessels. Additionally, activation of the nerves or mast cells in the area could result in release of vasoactive cytokines that can then stimulate lymphatic vessels to better mobilize fluid and inflammatory cells from the area (77–80). There are observational trials showing efficacy of acupuncture on breast cancer-associated lymphedema, supporting the idea that acupuncture can modulate lymphatic function (81–83). A recent randomized controlled trial examining the ability of acupuncture to further reduce lymphedema on top of current standard therapies such as lymphatic massage drainage and compression sleeves did not show additional benefits (84). However, whether acupuncture alone is at least as good as current standard therapies is not yet known. It should be of interest to better study whether acupuncture could modulate lymphatic function to aid in the treatment of autoimmune diseases.

While the objective of these manual therapies in lymphedema is to improve lymphatic flow and reduce inflammation and swelling in the affected tissues, improving lymphatic flow has the potential to also modulate immune cell activity in a number of ways. First, as mentioned in section Overview of the Lymphatic System, LECs can directly regulate immune cell function, and stimulation of lymphatic flow can modulate the ability of LECs to regulate immune cells (85). Second, lymphatic flow, by means of transporting antigen from the periphery, can impact the tolerance and activation of lymph node lymphocytes (5). Third, cytokines expressed in peripheral tissues can impact immune function in lymph nodes (86), potentially in part by lymphatic transport to the lymph nodes (87). Here, it is possible that cytokines transported to the draining nodes can both activate and regulate lymph node responses, suggesting that improving lymphatic flow can help reduce the duration and/or magnitude of ongoing autoimmune responses. Thus, in the study mentioned above examining the effects of MLD on hand edema in scleroderma patients (72), it would be interesting to understand whether MLD reduced autoantibody levels when edema was reduced. Manual therapies to improve lymphatic flow, then, may be a well-tolerated, relatively low-cost method to improve many facets of lymphatic function to reduce inflammation and autoimmunity in rheumatic diseases.

CONCLUSIONS AND FUTURE DIRECTIONS

The lymphatic system has not been well-studied in autoimmune diseases generally, but the existing evidence, especially in RA and, to a more limited extent, in systemic sclerosis suggests that there is at least dysfunction of lymphatic flow. Further studies focused on the consequences of dysfunctional flow as well as alterations in the direct effects of lymphatic vessels and LECs on innate and adaptive immune cells should provide insights into how best to target the lymphatics in autoimmune rheumatic diseases. Additionally, understanding the causes of lymphatic dysfunction in these diseases may help us better target upstream mediators and perhaps reveal that lymphatic targeting is a mechanism of action of some medications. Finally, as we consider new approaches to targeting lymphatics in autoimmune diseases, there may be value in better understanding older approaches in the context of Twenty-First century biomedical understanding of lymphatic and immune function to expand our therapeutic armamentarium for autoimmune diseases.

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REFERENCES

1. Loukas M, Bellary SS, Kuklinski M, Ferrauiola J, Yadav A, Shoja MM, et al. The lymphatic system: a historical perspective. *Clin Anat*. (2011) 24:807–16. doi: 10.1002/ca.21194
2. Oliver G, Detmar M. The rediscovery of the lymphatic system: old and new insights into the development and biological function of the lymphatic vasculature. *Genes Dev*. (2002) 16:773–83. doi: 10.1101/gad.975002
3. Oliver G, Alitalo K. The lymphatic vasculature: recent progress and paradigms. *Annu Rev Cell Dev Biol.* (2005) 21:457–83. doi: 10.1146/annurev.cellbio.21.012704.132338
4. Friedlaender MH, Baer H. Immunologic tolerance: role of the regional lymph node. *Science.* (1972) 176:312–4. doi: 10.1126/science.176.4032.312
5. Thomas SN, Rutkowski JM, Pasquier M, Kuan EL, Alitalo K, Randolph GJ, et al. Impaired humoral immunity and tolerance in K14-VEGFR-3-Ig mice that lack dermal lymphatic drainage. *J Immunol.* (2012) 189:2181–90. doi: 10.4049/jimmunol.1103545
6. Petrova TV, Koh GY. Organ-specific lymphatic vasculature: from development to pathophysiology. *J Exp Med.* (2018) 215:35–49. doi: 10.1084/jem.20171868
7. Baluk P, Fuxe J, Hashizume H, Romano T, Lashnits E, Butz S, et al. Functionally specialized junctions between endothelial cells of lymphatic vessels. *J Exp Med.* (2007) 204:2349–62. doi: 10.1084/jem.20062596
8. Oliver G. Lymphatic vasculature development. *Nat Rev Immunol.* (2004) 4:35–45. doi: 10.1038/nri1258
9. Muthuchamy M, Gashev A, Boswell N, Dawson N, Zawieja D. Molecular and functional analyses of the contractile apparatus in lymphatic muscle. *FASEB J.* (2003) 17:920–2. doi: 10.1096/fj.02-0626fje
10. Davis MJ, Davis AM, Ku CW, Gashev AA. Myogenic constriction and dilation of isolated lymphatic vessels. *Am J Physiol Heart Circ Physiol.* (2009) 296:H293–302. doi: 10.1152/ajpheart.01040.2008
11. Banerji S, Ni J, Wang SX, Clasper S, Su J, Tammi R, et al. LYVE-1, a new homologue of the CD44 glycoprotein, is a lymph-specific receptor for hyaluronan. *J Cell Biol.* (1999) 144:789–801. doi: 10.1083/jcb.144.4.789

12. Podgrabinska S, Braun P, Velasco P, Kloos B, Pepper MS, Skobe M. Molecular characterization of lymphatic endothelial cells. *Proc Natl Acad Sci USA.* (2002) 99:16069–74. doi: 10.1073/pnas.242401399
13. Tammela T, Alitalo K. Lymphangiogenesis: Molecular mechanisms and future promise. *Cell.* (2010) 140:460–76. doi: 10.1016/j.cell.2010.01.045
14. Christiansen AJ, Dieterich LC, Ohs I, Bachmann SB, Bianchi R, Proulx ST, et al. Lymphatic endothelial cells attenuate inflammation via suppression of dendritic cell maturation. *Oncotarget.* (2016) 7:39421–35. doi: 10.18632/oncotarget.9820
15. Schmaler M, Broggi MA, Lagarde N, Stocklin BF, King CG, Finke D, et al. IL-7R signaling in regulatory T cells maintains peripheral and allograft tolerance in mice. *Proc Natl Acad Sci USA.* (2015) 112:13330–5. doi: 10.1073/pnas.1510045112
16. Randolph GJ, Ivanov S, Zinselmeyer BH, Scallan JP. The lymphatic system: integral roles in immunity. *Annu Rev Immunol.* (2017) 35:31–52. doi: 10.1146/annurev-immunol-041015-055354
17. Steinskog ESS, Sagstad SJ, Wagner M, Karlsen TV, Yang N, Markhus CE, et al. Impaired lymphatic function accelerates cancer growth. *Oncotarget.* (2016) 7: 45789–802. doi: 10.18632/oncotarget.9953
18. Dieterich LC, Ikenberg K, Cetintas T, Kapaklikaya K, Hutmacher C, Detmar M. Tumor-associated lymphatic vessels upregulate PDL1 to inhibit T-cell activation. *Front Immunol.* (2017) 8:66. doi: 10.3389/fimmu.2017.00066
19. Dubrot J, Duraes FV, Potin L, Capotosti F, Brighouse D, Suter T, et al. Lymph node stromal cells acquire peptide-MHCII complexes from dendritic cells and induce antigen-specific CD4(+) T cell tolerance. *J Exp Med.* (2014) 211:1153–66. doi: 10.1084/jem.20132000
20. Scott DL, Wolfe F, Huizinga TW. Rheumatoid arthritis. *Lancet.* (2010) 376:1094–108. doi: 10.1016/S0140-6736(10)60826-4
21. Robertson MD, Hart FD, White WF, Nuki G, Boardman PL. Rheumatoid lymphadenopathy. *Ann Rheum Dis.* (1968) 27:253–60. doi: 10.1136/ard.27.3.253
22. Li J, Zhou Q, Wood RW, Kuzin I, Bottaro A, Ritchlin CT, et al. CD23(+)/CD21(hi) B-cell translocation and ipsilateral lymph node collapse is associated with asymmetric arthritic flare in TNF-Tg mice. *Arthritis Res Ther.* (2011) 13:R138. doi: 10.1186/ar3452
23. Zhou Q, Wood R, Schwarz EM, Wang YJ, Xing L. Near-infrared lymphatic imaging demonstrates the dynamics of lymph flow and lymphangiogenesis during the acute versus chronic phases of arthritis in mice. *Arthritis Rheum.* (2010) 62:1881–9. doi: 10.1002/art.27464
24. Guo R, Zhou Q, Proulx ST, Wood R, Ji RC, Ritchlin CT, et al. Inhibition of lymphangiogenesis and lymphatic drainage via vascular endothelial growth factor receptor 3 blockade increases the severity of inflammation in a mouse model of chronic inflammatory arthritis. *Arthritis Rheum.* (2009) 60:2666–76. doi: 10.1002/art.24764
25. Proulx ST, Kwok E, You Z, Beck CA, Shealy DJ, Ritchlin CT, et al. MRI and quantification of draining lymph node function in inflammatory arthritis. *Ann N Y Acad Sci.* (2007) 1117:106–23. doi: 10.1196/annals.1402.016
26. Li J, Kuzin I, Moshkani S, Proulx ST, Xing L, Skrombolas D, et al. Expanded CD23(+)/CD21(hi) B cells in inflamed lymph nodes are associated with the onset of inflammatory-erosive arthritis in TNF-transgenic mice and are targets of anti-CD20 therapy. *J Immunol.* (2010) 184:6142–50. doi: 10.4049/jimmunol.0903489
27. Moshkani S, Kuzin, II, Adewale F, Jansson J, Sanz I, Schwarz EM, et al. CD23+ CD21(high) CD1d(high) B cells in inflamed lymph nodes are a locally differentiated population with increased antigen capture and activation potential. *J Immunol.* (2012) 188:5944–53. doi: 10.4049/jimmunol.1103071
28. Bouta EM, Kuzin I, de Mesy Bentley K, Wood RW, Rahimi H, Ji RC, et al. Brief report: treatment of tumor necrosis factor-transgenic mice with anti-tumor necrosis factor restores lymphatic contractions, repairs lymphatic vessels, and may increase monocyte/macrophage egress. *Arthritis Rheumatol.* (2017) 69:1187–93. doi: 10.1002/art.40047
29. Bouta EM, Wood RW, Brown EB, Rahimi H, Ritchlin CT, Schwarz EM. *In vivo* quantification of lymph viscosity and pressure in lymphatic vessels and draining lymph nodes of arthritic joints in mice. *J Physiol.* (2014) 592:1213–23. doi: 10.1113/jphysiol.2013.266700
30. Zhou Q, Guo R, Wood R, Boyce BF, Liang Q, Wang YJ, et al. Vascular endothelial growth factor C attenuates joint damage in chronic inflammatory arthritis by accelerating local lymphatic drainage in mice. *Arthritis Rheum.* (2011) 63:2318–28. doi: 10.1002/art.30421
31. Bouta EM, Bell RD, Rahimi H, Xing L, Wood RW, Bingham CO III, et al. Targeting lymphatic function as a novel therapeutic intervention for rheumatoid arthritis. *Nat Rev Rheumatol.* (2018) 14:94–106. doi: 10.1038/nrrheum.2017.205
32. Aldrich MB, Sevick-Muraca EM. Cytokines are systemic effectors of lymphatic function in acute inflammation. *Cytokine.* (2013) 64:362–9. doi: 10.1016/j.cyto.2013.05.015
33. Liao S, Bouta EM, Morris LM, Jones D, Jain RK, Padera TP. Inducible nitric oxide synthase and $CD11b^{(+)}Gr1^{(+)}$ cells impair lymphatic contraction of tumor-draining lymphatic vessels. *Lymphat Res Biol.* (2018) doi: 10.1089/lrb.2018.0013. [Epub ahead of print].
34. Koller A, Mizuno R, Kaley G. Flow reduces the amplitude and increases the frequency of lymphatic vasomotion: role of endothelial prostanoids. *Am J Physiol.* (1999) 277:R1683–9.
35. Scallan JP, Wolpers JH, Muthuchamy M, Zawieja DC, Gashev AA, Davis MJ. Independent and interactive effects of preload and afterload on the pump function of the isolated lymphangion. *Am J Physiol Heart Circ Physiol.* (2012) 303:H809–24. doi: 10.1152/ajpheart.01098.2011
36. Proulx ST, Kwok E, You Z, Papuga MO, Beck CA, Shealy DJ, et al. Longitudinal assessment of synovial, lymph node, and bone volumes in inflammatory arthritis in mice by *in vivo* magnetic resonance imaging and microfocal computed tomography. *Arthritis Rheum.* (2007) 56:4024–37. doi: 10.1002/art.23128
37. Li J, Ju Y, Bouta EM, Xing L, Wood RW, Kuzin I, et al. Efficacy of B cell depletion therapy for murine joint arthritis flare is associated with increased lymphatic flow. *Arthritis Rheum.* (2013) 65:130–8. doi: 10.1002/art.37709
38. Rasmussen JC, Tan IC, Marshall MV, Adams KE, Kwon S, Fife CE, et al. Human lymphatic architecture and dynamic transport imaged using near-infrared fluorescence. *Transl Oncol.* (2010) 3:362–72. doi: 10.1593/tlo.10190
39. Rahimi H, Bell R, Bouta EM, Wood RW, Xing L, Ritchlin CT, et al. Lymphatic imaging to assess rheumatoid flare: mechanistic insights and biomarker potential. *Arthr Res Ther.* (2016) 18:194. doi: 10.1186/s13075-016-1092-0
40. Manzo A, Benaglio F, Vitolo B, Bortolotto C, Zibera F, Todoerti M, et al. Power doppler ultrasonographic assessment of the joint-draining lymph node complex in rheumatoid arthritis: a prospective, proof-of-concept study on treatment with tumor necrosis factor inhibitors. *Arthritis Res Ther.* (2016) 18:242. doi: 10.1186/s13075-016-1142-7
41. Manzo A, Caporali R, Vitolo B, Alessi S, Benaglio F, Todoerti M, et al. Subclinical remodelling of draining lymph node structure in early and established rheumatoid arthritis assessed by power Doppler ultrasonography. *Rheumatology.* (2011) 50:1395–400. doi: 10.1093/rheumatology/ker076
42. Rahimi H, Dieudonne G, Kheyfits V, Bouta EM, Wood RW, Barrett R, et al. Relationship between lymph node volume and pain following certolizumab therapy for rheumatoid arthritis flare: a pilot study. *Clin Med Insights Arthritis Musculoskelet Disord.* (2016) 9:203–8. doi: 10.4137/CMAMD.S40237
43. Campbell PM, LeRoy EC. Pathogenesis of systemic sclerosis: a vascular hypothesis. *Semin Arthritis Rheum.* (1975) 4:351–68. doi: 10.1016/0049-0172(75)90017-7
44. Rossi A, Sozio F, Sestini P, Renzoni EA, Khan K, Denton CP, et al. Lymphatic and blood vessels in scleroderma skin, a morphometric analysis. *Hum Pathol.* (2010) 41:366–74. doi: 10.1016/j.humpath.2009.08.009
45. Leu AJ, Gretener SB, Enderlin S, Bruhlmann P, Michel BA, Kowal-Bielecka O, et al. Lymphatic microangiopathy of the skin in systemic sclerosis. *Rheumatology.* (1999) 38:221–7. doi: 10.1093/rheumatology/38.3.221
46. Garber SJ, Wells AU, duBois RM, Hansell DM. Enlarged mediastinal lymph nodes in the fibrosing alveolitis of systemic sclerosis. *Br J Radiol.* (1992) 65:983–6. doi: 10.1259/0007-1285-65-779-983
47. El-Chemaly S, Pacheco-Rodriguez G, Ikeda Y, Malide D, Moss J. Lymphatics in idiopathic pulmonary fibrosis: new insights into an old disease. *Lymphat Res Biol.* (2009) 7:197–203. doi: 10.1089/lrb.2009.0014
48. Cui Y, Wilder J, Rietz C, Gigliotti A, Tang X, Shi Y, et al. Radiation-induced impairment in lung lymphatic vasculature. *Lymphat Res Biol.* (2014) 12:238–50. doi: 10.1089/lrb.2014.0012
49. Akhmetshina A, Beer J, Zwerina K, Englbrecht M, Palumbo K, Dees C, et al. Decreased lymphatic vessel counts in patients with systemic sclerosis:

association with fingertip ulcers. *Arthritis Rheum.* (2010) 62:1513–22. doi: 10.1002/art.27406
50. Manetti M, Milia AF, Guiducci S, Romano E, Matucci-Cerinic M, Ibba-Manneschi L. Progressive loss of lymphatic vessels in skin of patients with systemic sclerosis. *J Rheumatol.* (2011) 38:297–301. doi: 10.3899/jrheum.100767
51. Avraham T, Yan A, Zampell JC, Daluvoy SV, Haimovitz-Friedman A, Cordeiro AP, et al. Radiation therapy causes loss of dermal lymphatic vessels and interferes with lymphatic function by TGF-beta1-mediated tissue fibrosis. *Am J Physiol Cell Physiol.* (2010) 299:C589–605. doi: 10.1152/ajpcell.00535.2009
52. Vollmar B, Wolf B, Siegmund S, Katsen AD, Menger MD. Lymph vessel expansion and function in the development of hepatic fibrosis and cirrhosis. *Am J Pathol.* (1997) 151:169–75.
53. Yamauchi Y, Michitaka K, Onji M. Morphometric analysis of lymphatic and blood vessels in human chronic viral liver diseases. *Am J Pathol.* (1998) 153:1131–7. doi: 10.1016/S0002-9440(10)65657-X
54. Sakamoto I, Ito Y, Mizuno M, Suzuki Y, Sawai A, Tanaka A, et al. Lymphatic vessels develop during tubulointerstitial fibrosis. *Kidney Int.* (2009) 75:828–38. doi: 10.1038/ki.2008.661
55. Suzuki Y, Ito Y, Mizuno M, Kinashi H, Sawai A, Noda Y, et al. Transforming growth factor-β induces vascular endothelial growth factor-C expression leading to lymphangiogenesis in rat unilateral ureteral obstruction. *Kidney Int.* (2012) 81:865–79. doi: 10.1038/ki.2011.464
56. Matsui K, Nagy-Bojarsky K, Laakkonen P, Krieger S, Mechtler K, Uchida S, et al. Lymphatic microvessels in the rat remnant kidney model of renal fibrosis: aminopeptidase p and podoplanin are discriminatory markers for endothelial cells of blood and lymphatic vessels. *J Am Soc Nephrol.* (2003) 14:1981–9.
57. Stojan G, Petri M. Epidemiology of systemic lupus erythematosus: an update. *Curr Opin Rheumatol.* (2018) 30:144–50. doi: 10.1097/BOR.0000000000000480
58. Pons-Estel GJ, Ugarte-Gil MF, Alarcon GS. Epidemiology of systemic lupus erythematosus. *Expert Rev Clin Immunol.* (2017) 13:799–814. doi: 10.1080/1744666X.2017.1327352
59. Lee CK, Han JM, Lee KN, Lee EY, Shin JH, Cho YS, et al. Concurrent occurrence of chylothorax, chylous ascites, and protein-losing enteropathy in systemic lupus erythematosus. *J Rheumatol.* (2002) 29:1330–3.
60. Daniel A, Bagnato G, Vital E, Del Galdo F. Chylous ascites in a patient with an overlap syndrome: a surprising response to rituximab. *BMJ Case Rep.* (2017) 2017:bcr-2017-222339. doi: 10.1136/bcr-2017-222339
61. Manzella DJ, Dettori PN, Hertimian ML, Melero MJ. Chylous ascites and chylothorax as presentation of a systemic progression of discoid lupus. *J Clin Rheumatol.* (2013) 19:87–9. doi: 10.1097/RHU.0b013e3182847260
62. Dalvi SR, Yildirim R, Santoriello D, Belmont HM. Pseudo-pseudo Meigs' syndrome in a patient with systemic lupus erythematosus. *Lupus.* (2012) 21:1463–6. doi: 10.1177/0961203312461291
63. Rajasekhar L, Habibi S, Sudhakar P, Gumdal N. Lymphatic obstruction as a cause of extremity edema in systemic lupus erythematosus. *Clin Rheumatol.* (2013) 32 (Suppl. 1):S11–3. doi: 10.1007/s10067-009-1268-y
64. Calguneri M, Ozturk MA, Ozbalkan Z, Akdogan A, Ureten K, Kiraz S, et al. Frequency of lymphadenopathy in rheumatoid arthritis and systemic lupus erythematosus. *J Int Med Res.* (2003) 31:345–9. doi: 10.1177/147323000303100415
65. Shapira Y, Weinberger A, Wysenbeek AJ. Lymphadenopathy in systemic lupus erythematosus. Prevalence and relation to disease manifestations. *Clin Rheumatol.* (1996) 15:335–8. doi: 10.1007/BF02230354
66. Pinal-Fernandez I, Mammen AL. Dermatomyositis etiopathogenesis: a rebel soldier in the muscle. *Curr Opin Rheumatol.* (2018) 30:623–9. doi: 10.1097/BOR.0000000000000540
67. Sherzai D, Rao R, Nguyen C, Schulz E, Sherzai A. Lymphatic blockage and severe edema in dermatomyositis: lessons from this case and others. *Case Reports Clin Pathol.* (2014) 1:12–6. doi: 10.5430/crcp.v1n2p12
68. Fernandez-Flores A. Dilated lymphatics in gottron's papules. *Acta Dermatovenerol Croat.* (2010) 18:99–103.
69. Tiniakou E, Mammen AL. Idiopathic inflammatory myopathies and malignancy: a comprehensive review. *Clin Rev Allergy Immunol.* (2017) 52:20–33. doi: 10.1007/s12016-015-8511-x
70. Ezzo J, Manheimer E, McNeely ML, Howell DM, Weiss R, Johansson KI, et al. Manual lymphatic drainage for lymphedema following breast cancer treatment. *Cochrane Database Syst Rev.* (2015) 2015:Cd003475. doi: 10.1002/14651858.CD003475.pub2
71. Schaverien MV, Moeller JA, Cleveland SD. Nonoperative treatment of lymphedema. *Semin Plastic Surg.* (2018) 32:17–21. doi: 10.1055/s-0038-1635116
72. Bongi SM, Del Rosso A, Passalacqua M, Miccio S, Cerinic MM. Manual lymph drainage improving upper extremity edema and hand function in patients with systemic sclerosis in edematous phase. *Arthritis Care Res.* (2011) 63:1134–41. doi: 10.1002/acr.20487
73. Narahari SR, Ryan TJ, Mahadevan PE, Bose KS, Prasanna KS. Integrated management of filarial lymphedema for rural communities. *Lymphology.* (2007) 40:3–13.
74. Sands WA, McNeal JR, Murray SR, Stone MH. Dynamic compression enhances pressure-to-pain threshold in elite athlete recovery: exploratory study. *J Strength Cond Res.* (2015) 29:1263–72. doi: 10.1519/JSC.0000000000000412
75. Kephart WC, Mobley CB, Fox CD, Pascoe DD, Sefton JM, Wilson TJ, et al. A single bout of whole-leg, peristaltic pulse external pneumatic compression upregulates PGC-1alpha mRNA and endothelial nitric oxide sythase protein in human skeletal muscle tissue. *Exp Physiol.* (2015) 100:852–64. doi: 10.1113/EP085160
76. Li F, He T, Xu Q, Lin LT, Li H, Liu Y, et al. What is the Acupoint? A preliminary review of Acupoints. *Pain Med.* (2015) 16:1905–15. doi: 10.1111/pme.12761
77. Langevin HM, Yandow JA. Relationship of acupuncture points and meridians to connective tissue planes. *Anatom Record.* (2002) 269:257–65. doi: 10.1002/ar.10185
78. Zhang ZJ, Wang XM, McAlonan GM. Neural acupuncture unit: a new concept for interpreting effects and mechanisms of acupuncture. *Evid Based Complement Alternat Med.* (2012) 2012:429412. doi: 10.1155/2012/429412
79. Kunder CA, St John AL, Abraham SN. Mast cell modulation of the vascular and lymphatic endothelium. *Blood.* (2011) 118:5383–93. doi: 10.1182/blood-2011-07-358432
80. Zhang D, Ding G, Shen X, Yao W, Zhang Z, Zhang Y, et al. Role of mast cells in acupuncture effect: a pilot study. *Explore.* (2008) 4:170–7. doi: 10.1016/j.explore.2008.02.002
81. Kanakura Y, Niwa K, Kometani K, Nakazawa K, Yamaguchi Y, Ishikawa H, et al. Effectiveness of acupuncture and moxibustion treatment for lymphedema following intrapelvic lymph node dissection: a preliminary report. *Am J Chin Med.* (2002) 30:37–43. doi: 10.1142/S0192415X02000041
82. Cassileth BR, Van Zee KJ, Chan Y, Coleton MI, Hudis CA, Cohen S, et al. A safety and efficacy pilot study of acupuncture for the treatment of chronic lymphoedema. *Acupunct Med.* (2011) 29:170–2.
83. Alem M, Gurgel MS. Acupuncture in the rehabilitation of women after breast cancer surgery–a case series. *Acupunct Med.* (2008) 26:87–93.
84. Bao T, Iris Zhi W, Vertosick EA, Li QS, DeRito J, Vickers A, et al. Acupuncture for breast cancer-related lymphedema: a randomized controlled trial. *Breast Cancer Res Treat.* (2018) 170:77–87. doi: 10.1007/s10549-018-4743-9
85. Miteva DO, Rutkowski JM, Dixon JB, Kilarski W, Shields JD, Swartz MA. Transmural flow modulates cell and fluid transport functions of lymphatic endothelium. *Circ Res.* (2010) 106:920–31. doi: 10.1161/CIRCRESAHA.109.207274
86. Ding W, Beissert S, Deng L, Miranda E, Cassetty C, Seiffert K, et al. Altered cutaneous immune parameters in transgenic mice overexpressing viral IL-10 in the epidermis. *J Clin Invest.* (2003) 111:1923–31. doi: 10.1172/JCI200315722
87. Palframan RT, Jung S, Cheng G, Weninger W, Luo Y, Dorf M, et al. Inflammatory chemokine transport and presentation in HEV: a remote control mechanism for monocyte recruitment to lymph nodes in inflamed tissues. *J Exp Med.* (2001) 194:1361–73. doi: 10.1084/jem.194.9.1361

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Dendritic Cells and T Cells Interact Within Murine Afferent Lymphatic Capillaries

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Afferent lymphatic vessels contribute to immunity by transporting antigen and leukocytes to draining lymph nodes (LNs) and are emerging as new players in the regulation of peripheral tolerance. Performing intravital microscopy in inflamed murine ear skin we found that migrating dendritic cells (DCs) and antigen-experienced effector T cells spend considerable time arresting or clustering within afferent lymphatic capillaries. We also observed that intralymphatic T cells frequently interacted with DCs. When imaging polyclonal T cells during an ongoing contact-hypersensitivity response, most intralymphatic DC-T cell interactions were short-lived. Conversely, during a delayed-type-hypersensitivity response, cognate antigen-bearing DCs engaged in long-lived MHCII-(I-A/I-E)-dependent interactions with antigen-specific T cells. Long-lived intralymphatic DC-T cell interactions reduced the speed of DC crawling but did not delay overall DC migration to draining LNs. While further consequences of these intralymphatic interactions still need to be explored, our findings suggest that lymphatic capillaries represent a unique compartment in which adaptive immune interaction and modulation occur.

Keywords: dendritic cells, T cells, immune interactions, lymphatic vessels, adaptive immunity, migration

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INTRODUCTION

Afferent lymphatic vessels are present within most vascularized tissues and functionally convey lymph toward and into a draining LN. By transporting soluble inflammatory mediators, antigens and leukocytes, afferent lymphatic vessels establish an immunological connection between peripheral tissues and LNs. In addition to these traditional transport functions, several emerging studies highlight the role of lymphatic endothelium itself as a key modulator of peripheral immune responses (1–3).

The main cell types migrating via afferent lymphatic vessels are antigen-experienced $CD4^+$ T cells and antigen-presenting dendritic cells (DCs) (3, 4). While T cell recirculation through afferent lymphatic vessels is thought to contribute to immunesurveillance, DC migration is important for maintenance of tolerance and for induction of protective immunity in draining LNs (3). In this regard, DCs within the tissue take up antigen and migrate via afferent lymphatic vessels to a draining LN. Within the LN, naïve T cells survey arriving DCs for presentation of antigen. In the case that a naïve T cell encounters a cognate antigen, the T cell undergoes clonal expansion and differentiation into effector and memory T cells. Intravital microscopy has revealed that

such adaptive DC-T cell interactions progress through distinct phases of contact that depend on factors such as antigen recognition, timing of activation, signal strength, and the inflammatory environment (5, 6). At the end of the proliferation and differentiation phase, antigen-experienced effector, and memory T cells exit the LN via efferent lymphatic vessels and migrate to inflamed peripheral tissues. There, effector/memory T cells may be re-stimulated by antigen to perform local effector functions, or exit the tissue via afferent lymphatic vessels. Performing intravital microscopy in the murine ear skin we and others have recently described that both DCs and $CD4^+$ effector/memory T cells spend several hours actively patrolling within initial capillaries and are only passively transported to the draining LN once they reach the larger downstream collecting vessels (7–10). Considering the long time spent in lymphatic capillaries and the emerging knowledge of the immune-modulatory functions of lymphatic vessels (1, 3), we here set out to further characterize the intralymphatic migratory behavior of DCs and T cells and to specifically investigate whether these cells might interact inside lymphatic capillaries.

MATERIALS AND METHODS

Mouse Strains

Wilde-type (WT) C57BL/6 mice, VE-cadherin-Cre×RFP (7), hCD2-DsRed×Prox1-GFP (10), Prox1-Orange×CD11c-YFP (11, 12), and hCD2-DsRedxOTII (13, 14) mice were crossed and/or bred in specific-pathogen-free (SPF) facilities in-house. I-A/I-$E^{-/-}$ mice (15) were acquired from an SPF facility at University of Zurich Laboratory Animal Services Center (LASC), Schlieren. All experiments were approved by the Cantonal Veterinary Office Zurich.

Bone Marrow Chimeras

Bone marrow chimeras were generated as described in (7).

Generation of BM-DCs

WT or I-A/I-$E^{-/-}$ BM-DCs were generated as described in **Supplemental Experimental Procedures**.

Intravital Microscopy Specifications

Intravital microscopy of mouse ear pinna was performed as previously described (10). Exact imaging conditions and cell motility specifications are listed in **Supplemental Experimental Procedures**. For adoptive transfer experiments: five to six hours prior to imaging, mice were anesthetized using isoflurane (2–5%) and 500'000 to 750'000 WT or I-A/I-$E^{-/-}$ labeled BM-DCs, or CD11c-YFP BM-DCs, were adoptively transferred into the ear skin in 2-3 injections of up to 5 µl each.

Analysis of DC and T Cell Contacts

For DC - T cell contact analysis, individual DCs were followed frame-by-frame and contact with T cells manually annotated. Direct contact for longer than 2 min was considered an active interaction. Contacts shorter than 2 min were excluded from the analysis. A gap size of 2 min between contacts with the same T cell was considered a single continuing contact. Consequently, gaps in contact for more than 2 min were considered independent contacts. Using these criteria, a contact plot for each DC was generated. The length of each contact, DC occupancy and number of T cell contacts per DC were analyzed. DC occupancy index = a measure of the percentage time that a DC is contacted by a T cell/s during an imaging period.

Flow Cytometry

Flow cytometry was performed on ear skin, LNs or BM-DCs as described in **Supplemental Experimental Procedures**.

CHS-Induced or DTH-Induced Inflammation

See **Supplemental Experimental Procedures**.

Statistical Analysis

All cell tracking data are presented as medians and all other results presented as mean plus standard deviation. Data sets were analyzed using Prism 7 (GraphPad). Kruskal-Wallis test followed by post hoc analysis was used for multiple comparisons and Mann-Whitney *U*-test for simple comparisons.

RESULTS

DCs Patrol and Arrest Within Lymphatic Capillaries

We previously reported that DCs actively entered lymphatic capillaries and migrated in a semi-directed manner toward the collecting vessels (7, 9). Intriguingly, in these studies we also frequently observed DCs that remained arrested for long time periods. To assess the relative proportion of time that DCs spend arrested or patrolling, we classified DCs into four different groups, depending on whether they had spent the entire imaging period of 60 min (a) actively patrolling, (b) mainly patrolling, (c) mainly arrested, or (d) completely arrested. Imaging was performed in the ear skin of VE-cadherin-Cre × RFP mice, which feature red-fluorescent blood and lymphatic vessels (7), either upon adoptive transfer of LPS-matured YFP^+ bone marrow-derived DCs (BM-DCs; **Figure 1A**), or upon reconstitution of these mice with bone marrow from CD11c-YFP mice (BM chimeras, **Figure 1B**). In chimeric animals, endogenous YFP^+ DCs were induced to migrate into lymphatic vessels by intradermal LPS injection and topical imiquimod treatment. In both imaging setups only a small proportion of DCs (≈ 15%) continuously migrated or "patrolled" intravascularly, whereas a large proportion of DCs (≈ 75%) exhibited an intermittent arresting and patrolling behavior, and ∼10% remained completely arrested throughout the imaging period (**Figure 1C** and **Movie S1**). Most notably, in both setups, around 30% of DCs were arrested for more than half of the imaging period (**Figure 1C**, orange and red sectors combined).

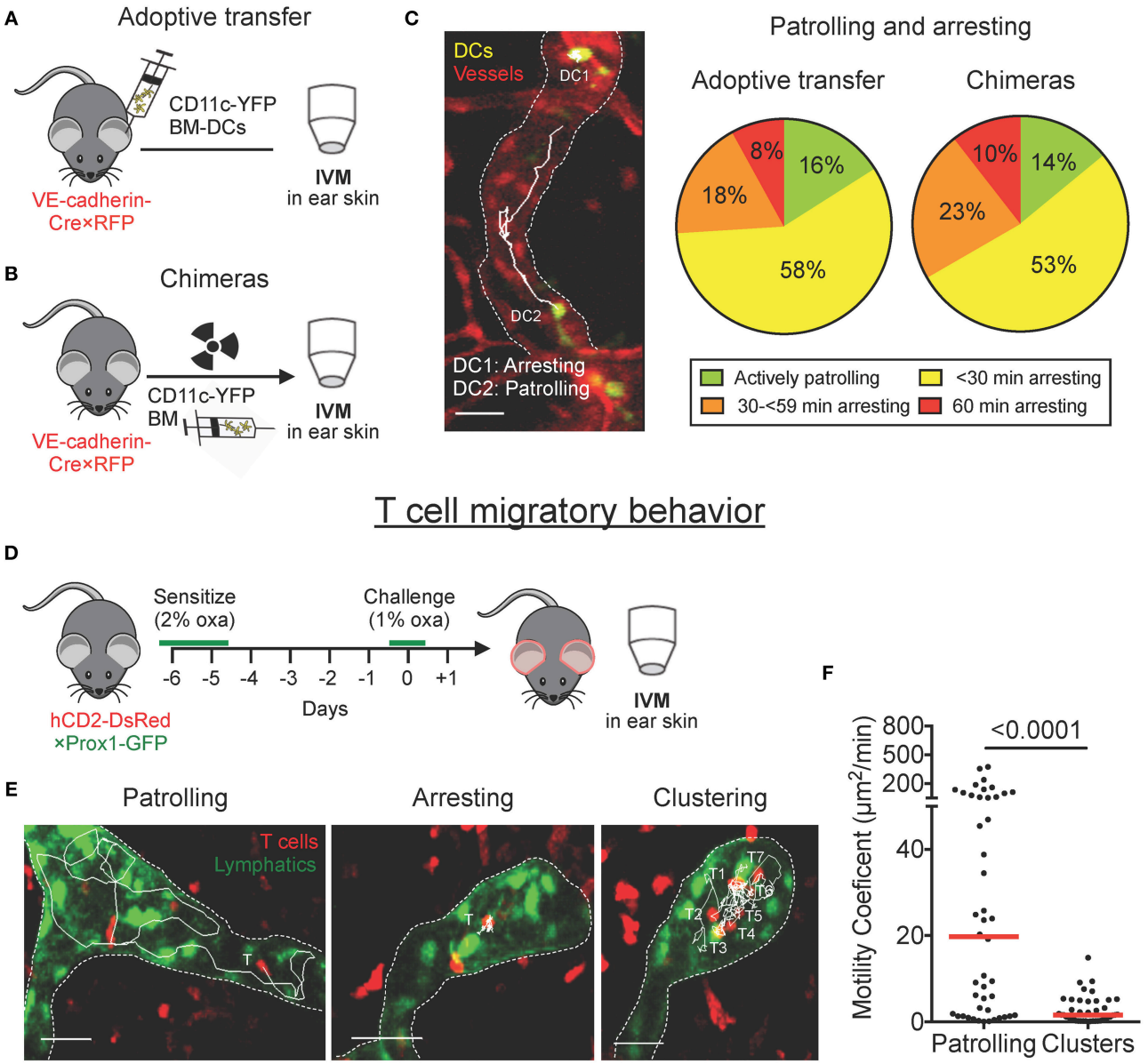

FIGURE 1 | DCs patrol and arrest, and T cells patrol, cluster and arrest in lymphatic capillaries in mouse ear skin. **(A–C)** Intravital microscopy was performed in the ear skin of VE-cadherin-Cre × RFP mice in which YFP$^+$ DCs had been adoptively transferred or in bone marrow chimeras. **(A,B)** Schematic diagrams of the experimental setups. **(C)** Representative image of YFP$^+$ DC probing and patrolling migratory behavior (scale bars: 30 μm). Tracks of individual DCs imaged over 60 min are shown as solid white lines. Stopping times of CD11c-YFP DCs were quantified manually and classified into four groups based upon their migratory behavior within an imaging period of 60 min. Pooled data from 6–9 mice. **(D–F)** Intravital microscopy was performed in CHS-inflamed ear skin of hCD2-DsRed × Prox1-GFP mice. **(D)** Schematic diagram of the experimental setup. **(E)** Representative images of DsRed$^+$ T cells migratory behavior inside lymphatic capillaries (scale bars: 30 μm). Tracks of individual T cells imaged over 30–45 min are shown as solid white lines. **(F)** Motility coefficient of patrolling T cells and T cells within clusters. Each dot represents a tracked cell. Median is shown as a red bar. Pooled data from 3 mice are shown.

T Cells Patrol, Cluster, and Frequently Arrest Within Lymphatic Capillaries

Performing intravital microscopy in contact hypersensitivity (CHS)-inflamed ear skin of hCD2-DsRed×Prox1-GFP mice, which feature red T cells and green lymphatic vessels (**Figure 1D**), we recently reported that, similarly to DCs, CD4$^+$ effector/memory T cells entered into and actively patrolled within lymphatic capillaries in mouse ear skin (10). After

further imaging and closer inspection of our videos, we also observed that several T cells remained arrested or clustered inside lymphatic capillaries (**Figure 1E**, **Movie S2**). Single-cell tracking analysis revealed that T cells in clusters were notably less motile than patrolling T cells (**Figure 1F**). Since clustering and swarming of T cells are hallmark phases of T cell activation in the draining LN (5, 6), we speculated that clustering or arresting T cells might be interacting with as yet "invisible" arrested DCs. In support of this "interaction hypothesis", we also occasionally observed T cells interacting with motile GFP^+ cells inside lymphatic capillaries of hCD2-DsRed×Prox1-GFP mice (**Figures S1A,B**, **Movie S3**). In flow cytometry a small fraction of GFP^+ cells were found to be $CD45^+$ cells and to express the DC markers CD11c and MHCII (I-A/I-E), indicating that the interacting cells might be DCs that had phagocytosed dying Prox1-GFP^+ lymphatic endothelial cells (**Figures S1C,D**).

Adoptively Transferred DCs Interact With T Cells Inside Lymphatic Capillaries During a CHS Response

To more definitively show that DCs interact with T cells inside lymphatic capillaries, we adoptively transferred YFP^+ LPS-matured BM-DCs (**Figure S2A**) into the ear skin of hCD2-DsRed×Prox1-GFP mice, which had been inflamed by induction of a CHS response to oxazolone. As previously reported (7, 9), YFP^+ DCs avidly entered and actively migrated within lymphatic capillaries. Most notably, YFP^+ DCs frequently interacted with T cells inside lymphatic capillaries (**Movie S4**).

The Majority of Intralymphatic Interactions Between T Cells and Transferred DCs Are Short-Lived and I-A/I-$E^{-/-}$-independent During a CHS Response

In LNs, migratory DCs are known to present processed antigen on MHCII (I-A/I-E) to circulating naïve T cells. To further characterize DC-T cell interactions inside dermal lymphatic capillaries, and to investigate their requirement for I-A/I-E, we adoptively transferred DeepRed-labeled wild-type (WT) or I-A/I-$E^{-/-}$ BM-DCs into the CHS-inflamed ear skin of hCD2-DsRed×Prox1-GFP mice (**Figures 2A,B**). Activated WT and I-A/I-$E^{-/-}$ DCs expressed similar levels of co-stimulatory molecules CD80 and CD86 (**Figures S2B,C**) and crawled with equal migratory speeds on lymphatic endothelial cell monolayers *in vitro* (**Figure S2D**) and in lymphatic capillaries of CHS-inflamed skin *in vivo* (**Figure 2C**). Both WT and I-A/I-$E^{-/-}$ DCs interacted with T cells inside lymphatic capillaries, and in most cases intralymphatic DC-T cell interactions were dynamic in nature: DCs interacted with several T cells during the imaging period and frequently interacted with more than one T cell simultaneously (**Figure 2B**, **Movie S5**). To quantify intralymphatic DC-T cell interactions, we generated contact plots whereby interacting DCs were analyzed frame by frame for contact with T cells (**Figure 2D**). This assessment revealed that the majority ($\approx$ 80%) of contacts were short-lived (<10 min), with only a handful ($\approx$ 5%) of contacts lasting longer than 30 min (**Figure 2E**). No long-lasting contacts were observed for I-A/I-$E^{-/-}$ DCs, but overall no major differences in T cell contact times between WT and I-A/I-$E^{-/-}$ DCs were observed (**Figure 2E**). However, WT DCs showed a tendency to be more occupied by T cells than I-A/I-$E^{-/-}$ DCs were (**Figure 2F**).

Adoptively Transferred Antigen-Presenting DCs Engage in Prolonged Interactions With Cognate Intralymphatic T Cells During a Delayed-Type Hypersensitivity (DTH) Response

Although not analyzed, most probably only a fraction of $DsRed^+$ T cells recruited into the skin was hapten-specific in our CHS model (**Figure 2**). Moreover, considering that we had not exposed DCs to the CHS-inducing agent oxazolone prior to adoptive transfer, cognate DC-T cell interactions were unlikely to have been observed by intravital microscopy in this model. To overcome this limitation, we switched to investigating DC-T cell interactions during a DTH response in which only T cell receptor (TCR) transgenic, cognate antigen-specific T cells were $DsRed^+$. To do so, we crossed TCR transgenic OTII mice, in which T cells are specific to ovalbumin-derived peptide $OVA_{323-339}$ presented on I-A/I-E (14), with hCD2-DsRed mice. $CD4^+$ T cells from hCD2-DsRed×OTII mice were transferred intravenously into Prox1-GFP mice, and mice were immunized with ovalbumin 1 day later (**Figure 3A**). After 4–7 days, ovalbumin was injected into the ears in order to elicit a DTH response (**Figure 3A**). Two days after elicitation, mouse ears were visibly reddened and ear thickness had increased (**Figure S3A**). By intravital microscopy we observed many $DsRed^+$ T cells within the tissue and inside lymphatic capillaries (**Figure S3B**). Characterization of the T cell population in DTH-inflamed ears revealed that $DsRed^+$OTII T cells constituted $\approx$ 5–20% of $CD4^+$ T cells in the ear skin (**Figures S3C,D,E**).

With a working DTH model, we adoptively transferred DeepRed-labeled, $OVA_{323-339}$-pulsed WT or I-A/I-$E^{-/-}$ BM-DCs into the DTH-inflamed ear skin of our model mice (**Figures 3A,B**). In comparison to the CHS model (**Figure 2**), contacts between WT DCs and T cells were less dynamic in nature: although WT DCs occasionally contacted more than one T cell, the majority of WT DCs engaged in long-lived contacts with a single T cell (**Figures 3D,E**, **Movie S6**). Most notably, more than 60% of contacts between WT DCs and T cells lasted longer than 30 min (**Figure 3E**). Conversely, the majority of contacts between I-A/I-$E^{-/-}$ DCs and T cells were short-lived, with <20% of contacts lasting longer than 30 min (**Figure 3E**). Moreover, whereas more than 65% of WT DCs were occupied by T cells for more than 80% of their track duration, only around 25% of I-A/I-$E^{-/-}$ DCs were equally occupied by T cells (**Figure 3F**). Consequently, intralymphatic I-A/I-$E^{-/-}$ DCs migrated faster than their WT counterparts (**Figure 3C**). However, in a competitive homing experiment, adoptively transferred WT and I-A/I-$E^{-/-}$ DCs migrated equally well to the draining LN (**Figure S4**).

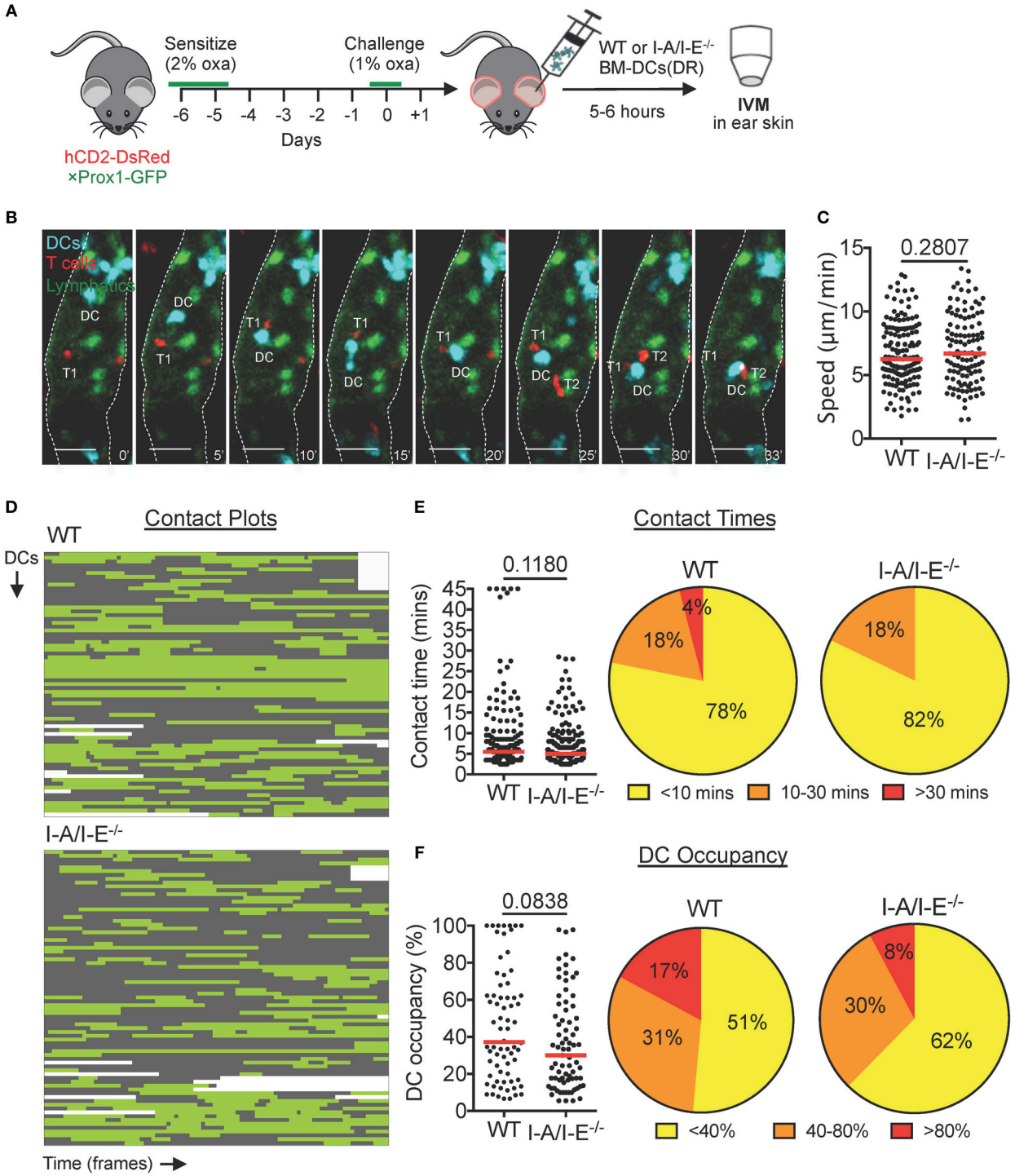

FIGURE 2 | DCs interact with T cells inside lymphatic capillaries and short interactions are I-A/I-E-independent in CHS-inflamed mouse ear skin. **(A–F)** Intravital microscopy was performed in CHS-inflamed ear skin of hCD2-DsRed×Prox1-GFP mice after adoptive transfer of DeepRed-labeled WT or I-A/I-E$^{-/-}$ BM-DCs. **(A)** Schematic diagram of the experimental setup. **(B)** Time-lapse images of a DeepRed^{+} WT DC (DC, cyan) contacting DsRed^{+} T cells (T1 and T2) inside a lymphatic capillary (scale bars: 30 µm). Times are shown in min. **(C)** Speed of WT and I-A/I-E$^{-/-}$ DCs within lymphatic capillaries. **(D)** Plots of contact between WT and I-A/I-E$^{-/-}$ DCs and T cells inside lymphatic capillaries. Each line is a DC indicating contact (green) and no contact (gray) with T cells. WT = 69 DCs, 174 contacts; I-A/I-E$^{-/-}$ = 77 DCs, 196 contacts. **(E)** Quantitative analysis of contact times from **(C)** are shown individually and after classification into three contact time groups. Median is shown as a red bar. **(F)** The occupancy of DCs by T cells from **(C)** are shown individually and after classification into three groups. Each dot in **(C,E,F)** represents a tracked cell. Medians are shown as red bars. Pooled data from 6 mice per group are shown.

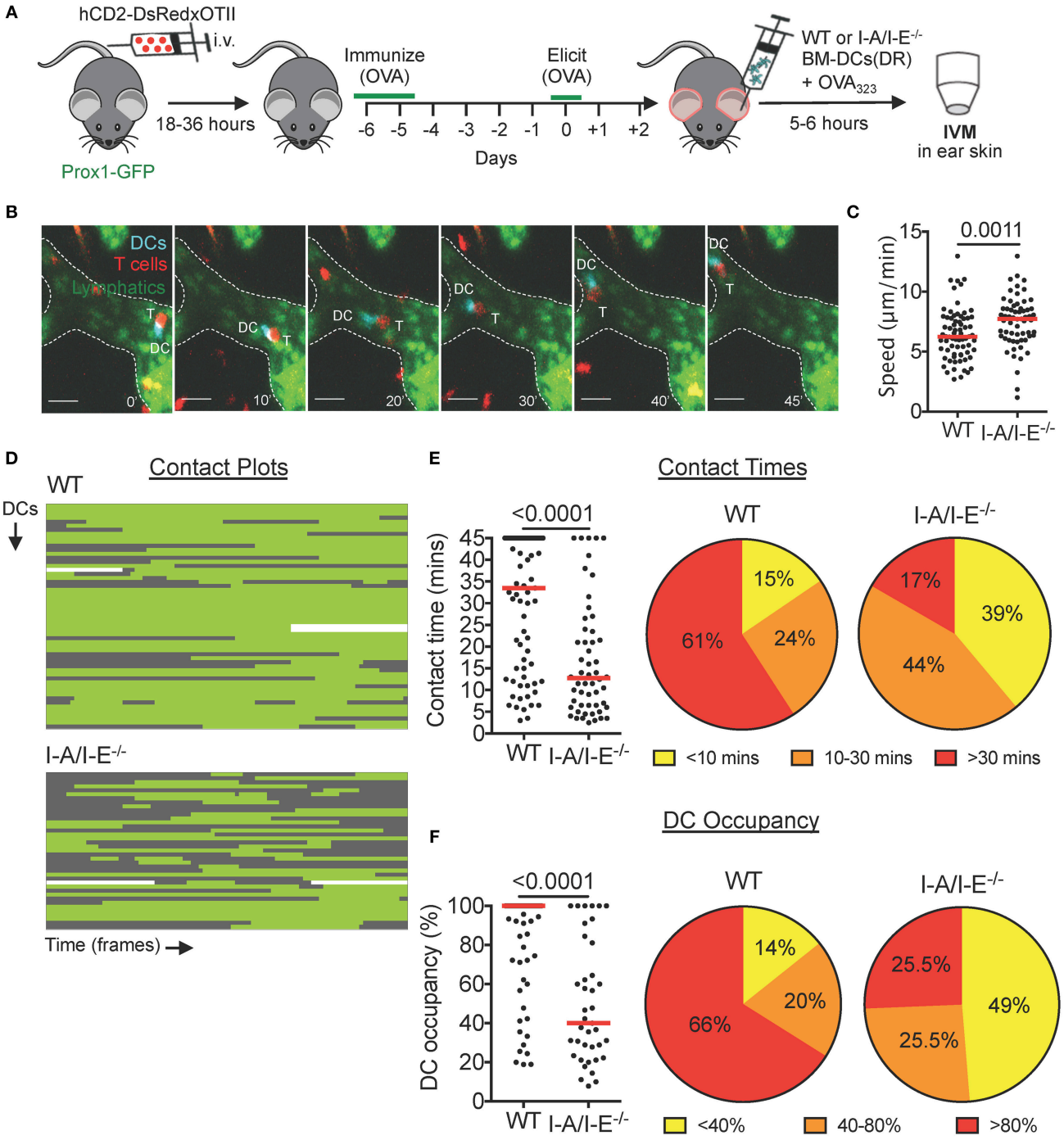

FIGURE 3 | Prolonged intralymphatic DC-T cell interactions are I-A/I-E-dependent in DTH-inflamed mouse ear skin. **(A–F)** Intravital microscopy was performed in DTH-inflamed ear skin of Prox1-GFP mice in which DeepRed-labeled WT or I-A/I-E$^{-/-}$ BM-DCs were adoptively transferred. **(A)** Schematic diagram of the experimental setup. **(B)** Time-lapse images of a DeepRed^{+} WT DC (DC, cyan) contacting a DsRed^{+} T cell (T) inside a lymphatic capillary (scale bars: 30 µm). Times are shown in min. **(C)** Speed of WT and I-A/I-E$^{-/-}$ DCs within lymphatic capillaries. **(D)** Plots of contact between WT and I-A/I-E$^{-/-}$ DCs and T cells inside lymphatic capillaries. Each line is a DC indicating contact (green) and no contact (gray) with T cells. WT = 56 DCs, 71 contacts; I-A/I-E$^{-/-}$ = 39 DCs, 54 contacts. **(E)** Quantitative analysis of contact times from **(C)** are shown individually and after classification into three contact time groups. **(F)** The occupancy of DCs by T cells from **(C)** are shown individually and after classification into three groups. Each dot in **(C,E,F)** represents a tracked cell. Medians are shown as red bars. Pooled data from 3–4 mice/group each are shown.

Endogenous DCs Interact With T Cells Inside Lymphatic Capillaries During a DTH Response to Ovalbumin

To investigate whether also endogenous DCs interact with T cells inside lymphatic capillaries, we established our hCD2-DsRed×OTII DTH model in Prox1-Orange×CD11c-YFP mice, which feature orange lymphatic vessels and yellow DCs (**Figure 4A**). Two days after challenge with ovalbumin, both endogenous YFP^+ DCs and *in vivo*-expanded $CD4^+$ hCD2-DsRed×OTII cells could be observed actively migrating and interacting inside lymphatic capillaries (**Figure 4B**, **Movie S7**). Analysis of contact plot data (**Figure 4C**) revealed that the majority of DCs engaged in short-lived contacts (<10 min) with T cells, with only a small percentage (5%) of DCs engaging in contacts longer than 30 min (**Figure 4D**). Although several DCs contacted more than one T cell, the majority of DCs engaged in a single contact with a T cell during an imaging period (**Figure 4C**). Consequently, the majority of DCs were occupied by a T cell/s for <40% of their track duration (**Figure 4E**).

T Cells, but Not DCs, Can Exit Lymphatic Capillaries in Murine Skin

Upon close inspection of videos generated from our CHS and DTH imaging setups, we occasionally observed T cells that exited lymphatic capillaries back into the surrounding tissue (**Figure 4F**, **Movie S8**). Egress across the endothelium was brief (≈ 2–5 min) and T cells visibly squeezed their cell body in order to exit the lymphatic lumen (**Figure 4F**, **Movie S8**). Although these events were not seen in every video, quantification in our endogenous DTH setup revealed that in total, ≈ 5–10% of intralymphatic T cells exited capillaries during an imaging period of 45 min (**Figure 4F**). Conversely, in all our imaging experience over the years, and quantitatively shown in our endogenous DTH setup (**Figure 4F**), we never observed a DC exit a lymphatic capillary.

DISCUSSION

In this study we have used intravital microscopy to further detail the behavior of DCs and T cells within dermal lymphatic capillaries during an ongoing immune response. In agreement with previous reports by us and by others (7–10) DCs and T cells actively migrated and patrolled within lymphatic capillaries. Moreover, we found that both cell types frequently arrested or clustered within lymphatic capillaries and that T cells, but not DCs, occasionally exited from the vessel lumen back into the tissue. Most intriguingly, we observed the occurrence of intralymphatic DC-T cell interactions.

Interactions between DCs and T cells are crucial for mounting an adaptive immune response. While they have mostly been studied in the draining LN during priming of naïve T cells (5, 6), only a few studies have investigated interactions of DCs with antigen-experienced effector/memory T cells in peripheral tissues like the skin (16–18). Given the spatial confinements of lymphatic vessels and the enhanced recruitment of activated DCs and T cells and drainage of antigen into the vessels during inflammatory processes, afferent lymphatic vessels might provide an ideal local compartment for adaptive modulation of the ongoing immune response. Intriguingly, several previous studies analyzing leukocyte subsets in afferent human lymph already reported the presence of cell aggregates comprising DCs and IFNγ-secreting $CD4^+$ T cells (4, 19–21), indicating that also in humans DC-T cell interactions might be occurring in afferent lymphatic vessels.

When eliciting a DTH response toward ovalbumin in mice with $DsRed^+$ TCR-transgenic OT-II T cells the majority of adoptively transferred $OVA_{323\text{-}339}$ peptide-presenting DCs engaged in long-lived, MHCII (I-A/I-E)-dependent interactions with TCR transgenic OTII T cells (**Figure 3**). By contrast, when imaging polyclonal $DsRed^+$ T cells 24 h after elicitation of a CHS response—a setup where likely only few of the adoptively transferred (unpulsed) DCs were presenting a cognate, haptenated antigen—numerous short-lived but only few long-lived DC-T cell interactions were observed (**Figure 2**). Similarly, imaging in our endogenous model of an ovalbumin-induced DTH response 48 h after ovalbumin injection (**Figure 4**), long-lived interactions only occurred in 5% of all cases. The reason why not more long-lived endogenous DC–T cell interactions occurred may be linked with the (unknown) level of $OVA_{323-339}$ peptide presentation: In contrast to the BM-DC transfer experiments, where imaging was carried out shortly after transfer of $OVA_{323-339}$ peptide pulsed DCs (**Figure 3**), likely much less $OVA_{323-339}$ was present on endogenous intralymphatic DCs when imaging 2 days after ovalbumin challenge (**Figure 4**). Overall antigen availability has been recognized as an important determinant of the duration of DC-T cell contacts in other studies (22–24). Moreover, somewhat in line with our findings, interactions within the tissue of DTH-inflamed rat ear skin were shown to progress from long-lived contacts during onset to less frequent short-lived contacts during the peak of the response (17).

At this point we do not know the specific subset of T cells involved in the observed intralymphatic DC-T cell interactions, and we can only speculate about the potential immunological significance of these interactions. Given current knowledge of T cell trafficking through inflamed afferent lymphatic vessels (3, 25) it is likely that intralymphatic DC-T cell interactions either involve $CD4^+$ effector T cells or regulatory T cells (T_{regs}). During an ongoing immune response, both effector T cells and T_{regs} are recruited into peripheral tissues irrespective of their antigenic specificity (18, 26, 27). By contrast, exit from the inflamed tissues via lymphatic vessels appears to be at least in part dependent on whether or not the T cell encountered its cognate antigen while surveying the interstitial space (28, 29). Particularly in the initial phase of a developing immune response (e.g., an infection), when antigen is still scarcely distributed, cognate effector T cells might not encounter their antigen on antigen-presenting cells scanned in the tissue and hence exit into lymphatics. In the case that an effector T cell now encountered a cognate antigen-presenting DC within lymphatic capillaries, the effector T cell could be re-activated and instructed to exit the lymphatic vessel back into surrounding tissue and continue searching for antigen in order to exert its effector functions in the tissue. Considering this scenario, it is intriguing that we

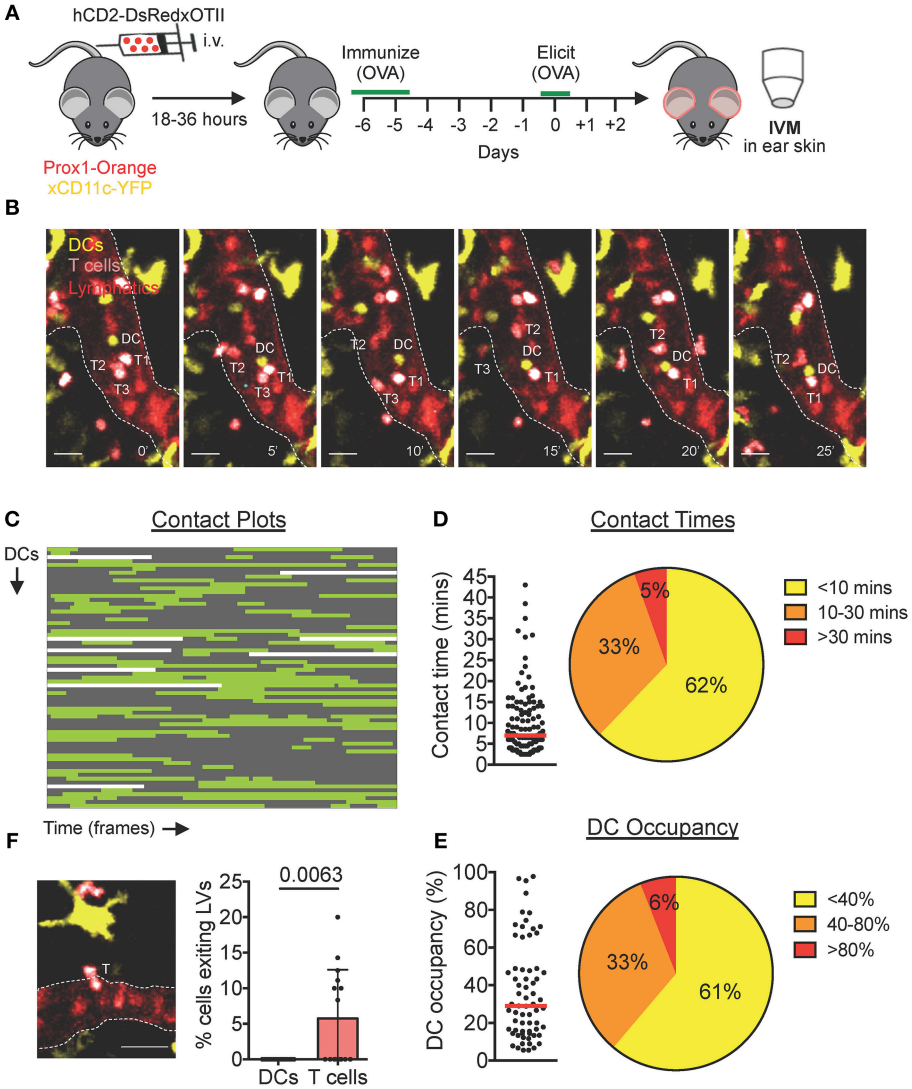

FIGURE 4 | Endogenous DCs interact with T cells inside lymphatic capillaries in DTH-inflamed mouse ear skin. **(A–F)** Intravital microscopy was performed in DTH-inflamed ear skin of Prox1-Orange×CD11c-YFP mice. **(A)** Schematic diagram of the experimental setup. **(B)** Time-lapse images of a YFP^+ DC (DC, yellow) contacting $DsRed^+$ T cells (T1 and T2) inside a lymphatic capillary (scale bars: 30 μm). A third T cell (T3) is shown exiting a lymphatic capillary. Times are shown in min. **(C)** Plots of contact between DCs and T cells inside lymphatic capillaries. Each line is a DC indicating contact (green) and no contact (gray) with T cells. Sixty seven DCs, 111 contacts. **(D)** Quantitative analysis of contact times from **(C)** are shown individually and after classification into three contact time groups. **(E)** The occupancy of DCs by T cells from **(C)** are shown individually and after classification into three groups. Each dot in **(D)** and **(E)** represents a tracked cell. Medians are shown as red bars. **(F)** Intravital microscopy snapshot of a $DsRed^+$ T cell (T) exiting a lymphatic capillary (scale bar: 30 μm) and quantification of the percentage of intralymphatic DCs or T cells that exited a lymphatic capillary during an imaging period. Each dot represents a movie analyzed. Mean and standard deviation are shown. Pooled data from 5 mice are shown.

found a substantial fraction of intralymphatic T cells exiting the vessel again (**Figure 4**). Overall, this exiting behavior could contribute to immunosurveillance, as these cells would take a "short-cut" back into tissue where their cognate antigen might be located, rather than recirculating through draining LNs, lymphatic vessels, and blood. In any case, our finding of T cells exiting back into the tissue asks for a revision of the current lymphatic trafficking paradigm, in which afferent lymphatic vessels have thus far exclusively been regarded as cellular tissue exit routes.

Interestingly, besides effector T cells, T_{regs} were found to constitute ~50% of T cells emigrating from CHS-inflamed skin to draining LNs via afferent lymphatic vessels (30, 31). Moreover, T_{regs} arriving via afferent lymphatic vessels were shown to contribute to the suppression of immunity in draining LNs and to be important for preventing exacerbated CHS-induced inflammation in skin (30, 31). In addition to directly suppressing T cell priming (32), T_{regs} are capable of suppressing the maturation phenotype of antigen-presenting DCs (33). Considering that afferent capillaries accumulate both DCs and

T_{regs} that are exiting from the tissue, it is perceivable that DC-T_{reg} interactions that modulate the DC phenotype might already take place in this compartment. Given the availability of T_{reg}-specific reporter mice, such as FoxP3-GFP mice (34), this hypothesis could be investigated in the near future.

We also observed that endogenous intralymphatic DCs or T cells frequently clustered amongst themselves. While we occasionally found several clustering T cells interacting with DCs, DC-T cell interactions do not seem to explain the formation of all or larger T cell clusters. Homotypic T cell-T cell clusters have previously been described within LNs and have been shown to augment T cell activation and differentiation via paracrine signaling of IL-2 and IFN-γ (35, 36). Although we cannot yet specifically determine the activation status of clustering T cells within lymphatic vessels, this might become possible in the future using new photoconvertible systems, such as Kaede mice (37).

Our simultaneous imaging of DCs and T cells also revealed that not all immotile DCs or T cells were necessarily engaging in interactions with other DCs or T cells, but that some cells simply remained arrested on the lymphatic endothelium for long time periods. It is tempting to speculate that DCs and T cells might be exchanging immune-modulatory signals with lymphatic endothelium during these lengthy arrest and interaction periods. Interestingly, autoantigen-presenting lymphatic endothelial cells in LNs have been identified as important players in the regulation of peripheral $CD8^+$ T cell tolerance (1, 38). Moreover, LN lymphatic endothelial cells have been shown to archive exogenous antigen derived from viral infections or vaccinations and pass it on to migratory DCs capable of antigen cross-presentation (39). Besides impacting $CD8^+$ T cell responses, emerging studies have also identified a role for LN lymphatic endothelial cells in modulating $CD4^+$ T cell responses, either by directly presenting antigen to T cells or by transferring self-antigen to DCs, leading to the induction of T cell anergy and tolerance (40, 41).

Of interest, we have observed that similarly to LN lymphatic endothelial cells, lymphatic endothelial cells in afferent lymphatic vessels upregulate PDL-1 and MHCII in response to inflammation [(42) and data not shown]. Together with our intravital microscopy observations of lengthy DC and T cell arresting, this could suggest that lymphatic endothelial cells in afferent lymphatics might exert similar immune-modulatory functions as in draining LNs. In fact, in the case of DCs, the production of prostaglandins (43) and ICAM-1 by lymphatic endothelial cells of afferent lymphatic vessels (44) were already suggested to impact maturation of migratory DCs. Moreover, dermal lymphatic vessels have recently been identified as important players in the regulation of peripheral $CD8^+$ T cell tolerance during tumor growth (2). Given the extensive time that DCs and T cells spend inside afferent lymphatics during their exit from the tissue, future studies should investigate the expanding role of afferent lymphatic endothelium in the immunomodulation of intralymphatic passengers.

In summary, our study for the first time reports the occurrence of adaptive DC-T cell interactions within lymphatic capillaries. Combined with the current literature, our findings provide several further pieces of evidence suggesting that afferent lymphatic vessels represent more than just a trafficking route to draining LNs but rather a new compartment for adaptive immune interactions and immune modulation.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the **Supplementary Files**.

AUTHOR CONTRIBUTIONS

MH designed research, performed research, analyzed data, and wrote the paper. AT designed research, performed research and analyzed, and discussed data. RM, ER, and PR performed research and analyzed and discussed data. FK provided mice and discussed data. CH designed research, analyzed data, and wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.00520/full#supplementary-material

REFERENCES

1. Humbert M, Hugues S, Dubrot J. Shaping of peripheral T cell responses by lymphatic endothelial cells. *Front Immunol.* (2016) 7:684. doi: 10.3389/fimmu.2016.00684
2. Lane RS, Femel J, Breazeale AP, Loo CP, Thibault G, Kaempf A, et al. IFNγ-activated dermal lymphatic vessels inhibit cytotoxic T cells in melanoma and inflamed skin. *J Exp Med.* (2018) 215:3057–74 doi: 10.1084/jem.20180654
3. Permanyer M, Bosnjak B, Forster R. Dendritic cells, T cells and lymphatics: dialogues in migration and beyond. *Curr Opin Immunol.* (2018) 53:173–9. doi: 10.1016/j.coi.2018.05.004
4. Yawalkar N, Hunger RE, Pichler WJ, Braathen LR, Brand CU. Human afferent lymph from normal skin contains an increased number of mainly memory / effector CD4(+) T cells expressing activation, adhesion and co-stimulatory molecules. *Eur J Immunol.* (2000) 30:491–7. doi: 10.1002/1521-4141(200002)30:2<491::AID-IMMU491>3.0.CO;2-H

5. Miller MJ, Safrina O, Parker I, Cahalan, MD. Imaging the single cell dynamics of CD4+ T cell activation by dendritic cells in lymph nodes. *J Exp Med*. (2004) 200:847–856. doi: 10.1084/jem.20041236
6. Mempel TR, Henrickson SE, Von Andrian UH. T-cell priming by dendritic cells in lymph nodes occurs in three distinct phases. *Nature*. (2004) 427:154–9. doi: 10.1038/nature02238
7. Nitschke M, Aebischer D, Abadier M, Haener S, Lucic M, Vigl B, et al. Differential requirement for ROCK in dendritic cell migration within lymphatic capillaries in steady-state and inflammation. *Blood*. (2012) 120:2249–58. doi: 10.1182/blood-2012-03-417923
8. Tal O, Lim HY, Gurevich I, Milo I, Shipony Z, Ng LG, et al. DC mobilization from the skin requires docking to immobilized CCL21 on lymphatic endothelium and intralymphatic crawling. *J Exp Med*. (2011) 208:2141–53. doi: 10.1084/jem.20102392
9. Russo E, Teijeira A, Vaahtomeri K, Willrodt AH, Bloch JS, Nitschke M, et al. Intralymphatic CCL21 promotes tissue egress of dendritic cells through afferent lymphatic vessels. *Cell Rep*. (2016) 14:1723–34. doi: 10.1016/j.celrep.2016.01.048
10. Teijeira A, Hunter MC, Russo E, Proulx ST, Frei T, Debes GF, et al. T cell migration from inflamed skin to draining lymph nodes requires intralymphatic crawling supported by ICAM-1/LFA-1 interactions. *Cell Rep*. (2017) 18:857–65. doi: 10.1016/j.celrep.2016.12.078
11. Hagerling R, Pollmann C, Kremer L, Andresen V, Kiefer F. Intravital two-photon microscopy of lymphatic vessel development and function using a transgenic Prox1 promoter-directed mOrange2 reporter mouse. *Biochem Soc Trans*. (2011) 39:1674–81. doi: 10.1042/BST20110722
12. Lindquist RL, Shakhar G, Dudziak D, Wardemann H, Eisenreich T, Dustin ML, et al. Visualizing dendritic cell networks *in vivo*. *Nat Immunol*. (2004) 5:1243–50. doi: 10.1038/ni1139
13. Veiga-Fernandes H, Coles MC, Foster KE, Patel A, Williams A, Natarajan D, et al. Tyrosine kinase receptor RET is a key regulator of Peyer's patch organogenesis. *Nature*. (2007) 446:547–51. doi: 10.1038/nature05597
14. Barnden MJ, Allison J, Heath WR, Carbone FR. Defective TCR expression in transgenic mice constructed using cDNA-based alpha- and beta-chain genes under the control of heterologous regulatory elements. *Immunol Cell Biol*. (1998) 76:34–40. doi: 10.1046/j.1440-1711.1998.00709.x
15. Kontgen F, Suss G, Stewart C, Steinmetz M, Bluethmann H. Targeted disruption of the MHC class II Aa gene in C57BL/6 mice. *Int Immunol*. (1993) 5:957–64. doi: 10.1093/intimm/5.8.957
16. Natsuaki Y, Egawa G, Nakamizo S, Ono S, Hanakawa S, Okada T, et al. Perivascular leukocyte clusters are essential for efficient activation of effector T cells in the skin. *Nat Immunol*. (2014) 15:1064–9. doi: 10.1038/ni.2992
17. Matheu MP, Beeton C, Garcia A, Chi V, Rangaraju S, Safrina O, et al. Imaging of effector memory T cells during a delayed-type hypersensitivity reaction and suppression by Kv1.3 channel block. *Immunity*. (2008) 29:602–14. doi: 10.1016/j.immuni.2008.07.015
18. Egawa G, Honda T, Tanizaki H, Doi H, Miyachi Y, Kabashima K. *In vivo* imaging of T-cell motility in the elicitation phase of contact hypersensitivity using two-photon microscopy. *J Invest Dermatol*. (2011) 131:977–9. doi: 10.1038/jid.2010.386
19. Brand CU, Hunziker T, Schaffner T, Limat A, Gerber HA, Braathen LR. Activated immunocompetent cells in human skin lymph derived from irritant contact dermatitis: an immunomorphological study. *Br J Dermatol*. (1995) 132:39–45. doi: 10.1111/j.1365-2133.1995.tb08622.x
20. Olszewski WL, Grzelak I, Ziolkowska A, Engeset A. Immune cell traffic from blood through the normal human skin to lymphatics. *Clin Dermatol*. (1995) 13:473–83. doi: 10.1016/0738-081X(95)00087-V
21. Brand CU, Hunger RE, Yawalkar N, Gerber HA, Schaffner T, Braathen LR. Characterization of human skin-derived CD1a-positive lymph cells. *Arch Dermatol Res*. (1999) 291:65–72. doi: 10.1007/s004030050385
22. Henrickson SE, Mempel TR, Mazo IB, Liu B, Artyomov MN, Zheng H, et al. T cell sensing of antigen dose governs interactive behavior with dendritic cells and sets a threshold for T cell activation. *Nat Immunol*. (2008) 9:282–91. doi: 10.1038/ni1559
23. Henrickson SE, Perro M, Loughhead SM, Senman B, Stutte S, Quigley M, et al. Antigen availability determines CD8(+) T cell-dendritic cell interaction kinetics and memory fate decisions. *Immunity*. (2013) 39:496–507. doi: 10.1016/j.immuni.2013.08.034
24. Egen JG, Rothfuchs AG, Feng CG, Horwitz MA, Sher A, Germain RN. Intravital imaging reveals limited antigen presentation and T cell effector function in mycobacterial granulomas. *Immunity*. (2011) 34:807–19. doi: 10.1016/j.immuni.2011.03.022
25. Hunter MC, Teijeira A, Halin C. T Cell Trafficking through Lymphatic Vessels. *Front Immunol*. (2016) 7:613. doi: 10.3389/fimmu.2016.00613
26. Stephens R, Randolph DA, Huang G, Holtzman MJ, Chaplin DD. Antigen-nonspecific recruitment of Th2 cells to the lung as a mechanism for viral infection-induced allergic asthma. *J Immunol*. (2002) 169:5458–67. doi: 10.4049/jimmunol.169.10.5458
27. Ghani S, Feuerer M, Doebis C, Lauer U, Loddenkemper C, Huehn J, et al. T cells as pioneers: antigen-specific T cells condition inflamed sites for high-rate antigen-non-specific effector cell recruitment. *Immunology*. (2009) 128:e870–e880. doi: 10.1111/j.1365-2567.2009.03096.x
28. Gomez D, Diehl MC, Crosby EJ, Weinkopff T, Debes GF. Effector T cell egress via afferent lymph modulates local tissue inflammation. *J Immunol*. (2015) 195:3531–6. doi: 10.4049/jimmunol.1500626
29. Mcnamee EN, Masterson JC, Veny M, Collins CB, Jedlicka P, Byrne FR, et al. Chemokine receptor CCR7 regulates the intestinal TH1/TH17/Treg balance during Crohn's-like murine ileitis. *J Leukoc Biol*. (2015) 97:1011–22. doi: 10.1189/jlb.3HI0614-303R
30. Tomura M, Honda T, Tanizaki H, Otsuka A, Egawa G, Tokura Y, et al. Activated regulatory T cells are the major T cell type emigrating from the skin during a cutaneous immune response in mice. *J Clin Invest*. (2010) 120:883–93. doi: 10.1172/JCI40926
31. Ikebuchi R, Teraguchi S, Vandenbon A, Honda T, Shand FH, Nakanishi Y, et al. A rare subset of skin-tropic regulatory T cells expressing Il10/Gzmb inhibits the cutaneous immune response. *Sci Rep*. (2016) 6:35002. doi: 10.1038/srep35002
32. Schmidt A, Oberle N, Krammer PH. Molecular mechanisms of treg-mediated T cell suppression. *Front Immunol*. (2012) 3:51. doi: 10.3389/fimmu.2012.00051
33. Dhainaut M, Moser M. Mechanisms of surveillance of dendritic cells by regulatory T lymphocytes. *Prog Mol Biol Transl Sci*. (2015) 136:131–54. doi: 10.1016/bs.pmbts.2015.08.003
34. Bettelli E, Carrier Y, Gao W, Korn T, Strom TB, Oukka M, et al. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature*. (2006) 441:235–8. doi: 10.1038/nature04753
35. Sabatos CA, Doh J, Chakravarti S, Friedman RS, Pandurangi PG, Tooley AJ, et al. A synaptic basis for paracrine interleukin-2 signaling during homotypic T cell interaction. *Immunity*. (2008) 29:238–48. doi: 10.1016/j.immuni.2008.05.017
36. Gerard A, Khan O, Beemiller P, Oswald E, Hu J, Matloubian M, et al. Secondary T cell-T cell synaptic interactions drive the differentiation of protective CD8+ T cells. *Nat Immunol*. (2013) 14:356–63. doi: 10.1038/ni.2547
37. Tomura M, Yoshida N, Tanaka J, Karasawa S, Miwa Y, Miyawaki A, et al. Monitoring cellular movement *in vivo* with photoconvertible fluorescence protein "Kaede" transgenic mice. *Proc Natl Acad Sci USA*. (2008) 105:10871–6. doi: 10.1073/pnas.0802278105
38. Rouhani SJ, Eccles JD, Tewalt EF, Engelhard VH. Regulation of T-cell tolerance by lymphatic endothelial cells. *J Clin Cell Immunol*. (2014) 5:1000242. doi: 10.4172/2155-9899.1000242
39. Kedl RM, Lindsay RS, Finlon JM, Lucas ED, Friedman RS, Tamburini BAJ. Migratory dendritic cells acquire and present lymphatic endothelial cell-archived antigens during lymph node contraction. *Nat Commun*. (2017) 8:2034. doi: 10.1038/s41467-017-02247-z
40. Dubrot J, Duraes FV, Potin L, Capotosti F, Brighouse D, Suter T, et al. Lymph node stromal cells acquire peptide-MHCII complexes from dendritic cells and induce antigen-specific CD4(+) T cell tolerance. *J Exp Med*. (2014) 211:1153–66. doi: 10.1084/jem.20132000
41. Rouhani SJ, Eccles JD, Riccardi P, Peske JD, Tewalt EF, Cohen JN, et al. Roles of lymphatic endothelial cells expressing peripheral tissue antigens in CD4 T-cell tolerance induction. *Nat Commun*. (2015) 6:6771. doi: 10.1038/ncomms7771
42. Vigl B, Aebischer D, Nitschke M, Iolyeva M, Rothlin T, Antsiferova O, et al. Tissue Inflammation Modulates Gene Expression Of Lymphatic Endothelial

cells and dendritic cell migration in a stimulus-dependent manner. *Blood*. (2011) 118:205–15. doi: 10.1182/blood-2010-12-326447

43. Christiansen AJ, Dieterich LC, Ohs I, Bachmann SB, Bianchi R, Proulx ST, et al. Lymphatic endothelial cells attenuate inflammation via suppression of dendritic cell maturation. *Oncotarget*. (2016) 7:39421–35. doi: 10.18632/oncotarget.9820
44. Podgrabinska S, Kamalu O, Mayer L, Shimaoka M, Snoeck H, Randolph GJ, et al. Inflamed lymphatic endothelium suppresses dendritic cell maturation and function via Mac-1/ICAM-1-dependent mechanism. *J Immunol*. (2009) 183:1767–79. doi: 10.4049/jimmunol.0802167

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Mesenteric Lymphatic Alterations Observed During DSS Induced Intestinal Inflammation Are Driven in a TLR4-PAMP/DAMP Discriminative Manner

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Background: Inflammatory bowel disease (IBD) is characterized by both acute and chronic phase inflammation of the gastro-intestinal (GI) tract that affect a large and growing number of people worldwide with little to no effective treatments. This is in part due to the lack of understanding of the disease pathogenesis and also the currently poorly described involvement of other systems such as the lymphatics. During DSS induced colitis, mice also develop a severe inflammation of terminal ileum with many features similar to IBD. As well as inflammation within the ileum we have previously demonstrated lymphatic remodeling within the mesentery and mesenteric lymph nodes of DSS-treated mice. The lymphatic remodeling includes lymphangiogenesis, lymphatic vessel dilation and leakiness, as well as cellular infiltration into the surrounding tissue and peripheral draining lymph nodes.

Methods: Intestinal inflammation was induced in C57BL/6 mice by administration of 2.5% DSS in drinking water for 7 days. Mice were treated with TLR4 blocker C34 or Polymyxin-B (PMXB) daily from days 3 to 7 of DSS treatment via I.P. injection, and their therapeutic effects on disease activity and lymphatic function were examined. TLR activity and subsequent effect on lymphangiogenesis, lymphadenopathy, and mesenteric lymph node cellular composition were assessed.

Results: DSS Mice treated with TLR4 inhibitor, C34, had a significantly improved disease phenotype characterized by reduced ileal and colonic insult. The change correlated with significant reduction in colonic and mesenteric inflammation, resolved mesenteric lymphangiectasia, and $CD103^+$ DC migration similar to that of healthy control. PMXB treatment however did not resolve inflammation within the colon or associated mesenteric lymphatic dysfunction but did however prevent lymphadenopathy within the MLN through alteration of CCL21 gradients and $CD103^+$ DC migration.

Conclusions: TLR4 appears to mediate several changes within the mesenteric lymphatics, more specifically it is shown to have different outcomes whether stimulation occurs through pathogen derived factors such as LPS or tissue derived DAMPs, a novel phenomenon.

Keywords: lymphatics, mesentery, toll-like receptors, inflammation, lipopolysaccharides, colitis

INTRODUCTION

IBD constitutes of two major phenotypes of gastrointestinal diseases, Ulcerative colitis and Crohn's disease. Both diseases have an inflammatory component which results in impaired nutrient absorption, cell recruitment, and chronic inflammation. During the pathogenesis of IBD, a major alteration occurring within the hosts GI tract is focused upon the lymphatics.

Severe lymphatic remodeling has been observed within the intestinal wall of IBD patients, at the stage of the initial (lacteal) lymphatics, through to the collecting mesenteric lymphatics, and mesenteric lymph nodes (1–5). However, what effect these alterations are having upon disease progression is still not fully understood. The expansion of the lymphatic network, also known as lymphangiogenesis, is mediated through the binding of the lymphatic vascular endothelial selective growth factors VEGF-C and VEGF-D to VEGFR3, and is a common feature in Crohn's disease (6–8). Blockade of lymphangiogenesis through anti-VEGFR3 antibodies do not provide any therapeutic benefit but rather exacerbates submucosal oedema in animal models of IBD (9, 10), while stimulation of lymphatic functions with VEGF-C ameliorates experimental IBD (10). Therefore, it presents the idea that lymphangiogenesis may in fact be a reparative measure in response to inflammation and pro-lymphangiogenic factors, such as VEGF-C, may provide novel strategies for the treatment of chronic inflammatory diseases. Lymphangiectasia, the dilation of lymphatic vessels, is a common sign of collecting lymphatic vessel disruption. It has been shown to be associated in case of inflammation, and intestinal inflammation in particular, with increase permeability of the lymphatics (3, 4). This can result in oedema, hypoproteinaemia, lymphocytopenia, and immunologic anomalies. Another great concern associated with the leakage is lipid absorption issues resulting in weight loss and fat deposition within the mesothelium. To what extent the fat contributes to inflammation, and the effect it has on resident cells, is still not fully elucidated however has been suggested that lymphatic-associated fat can be a source of inflammatory material and may play a greater role in disease pathogenesis than first expected (11–13).

Toll-like receptors (TLRs) play a key role in mucosal innate immunity and may be involved in the pathogenesis of IBD (14). An evolutionarily conserved family of transmembrane pattern recognition receptors, TLRs recognize pathogen-associated molecular patterns (PAMPs) conserved between microbes (15). Activation of TLRs results in the induction of cytokines, chemokines, and antimicrobial molecules, all important factors in the initial innate response aiding in priming the adaptive immune system (16, 17). TLR4 binds the gram-negative bacterial cell wall component lipopolysaccharide (LPS) and through co-receptor MD-2, interaction triggers both MyD88-dependent and independent pathway leading to the translocation of NF-κB and subsequent production of inflammatory cytokines and proteins (18, 19). TLR4-mediated signaling is important for the recruitment of immune cells to the site of inflammation promoting reparative mechanisms, but can be described as a double-edged sword, as aberrant stimulation can induce chronic inflammation (20).

The lymphatic system is a complex network of specialized vessels involved in tissue fluid homeostasis. Lymphatic vessels drain fluid from tissues and associated organs, and propel it unidirectionally as nutrient- and cell-rich "lymph" back into peripheral blood circulation. Initial lymphatic vessels comprised of closed-end, lymphatic capillaries, which branch into tissue then amalgamate to form larger collecting vessels, which, through the presence of smooth muscle cells surrounding the endothelium wall, propel lymph via peristaltic-like contractions toward the draining lymph node. Formation of lymph is believed to occur through the swelling of the interstitium, respiration, arterial pulsations and skeletal movement. Increased interstitial pressure opens the initial lymphatic vessels through small anchoring filaments attaching endothelial cells to the extra-cellular matrix. During inflammatory diseases such as IBD, increased localized swelling within a tissue creates an increased burden upon the draining lymphatics. Within IBD, disruptions in the mesenteric lymphatic architecture has been correlated to worsened disease progression, putting the changes under scrutiny for their potential contribution to pathogenesis (21, 22).

We aimed to determine whether lymphatic disruption in the mouse model of DSS-induced ileitis/colitis, was in portion driven by TLR4. In order to block TLR4 activity directly and indirectly, two drugs were used. The first, Polymyxin-B, inhibits TLR4 recognition of LPS by binding the lipid-A component of LPS preventing recognition by the receptor. The other, C34, is a direct chemical inhibitor of TLR4 binding to the receptor in an antagonistic and competitive manner. Identified by Neal and colleagues, C34 was a potent TLR4 blocker in enterocytes and macrophages *in vitro*, and reduced systemic inflammation in mouse models of endotoxemia and necrotizing enterocolitis (23). These treatments allowed us to differentiate between the activation of TLR4 by LPS or by TLR4-directed agonists of another source.

MATERIALS AND METHODS

Mice

All mice used were housed at constant temperature (22°C) on a 12:12-h light-dark cycle, with food and water *ad libitum*.

The animal handling and experiments were approved by the University of Calgary Animal Care and Ethics Committee and conformed to the guidelines established by the Canadian Council on Animal Care.

Cell Culture

HEK293 (TLR4/MD2/CD14) dual reporter cells (Invivogen, USA Cat. No HKD-mTLR4ni) were maintained in supplemented DMEM (High Glucose) 10% FCS with Normocin, Hygromycin Gold, and Zeocin as per manufacturers instruction. Cells were passaged every 3–5 days at 80% confluency and maintained in a 37°C incubator, 5% CO_2 atmosphere. All cells were used between passages 3–8 and experiments were performed on 3 or more distinct passages of cells.

Induction of Colitis and Administration of Treatments

Acute DSS

Six-week-old C57BL/6 mice were obtained from Jackson Laboratories. Colitis was induced in these mice by administration of 2.5% (weight/vol) dextran sulfate sodium (DSS; Affymetrix, Cleveland, Ohio, USA) in drinking water for 7 days. Sham mice were given normal drinking water. I.P. injections of C34 (50 mg/kg) (Tocris, USA, Cat. No 5373) or Polymyxin-B (50 mg/kg) (Sigma Aldrich, USA, Cas. No 1405-20-5) were administered from days 3 to 7 diluted to 200 µl total in saline, control mice received saline only. Mice were euthanized by exposure to isoflurane and cervical dislocation.

Disease Evaluation

In order to assess the severity of DSS-induced inflammation a multi-parameter approach was used in order to quantify inflammation by region. Colon shortening, a common sign of inflammation-driven fibrosis, was measured as a marker of colonic inflammation. Differences in weight were calculated as the percentage weight loss pre- and post-treatment (SHAM/DSS/DSS + treatment). Additionally, fecal matter consistency and visual blood presence were assessed. All of these factors were evaluated, and using the scoring system detailed in **Table 3**, the disease activity score (DAI) was calculated (see **Table 1**) [adapted from (24)].

TABLE 1 | Disease activity index scoring.

Symptom/score	Characteristic
BODY WEIGHT LOSS	
0	No negative change in weight
1	1–5% loss of body weight
2	5–10% loss of body weight
3	10–20% loss of body weight
4	>20% loss of body weight
STOOL CONSISTENCY	
0	Normal
1	Loose consistency
2	Watery
3	Slimy diarrhea
4	Severe diarrhea
BLOOD PRESENCE IN STOOL	
0	No blood
2	Red feces
4	Visible bleeding

Alterations in Lymphatics

Lymphangiectasia

Lymphatic vessels were identified as initials by positive staining with CCL21 (R&D systems, USA, Cat. No AF457) and as collectors by staining with αSMA (Sigma-Aldrich, USA, Cat. No C6198) in whole-mount mesenteric preparations. Vessel diameters were measured in 3–5 vessels per sample at 3 random sites along each vessel.

Lymphadenopathy

Mesenteric lymph nodes (MLNs) were isolated from the mouse, cleaned of fat and connective tissue and measured for both size (lengthways) and weight.

Lymphangiogenesis

Lymphangiogenesis was measured via CCL21 staining of mesenteric whole-mounts. Vessel branch points and numbers were determined in a fixed area of interest kept uniform between samples.

Whole Mount Immunofluorescence

Whole mount mesenteries were fixed on sylgard coated dishes and fixed with 4% PFA for 1 h at room temperature. Tissues were washed, permeabilized in PBST (PBS + 0.03% Triton X-100) and blocked with 2–3% BSA in PBST. Primary antibodies incubation occurred for 24 h at 4°C. Samples were washed three times in PBST (PBS + 0.01% Triton X-100) for 10 minutes per wash and then incubated with secondary antibodies (in 2% BSA containing 0.01% Triton X-100) for 1–2 h. Samples were washed as previously described before preparation for optical clearing. Fat-clearing was obtained by serial ethanol dehydration followed by methyl salicylate (MeS) immersion for 15 min. Immediately after clearing, samples were mounted with DAPI containing mounting medium and installed with a coverslip for imaging. The imaging occurred within 2 h of clearing due to fluorescent diminishment. Vessel diameter and branching was quantified using the LASX software attached to a Leica SP8 confocal microscope.

Quantitative Real-Time Polymerase Chain Reaction (qPCR)

The total RNA isolated from given samples was purified using the QIAGEN RNA total cleanup kits as per manufacturers instruction. One hundred nanogram of the RNA was converted using EvaGreen RT conversion kit in a gradient thermocycler as per manufacturers description. One nanogram per microliter of the converted cDNA samples was added to EvaGreen SYBR qPCR master-mix and qPCR analysis was performed in an ABI StepOne Plus PCR system. Annealing temperatures were kept at 60°C, and

TABLE 2 | RT-PCR primer sequences.

Gene	Sense primer (5′-3′)	Antisense primer (5′-3′)	Product size (bp)	References
TLR2	AAGAGGAAGCCCAAGAAAGC	CGATGGAATCGATGATGTTG	199	(25)
TLR3	CACAGGCTGAGCAGTTTGAA	TTTCGGCTTCTTTTGATGCT	190	(25)
TLR4	ACCTGGCTGGTTTACACGTC	CTGCCAGAGACATTGCAGAA	201	(25)
TLR5	AAGTTCCGGGGAATCTGTTT	GCATAGCTGAGCCTGTTTC	201	(25)
TLR7	AATCCACAGGCTCACCCATA	CAGGTACCAAGGGATGTCCT	142	(25)
TLR8	GACATGGCCCCTAATTTCCT	GACCCAGAAGTCCTCATGGA	195	(25)
TLR9	ACTGAGCACCCCTGCTTCTA	AGATTAGTAGCGGCAGGAA	198	(25)
VEGFR3	TCTGCTACAGCTTCCAGGTGG	GCAGCCAGGTCTCTGTGGAT	200	(26)
VEGFC	TGTGCTTCTTGTCTCTGGCG	CCTTCAAAAGCCTTGACCTCG	148	N/A
CCL21	GGTTCTGGCCTTTGGCATC	AGGCAACAGTCCTGAGCCC	262	(27)
GAPDH	CTCATGACCACAGTCCATGC	CACATTGGGGGTAGGAACAC	201	(25)

qPCR primer sets used for the amplification of genes of interest.

TABLE 3 | TLR4 mRNA expression changes in murine mesentery during DSS treatment.

	Fold change sham DSS	*P*-value
TLR2	2.359 ± 1.313	>0.9999
TLR3	0.3314 ± 0.185	>0.9999
TLR4	221.7 ± 80.93	<0.0001
TLR5	99.15 ± 24.27	0.0324
TLR7	2.565 ± 0.6062	>0.9999
TLR8	5.123 ± 1.245	>0.9999
TLR9	4.959 ± 1.44	>0.9999

DSS induces expression of TLR4 within the mesentery. Mesenteric lymphatic samples from SHAM and DSS treated mice (2.5% 7d) were analyzed for TLR expression using qPCR. Unpaired Mann-Whitney test, two-tailed.

40 cycles of amplification were performed to produce a sufficient read. Sequences of primers used are detailed in **Table 2**.

Total LPS Isolation From Murine Feces

Samples were homogenized in PBS and subsequently filtered in order to remove non-soluble components. Protein content of the fecal homogenate was determined through the Precision Red Protein Quantification assay (Cytoskeleton, Inc., USA Cat. No ADV02). The concentration of samples was then equilibrated to 1 mg/ml through addition of supplemented DMEM, before being assayed for endotoxin content using the chromogenic-Limulus amebocyte lysate (LAL) assay (ThermoFisher, USA, Cat. No 88282). Equal volumes (3.5 ml) of quantified fecal homogenate was then passed through a high capacity endotoxin removal spin column (Pierce, USA. Cat No 88274) reducing LPS content from an average of 87.67 EU/mg/ml (±7.36) to <1.71 EU/mg/ml (±0.39). The bound LPS was removed for later studies.

HEK-TLR4 Cell Stimulation

Cells were maintained in Normocin, Hygromycin Gold, Zeocin DMEM-high glucose media as per manufacturers instruction. During stimulation Hygromycin Gold and Zeocin were not present in the media. "Endotoxin-low" samples isolated using the PMXB columns were added to HEK293 (TLR4/MD2/CD14) dual reporter cells (Invivogen, USA Cat. No HKD-mTLR4ni) in order to assess the stimulatory capacity of the material through TLR4. Samples were diluted 1:10 in supplemented DMEM to remove toxic effects of salt exchange which occurred during the LPS removal process reducing endotoxin levels to below threshold (<0.1EU/ml) a value at which comparable LPS concentrations do not activate the cells.

Statistics

Data are expressed as the mean ± one standard error of the mean (SEM). Sample size varies from 3 to 9 as indicated, performed as a minimum experimental triplicate. Statistical significance was assessed through the use of two-tailed unpaired Student's *t*-test for parametric data, while the Mann-Whitney test was performed for non-parametric data. Multiple analyses were performed using a one-way Anova with *post-hoc* Tukey test where indicated. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

RESULTS

DSS Alters TLR mRNA Expression Within the Mesentery

Previous findings have demonstrated DSS directly impacting on the expression of TLRs within the inflamed colon mimicking that found in the patient cohort (28–31). We wanted to ascertain whether there the same was true for the murine mesentery. In order to do so, qPCR screening of all known murine TLRs, was assessed. Total RNA from the mesentery of sham-control mice, DSS, and other treatments were isolated and converted to cDNA before analysis via SYBR-green qPCR. Of the 14-known murine TLRs only 7 were detectable within the murine mesentery samples (**Table 3**). Analysis of expression within sham controls provided a baseline for subsequent comparison with DSS which demonstrated a significant upregulation in TLR4 ($P < 0.0001$). We hypothesized that TLR4, which recognize the bacterial component LPS, could be involved heavily in lymphatic-driven inflammation and dysfunction during DSS induced colitis. Additionally, during the progression of DSS

induced colitis, the epithelium of the gut is severely disrupted allowing a vast influx of lumenally-derived microbial content into the mesenteric lymphatics.

DAMPs Created Within the Colon During DSS Treatment Activate Cells in a TLR4 Dependent Manner

In order to discern what, within the GI tract, could be activating upon TLR4 directly, samples of colonic fecal matter were collected from sham and DSS treated mice for analysis. Data shown in **Figure 1A** show protein normalized samples and their subsequent LAL-determined endotoxin levels per milligram of fecal matter from both sham and DSS treated mice. The levels of endotoxin present in the sample are denoted as: PRE (before) and POST (after) endotoxin removal via the Polymyxin-B column. **Figure 1B** demonstrates that the induction of the NF-κBSEAP (Secreted Embryonic Alkaline Phosphatase) and IL-8 luciferin reporters found within the HEK-TLR4 reporter cells can be driven by substances within the murine fecal matter. A significant reduction in the induction of gene expression can be seen through the removal of LPS. However, a proportion of the (POST) DSS sample can still induce both NF-κB and IL-8 gene expression suggesting other molecules are being recognized by TLR4.

TLR4 Blockade Through C34 Treatment Ameliorates the Progression of DSS Induced Disease Activity

Being home to the majority of the microbiome, the gut must function effectively as a barrier in order to prevent the influx of microbial pathogens into the normally sterile sub-mucosa. The multi-layered composition of the intestinal tract aids in this function through the secretion of mucins, the epithelial barrier itself and the rapid response of immune cells a site of breach (32). During DSS induced colitis, the breakdown of the epithelial barrier leaves the potential for invasion of commensal bacterium, fungi, viruses and dietary substances to permeate the pseudo-sterile barrier (33, 34). Activation of the resident macrophages, dendritic cells and mast cells within the epithelial sub-lining promotes the recruitment of neutrophils, the induction of pro-reparative measures, and the clearance of antigens to the lymph node in an attempt to create and effective immune response to the infection (35, 36).

With high levels of bacterial LPS and DAMPs present within the intestinal luminal space, a potential to activate a TLR4 mediated innate immune response is rife. Inflammatory molecules induced by TLR4 activation are documented to negatively impact on lymphatic function, thus potentially reducing flow of antigens to the lymph node and subsequent immunosuppression (37). Therefore, we attempted to determine the effect of inhibition of TLR4 and subsequent effect on inflammation within the local drainage lymphatic system. Mice treated with 2.5% DSS for 7 days, received a daily I.P injection of either saline, C34 or PMXB from days 3 to 7 (See Methods). DSS colitis in mice is characterized by the development of diarrhea, colonic inflammation, and subsequent weight loss. Fecal consistency, blood presence in feces and the extent of the colon shortening was converted to a DAI and recorded as described in Methods (see **Table 3**). When compared to sham controls, treatment with C34 significantly reduced weight loss (**Figure 2A**), reduced disease activity score (**Figure 2B**) and reduced colon shortening (**Figure 2C**). However, treatment with PMXB did not aid significantly in the characteristic disease phenotype. We also tested an alternative drug delivery method via oral gavage of the treatments in the same dosage and time frame, however, neither treatment alleviated any tested condition (Data not shown).

TLR4 Modulates Lymphatic Alterations Within the Mesentery in an LPS-Independent Manner

The promotion of lymphangiogenesis within the mesentery of DSS treated animals is a well-documented phenomenon (3, 10). Alongside lymphangiogenesis, vessel dilation mediated by iNOS-dependent production of nitric oxide is a common feature associated with inflammation within the tissue surrounding the lymphatics (37). The dilation often correlates with increased vessel permeability and particulate exchange, although the effectors regulating this process are still unknown. This "leakiness" can disrupt the flow of antigen-bearing immune cells to the lymph node and cause lymph and its content to spill out into the surrounding tissue. We hypothesized that one of the functions of mesenteric lymphatic network expansion is to resorb this lost material.

Mesenteric sections were isolated from sham and DSS mice treated or not with C34 or PMXB, fixed as whole-mount and stained with lymphatic vessel markers. The initial-lymphatic endothelial marker CCL21, and collecting lymphatic marker αSMA, were used to highlight the border of the lymphatic endothelium and/or smooth muscle layer, allowing vessel size measurement (**Figure 3**). As illustrated in **Figure 3**, sham-control collecting lymphatic vessels average luminal diameter and branch points were assessed creating baseline values. DSS treated samples showed extensive expansion of the lymphatic network and a significant increase in lymphatic vessel diameter. Restoration of the normal phenotype was successfully achieved through administration with C34. However, PMXB had no significant effect. This finding was compounded through the analysis of CCL21 mRNA levels within the affected tissue, whereby C34 reduced expression to that of sham control whilst PMXB had no effect on transcript levels. Altogether, these data show mesenteric lymphangiogenesis and lymphangiectasia can be resolved through the I.P. blockade of TLR4 using C34.

TLR4 Activation Modulates Lymphangiogenic and Inflammatory Molecules Within the Mesentery

The inflammation associated with DSS-induced intestinal inflammation promotes lymphangiogenesis within the mesentery as well as altering lymphatic structure through dilation of the collecting lymphatic vessels (**Figure 2**). Analysis of mRNA levels of poignant lymphatic markers (**Figure 4**) revealed

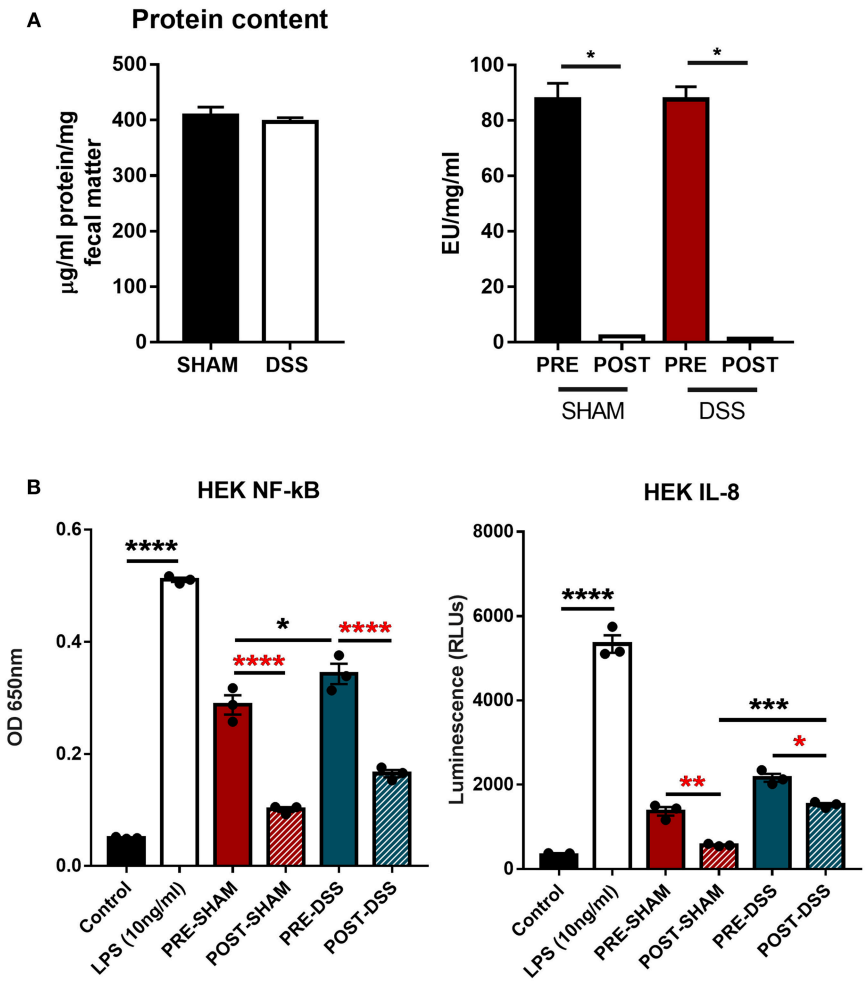

FIGURE 1 | TLR4 activation *in vitro* is triggered by a ligand other than LPS. **(A)** Fecal matter samples from sham and DSS (2.5% 7d) mice were isolated, normalized by protein content, and treated in an endotoxin removal column for 72 h at 4°C (post-sham and post-DSS samples). **(B)** Percentage NF-κB and IL-8 response from samples removed of endotoxin displayed DSS treated mice having a large proportion of activation due to non-LPS derived products. Data is represented as the mean ±SEM of 3 experimental replicates. EU, endotoxin unit. Two-tailed Student's *t*-test and one-way Anova (Tukey *post-hoc* test) and were used in A and B, respectively. $^{*}P < 0.05$, $^{***}P < 0.001$, and $^{****}P < 0.0001$.

significant increases in LYVE-1 ($P < 0.001$) and CCL21 ($P < 0.05$) transcription during DSS treatment with the effect ameliorated by treatment with C34. Interestingly, the widely accepted universal lymphatic endothelial marker PROX-1, was not significantly induced through DSS treatment. Rather, inhibition of TLR4 signaling through C34 treatment induced PROX-1 transcription suggesting that TLR4 regulates the lymphangiogenic transcription factor in an activation dependent manner. Additionally, COX2 ($P < 0.0001$) and iNOS ($P < 0.01$) mRNA levels spiked during DSS treatment, indicating signs of mesenteric dysfunction, with both treatments ameliorating this induction suggesting an anti-inflammatory effect of TLR4 inhibition within the mesentery itself. Interestingly VEGFR3 expression was not significantly increased in the DSS group compared to sham but was rather significantly reduced through C34 ($P < 0.01$) and PMXB ($P < 0.01$). Furthermore, analysis of common inflammatory markers (TNFα, IL-1β and IL-6) in paired mesenteric samples showed no significant induction within any group at day 7, inferring the passage of the acute inflammatory phase within that region (Data not shown).

LPS Drives Lymph Node Expansion and Cellular Migration During DSS Treatment

Lymphadenopathy, or swelling on the lymph node, is a common occurrence during the response to infection presenting in either a localized (regional) or diffuse (generalized) phenotype (38). Immune cells within the mesentery can become activated and promote expression of chemotactic agents within the collecting lymphatics such as CCL21 which, though production of a gradient, attracts $CCR7^{+}$ cells, such as dendritic cells, from the lamina propria to the mesenteric lymph node for antigen presentation (39, 40). These cells accumulate within the lymph node after trafficking antigens from a peripheral site of inflammation, and subsequently produce a wide array

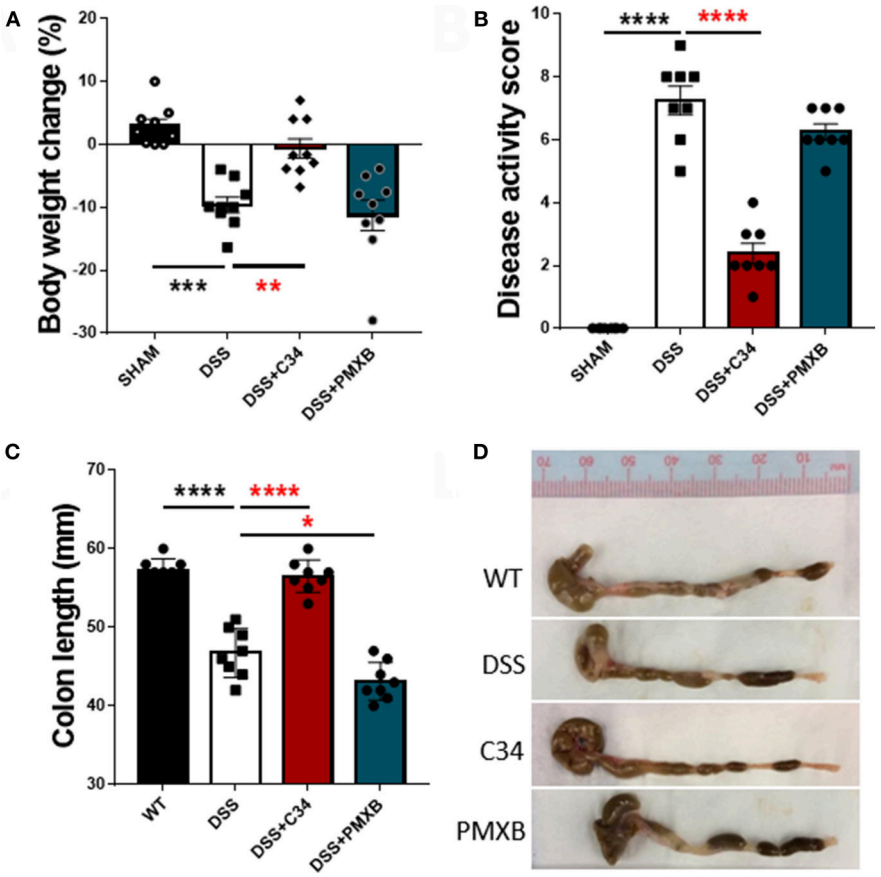

FIGURE 2 | Total TLR4 blockade within the peritoneum ameliorates DSS induced colitis *in vivo*. Mice were treated with DSS 2.5% and additionally with either C34 (50 mg/kg) or PMXB (50 mg/kg) from days 3 to 7. Percent body weight change **(A)**, clinical disease activity score **(B)** and colon length **(C, D)** were measured as macroscopic scores for disease response measures. Data are expressed as the average ±SEM of 3 independent experiments ($n = 8$ in each group). Images are representatives from those experiments. Statistics analyzed using one-way Anova (Tukey *post-hoc* test), $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$.

of proliferative and chemoattractant agents, which, result in structural remodeling of the node (41, 42). TLR4 activation is known to induce TNF-a production, IL-8 secretion, and matrix protease secretion from a wide variety of cells, including fibroblastic reticular cells and macrophages, key players in lymph node remodeling (43, 44).

Examination of the lymph node corroborated that DSS had a distinct effect on lymphadenopathy significantly increasing the MLN size ($P < 0.0001$), weight ($P < 0.001$), and cellular content ($P < 0.0001$) (**Figure 5A**). This effect was not abrogated through C34 blockade of TLR4, however, PMXB treatment reduced significantly the MLN size ($P < 0.0001$), weight ($P < 0.0001$), and cell count ($P < 0.0001$) suggesting the possible involvement of a non-TLR4 dependent LPS interaction in lymphadenopathy. Altogether, these results suggest that TLR4 does not directly influence lymphadenopathy in a DSS model of colonic inflammation but LPS does, and it does so in a TLR4-independent manner. DSS treatment also significantly upregulated CCL21 expression within the MLN ($P < 0.05$) (**Figure 5B**). C34 in combination with DSS treatment had no effect on CCL21 expression in the MLN, however PMXB treatment significantly reduced it ($P < 0.01$), impacting the recruitment of $CCR7^{+}$ $CD103^{+}$ DCs accumulation ($P < 0.05$) (**Figure 5C**).

DISCUSSION

Within the intestine there is a delicate balance between innate immune activation and inflammation. Invasive pathogens can be recognized by a myriad of pattern recognition receptors and induce an inflammatory response, a feature critical to the successful clearance of aforementioned pathogen (45). However, in a system that has the potential to be exposed to an enormous volume of these microbes, a correct magnitude of response is paramount (46). Patients suffering from IBD have an exacerbated and possibly unregulated inflammation within the intestinal tract, a feature that does not resolve normally as expected, but rather perpetuates in a chronic fashion. During the progression of IBD, and perhaps its conception, the structure of the gastrointestinal-associated and mesenteric lymphatic vascular environment changes drastically (22, 47–49). Whether this phenomenon is a direct cause of IBD or a causal agent of IBD

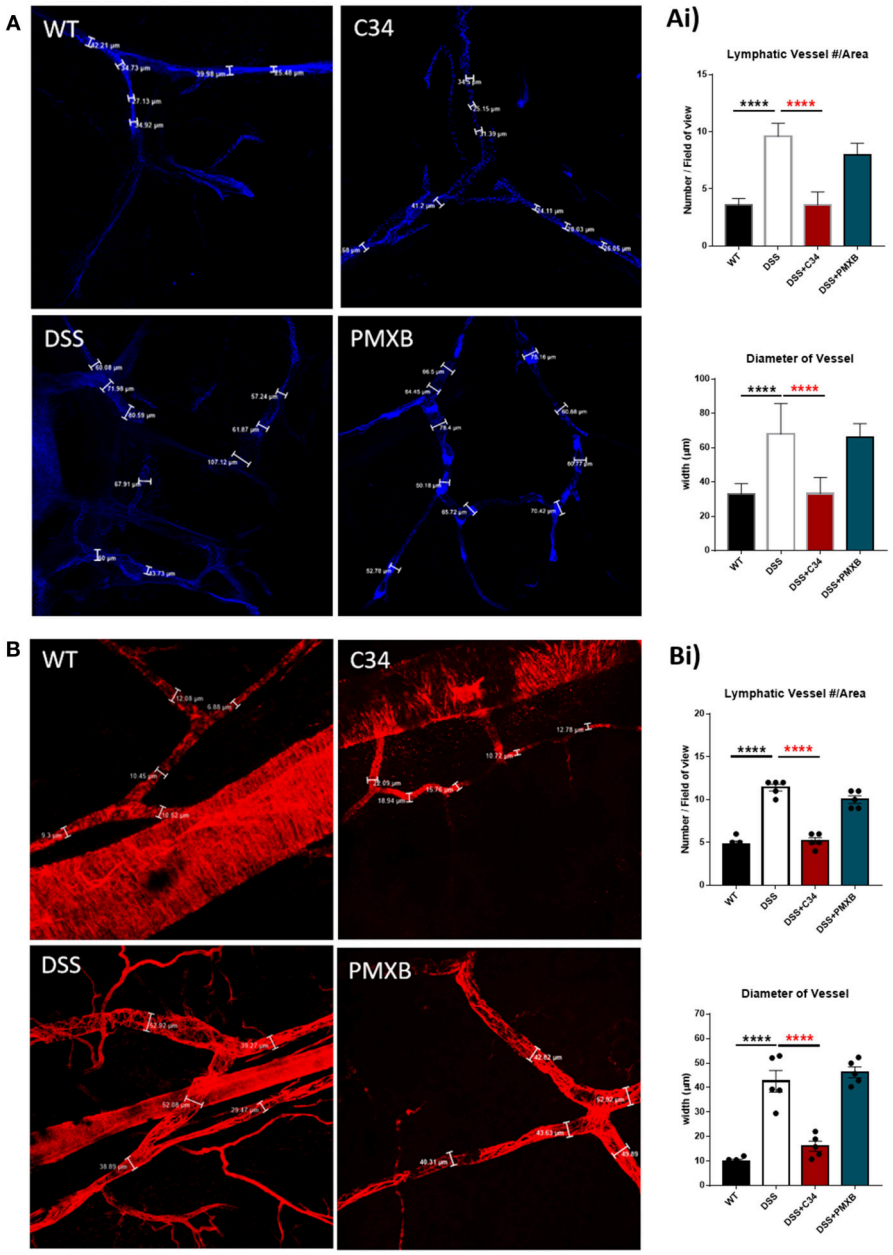

FIGURE 3 | TLR4 blockade ameliorates lymphatic alterations within the mesentery of DSS treated mice. Mice treated with C34 TLR4 total inhibitor reduced lymphangiogenesis and lymphangiectasia within the mesentery however, PMXB treated mice had no significant reduction. Images are representative staining of **(A)** CCL21 positive mesenteric initial lymphatics and **(B)** αSMA positive mesenteric collecting lymphatics, $n = 3$ for each group. Measurements were taken at 3 random points along the vessel width and averaged for each mouse. Branching points were identified and calculated per field of view. Data are expressed as mean ±SEM of 2–3 separate experiments. One-way ANOVA with Tukey *post-hoc* test $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$.

is debated. However, from our own lympho-centric opinion, the lymphatic dysfunction itself could cause both. Increased intestinal permeability, combined with reduced lymphatic function could lead to a stagnation of material within the effected intestinal region leading to a hot-spot of inflammation. In the DSS model, intestinal permeability is increased through the chemical ablation of the intestinal epithelium via the formation of nano-lipocomplexes between medium-chain-length fatty acids and DSS, highly abundant in the colon, therefore greatly isolating its effector venue (50). Loss of this barrier allows a vast milieu of microbial and dietary content to enter the submucosa and be directly exposed to the lymphatic system via the initial lacteal vessels. Within this transport system the exposure of immune and stromal cells to bacterial products such as LPS cause a

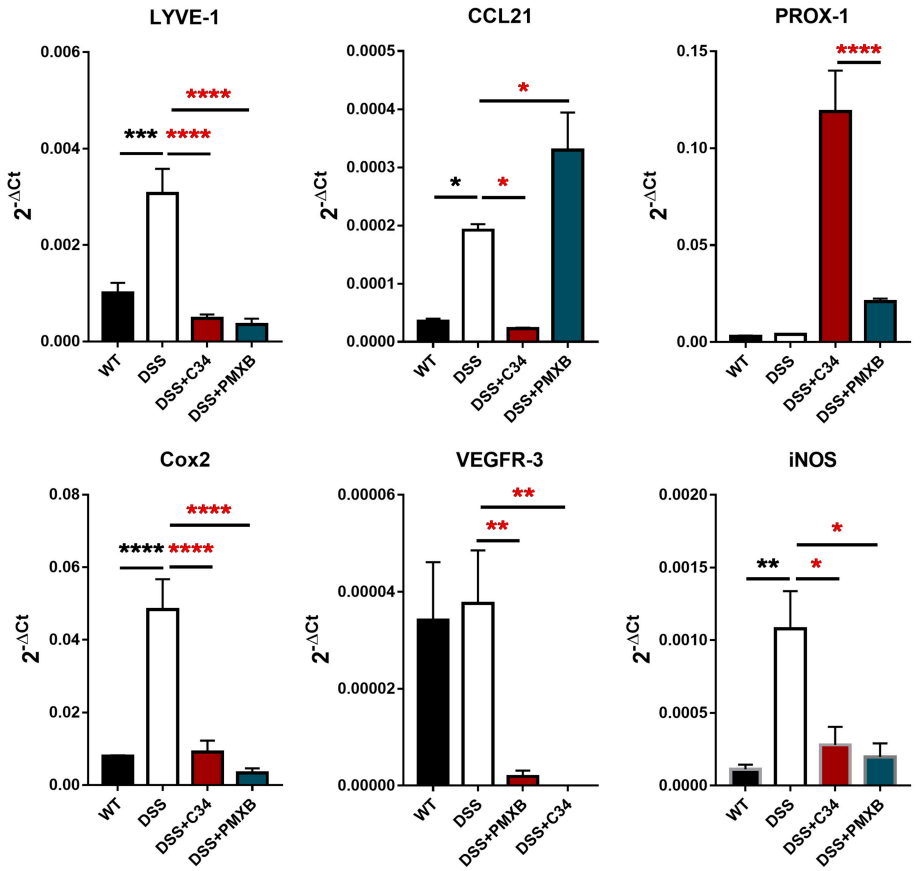

FIGURE 4 | DSS alters lymphatic and inflammatory modulators within the mesentery and is altered through TLR4 blockade. mRNA induction of lymphatic markers LYVE-1, CCL21, Prox-1, VEGFR3 and inflammatory markers COX2 and iNOS were measured on total extractions of mesenteric preparations from SHAM, DSS treated, and DSS + treatment groups. GAPDH was used for normalization to a housekeeping gene and values are expressed as such. Data is mean ±SEM from 5 individuals from 3 separate experiments. One-way ANOVA with Tukey-*post-hoc* test $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$.

large inflammatory response, tissue remodeling and cellular proliferation/recruitment, a phenomenon well-documented in the lung (51, 52). During the progression of IBD extra-cellular matrix remodeling is common, the role of this remodeling however, is poorly understood and it is thought that extracellular molecules produced during this destructive/reparative stage may in fact perpetuate inflammation (53). We have previously demonstrated that even after the removal of DSS, remodeled lymphatics persist, a phenomenon that is evident with the IBD patient population (3).

TLR4 has been implicated in the pathogenesis of many inflammatory diseases including IBD, as through the recognition of LPS and a wide array of previously mentioned PAMPs and DAMPs, a large inflammatory stimulus can be generated (54). Our data reveals not only the TLR4-driven lymphatic alterations during DSS-induced colitis, but the gross-mechanisms which they act. Through the use of a competitive TLR4 antagonist (C34) and Polymyxin we were able to selectively differentiate between total TLR4-driven lymphatic alterations/consequences during DSS induced colitis, and those driven by LPS. Data presented, indicates that substances separate from LPS, i.e., TLR4-DAMPs, and other PRR PAMPs, modulate inflammation and lymphatics to a much greater extent than previously estimated. This hypothesis was confirmed through the detection and analysis of TLR4 activating material within the colon of DSS mice separate from the LPS content. Currently, specific DAMPs have not been elucidated in this system, however it would be feasible to expect well-published TLR4 DAMPs such as Tenacin-C, HMGB1, HSP90, or S100 proteins to be candidates as levels are known to drastically rise during tissue damage (55–57).

A key marker of correct lymphatic response was the potent lymphadenopathy seen during DSS treatment. This effect was disrupted through the PMXB treatment, accredited to the lack of $CD103^{+}$ DC migration to the lymph node. However, through C34 inhibition of TLR4, CCL21 (a potent lymphatic chemokine) was downregulated in the mesentery but not the lymph node, creating a gradient for increased movement of $CCR7^{+}$ $CD103^{+}$ DCs. This gives partial explanation to the reduced cellular content of the lymph node and significant reduction in lymphadenopathy which was ameliorated through PMXB treatment where the CCL21 content in the lymph node is

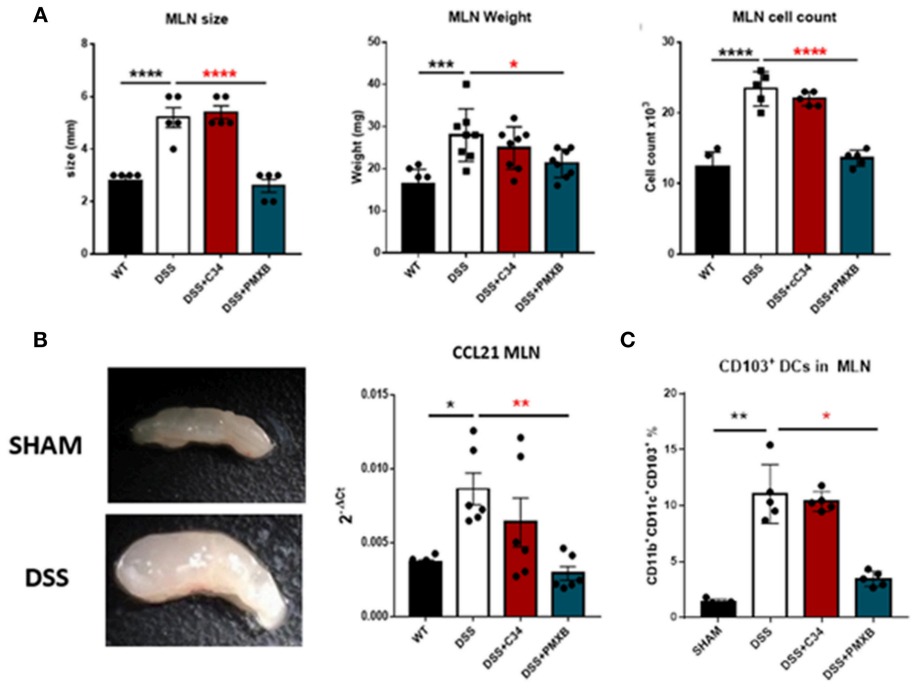

FIGURE 5 | LPS drives lymphadenopathy during DSS treatment augmenting CD103$^+$ DC recruitment to the Mesenteric lymph node. **(A)** Isolated MLNs were measured for size, weight and cellularity. **(B)** mRNA expression of CCL21 within the MLN and mesentery during treatments. **(C)** MLN accumulation of CD11c$^+$CD11b$^+$CD103$^+$ Dendritic cells were determined through flow cytometric analysis and quantified within the lymph node in each condition. Data are mean ±SEM of 3 experimental replicates (n = 5–8). One-way Anova with Tukey *post-hoc* tests were performed as necessary. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$.

downregulated. Therefore, any CCL21-driven chemotaxis within the mesentery has no directionality. This also suggests that the lymphadenopathy, whilst caused by LPS, is not driven through TLR4, a novel finding in this context.

We also present evidence supporting our hypothesis that restoration of lymphatic function to a "normal" phenotype, significantly aids in the reparation of DSS-induced disease activity. Achieved through TLR4 blockade via C34, mesenteric lymphatic disruption was significantly reduced, evidenced by reduced lymphangiogenesis and lymphangiectasia. We note that this finding, of reduced lymphangiogenesis improving DSS-induced phenotype, is in somewhat opposition to D'Alessio's, work whereby lymphangiogenesis was beneficial to their model of IBD (10). This discrepancy could be solely contributed to the timing of the treatments or the target itself. Our experimental method and timings were designed to modulate inflammation after its genesis rather than in a preventative capacity. We attempted to modulate TLR4-associated inflammation and therefore the subsequent lymphatic remodeling, whereas D'Alessio and colleagues focused intentionally on promoting lymphatic remodeling through the overexpression of VEGFC, a method that likely had many targets separate from VEGFR3 induced lymphangiogenesis.

We know with certainty that alterations occur within the lymphatics of patients with of IBD and we are able to mimic them in murine models of DSS-induced intestinal inflammation. However, what is not yet understood is whether TLR4 could be a potential target for IBD in humans. Targeting such an important receptor undoubtedly has its risks but data presented in this paper suggest the plasticity of the receptor in delineating pathogenic material from self, a phenomenon that could be utilized in the future for the development of novel treatment of IBD.

DATA AVAILABILITY

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

Project planning and experimentation was solely carried out by MS. Interpretation of data, technical advice, and manuscript proofreading was performed by P-YvdW and SL.

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REFERENCES

1. Kaiserling E, Krober S, Geleff S. Lymphatic vessels in the colonic mucosa in ulcerative colitis. *Lymphology.* (2003) 36:52–61.
2. Wen J, Tang Q, Wu J, Wang Y, Cai W. Primary intestinal lymphangiectasia: four case reports and a review of the literature. *Dig Dis Sci.* (2010) 55:3466–72. doi: 10.1007/s10620-010-1161-1
3. Rehal S, Stephens M, Roizes S, Liao S, von der Weid P-Y. Acute small intestinal inflammation results in persistent lymphatic alterations. *Am J Physiol Gastrointest Liver Physiol.* (2017) 314:G408–17. doi: 10.1152/ajpgi.00340.2017
4. Rehal S, von der Weid PY. TNFDeltaARE mice display abnormal lymphatics and develop tertiary lymphoid organs in the mesentery. *Am J Pathol.* (2017) 187:798–807. doi: 10.1016/j.ajpath.2016.12.007
5. Kovi J, Duong HD, Hoang CT. Ultrastructure of intestinal lymphatics in Crohn's disease. *Am J Clin Pathol.* (1981) 76:385–94.
6. Pedica F, Ligorio C, Tonelli P, Bartolini S, Baccarini P. Lymphangiogenesis in Crohn's disease: an immunohistochemical study using monoclonal antibody D2–40. *Virchows Arch.* (2008) 452:57–63. doi: 10.1007/s00428-007-0540-2
7. von der Weid PY, Rehal S, Ferraz JG. Role of the lymphatic system in the pathogenesis of Crohn's disease. *Curr Opin Gastroenterol.* (2011) 27:335–41. doi: 10.1097/MOG.0b013e3283476e8f
8. Rahier JF, De Beauce S, Dubuquoy L, Erdual E, Colombel JF, Jouret-Mourin A, et al. Increased lymphatic vessel density and lymphangiogenesis in inflammatory bowel disease. *Aliment Pharmacol Ther.* (2011) 34:533–43. doi: 10.1111/j.1365-2036.2011.04759.x
9. Jurisic G, Sundberg JP, Detmar M. Blockade of VEGF receptor-3 aggravates inflammatory bowel disease and lymphatic vessel enlargement. *Inflamm Bowel Dis.* (2013) 19:1983–9. doi: 10.1097/MIB.0b013e31829292f7
10. D'Alessio S, Correale C, Tacconi C, Gandelli A, Pietrogrande G, Vetrano S, et al. VEGF-C–dependent stimulation of lymphatic function ameliorates experimental inflammatory bowel disease. *J Clin Invest.* (2014) 124:3863–78. doi: 10.1172/JCI72189
11. Gewirtz AT. Deciphering the role of mesenteric fat in inflammatory bowel disease. *Cell Mol Gastroenterol Hepatol.* (2015) 1:352–3. doi: 10.1016/j.jcmgh.2015.05.004
12. Peyrin-Biroulet L, Gonzalez F, Dubuquoy L, Rousseaux C, Dubuquoy C, Decourcelle C, et al. Mesenteric fat as a source of C reactive protein and as a target for bacterial translocation in Crohn's disease. *Gut.* (2012) 61:78–85. doi: 10.1136/gutjnl-2011-300370
13. Goncalves P, Magro F, Martel F. Metabolic inflammation in inflammatory bowel disease: crosstalk between adipose tissue and bowel. *Inflamm Bowel Dis.* (2015) 21:453–67. doi: 10.1097/MIB.0000000000000209
14. Nell S, Suerbaum S, Josenhans C. The impact of the microbiota on the pathogenesis of IBD: lessons from mouse infection models. *Nat Rev Microbiol.* (2010) 8:564–77. doi: 10.1038/nrmicro2403
15. Delneste Y, Beauvillain C, Jeannin P. [Innate immunity: structure and function of TLRs]. *Med Sci.* (2007) 23:67–73. doi: 10.1051/medsci/200723167
16. Kawai T, Akira S. TLR signaling. *Cell Death Differ.* (2006) 13:816–25. doi: 10.1038/sj.cdd.4401850
17. Kawasaki T, Kawai T. Toll-like receptor signaling pathways. *Front Immunol.* (2014) 5:461. doi: 10.3389/fimmu.2014.00461
18. Dziarski R, Gupta D. Role of MD-2 in TLR2- and TLR4-mediated recognition of Gram-negative and Gram-positive bacteria and activation of chemokine genes. *J Endotoxin Res.* (2000) 6:401–5. doi: 10.1177/09680519000060050101
19. Park BS, Song DH, Kim HM, Choi BS, Lee H, Lee JO. The structural basis of lipopolysaccharide recognition by the TLR4-MD-2 complex. *Nature.* (2009) 458:1191–5. doi: 10.1038/nature07830
20. Murad S. Toll-like receptor 4 in inflammation and angiogenesis: a double-edged sword. *Front Immunol.* (2014) 5:313. doi: 10.3389/fimmu.2014.00313
21. Alexander JS, Chaitanya GV, Grisham MB, Boktor M. Emerging roles of lymphatics in inflammatory bowel disease. *Ann N Y Acad Sci USA.* (2010) 1207 (Suppl. 1):E75–85. doi: 10.1111/j.1749-6632.2010.05757.x
22. McNamee EN, Rivera-Nieves J. Defective lymphatics in Crohn's disease: tertiary lymphoid follicles plug the gap. *Gastroenterology.* (2017) 152:908–10. doi: 10.1053/j.gastro.2017.01.022
23. Neal MD, Jia H, Eyer B, Good M, Guerriero CJ, Sodhi CP, et al. Discovery and validation of a new class of small molecule Toll-like receptor 4 (TLR4) inhibitors. *PLoS ONE.* (2013) 8:e65779. doi: 10.1371/journal.pone.0065779
24. Agus A, Denizot J, Thévenot J, Martinez-Medina M, Massier S, Sauvanet P, et al. Western diet induces a shift in microbiota composition enhancing susceptibility to Adherent-Invasive E. *coli infection and intestinal inflammation Sci Rep.* (2016) 6:19032. doi: 10.1038/srep19032
25. Renshaw M, Rockwell J, Engleman C, Gewirtz A, Katz J, Sambhara S. Cutting edge: impaired Toll-like receptor expression and function in aging. *J Immunol.* (2002) 169:4697–701. doi: 10.4049/jimmunol.169.9.4697
26. Hirakawa S, Hong Y-K, Harvey N, Schacht V, Matsuda K, Libermann T, et al. Identification of vascular lineage-specific genes by transcriptional profiling of isolated blood vascular and lymphatic endothelial cells. *Am J Pathol.* (2003) 162:575–86. doi: 10.1016/S0002-9440(10)63851-5
27. Miyagaki T, Sugaya M, Okochi H, Asano Y, Tada Y, Kadono T, et al. Blocking MAPK signaling downregulates CCL21 in lymphatic endothelial cells and impairs contact hypersensitivity responses. *J Invest Dermatol.* (2011) 131:1927–35. doi: 10.1038/jid.2011.135
28. Szebeni B, Veres G, Dezsõfi A, Rusai K, Vannay Á, Mraz M, et al. Increased expression of Toll-like receptor (TLR) 2 and TLR4 in the colonic mucosa of children with inflammatory bowel disease. *Clin Exp Immunol.* (2008) 151:34–41. doi: 10.1111/j.1365-2249.2007.03531.x
29. Zheng B, Morgan ME, van de Kant HJG, Garssen J, Folkerts G, Kraneveld AD. Transcriptional modulation of pattern recognition receptors in acute colitis in mice. *Biochim Biophys Acta (BBA) Mol Basis Dis.* (2013) 1832:2162–72. doi: 10.1016/j.bbadis.2013.07.004
30. Tan Y, Zou KF, Qian W, Chen S, Hou XH. Expression and implication of toll-like receptors TLR2, TLR4 and TLR9 in colonic mucosa of patients with ulcerative colitis. *J Huazhong Univ Sci Technolog Med Sci.* (2014) 34:785–90. doi: 10.1007/s11596-014-1353-6
31. Fukata M, Chen A, Vamadevan AS, Cohen J, Breglio K, Krishnareddy S, et al. Toll-like receptor-4 (TLR4) promotes the development of colitis-associated colorectal tumors. *Gastroenterology.* (2007) 133:1869–81. doi: 10.1053/j.gastro.2007.09.008
32. König J, Wells J, Cani PD, García-Ródenas CL, MacDonald T, Mercenier A, et al. Human intestinal barrier function in health and disease. *Clin Transl Gastroenterol.* (2016) 7:e196. doi: 10.1038/ctg.2016.54
33. Midtvedt T, Bylund-Fellenius AC. The role of intestinal bacteria, bacterial translocation and endotoxin in dextran sodium sulphate-induced colitis in the mouse AU - axelsson, L.-G. *Microbial Ecol Health Dis.* (1996) 9:225–37. doi: 10.3109/08910609609166463
34. Chassaing B, Aitken JD, Malleshappa M, Vijay-Kumar M. Dextran sulfate sodium (DSS)-induced colitis in mice. *Curr Protoc Immunol.* (2014) 104:15–25. doi: 10.1002/0471142735.im1525s104
35. Grainger JR, Konkel JE, Zangerle-Murray T, Shaw TN. Macrophages in gastrointestinal homeostasis and inflammation. *Pflugers Arch.* (2017) 469:527–39. doi: 10.1007/s00424-017-1958-2
36. Gross M, Salame T-M, Jung S. Guardians of the gut—murine intestinal macrophages and dendritic cells. *Front Immunol.* (2015) 6:254–254. doi: 10.3389/fimmu.2015.00254
37. Liao S, Cheng G, Conner DA, Huang Y, Kucherlapati RS, Munn LL, et al. Impaired lymphatic contraction associated with immunosuppression. *Proc Natl Acad Sci USA.* (2011) 108:18784. doi: 10.1073/pnas.1116152108
38. Heitman B, Irizarry A. Infectious disease causes of lymphadenopathy: localized versus diffuse. *Lippincotts Prim Care Pract.* (1999) 3:19–38.
39. Jang MH, Sougawa N, Tanaka T, Hirata T, Hiroi T, Tohya K, et al. CCR7 is critically important for migration of dendritic cells in intestinal lamina propria to mesenteric lymph nodes. *J Immunol.* (2006) 176:803–10. doi: 10.4049/jimmunol.176.2.803
40. Seth S, Oberdörfer L, Hyde R, Hoff K, Thies V, Worbs T, et al. CCR7 essentially contributes to the homing of plasmacytoid dendritic cells to lymph nodes under steady-state as well as inflammatory conditions. *J Immunol.* (2011) 186:3364. doi: 10.4049/jimmunol.1002598
41. Kumar V, Scandella E, Danuser R, Onder L, Nitschke M, Fukui Y, et al. Global lymphoid tissue remodeling during a viral infection is orchestrated by a B cell-lymphotoxin-dependent pathway. *Blood.* (2010) 115:4725–33. doi: 10.1182/blood-2009-10-250118

42. Gregory JL, Walter A, Alexandre YO, Hor JL, Liu R, Ma JZ, et al. Infection programs sustained lymphoid stromal cell responses and shapes lymph node remodeling upon secondary challenge. *Cell Rep.* (2017) 18:406–18. doi: 10.1016/j.celrep.2016.12.038
43. Kang S, Lee SP, Kim KE, Kim HZ, Memet S, Koh GY. Toll-like receptor 4 in lymphatic endothelial cells contributes to LPS-induced lymphangiogenesis by chemotactic recruitment of macrophages. *Blood.* (2009) 113:2605–13. doi: 10.1182/blood-2008-07-166934
44. Genovese L, Brendolan A. Lymphoid tissue mesenchymal stromal cells in development and tissue remodeling. *Stem Cells Int.* (2016) 2016:8419104. doi: 10.1155/2016/8419104
45. Takeuchi O, Akira S. Pattern recognition receptors and inflammation. *Cell.* (2010) 140:805–20. doi: 10.1016/j.cell.2010.01.022
46. Suresh R, Mosser DM. Pattern recognition receptors in innate immunity, host defense, and immunopathology. *Adv Physiol Edu.* (2013) 37:284–91. doi: 10.1152/advan.00058.2013
47. Shen W, Li Y, Zou Y, Cao L, Cai X, Gong J, et al. Mesenteric adipose tissue alterations in Crohn's disease are associated with the lymphatic system. *Inflamm Bowel Dis.* (2018) 25:283–93. doi: 10.1093/ibd/izy306
48. Bronsart L, Nguyen L, Habtezion A, Contag C. Reactive oxygen species imaging in a mouse model of inflammatory bowel disease. *Mol Imaging Biol.* (2016) 18:473–8. doi: 10.1007/s11307-016-0934-0
49. Behr MA. The path to Crohn's disease: is mucosal pathology a secondary event?. *Inflamm Bowel Dis.* (2010) 16:896–902. doi: 10.1002/ibd.21171
50. Laroui H, Ingersoll SA, Liu HC, Baker MT, Ayyadurai S, Charania MA, et al. Dextran sodium sulfate (DSS) induces colitis in mice by forming nano-lipocomplexes with medium-chain-length fatty acids in the colon. *PLoS ONE.* (2012) 7:e32084. doi: 10.1371/journal.pone.0032084
51. Brass DM, Savov JD, Gavett SH, Haykal-Coates N, Schwartz DA. Subchronic endotoxin inhalation causes persistent airway disease. *Am J Physiol Lung Cell Mol Physiol.* (2003) 285:L755–L761. doi: 10.1152/ajplung.00001.2003
52. Brass DM, Hollingsworth JW, Cinque M, Li Z, Potts E, Toloza E, et al. Chronic LPS inhalation causes emphysema-like changes in mouse lung that are associated with apoptosis. *Am J Respir Cell Mol Biol.* (2008) 39:584–90. doi: 10.1165/rcmb.2007-0448OC
53. Shimshoni E, Yablecovitch D, Baram L, Dotan I, Sagi I. ECM remodelling in IBD: innocent bystander or partner in crime? *The emerging role of extracellular molecular events in sustaining intestinal inflammation Gut.* (2015) 64:367–72. doi: 10.1136/gutjnl-2014-308048
54. Lu Y, Li X, Liu S, Zhang Y, Zhang D. Toll-like receptors and inflammatory bowel disease. *Front Immunol.* (2018) 9:72. doi: 10.3389/fimmu.2018.00072
55. Midwood K, Sacre S, Piccinini AM, Inglis J, Trebaul A, Chan E, et al. Tenascin-C is an endogenous activator of Toll-like receptor 4 that is essential for maintaining inflammation in arthritic joint disease. *Nat Med.* (2009) 15:774–80. doi: 10.1038/nm.1987
56. Kim S, Kim SY, Pribis JP, Lotze M, Mollen KP, Shapiro R, et al. Signaling of high mobility group box 1 (HMGB1) through toll-like receptor 4 in macrophages requires CD14. *Mol Med.* (2013) 19:88–98. doi: 10.2119/molmed.2011.00001
57. Ehrchen JM, Sunderkotter C, Foell D, Vogl T, Roth J. The endogenous Toll-like receptor 4 agonist S100A8/S100A9 (calprotectin) as innate amplifier of infection, autoimmunity, and cancer. *J Leukoc Biol.* (2009) 86:557–66. doi: 10.1189/jlb.1008647

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Halted Lymphocyte Egress via Efferent Lymph Contributes to Lymph Node Hypertrophy During Hypercholesterolemia

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Dyslipidemia is a central component of atherosclerosis and metabolic syndrome linked to chronic inflammation and immune dysfunction. Previously, we showed that hypercholesterolemic apolipoprotein E knock out (apoE$^{-/-}$) mice exhibit systemic effects including skin inflammation and hypertrophic lymph nodes (LNs). However, the mechanisms accounting for LN hypertrophy in these mice remain unknown. Here, we show that hypercholesterolemia led to the accumulation of lymphocytes in LNs. We excluded that the increased number of lymphocytes in expanded LNs resulted from increased lymphocyte proliferation or entry into those LNs. Instead, we demonstrated that the egress of lymphocytes from the enlarged LN of apoE$^{-/-}$ mice was markedly decreased. Impairment in efferent lymphatic emigration of lymphocytes from LNs resulted from an aberrant expansion of cortical and medullary sinuses that became hyperplastic. Moreover, CCL21 was more abundant on these enlarged sinuses whereas lymph levels of sphingosine 1 phosphate (S1P) were decreased in apoE$^{-/-}$ mice. Normal LN size, lymphatic density and S1P levels were restored by reversing hypercholesterolemia. Thus, systemic changes in cholesterol can sequester lymphocytes in tissue draining LNs through the extensive remodeling of lymphatic sinuses and alteration of the balance between retention/egress signals leading to LN hypertrophy which subsequently may contribute to poor immunity. This study further illustrates the role of lymphatic vessels in immunity through the regulation of immune cell trafficking.

Keywords: lymph node, lymphatic vessel, lymphocyte egress, mouse model, dyslipidemia

INTRODUCTION

Lymph nodes (LNs) are highly specialized organs that play an essential role in immune priming and function. LN comprises of three main regions namely the cortex, paracortex, and the medulla. Lymph enters the LN via the afferent LVs and reaches the subcapsular sinuses first before it drains through the cortical sinuses in the cortex region, and into the medullary sinuses in the medulla region, and then eventually exits the LN via efferent lymphatic vessels. Protein, lipid, antigens,

microorganisms, and immune cells such as lymphocytes and dendritic transported via the lymph enter the subcapsular sinus of the LN. Subcapsular and medullary sinuses are directly connected and thus lymph can pass through LN without filtering through the cortex (1). In most cases, lymphocytes enter the LN via the high endothelial venules (2), except for a small proportion of memory lymphocytes that enter via the lymphatics (3, 4). Activated and naïve lymphocytes in the LN matrix would eventually need to enter the sinuses so that they can be transported by the efferent lymph to reach the effector sites or return back to the circulation for immune surveillance, respectively. Lymphatic endothelial cells (LECs) in the cortical sinuses control lymphocyte trafficking within the LN by accumulating lymphocytes for further transit to medullary sinuses (5). Furthermore, lymphocytes can migrate from the lymphatic sinuses back to the LN parenchyma (5). Lymph borne lymphocytes can also be passively collected into the peripheral medullary sinuses, and then enter the LN parenchyma in a CCR7-independent manner by moving into adjacent peripheral medullary cords (6). The medulla is formed of a three-dimensional labyrinthine structure of sinus channels starting as cortical sinusoids and expand to become wider medullary sinuses that finally drain collectively into the efferent lymphatic vessel (7). In addition to cortical sinuses, medullary sinuses have been proposed as exit routes for the egress of lymphocytes from LNs (5, 8, 9). Lymphocyte egress are governed by mechanisms controlling the entry of lymphocytes into the efferent lymphatic vessels including the regulation of CCR7 and sphingosine-1-phosphate receptor-1 expression (10–12), or those regulating lymphatic endothelial barriers (13, 14). It is well-established that lymphocyte egress from LNs via cortical and medullary sinuses is dependent on signals generated by lymph-borne S1P (8, 9, 15) produced by LECs via S1P kinase 1 kinase (16). 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/CCL21-retention signals from LN parenchyma into medullary and cortical sinuses and ultimately facilitates T cell egress (10). Thus, the structural integrity and functions of these lymphatic sinuses have to be maintained as they are not only just passive carrier of lymph, but active player in the regulation of lymphocyte egress.

Local immune responses and inflammation are associated with alterations in the trafficking of lymphocytes through activated LNs. Indeed, the entry of lymphocytes into the draining LNs is increased whereas their exit into the efferent lymph is transiently blocked from few hours to days depending on the nature of the antigen and inflammation (17–20). In contrast to our knowledge about the mechanisms controlling lymphocyte retention within the inflamed LN after the initiation of an immune response (8, 21, 22), little is known about those reestablishing lymphocyte egress to steady-state levels. We showed previously that the expansion of cortical and medullary sinuses in the stimulated LNs at later stages of inflammation contribute to the reestablishment of lymphocyte egress rates (23). Reestablishment of steady-state egress from inflamed LNs may help to content the enhanced lymphocyte entry into LNs when inflammation is prolonged. This may prevent these LNs from becoming a "sink" that may compromise efficient lymphocyte recirculation and the timely induction of a suitable immune response (24, 25). This raises the possibility that LN hypertrophy and hyperactivation occurring with autoimmune and chronic inflammatory diseases may arise when lymphocytes fail to emigrate from the inflamed LNs in a timely manner. Notably, we and others reported in mice that hypercholesterolemia associated with atherosclerosis disease leads to systemic effects including skin inflammation, hypertrophic LNs, and compromised immunity (26–33). However, how hypercholesterolemia lead to LN hypertrophy in still remains an open question. Here, we explored how lymphocyte trafficking and LN structure and function are affected by hypercholesterolemia that occurs in mice lacking apoE (apoE$^{-/-}$). The study reveals an accumulation of lymphocytes in enlarged skin draining LNs which results from a severe blockade of their egress rather than increased lymphocyte proliferation or entry within the enlarged LNs. We further show that hypercholesterolemia induces an aberrant remodeling of the cortical and medullary sinuses and an imbalance between retention and egress signals that account for the impaired lymphocyte egress in dyslipidemic mice.

METHODS

Animals

Male CD45.2 C57BL/6 mice deficient in apolipoprotein E (apoE$^{-/-}$ mice) and low-density lipoprotein receptor (Ldlr$^{-/-}$), CD45.2 wild type C57BL/6 (WT), and CD45.1 C57BL/6 WT were obtained from The Jackson Laboratory (Bar Harbor, ME). All mice were given a chow diet (18% protein and >5% fat, Harlan Teklad, Madison, WI) until 6 weeks of age. At 6 weeks of age, the diet was switched to a high fat and cholesterol rich diet containing 21.2% fat and 0.2% cholesterol (Harlan Teklad) until sacrifice. In some experiments, 13–16 weeks old male apoE$^{-/-}$ and WT mice fed a high fat diet were treated daily with ezetimibe (5 mg/kg; Kemprotec) or corn oil (vehicle) via oral gavage corn oil for 12 weeks prior sacrifice as previously described (34). All mice were housed under specific pathogen free conditions with unrestricted access to food and water in the animal housing unit of the National University of Singapore. All studies were performed under protocols approved by the Institutional Animal Care and Use Committee (IACUC) of the National University of Singapore.

Adoptive Cell Transfers

For adoptive transfers, CD45.2 recipient mice were intravenously injected with 2×10^7 of CD45.1 spleen and LN cells. In short term homing experiments to assess lymphocyte entry into skin draining LNs (axillary and brachial), CD45.1, or CD45.2 cells were adoptively transferred into CD45.2 or CD45.1 recipient mice, respectively and allowed to equilibrate for 2.5 h before sacrifice. At this time point, entry from the peripheral blood into skin draining LNs directly determined the CD45.1 cell numbers within recipient LNs (23, 35). In experiments where donor lymphocytes from CD45.1 WT and CD45.2

apoE$^{-/-}$ mice were co-transferred, lymphocytes were labeled with carboxyfluorescein succinimidyl ester (CFSE). To assess lymphocyte egress from skin draining LN (axillary and brachial), CD45.1, or CD45.2 cells were adoptively transferred into CD45.2 or CD45.1 recipient mice, respectively and allowed to equilibrate for 24 h. Following equilibrium, half of the mice were sacrificed (T_0) while the other half of the mice were treated with anti-CD62L antibody and sacrificed 20 h after administration (T_{20}). Intraperitoneal administration of anti-CD62L antibody at a dose of 100 μg per mouse (clone Mel-14 hybridoma from American Type Culture Collection) blocked further entry of circulating lymphocytes into LNs without affecting lymphocyte egress from LNs (8, 10). Therefore, the population of transferred lymphocytes remaining within skin draining LNs at T_{20} compared to T_0 will be a measure of lymphocyte egress from LNs (23).

Spleen, LN, and Lymph Cell Suspensions

Cell suspensions from skin draining LNs and spleens were prepared by mechanical disruption. For quantification of LECs by flow cytometry, LNs were first digested with 4 mg/mL collagenase IV (Roche) in calcium and magnesium free HBSS at 37°C for 45 min with gentle agitation. After 45 min, EDTA was added to the digestion mixture to a working concentration of 10 mM and the lymph nodes were further digested for 5 min at 37°C in a final digestion step. For lymph cell collection, mice were fasted overnight prior sacrifice and lymph was collected as described by Matloubian et al. (36). The peritoneal cavity of anesthetized mice was exposed ventrally and the cysterna chili identified. Lymph was subsequently drawn from the cysterna chili with the use of extended length gel-loading pipette tips (Neptune Scientific).

Flow Cytometry Analysis

Flow cytometric analysis of skin draining LN (brachial and axillary) cell suspensions stained for CD45, podoplanin and CD31 allowed differentiation and quantification of LECs (CD45$^-$, CD31$^+$, podoplanin$^+$) (23). Antibodies used included the following: rat anti-mouse CD31 (Serotec) detected with anti-rat-APC, hamster anti-mouse podoplanin (clone 8.1.1; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA) detected with anti-hamster PE and PerCPcy5.5—conjugated anti-mouse CD45.2 (BD Biosciences). FACS analysis was also employed to quantify congenic transferred lymphocytes T and B cell populations in LNs, and lymph. Antibodies used included the following: FITC or PerCPCy5.5—or Pacific Blue conjugated anti-B220, APC-conjugated anti-CD3e, PerCPCy5.5-conjugated anti-CD45.2, biotin–conjugated anti-CD45.1 revealed with Streptavidin-PE. Live and dead cells were discriminated during flow cytometry using a LIVE/DEAD® Fixable Aqua Dead Cell Stain Kit (Molecular Probes, Invitrogen). Cell counts were determined during flow cytometry using Count Bright® Absolute Counting Beads (Molecular Probes, Invitrogen). FACS analysis was performed using a CyAn ADP Analyzer (Beckman Coulter) and data were analyzed with Flowjo software (Treestar).

Immunohistochemistry

Skin draining LNs (axillary and brachial) were either freshly embedded in tissue freezing medium or fixed overnight in 2% paraformaldehyde/30% sucrose solution at 4°C and embedded in tissue freezing medium. 6–8 μm thick cryostat sections were cut for imaging by the fluorescence microscope. Primary antibodies used included biotinylated or purified anti-B220 (eBiosciences), anti-TCRβ (BD Biosciences), anti-LYVE-1 (Upstate), anti-CD31 (Serotec), anti-Ki67 (Dako), anti-CCL21 (R&D Systems), anti-collagen type IV (Cosmo Bio), FITC-conjugated anti-CD169 (Serotec), biotinylated anti-CD45.1 (eBiosciences) antibodies. Secondary antibodies used included Dylight647-conjugated streptavidin, Cy2 or Cy3-conjugated anti-rat IgG, Dylight647 or Dylight549-conjugated anti-armenian hamster IgG, Dylight 647-conjugated anti-goat IgG and Cy2, Cy3, or Dylight647-conjugated anti-rabbit IgG antibodies (Jackson Immunoresearch). Endogenous avidin and biotin were quenched using the Avidin/Biotin blocking kit (Vector Laboratories).

Microscopy, Image Analysis, and Lumen Area Measurements

To anatomically locate the lymphatic sinuses in the paracortex and medulla of the skin draining LNs images of LN sections stained for LYVE-1 and B220 were captured with a fluorescence microscope (Axio imager.Z1, Axiocam HRM camera; Carl Zeiss Micro Imaging, Inc., Jena, Germany). Lymphatics were identified in cortical or medullary sinuses if they were found in the LN paracortex or medulla and to contain B cells within their lumen (23). To determine lumen area of lymphatic sinuses, images were acquired from 6 representative LN sections per mouse. Within each section, the area of 15 lymphatic vessels in the cortex region and 10 lymphatic vessels in the medulla region were quantified. Therefore, a total of 90 cortical and 60 medullary sinuses were sampled per mouse. Lumen area was obtained by using the "measure" tool of the Axiovision software (version 4.8; Carl Zeiss Micro Imaging, Inc.).

S1P Measurement by Liquid Chromatography-Mass Spectrometry

The lymph samples were centrifuged at 14,000 g for 10 min at 4°C and the supernatant was obtained for subsequent S1P extraction. Five microliter of lymph was mixed with 5 μl ISTD and 90 μl methanol. The samples were sonicated for 30 min at room temperature and centrifuged at 14,000 g for 10 min. The supernatant was recovered and 10 μl of TMS-Diazomethane (2M in hexane) was added. The sample incubated for 20 min at room temperature under gentle mixing at 750 rpm. The reaction was stopped by adding 1 μl of acetic acid. The derivatized samples were dried in speedvac and reconstituted in 100 μl of mobile phase before injecting 1 μl of sample into the LC-MS system. All solvents for LC-MS analysis were LC-MS grade and were purchased from Fisher Scientific and Merck Millipore. Lipid standards: isotope labeled standard D-erythro-Sphingosine-1-phosphate (13C2D2–S1P,) were purchased from Toronto Research Chemicals. All LC-MS/MS experiments were

performed using Agilent 1200 series HPLC-Chip systems connected to the Agilent 6490 QQQ mass spectrometer as described in our previous report (37). A customized HILIC-chip containing Amide-80 stationary phase (Tosoh Bioscience, LLC. Montgomeryville, PA, 5 Nm particle size, 80 Å pore size) was used for the chromatographic separation, including a 160 nl trapping column and a 75 Nm × 150 mm analytical column (Agilent Technologies Corp., Santa Clara CA). Solvents used for HILIC HPLC: 50% acetonitrile in water containing 25 mM ammonium formate pH 4.6 (solvent A), 95% acetonitrile containing 25 mM ammonium formate pH 4.6. The pH value was adjusted with formic acid. Analytes were eluted with the following gradient: 100% B from 0 to 1.5 min, 40% B from 1.5 to 8.5 min, 30% B from 8.5 to 10.5 min, 0% B from 11.5 to 13.0 min, 100% B from 13.1 to 19 min. The chip cube was operated with back flush mode and samples were injected through the enrichment column at 4 µl/min. The valve was switched 1.5 min after injection to place the enrichment column in line with the analytical column at a flow rate of 400 nl/min. Murine lymph samples were spiked with known amounts of internal standard (S1P-13C2D2). The Agilent 6490 triple quadrupole (QQQ) mass spectrometer was operated in positive mode for MRM where the CID fragment at m/z 60 was used as a "quantifier" and m/z 113 was used as a "qualifier." These ions were present after fragmentation of all species. Quantification was performed according to the internal standard method, comparing peak areas of the endogenous S1P extracted with MassHunter Quant (Agilent) to the ISTD peak.

CCL21 ELISA

Skin draining LNs were harvested and homogenized in lysis buffer (RIPA buffer, Sigma Chemicals) with a protease inhibitor cocktail (Roche Diagnostics). Homogenates were centrifuged for 10 min at 4°C at 14,000 g, and supernatants were assayed using commercial CCL21 (R&D Systems) ELISA kits as per manufacturer's protocols.

Quantitative RT-PCR

Total RNA from skin draining LNs was homogenized and extracted using TRIZOL® reagent (Invitrogen) and NucleoSpin® RNA II kit (Macherey-Nagel). First strand cDNA was synthesized using TaqMan® Reverse Transcription Reagents (Applied Biosystems). Real-time PCR were performed using iTaq™ SYBR® Green supermix with ROX (Biorad) on a 7500 Real-Time PCR System (Applied Biosystems). Expression of genes of interest expression was normalized to the expression of GAPDH. The following primers were used: *Sphk1*, forward 5′-AACTTGACTGTCCATACCTGGTTC-3′ and reverse 5′-CACATACCATCAGCTCTCCATCC-3′; *Sgpl1*, forward, 5′-CCTGTTGGGCCGCCTTGATGC-3′ and reverse 5′-AAATTCCACCCCTTAGC-3′.

Statistical Analysis

Statistical analysis was performed with Prism 5 (Graph-Pad Software, Inc.). All values were expressed as the mean of n samples ± SD. Statistical significances were determined using the unpaired two-tailed t-test. Whenever more than two groups were compared, the one-way ANOVA test with Bonferroni's post-test was applied. For all tests, a $p < 0.05$ was considered significant.

RESULTS

Lymphocytes Accumulate in Hypertrophic LN From apoE$^{-/-}$ Mice

Consistent with our previous report (33), the substantial increase in skin draining LN cellularity was evident in 22 to 28 weeks old apoE$^{-/-}$ mice fed a diet rich in fat and cholesterol compared to age-matched WT mice but not in 6 week old apoE$^{-/-}$ mice (**Figures 1A,B**). LN hypertrophy was also observed in Ldlr$^{-/-}$ mice, another hypercholesterolemic mouse model (**Supplemental Figure 1**). Flow cytometry analysis revealed T and B cells accumulation in the enlarged LNs of apoe$^{-/-}$ mice (**Figure 1C**) with a proportionally greater increase in B cells compared to T cells (**Figure 1D**). The number of CD4$^+$ and CD8$^+$ T cells increased in hypertrophic LN of *Apoe*$^{-/-}$ mice but the ratio of CD4$^+$/CD8$^+$ T cells was similar to WT mice (data not shown).

Lymphocyte Proliferation and Entry Within Expanded LNs From apoE$^{-/-}$ Mice Are Not Altered

Accumulation of lymphocytes in an activated LN may result from increased proliferation within LN, increased entry of lymphocytes from the blood into the LN or, conversely, a decreased efferent lymphatic emigration from the enlarged LN. Therefore, we sought to determine which of these possibilities could account for LN hypertrophy in apoE$^{-/-}$ mice. We excluded the possibility of lymphocyte proliferation contributing to the LN hypertrophy in apoE$^{-/-}$ mice since we did not detect any obvious differences for proliferative marker Ki-67 in apoE$^{-/-}$ and WT LN sections co-stained with TCRβ or B220 to detect T cells and B cells, respectively (**Figure 2A**). To determine whether lymphocyte trafficking into the enlarged LNs of apoE$^{-/-}$ mice is affected, we quantitated lymphocyte entry into LNs in short-term homing assays (23). 2.5 hours after adoptive transfer of CD45.1 WT lymphocytes into CD45.2 apoE$^{-/-}$ or WT mice, CD45.1-transferred T cells accumulated similarly in the LN of WT and apoE$^{-/-}$ recipient mice (**Figure 2B**) whereas the number of transferred B cells was increased in apoE$^{-/-}$ LN compared to WT LN. Despite subtle differences in T and B cell trafficking, the overall entry of CD45.1-transferred lymphocytes into apoE$^{-/-}$ and WT LN was similar (**Figure 2B**), indicating that lymphocyte entry into the hypertrophic LN of *Apoe*$^{-/-}$ mice was not augmented. To substantiate these findings, we performed reverse adoptive transfer experiments. Lymphocytes, T or B cells isolated from CD45.1 WT and CD45.2 apoE$^{-/-}$ mice were labeled with CFSE and co-transferred into CD45.1 WT recipient mice. Unexpectedly, we found that the entry of apoE$^{-/-}$ lymphocytes into WT LN was decreased compared to WT lymphocytes (**Figure 2C**). Further analysis revealed that apoE$^{-/-}$ T cell entry was decreased whereas input of apoE$^{-/-}$ B cells into WT LN was comparable to WT B cells. Taken together, these data indicate that lymphocyte accumulation in enlarged LN

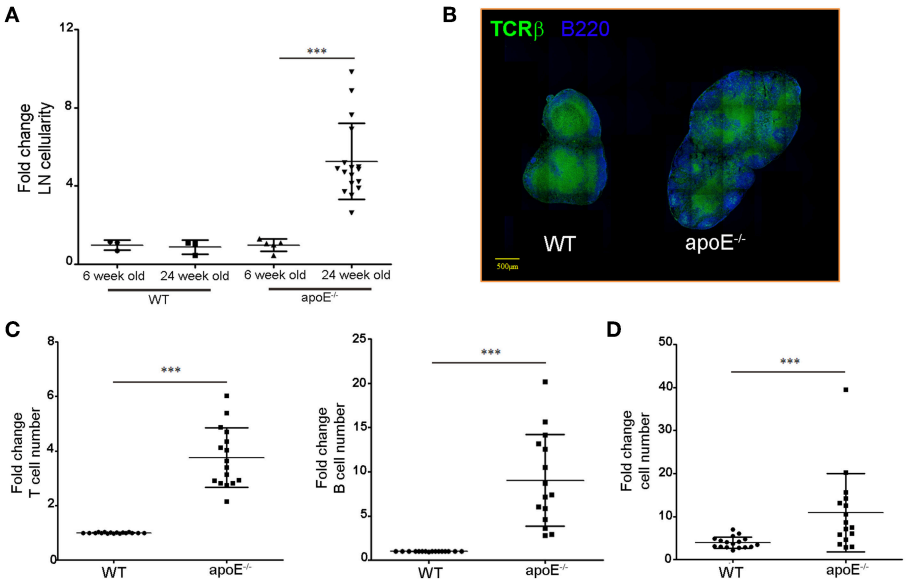

FIGURE 1 | Dyslipidemia is associated with LNs hypertrophy. **(A)** Lymph node cellularity was determined in 6 and 24 weeks old WT and apoE$^{-/-}$ mice and was expressed as fold change over WT mice. Data is pooled from four independent experiments with 4–5 mice per group in each experiment. **(B)** Immunoreactivity for B220 and TCRβ was examined in LN sections from WT and apoE$^{-/-}$ mice at 22 to 28 weeks of age. **(C)** T and B cell numbers were examined by flow cytometry and results were expressed as fold change over WT mice. **(D)** The fold change in T and B cells over WT mice was examined. ***$p < 0.0005$.

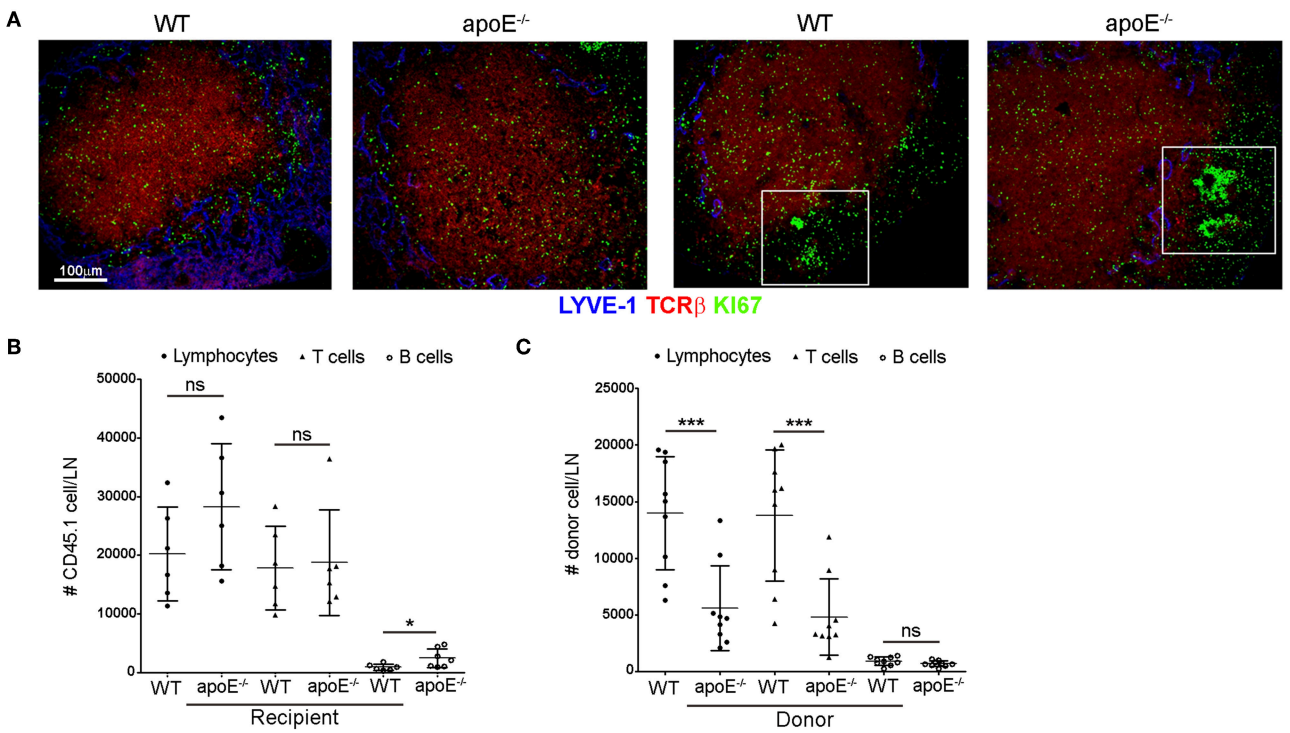

FIGURE 2 | Proliferation and entry of lymphocytes in apoE$^{-/-}$ mice are not affected by dyslipidemia. **(A)** LNs sections from 22 to 28 weeks old WT and apoE$^{-/-}$ mice were double stained for TCRβ and Ki-67. Box demarcates B cell follicle **(B)** Short term homing experiments were performed to assess the entry of CD45.1 WT lymphocytes, T or B cells into LNs from CD45.2 WT and apoE$^{-/-}$ recipient mice. **(C)** The entry of CD45.2 CFSE-labeled WT or apoE$^{-/-}$ lymphocytes, T or B cells into WT LNs was examined. $n = 9$ mice per group. *$p < 0.05$; ***$p < 0.0005$.

of apoE$^{-/-}$ mice is not due to increased lymphocyte entry into the LNs.

The Efferent Lymphatic Emigration of Lymphocytes From LN Is Markedly Blocked in apoE$^{-/-}$ Mice

Since we rule out the involvement of lymphocyte entry and proliferation to impact lymphocyte accumulation in LNs of Apoe$^{-/-}$ mice, we hypothesized that the increased number of lymphocytes in skin draining LNs from apoE$^{-/-}$ mice could result from impaired egress of these cells from the enlarged LN via efferent lymphatic vessels. In support of this hypothesis, we found a marked decrease in the number of total lymphocytes, T and B cells in efferent lymph from apoE$^{-/-}$ mice collected at the cysterna chyli (36, 38) compared to WT efferent lymph (**Figure 3A**). This prompted us to assess the exit of lymphocytes from LN using long-term adoptive transfer assays (23, 36). We first evaluated the capacity of CD45.1 WT donor lymphocytes to egress from CD45.2 WT and apoE$^{-/-}$ LN mice. The data were expressed as mean fraction of egressed cells—defined by dividing the mean CD45.1 T cell that have exited (T_{20}) by the mean population of CD45.1 T cells present at baseline (T_0) (23). This experiment revealed that the egress of WT transferred lymphocytes from hypertrophic apoE$^{-/-}$ recipient LN was severely abrogated compared to WT recipient LN (**Figure 3B**). This phenomenon was observed for both T and B cell egress. Next, we compared the egress of CD45.2 apoE$^{-/-}$ lymphocytes from CD45.1 WT recipient LN with the egress capacity of CD45.2 WT lymphocytes. No significant difference was observed between the egress index of WT and apoE$^{-/-}$ lymphocyte, T or B cells (**Figure 3C**). Considering that no other alternative possibilities could account for the greatly increased numbers of lymphocyte in the hypertrophic LN from apoE$^{-/-}$ mice, we conclude that increased lymphocytes accumulation in expanded skin draining LN occurs because less lymphocytes emigrate from those LNs into the efferent lymph.

Lymphangiogenesis Is Induced in Hypertrophic LNs

Our long-term adoptive transfer assays indicate that the impaired egress of lymphocytes from apoE$^{-/-}$ hypertrophic LN was not due to intrinsic defects in lymphocytes but rather to environmental changes in the enlarged LN. We hypothesized that the hypercholesterolemic environment in apoE$^{-/-}$ mice induces LN remodeling compromising the egress of lymphocytes, which in turn promotes subsequent LN hypertrophy. As the lymphatic vessels are critical routes for lymphocyte egress from LNs and inflammation has been shown to induce lymphangiogenesis in activated LN such as upon immunization (23, 38), we examined LN lymphangiogenesis in apoE$^{-/-}$ mice. We employed flow cytometry to quantify LECs in LNs (**Figure 4A**). Hypercholesterolemia in apoE$^{-/-}$ mice stimulated a significant increase in LEC numbers over WT LN (**Figure 4B**). This marked expansion of lymphatic network in hypertrophic skin draining LNs from apoE$^{-/-}$ mice was also apparent in LN sections immunostained for LYVE-1 which identifies lymphatic sinuses in the medulla and cortex (**Figure 4C**). Moreover, immunostaining of LN sections for proliferative marker Ki67 revealed that the lymphatic expansion resulted from proliferation of pre-existing lymphatics (**Figure 4D**).

Expanded Cortical and Medullary Sinuses Are Overly Dilated in Hypertrophic LN

We next investigated whether the extensive expansion of cortical and medullary sinuses observed in apoE$^{-/-}$ LN was accompanied by any morphological changes in the sinuses. A close-up examination using LYVE-1 revealed that majority of the cortical and medullary sinuses in hypertrophic LN from apoE$^{-/-}$ mice were notably dilated with open lumens compared to those in WT LN with more partially collapsed state (**Figure 5A**). This was further supported by the marked increase in lumen area of cortical and medullary sinuses in apoE$^{-/-}$ mice LN compared to WT control LN (**Figure 5B**). Moreover, the dilated sinuses were more irregular and tortuous with occasional focal absence of LYVE-1 expression (**Figure 5C**). These morphological changes resemble those described in certain tumor-associated vessels having regions lacking endothelial cell markers and termed "mosaic vessels" (39). Additional co-staining of LN sections with LYVE-1 and collagen type IV or CD31 that identify the basement membrane and endothelial cells of sinuses, respectively, confirmed the focal absence of LYVE-1 expression in the cortical and medullary sinuses of apoE$^{-/-}$ mice compared to WT mice although collagen type IV (**Figure 5D**) and CD31 (**Figure 5E**) remained intact in those regions. These data indicated extensive structural changes in the LN cortical and medullary sinuses from dyslipidemic mice.

The Balance Between Retention and Egress Signals Is Altered in Hypetrophic LNs

We next investigated whether these structural alterations may compromise the entry of lymphocytes into the sinuses. To address this question, we stained LN sections from long-term adoptive transfer experiments for CD45.1 at T_0 and T_{20h} to identify the WT donor lymphocytes in LNs from WT and apoE$^{-/-}$ mice. While we failed to capture transferred lymphocytes within collapsed sinuses in WT LN (data not shown), numerous transferred lymphocytes were detected within the dilated sinuses in apoE$^{-/-}$ LN (**Figure 6A**). Thus, although the lymphocytes were able to migrate into the exit structures of enlarged LN they failed to be captured by the lymph flow and transported to the efferent lymph (**Figures 3A,B**). This may suggest an imbalance between CCL21-mediated retention and S1P-mediated exit signals in the enlarged LN. Measurement of total CCL21 levels by ELISA that reflects the intracellular or secreted (inactive) CCL21 and the extracellular or gradient-forming (active) CCL21 revealed a significant increase in this chemokine in LN from apoE$^{-/-}$ mice (**Figure 6B**). Microscopic examination of CCL21 distribution in LN from apoE$^{-/-}$ mice by immunostaining revealed lower levels of CCL21 associated with non-endothelial cells which might reflect its continuous secretion but higher levels of active CCL21 bound to the basement

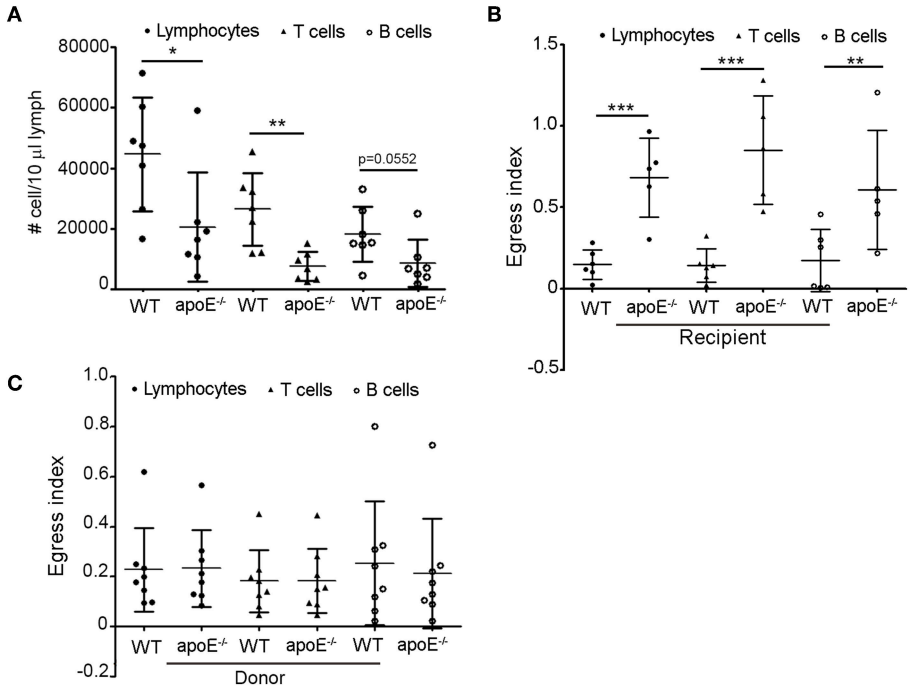

FIGURE 3 | Lymphocyte egress from apoE$^{-/-}$ mice LN is compromised **(A)** Lymphocytes, T and B cell were enumerated in lymph from 22 to 28 weeks old WT and apoE$^{-/-}$ mice by flow cytometry. **(B)** Using lymphocyte adoptive transfer assay, the egress of transferred CD45.1 WT lymphocytes, T or B cells from WT or apoE$^{-/-}$ CD45.2 recipient LNs was assessed. Results were expressed as mean fraction of egressed T cells from LNs. **(C)** The egress of transferred CD45.2 WT or apoE$^{-/-}$ lymphocytes, T or B cells from WT CD45.1 recipient LNs was assessed. Results were expressed as mean fraction of egressed T cells from LNs. $n = 7$–10 mice per group $^{*}p < 0.05$; $^{**}p < 0.005$; $^{***}p < 0.0005$.

membrane of the dilated cortical and medullary sinuses but also of LYVE-1^{-} vessels (**Figure 6C**). Together, this finding suggest that CCL21 is more actively released in LN of apoE$^{-/-}$ mice compared to WT mice. Moreover, quantification of S1P in lymph from WT and apoE$^{-/-}$ mice by mass spectrometry revealed a significant decrease in lymph S1P levels in dyslipidemic mice (**Figure 6D**). This reduction in lymph S1P in apoE$^{-/-}$ mice likely resulted from decreased production in LN as supported by reduced expression of LN *Sphk1* but also from increased S1P degradation by S1P lyase whose expression was markedly increased in apoE$^{-/-}$ mice LN (**Figure 6E**). Together these data indicate that the decreased egress of lymphocyte from hypertrophic LN observed in apoe$^{-/-}$ mice is due to reduced S1P exit signal and conversely, increased CCL21 retention signal.

Reversing Dyslipidemia in apoE$^{-/-}$ Restores Lymphocyte Egress

Next, we tested whether reduction of circulating cholesterol levels in apoE$^{-/-}$ mice could restore lymphocyte egress. Consistent with our and others work (34, 40, 41) daily gavage of ezetimibe, a FDA approved cholesterol-lowering drug, reduced total plasma cholesterol levels in apoE$^{-/-}$ mice but did not affect plasma cholesterol levels in WT mice (**Table 1**). Following ezetimibe treatment, LN cellularity was significantly reduced in apoE$^{-/-}$ mice compared to untreated apoE$^{-/-}$ mice whereas LN cellularity in WT mice was not affected (**Figure 7A**). In ezetimibe treated apoE$^{-/-}$ mice, lymphangiogenesis was reduced (**Figure 7B**) and the number of LECs was reversed to WT LEC number (**Figure 7C**). Since ezetimibe did not have any effect in WT mice, vehicle-treated WT mice were used as control for comparison with vehicle-treated apoE$^{-/-}$ mice in the subsequent experiments. Ezetimibe treatment also affected the dilation of lymphatic sinuses in apoE$^{-/-}$ mice. Indeed, the lumen area of cortical and medullary sinuses was not equivalent to WT mice but was significantly improved over untreated apoE$^{-/-}$ mice (**Figure 7D**). Furthermore, the expression of *Sgpl1* in LN from ezetimibe treated apoE$^{-/-}$ mice was improved to WT expression whereas the expression of *Sphk1* was significantly increased compared to untreated apoE$^{-/-}$ mice (**Figure 7E**). Importantly, these changes in ezetimibe treated apoE$^{-/-}$ mice resulted in the restoration of lymph S1P levels comparable to WT controls (**Figure 7F**). In contrast, the expression of CCL21 surrounding the cortical and medullary in LN from ezetimibe treated apoE$^{-/-}$ mice was not significantly different compared to untreated apoE$^{-/-}$ mice (data not shown). Nevertheless, the amelioration of cortical and medullary sinuses dilatation and the reversal of LEC numbers and lymph S1P levels in ezetimibe treated apoE$^{-/-}$ mice were sufficient to override CCL21 retention signal since lymphocyte egress from LN was partially restored in these mice, increasing lymphocyte egress index by 50% (**Figure 7G**). This effect was mainly due to the improvement of T cell egress but not B cell.

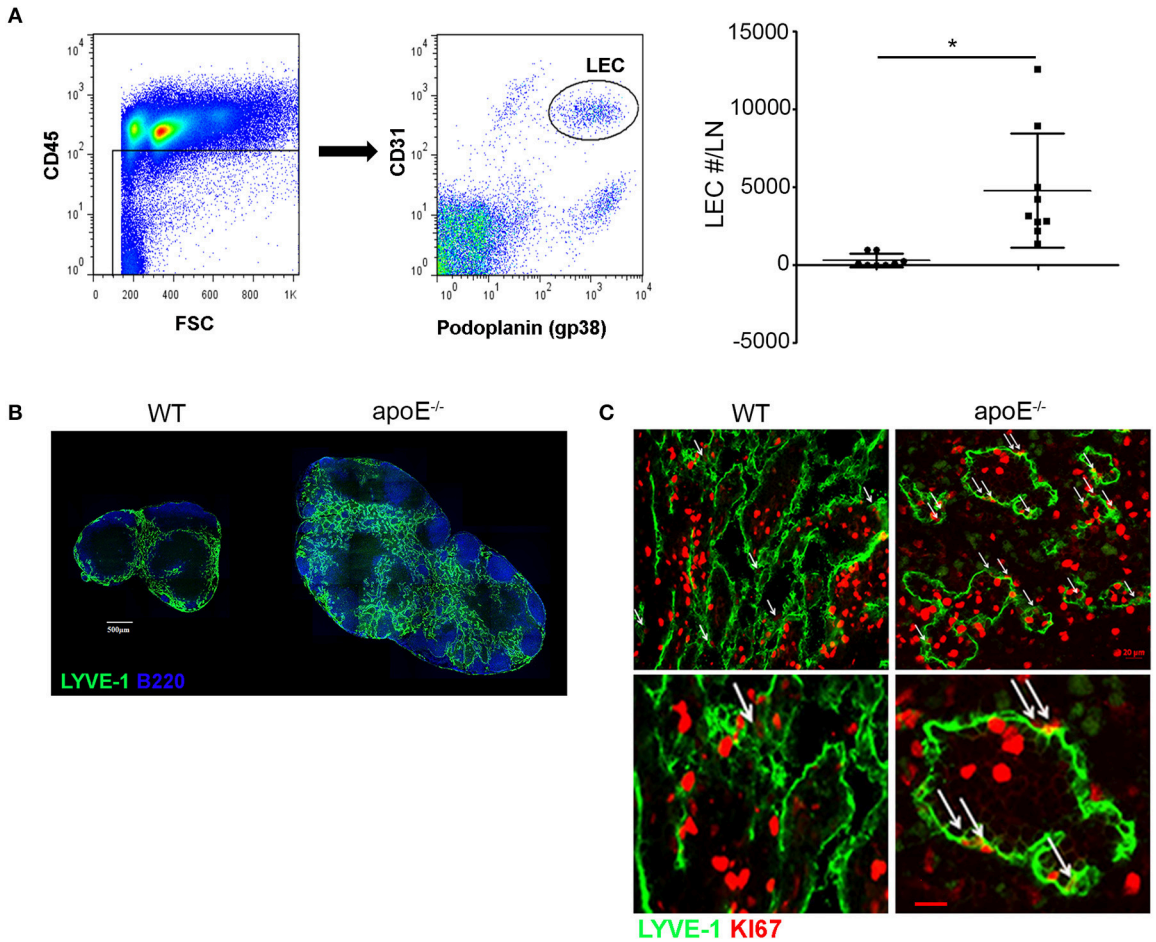

FIGURE 4 | Dyslipidemia induces LN lymphangiogenesis **(B)** LN sections from WT and apoE$^{-/-}$ mice at 22 to 28 weeks of age were stained for B220 and LYVE-1. Images are representative of four independent experiments ($n = 3$–4 mice per group). **(A)** LN cells were stained for CD45, podoplanin and CD31 to identify LECs by FACS analysis. The number of LEC was enumerated in LNs from WT and apoE$^{-/-}$ mice at 22 to 28 weeks of age and expressed as fold change over WT mice. Data is pooled from three independent experiments with 3–5 mice per group in each experiment; $^{*}p < 0.05$. **(C)** LYVE-1^{+} vessels were analyzed for co-expression of the proliferative marker KI67. Arrows indicate co-localization of lymphatics with Ki67. Ki67^{bright} dividing lymphocytes were also observed. Images are representative of three to four independent experiments ($n = 3$–4 mice per group).

DISCUSSION

Here, we elucidate the mechanisms whereby hypercholesterolemia in mouse models leads to the accumulation of lymphocytes in skin draining LNs and subsequent LN hypertrophy. We show that the increased number of lymphocytes does not result from increased proliferation or input of lymphocyte within the enlarged LN but from the severe impairment of lymphocytes to emigrate from those LN via efferent lymph. The adoptive transfer experiments used to determine lymphocyte egress also revealed that defects in extrinsic microenvironment surrounding lymphocytes rather than intrinsic defects in lymphocytes account for the impaired lymphocyte egress in apoE$^{-/-}$ mice compared to WT mice. Lymphatic vessels are essential for lymphocyte trafficking. We showed previously that skin lymphatic transport is severely compromised in hypercholesterolemic apoE$^{-/-}$ and Ldlr$^{-/-}$ mice which also exhibit LN hypertrophy and impaired skin DC migration (34, 42). Therefore, we hypothesized that hypercholesterolemia may alter the lymphatic sinuses of the activated LN.

Consistent with this hypothesis, we found a pronounced expansion of the lymphatic network in LNs from apoE$^{-/-}$ mice as denoted by a >20-fold increase in LEC numbers and the expression of proliferative marker Ki67. This expansion affects predominantly the cortical and medullary sinuses. Further microscopic examination revealed structural abnormalities in this expanded sinuses network including tortuous morphology, severe dilation and local loss of LYVE-1 expression. Such "mosaic" vessels have also been observed in various tumors as a result of extensive intra-tumoral lymphangiogenesis (39, 43) and have been shown to be "leaky" and as a consequence poor lymph carrier (43). Thus, it is possible that these structural abnormalities in the overly expanded sinuses of apoE$^{-/-}$ LN may lead to

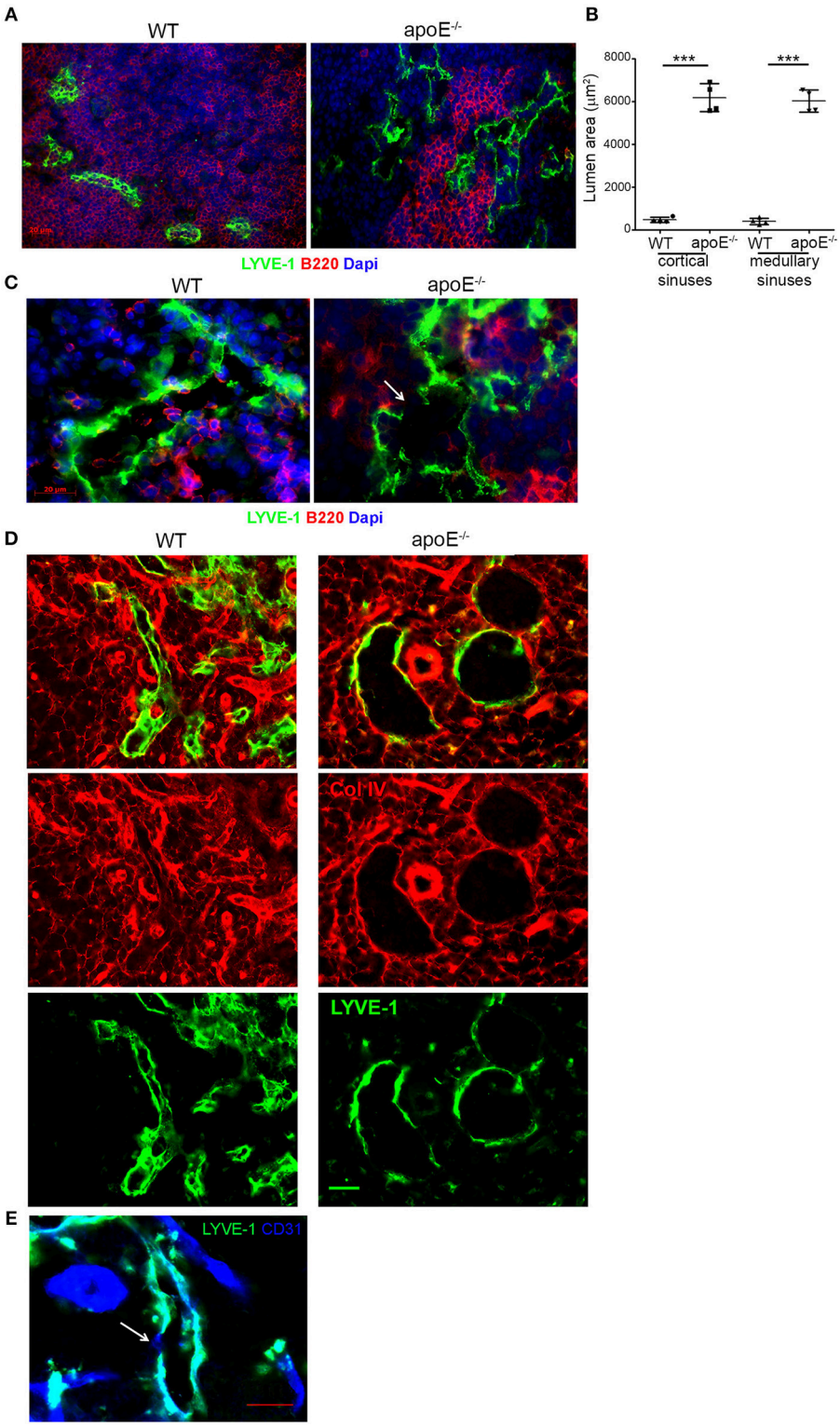

FIGURE 5 | The extended lymphatic network in apoE$^{-/-}$ mice LNs exhibits structural abnormalities. **(A)** Immunoreactivity for LYVE-1 was examined in LN sections from WT and apoE$^{-/-}$ mice at 22–28 weeks of age. **(B)** Lumen area of cortical and medullary sinuses was determined on LN sections from 22–28 week-old WT and apoE$^{-/-}$ mice. $n = 4$ mice per group; ***$p < 0.0005$ **(C)** Immunoreactivity for LYVE-1 and B220, **(D)** LYVE-1 and collagen (Col) type IV or **(E)** LYVE-1 and CD31 was examined in LN sections from apoE$^{-/-}$ mice at 22–28 weeks of age. Images are representative of 3–4 independent experiments ($n = 3$–5 mice per group). Arrows indicate loss of LYVE-1 expression.

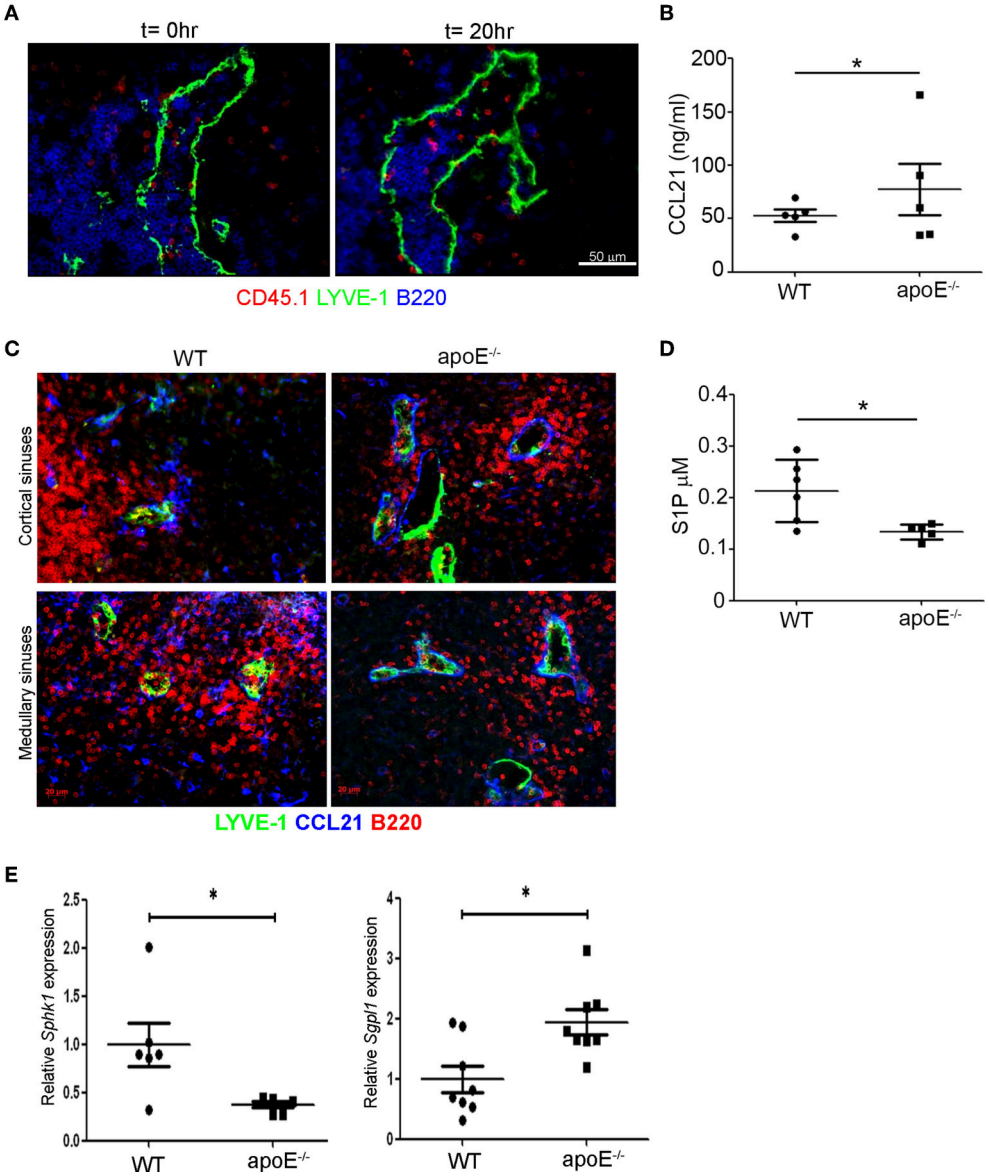

FIGURE 6 | The balance between retention and exit signals is altered in apoE$^{-/-}$ mice LNs. **(A)** Immunoreactivity for LYVE-1, B220, and CD45.1 was examined on LN sections from long-term adoptive transfer at $t = 0$ and $t = 20$ h to determine the localization of CD45.1 transferred lymphocytes in enlarged LNs from apoE$^{-/-}$ mice. Images are representative of three to four independent experiments ($n = 3$–4 mice per group) **(B)** CCL21 protein content was analyzed in homogenates of 22 to 28 weeks-old WT and apoE$^{-/-}$ mice LNs by ELISA. **(C)** Immunoreactivity for CCL21 and LYVE-1 was examined in LN sections from 22 to 28 weeks-old WT and apoE$^{-/-}$ mice. Images are representative of three to four independent experiments ($n = 3$–5 mice per group) **(D)** S1P content was analyzed in efferent lymph of 22 to 28 weeks-old WT and apoE$^{-/-}$ mice by mass spectrometry. **(E)** Quantitative RT-PCR analysis was performed on whole LN RNA to examine expression of *Sph1k* and *Spgl1* in WT and apoE$^{-/-}$ mice at 22 to 28 weeks of age. $n = 6$–10 mice per group; $^*p < 0.05$.

TABLE 1 | Effect of ezetimibe on total cholesterol.

Mice	Treatment	Total cholesterol
Wild-type	Vehicle	34.95 ± 4.72
	Ezetimibe	22.6 ± 2.49
Apoe$^{-/-}$	Vehicle	1882 ± 188.70
	Ezetimibe	791.7 ± 111.50***

*Values are mean ± SEM. ***$p < 0.0001$.*

"leaky" sinuses and disturb efferent lymph flow which is critical for optimal lymphocyte transport from the LN (44). Altered efferent flow may also diminish the transport of lymphocytes within efferent lymph which is consistent with the poor number of lymphocyte collected in efferent lymph from apoE$^{-/-}$ mice and conversely, increase the opportunity for CCL21 to attract lymphocytes back to the LN parenchyma. Notably, numerous adoptively transferred lymphocytes in apoE$^{-/-}$ mice were found within and surrounding cortical and medullary sinuses where

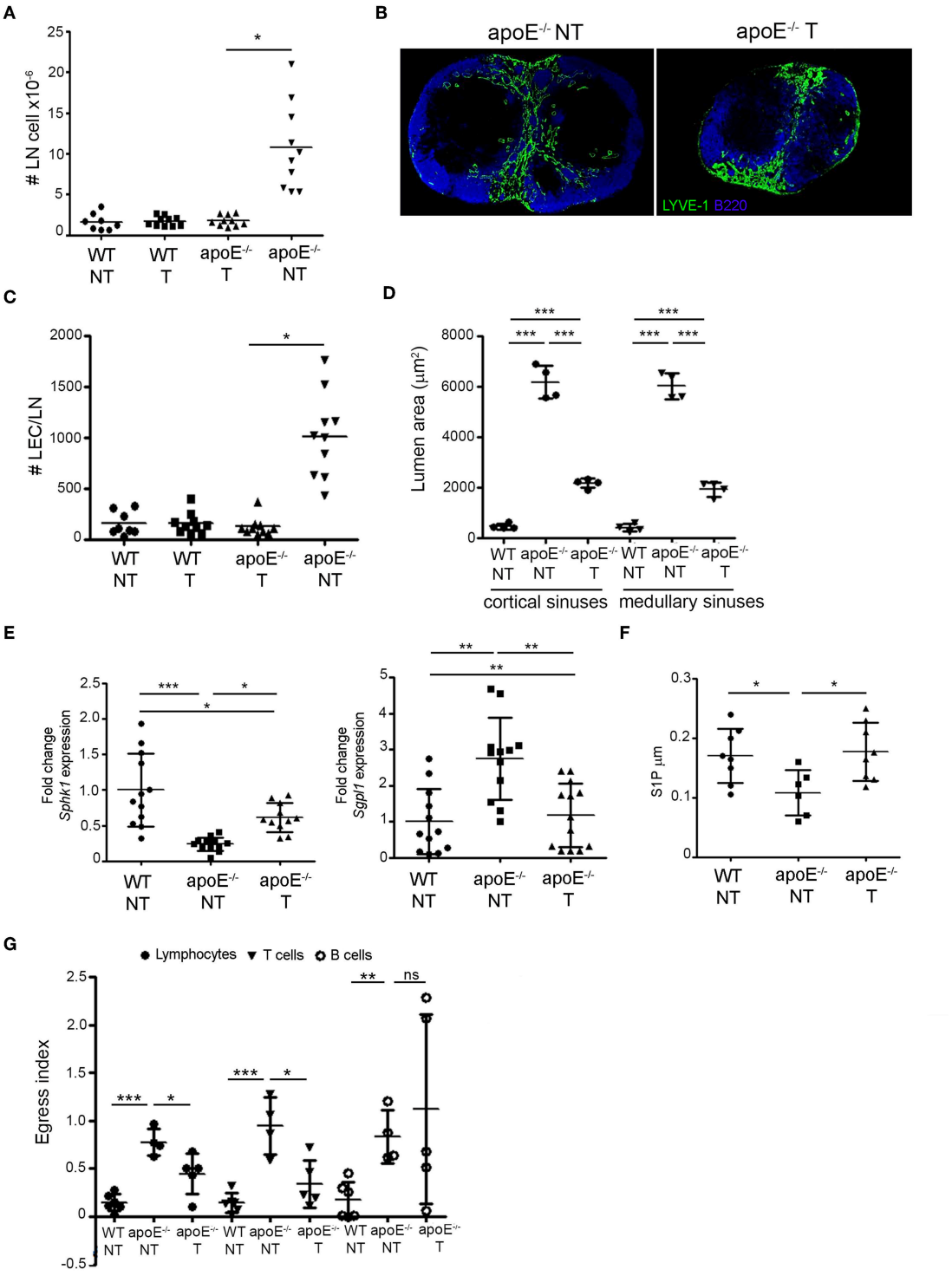

FIGURE 7 | Reversing dyslipidemia improves lymphocyte egress and LN hypertrophy. **(A)** WT and apoE$^{-/-}$ mice were treated with eztimibe (treated, T) or vehicle (non-treated, NT) and LN cellularity expressed as fold change over WT mice was determined. $n = 8$–10 mice per group; $^*p < 0.05$. **(B)** Immunoreactivity for B220 and LYVE-1 was examined in LN sections from NT and T apoE$^{-/-}$ mice. Images are representative of three independent experiments ($n = 3$–5). **(C)** The number of LECs was determined in LN suspensions from NT WT, NT and T apoE$^{-/-}$ mice by flow cytometry and expressed as fold change over baseline. $n = 8$–10; $^*p < 0.05$. **(D)** Lumen area of cortical and medullary sinuses was determined on LN sections NT WT, NT, and T apoE$^{-/-}$ mice. $n = 4$ mice per group; $^{***}p < 0.0005$. **(E)** Quantitative RT-PCR analysis was performed on whole LN RNA to examine expression of *Sph1k* and *Spgl1* in NT WT, NT, and T apoE$^{-/-}$ mice. Data is pooled

(Continued)

FIGURE 7 | from three independent experiments with 4 mice per group in each experiment; $^{*}p < 0.05$; $^{**}p < 0.005$; $^{***}p < 0.0005$. **(F)** S1P content was analyzed in efferent lymph of NT WT, NT and T apoE$^{-/-}$ mice by mass spectrometry. Data is pooled from three independent experiments with 3–4 mice per group in each experiment **(G)** Egress of adoptively transferred CD45.1 lymphocytes, T and B cell from NT WT, NT and T apoE$^{-/-}$ mice was examined. $n = 4$–6 mice per group; $^{*}p < 0.05$; $^{**}p < 0.005$; $^{***}p < 0.0005$.

CCL21 was more abundant compared to WT mice. Although the measure of lymph flow will require further investigations which are beyond the scope of this study, our previous data on skin lymphatics in apoE$^{-/-}$ mice showing that they are leaky and exhibit abnormal valve (34) support the idea of altered efferent lymph flow in these hypercholesterolemic mice. The extensive remodeling of lymphatic sinuses observed in apoE$^{-/-}$ mice was also associated with a significant decrease in lymph S1P levels. This decrease may result from a reduced local production of S1P by LECs as supported by lower expression of S1PK but also from an increased degradation by S1P lyase whose expression was significantly higher in apoE$^{-/-}$ LN compared to WT LN. However, we cannot exclude the possible contribution of systemic abnormalities in sphingolipids as shown in a lipidomic study by Chen et al., reporting elevated plasma levels of sphingolipids and abnormal sphingolipid metabolism in apoE$^{-/-}$ mice (45).

Finally, we provide evidence that hypercholesterolemia accounts in part for the impaired lymphocyte egress, extensive remodeling of lymphatic sinuses and decrease in lymph S1P levels as these defects were restored by reversing hypercholesterolemia with the lowering-cholesterol drug ezetimibe. This is in line with our previous report demonstrating that ezetimibe improves lymphatic drainage and skin dendritic cell migration in both apoE$^{-/-}$ and Ldlr$^{-/-}$ mouse models (34). By analogy to arterial alterations induced by hypercholesterolemia, the accumulation of lipoprotein including LDL and oxidized LDL may compromise the structural integrity of lymphatic vessels by for example altering the expression of tight junction assembly (46) or may induce lymphatic dysfunction by affecting smooth muscle contraction of efferent lymphatic vessels through the modulation of nitric oxide levels (47). However, hypercholesterolemia in apoE$^{-/-}$ mouse is often associated with systemic inflammation including in skin (26, 27, 33). Therefore, inflammation that can affect lymphatic vessel structure and function may also account for lymphatic alterations and impaired lymphocyte trafficking. Although ezetimibe has been shown to reduce atherosclerosis progression mainly through its effect on plasma LDL cholesterol by inhibiting its absorption by the intestine (40, 41), it is possible that ezetimibe may indirectly affect inflammation associated with hypercholesterolemia in apoE$^{-/-}$ mice by reducing LDL cholesterol and subsequently inflammatory modified LDL. Therefore, it would be interesting to investigate whether anti-inflammatory strategies alone without affecting hypercholesterolemia would be sufficient to improve lymphocyte egress and lymphatic function in apoE$^{-/-}$ mice. That the improvement of lymphocyte egress in apoE$^{-/-}$ mice by ezetimibe treatment resulted from the improved T cell egress but not that of B cell suggests that the egress of B cells may rely on additional signals than S1P. In fact, most of the knowledge on lymphocyte egress is based on studies on T cell egress.

It is now apparent that the expansion of lymphatic vessels in LN can modulate the immune response during inflammation (38, 48, 49). All of these studies however focused on lymphangiogenesis occurring at early phases of inflammation. More recently, we reported a biphasic remodeling of lymphatic vessels during the course of inflammation, the subcapsular sinuses being expanded first followed by the cortical and medullary sinuses (23). Notably, this differential remodeling is biologically and functionally important. Indeed, the early expansion of subcapsular sinuses enhances DC migration from the periphery into the inflamed LN (38) whereas the expansion of cortical and medullary sinuses at later phases of inflammation reestablishes the steady-state egress of lymphocytes from those LNs (23). Therefore, the increased accumulation of lymphocytes into LNs during inflammation has to be accompanied by a proportional increase in lymphocyte exit in the efferent lymph to prevent LN hypertrophy. Here, we provide evidence that under certain circumstances such as chronic inflammation associated with hypercholesterolemia, the aberrant expansion of cortical and medullary sinuses can conversely inhibit the emigration of lymphocyte from the inflamed LNs leading to subsequent LN hypertrophy.

Activated antigen-specific lymphocytes must leave the LN to migrate into the effector sites in order to exert their appropriate immune responses (9). Naïve lymphocyte must also exit the LN via efferent lymph to reach the blood circulation for immune surveillance. Therefore, the impairment of lymphocyte egress is expected to affect the normal function of both naïve and effector lymphocytes and subsequently, compromised immune responses. This scenario is likely relevant to hypercholesterolemic mouse models. Indeed, apoE$^{-/-}$ and Ldlr$^{-/-}$ mice show impaired priming when immunologically challenged (33) and reduced capacity for clearance of bacteria (29, 30, 50), fungi (28), and virus (31). In addition, this increased susceptibility to infection does not occur in young apoE$^{-/-}$ mice, but increases with age (31, 51), consistent with the later onset of LN hypertrophy and decreased lymphocyte egress. Thus, the increased susceptibility to infection in hypercholesterolemic mice may not only result from impaired DC migration from peripheral tissue into the draining LN as we proposed previously (33) but also from the impairment in efferent lymphatic emigration of lymphocytes from the enlarged LN. This is supported by the study of Ludewig et al. (31) showing that apoE$^{-/-}$ mice infected with LCMV exhibit impaired migration of virus specific CD8 cytotoxic T cells into the blood and liver.

In conclusion, we provide evidence that dyslipidemia severely compromises the efferent lymphatic emigration

of lymphocyte from LN which subsequently leads to LN hypertrophy by altering the structure and function of lymphatic vessels. Since LN hypertrophy is often associated with chronic inflammatory and autoimmune diseases such as systemic lupus erythematosus and rheumatoid arthritis, our findings may also be relevant to other chronic diseases than those related to dyslipidemia. This study further illustrates the importance of maintaining healthy lymphatic vessel for optimal immunity.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and the **Supplementary Files**.

AUTHOR CONTRIBUTIONS

SL, MT, YL, KT, FT, PN, MW, CT, and VA designed the study and developed the methodology. SL, MT, YL, KT, FT, PN, and CT performed and analyzed the experiments and VA wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.00575/full#supplementary-material

REFERENCES

1. Gretz JE, Norbury CC, Anderson AO, Proudfoot AE, Shaw S. Lymph-borne chemokines and other low molecular weight molecules reach high endothelial venules via specialized conduits while a functional barrier limits access to the lymphocyte microenvironments in lymph node cortex. *J Exp Med.* (2000) 192:1425–40. doi: 10.1084/jem.192.10.1425
2. Girard JP, Springer TA. High endothelial venules (HEVs): specialized endothelium for lymphocyte migration. *Immunol Today.* (1995) 16:449–57. doi: 10.1016/0167-5699(95)80023-9
3. Mackay CR, Marston WL, Dudler L. Naive and memory T cells show distinct pathways of lymphocyte recirculation. *J Exp Med.* (1990) 171:801–17.
4. Sallusto F, Lenig D, Forster R, Lipp M, Lanzavecchia A. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature.* (1999) 401:708–12.
5. Grigorova IL, Panteleev M, Cyster JG. Lymph node cortical sinus organization and relationship to lymphocyte egress dynamics and antigen exposure. *Proc Natl Acad Sci USA.* (2010) 107:20447–52. doi: 10.1073/pnas.1009968107
6. Braun A, Worbs T, Moschovakis GL, Halle S, Hoffmann K, Bolter J, et al. Afferent lymph-derived T cells and DCs use different chemokine receptor CCR7-dependent routes for entry into the lymph node and intranodal migration. *Nat Immunol.* (2011) 12:879–87. doi: 10.1038/ni.2085
7. Forster R, Braun A, Worbs T. Lymph node homing of T cells and dendritic cells via afferent lymphatics. *Trends Immunol.* (2012) 33:271–80. doi: 10.1016/j.it.2012.02.007
8. Pham TH, Okada T, Matloubian M, Lo CG, Cyster JG. S1P1 receptor signaling overrides retention mediated by G alpha i-coupled receptors to promote T cell egress. *Immunity.* (2008) 28:122–33. doi: 10.1016/j.immuni.2007.11.017
9. Grigorova IL, Schwab SR, Phan TG, Pham TH, Okada T, Cyster JG. Cortical sinus probing, S1P1-dependent entry and flow-based capture of egressing T cells. *Nat Immunol.* (2009) 10:58–65. doi: 10.1038/ni.1682
10. Lo CG, Xu Y, Proia RL, Cyster JG. Cyclical modulation of sphingosine-1-phosphate receptor 1 surface expression during lymphocyte recirculation and relationship to lymphoid organ transit. *J Exp Med.* (2005) 201:291–301. doi: 10.1084/jem.20041509
11. Schwab SR, Pereira JP, Matloubian M, Xu Y, Huang Y, Cyster JG. Lymphocyte sequestration through S1P lyase inhibition and disruption of S1P gradients. *Science.* (2005) 309:1735–9. doi: 10.1126/science.1113640
12. Pappu R, Schwab SR, Cornelissen I, Pereira JP, Regard JB, Xu Y, et al. Promotion of lymphocyte egress into blood and lymph by distinct sources of sphingosine-1-phosphate. *Science.* (2007) 316:295–8. doi: 10.1126/science.1139221
13. Wei SH, Rosen H, Matheu MP, Sanna MG, Wang SK, Jo E, et al. Sphingosine 1-phosphate type 1 receptor agonism inhibits transendothelial migration of medullary T cells to lymphatic sinuses. *Nat Immunol.* (2005) 6:1228–35. doi: 10.1038/ni1269
14. Sanna MG, Wang SK, Gonzalez-Cabrera PJ, Don A, Marsolais D, Matheu MP, et al. Enhancement of capillary leakage and restoration of lymphocyte egress by a chiral S1P1 antagonist *in vivo*. *Nat Chem Biol.* (2006) 2:434–41. doi: 10.1038/nchembio804
15. Schwab SR, Cyster JG. Finding a way out: lymphocyte egress from lymphoid organs. *Nat Immunol.* (2007) 8:1295–301. doi: 10.1038/ni1545
16. Pham TH, Baluk P, Xu Y, Grigorova I, Bankovich AJ, Pappu R, et al. Lymphatic endothelial cell sphingosine kinase activity is required for lymphocyte egress and lymphatic patterning. *J Exp Med.* (2010) 207:17–27. doi: 10.1084/jem.20091619
17. Hall JG, Morris B. The immediate effect of antigens on the cell output of a lymph node. *Br J Exp Pathol.* (1965) 46:450–4.
18. Cahill RN, Frost H, Trnka Z. The effects of antigen on the migration of recirculating lymphocytes through single lymph nodes. *J Exp Med.* (1976) 143:870–88. doi: 10.1084/jem.143.4.870
19. Mackay CR, Marston W, Dudler L. Altered patterns of T cell migration through lymph nodes and skin following antigen challenge. *Eur J Immunol.* (1992) 22:2205–10. doi: 10.1002/eji.1830220904
20. Palframan RT, Jung S, Cheng G, Weninger W, Luo Y, Dorf M, et al. Inflammatory chemokine transport and presentation in HEV: a remote control mechanism for monocyte recruitment to lymph nodes in inflamed tissues. *J Exp Med.* (2001) 194:1361–73. doi: 10.1084/jem.194.9.1361
21. Young AJ. The physiology of lymphocyte migration through the single lymph node *in vivo*. *Semin Immunol.* (1999) 11:73–83. doi: 10.1006/smim.1999.0163
22. Shiow LR, Rosen DB, Brdickova N, Xu Y, An J, Lanier LL, et al. CD69 acts downstream of interferon-alpha/beta to inhibit S1P1 and lymphocyte egress from lymphoid organs. *Nature.* (2006) 440:540–4. doi: 10.1038/nature04606

23. Tan KW, Yeo KP, Wong FH, Lim HY, Khoo KL, Abastado JP, et al. Expansion of cortical and medullary sinuses restrains lymph node hypertrophy during prolonged inflammation. *J Immunol.* (2012) 188:4065–80. doi: 10.4049/jimmunol.1101854
24. Von Andrian UH, Mempel TR. Homing and cellular traffic in lymph nodes. *Nat Rev Immunol.* (2003) 3:867–78. doi: 10.1038/nri1222
25. Junt T, Scandella E, Ludewig B. Form follows function: lymphoid tissue microarchitecture in antimicrobial immune defence. *Nat Rev Immunol.* (2008) 8:764–75. doi: 10.1038/nri2414
26. Feingold KR, Elias PM, Mao-Qiang M, Fartasch M, Zhang SH, Maeda N. Apolipoprotein E deficiency leads to cutaneous foam cell formation in mice. *J Invest Dermatol.* (1995) 104:246–50. doi: 10.1111/1523-1747.ep12612790
27. Van Ree JH, Gijbels MJ, Van Den Broek WJ, Hofker MH, Havekes LM. Atypical xanthomatosis in apolipoprotein E-deficient mice after cholesterol feeding. *Atherosclerosis.* (1995) 112:237–43. doi: 10.1016/0021-9150(94)05419-J
28. Netea MG, Demacker PN, De Bont N, Boerman OC, Stalenhoef AF, Van Der Meer JW, et al. Hyperlipoproteinemia enhances susceptibility to acute disseminated *Candida albicans* infection in low-density-lipoprotein-receptor-deficient mice. *Infect Immun.* (1997) 65:2663–7.
29. Roselaar SE, Daugherty A. Apolipoprotein E-deficient mice have impaired innate immune responses to *Listeria monocytogenes in vivo*. *J Lipid Res.* (1998) 39:1740–3.
30. De Bont N, Netea MG, Demacker PN, Verschueren I, Kullberg BJ, Van Dijk KW, et al. Apolipoprotein E knock-out mice are highly susceptible to endotoxemia and *Klebsiella pneumoniae* infection. *J Lipid Res.* (1999) 40:680–5.
31. Ludewig B, Jaggi M, Dumrese T, Brduscha-Riem K, Odermatt B, Hengartner H, et al. Hypercholesterolemia exacerbates virus-induced immunopathologic liver disease via suppression of antiviral cytotoxic T cell responses. *J Immunol.* (2001) 166:3369–76. doi: 10.4049/jimmunol.166.5.3369
32. Van Lenten BJ, Wagner AC, Anantharamaiah GM, Garber DW, Fishbein MC, Adhikary L, et al. Influenza infection promotes macrophage traffic into arteries of mice that is prevented by D-4F, an apolipoprotein A-I mimetic peptide. *Circulation.* (2002) 106:1127–32. doi: 10.1161/01.CIR.0000030182.35880.3E
33. Angeli V, Llodra J, Rong JX, Satoh K, Ishii S, Shimizu T, et al. Dyslipidemia associated with atherosclerotic disease systemically alters dendritic cell mobilization. *Immunity.* (2004) 21:561–74. doi: 10.1016/j.immuni.2004.09.003
34. Lim HY, Thiam CH, Yeo KP, Bisoendial R, Hii CS, Mcgrath KC, et al. Lymphatic vessels are essential for the removal of cholesterol from peripheral tissues by SR-BI-mediated transport of HDL. *Cell Metab.* (2013) 17:671–84. doi: 10.1016/j.cmet.2013.04.002
35. Halin C, Scimone ML, Bonasio R, Gauguet JM, Mempel TR, Quackenbush E, et al. The S1P-analog FTY720 differentially modulates T-cell homing via HEV: T-cell-expressed S1P1 amplifies integrin activation in peripheral lymph nodes but not in Peyer patches. *Blood.* (2005) 106:1314–22. doi: 10.1182/blood-2004-09-3687
36. Matloubian M, Lo CG, Cinamon G, Lesneski MJ, Xu Y, Brinkmann V, et al. Lymphocyte egress from thymus and peripheral lymphoid organs is dependent on S1P receptor 1. *Nature.* (2004) 427:355–60. doi: 10.1038/nature02284
37. Narayanaswamy P, Shinde S, Sulc R, Kraut R, Staples G, Thiam CH, et al. Lipidomic "deep profiling": an enhanced workflow to reveal new molecular species of signaling lipids. *Anal Chem.* (2014) 86:3043–7. doi: 10.1021/ac4039652
38. Angeli V, Ginhoux F, Llodra J, Quemeneur L, Frenette PS, Skobe M, et al. B cell-driven lymphangiogenesis in inflamed lymph nodes enhances dendritic cell mobilization. *Immunity.* (2006) 24:203–15. doi: 10.1016/j.immuni.2006.01.003
39. Di Tomaso E, Capen D, Haskell A, Hart J, Logie JJ, Jain RK, et al. Mosaic tumor vessels: cellular basis and ultrastructure of focal regions lacking endothelial cell markers. *Cancer Res.* (2005) 65:5740–9. doi: 10.1158/0008-5472.CAN-04-4552
40. Davis HR Jr, Compton DS, Hoos L, Tetzloff G. Ezetimibe, a potent cholesterol absorption inhibitor, inhibits the development of atherosclerosis in ApoE knockout mice. *Arterioscler Thromb Vasc Biol.* (2001) 21:2032–8. doi: 10.1161/hq1201.100260
41. Kuhlencordt PJ, Padmapriya P, Rutzel S, Schodel J, Hu K, Schafer A, et al. Ezetimibe potently reduces vascular inflammation and arteriosclerosis in eNOS-deficient ApoE ko mice. *Atherosclerosis.* (2009) 202:48–57. doi: 10.1016/j.atherosclerosis.2008.03.021
42. Lim HY, Rutkowski JM, Helft J, Reddy ST, Swartz MA, Randolph GJ, et al. Hypercholesterolemic mice exhibit lymphatic vessel dysfunction and degeneration. *Am J Pathol.* (2009) 175:1328–37. doi: 10.2353/ajpath.2009.080963
43. Jain RK. Normalization of tumor vasculature: an emerging concept in antiangiogenic therapy. *Science.* (2005) 307:58–62. doi: 10.1126/science.1104819
44. Cyster JG, Schwab SR. Sphingosine-1-phosphate and lymphocyte egress from lymphoid organs. *Annu Rev Immunol.* (2012) 30:69–94. doi: 10.1146/annurev-immunol-020711-075011
45. Chen Y, Wen S, Jiang M, Zhu Y, Ding L, Shi H, et al. Atherosclerotic dyslipidemia revealed by plasma lipidomics on ApoE(-/-) mice fed a high-fat diet. *Atherosclerosis.* (2017) 262:78–86. doi: 10.1016/j.atherosclerosis.2017.05.010
46. Demaio L, Rouhanizadeh M, Reddy S, Sevanian A, Hwang J, Hsiai TK. Oxidized phospholipids mediate occludin expression and phosphorylation in vascular endothelial cells. *Am J Physiol Heart Circ Physiol.* (2006) 290:H674–83. doi: 10.1152/ajpheart.00554.2005
47. Shimokawa H. Primary endothelial dysfunction: atherosclerosis. *J Mol Cell Cardiol.* (1999) 31:23–37. doi: 10.1006/jmcc.1998.0841
48. Kataru RP, Jung K, Jang C, Yang H, Schwendener RA, Baik JE, et al. Critical role of CD11b+ macrophages and VEGF in inflammatory lymphangiogenesis, antigen clearance, and inflammation resolution. *Blood.* (2009) 113:5650–9. doi: 10.1182/blood-2008-09-176776
49. Huggenberger R, Ullmann S, Proulx ST, Pytowski B, Alitalo K, Detmar M. Stimulation of lymphangiogenesis via VEGFR-3 inhibits chronic skin inflammation. *J Exp Med.* (2010) 207:2255–69. doi: 10.1084/jem.20100559
50. Martens GW, Arikan MC, Lee J, Ren F, Vallerskog T, Kornfeld H. Hypercholesterolemia impairs immunity to tuberculosis. *Infect Immun.* (2008) 76:3464–72. doi: 10.1128/IAI.00037-08
51. Moazed TC, Kuo C, Grayston JT, Campbell LA. Murine models of *Chlamydia pneumoniae* infection and atherosclerosis. *J Infect Dis.* (1997) 175:883–90. doi: 10.1086/513986

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REVIEW
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Tumor-Associated Lymphatic Vessel Features and Immunomodulatory Functions

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The lymphatic system comprises a network of lymphoid tissues and vessels that drains the extracellular compartment of most tissues. During tumor development, lymphatic endothelial cells (LECs) substantially expand in response to VEGFR-3 engagement by VEGF-C produced in the tumor microenvironment, a process known as tumor-associated lymphangiogenesis. Lymphatic drainage from the tumor to the draining lymph nodes consequently increases, powering interstitial flow in the tumor stroma. The ability of a tumor to induce and activate lymphatic growth has been positively correlated with metastasis. Much effort has been made to identify genes responsible for tumor-associated lymphangiogenesis. Inhibition of lymphangiogenesis with soluble VEGFR-3 or with specific monoclonal antibodies decreases tumor spread to LNs in rodent models. Importantly, tumor-associated lymphatics do not only operate as tumor cell transporters but also play critical roles in anti-tumor immunity. Therefore, metastatic as well as primary tumor progression can be affected by manipulating tumor-associated lymphatic remodeling or function. Here, we review and discuss our current knowledge on the contribution of LECs immersed in the tumor microenvironment as immunoregulators, as well as a possible functional remodeling of LECs subsets depending on the organ microenvironment.

Keywords: lymphatic vessels, anti-tumor immune response, metastasis, lymphangiogenesis, tumor microenvironment

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INTRODUCTION

Over the last few years, immunotherapy has evolved into a very promising new approach for fighting tumor progression. However, the proportion of cancer patients that positively respond to these treatments is still limited. Indeed, tumor cells foster mechanisms to escape immunosurveillance either by inducing poorly immunogenic tumors (immunoselection) or by setting up a tolerogenic environment that inhibits immune effector cells (immunosubversion) [reviewed in (1–3)]. Therefore, manipulations aiming at boosting anti-tumor immune cell responses and in particular tumor-specific T cell priming currently represent an extensive axis of investigations.

During tumor development, lymphatic endothelial cells (LECs), the principal components of lymphatic vessels (LVs), undergo active modifications that facilitate metastatic dissemination, and induce immunoregulation. LEC phenotype and functions are strongly altered by inflammation or infections, which may directly influence on-going immune responses (4). In particular, it has been suggested that LECs immersed in the tumor microenvironment (TME) can act as immunoregulators of the anti-tumor T cell response (5). *In vitro* studies have further shown that, tumor derived LECs exhibit altered gene expression profiles compared to dermal derived LECs (6) and upregulate PD-L1 to inhibit T cell activation (7, 8). On the other hand, a recent study has suggested that tumor-associated (TA) LVs might be beneficial for the efficacy of anti-PD-1 immunotherapy (9). Therefore, depending on the stage of tumor progression and on the immunological settings (immune evasion/immunosubversion or immunotherapy), LV might display positive and/or negative effects on tumor immunity. It is thus urgent to decipher precisely the roles for LVs in tumor cell dissemination and anti-tumor T cell immunity. In this review, we discuss the ability of LECs to shape tumor development through their contribution to tumor cell spreading and regulation of anti-tumoral T cell responses.

LYMPHATIC VESSELS AS IMMUNOREGULATORS IN NON-TUMOR CONTEXT

LVs develop as a hierarchical vasculature facilitating a unidirectional drainage system of fluid and cells from tissues toward draining lymph nodes (LNs) (10). They interlace the blood vessel circulation and play a crucial role in lipid absorption, tissue fluid homeostasis and immunity (11). The lymphatic system is a linear and blind-ended circuit. Initial lymphatic capillaries are composed of a single layer of LECs with minimal basement membrane and are not covered by pericytes or smooth muscle cells. This particular organization of LECs is highly permeable for the uptake of cells, macromolecules and interstitial fluids (12). Lymphatic capillaries drain to collecting lymphatics defined by pericyte and smooth muscle cell coverage, continuous basement membrane with "zipper-like" junctions, and a system of valves preventing retrograde flow (12, 13). Our knowledge of multiple LV functions has quickly evolved, based on the identification of LEC markers such as the transcription factor Prox-1 and the surface protein LYVE-1, that are not expressed by others endothelial cells. Prox-1 is primordial for the development and the maintenance of LECs (14–16). LYVE-1 is enriched in lymphatic junctions, highly expressed in initial lymphatics, but mostly absent from LV collectors [reviewed in (12)]. This molecule is implicated in dendritic cells (DCs) trafficking within LVs (17). LECs also express GP38 (podoplanin) and platelet endothelial cell adhesion molecule (PECAM-1 or CD31) that are markers shared with fibroblastic reticular cells (FRCs) and blood endothelial cells (BECs), respectively. An important function of lymphatics is to transport immune cells from peripheral tissues to LNs and therefore to participate to immune response initiation (18–21). Transcriptomic analysis of *ex vivo* LN stromal cell (LNSC) subsets in distinct immunological situations established that FRCs, BECs, and LECs express a multitude of immune mediators and growth factors that may influence the immune system. LNSCs are strongly modulated by inflammation or infections, and may contribute as active participants of on-going immune responses. In addition, a more precise characterization of these cells within distinct conditions suggested that LNSCs are specialized for their unique microenvironment (4). This might reflect a functional specialization of LNSC subsets depending on the organ microenvironment. Apart from their effect on tissue drainage and immune cell migration, LECs regulate T cell responses through different mechanisms (22). First, different studies in mice showed that steady-state LN LECs participate to peripheral T cell tolerance by presenting endogenously expressed tissue-restricted antigens (17, 18) through MHC class I (MHCI) molecules and eliminating autoreactive $CD8^+$ T cells (23–25). LN LECs can also cross-present exogenous antigens onto MHCI molecules, and further drive the apoptosis of antigen-specific $CD8^+$ T cells (26). Whether LN LECs have an impact on peripheral $CD4^+$ T cell responses in different immunological settings remains largely unknown and controversial. On the one hand, Rouhani et al. showed that LECs were unable to load MHC class II (MHCII) molecules with antigenic peptides due to their lack of H2-M expression at steady-state (27). However, LECs express the promoter IV (pIV) of CIITA, the master regulator for MHCII molecule expression (28). CIITA pIV being inducible by IFN-γ (29), LECs might require exposure to IFN-γ to upregulate H-2M molecules and be capable of MHCII-restricted antigen presentation. On the other hand, we published that surface MHCII expression on LNSCs results from the combination of both endogenous and acquired molecules. *In vitro* and *in vivo*, LNSCs further present peptide-MHCII complexes acquired from DCs to $CD4^+$ T cells to induce their dysfunction. In particular, LECs specifically induce $CD4^+$ T cell death, whereas LECs, BECs and FRCs all induce T cell anergy (28). Moreover, our recent studies demonstrate that the loss of MHCII expression on LNSCs in murine LNs impairs peripheral $CD4^+$ T cell tolerance, and alters regulatory T cell populations, resulting in signs of spontaneous autoimmunity in elderly (30). Their lack of costimulatory molecules could explain LNSC implication in T cell tolerance. By releasing the sphingosine 1-phosphate (S1P), LECs play also an important role in the egress of activated T cells from LNs (31, 32). In addition, LEC-derived S1P is involved in naïve T cells survival, its signaling further providing sufficient energy to maintain their steady-state recirculation (33). LECs are also capable of preventing T cell activation and proliferation in a negative regulatory feedback process. Indeed, LECs from LNs produce nitric oxide in response to inflammatory signals (IFN-γ and TNF) produced by T cells, inhibiting back T cell activation (34). Finally, during inflammation, LECs present in collecting LV or in the skin suppress DC maturation via a Mac-1/ICAM-1 dependent mechanism (35), or through prostacyclin synthesis, respectively (36), leading to subsequent dampening of T cell activation.

TUMOR-ASSOCIATED LYMPHANGIOGENESIS AND METASTASIS

The mortality linked to solid tumors is mainly associated with their capacity to disseminate to distant organs in a process known as metastasis (37). LVs are essential in tumor cell spreading as they function as "highways" connecting primary tumors to secondary lymphoid organs. The process of lymphatic proliferation, sprouting and enlargement during tumor progression, known as tumor lymphangiogenesis, and its implication in the spread of the disease has been studied for many years. TA-lymphangiogenesis correlates with metastasis and poor prognosis in several cancer types [as depicted in (10)], illustrating the relevance of lymphatic vasculature to cancer biology. A retrospective analysis of melanoma patients with lung metastases showed that high LV density and lymphatic invasion in metastatic regions were associated with poor prognosis (38). Moreover, LVs and immune cell infiltrates positively correlate in human metastatic cutaneous melanoma and colorectal cancer (39, 40). Therefore, therapies aiming at blocking tumor lymphangiogenesis are being considered as promising approaches for the treatment of such malignancies [as discussed in (41)]. Importantly, several inhibitors targeting distinct actors of lymphangiogenesis have been developed in murine tumor models and could be translated into clinic to reduce metastasis. Accordingly, the inhibition of the prolymphangiogenic VEGFR-3 signaling by using VEGFR-3 blocking antibodies or VEGF-C/D trap reduces LN, and/or distant organ metastasis in different tumor mouse models (42–45). Conversely, overexpression of two lymphangiogenic factors VEGF-C and VEGF-D increases metastasis dissemination to sentinel LNs (46–49). In particular, molecules or antibodies blocking VEGF-C/VEGFR3 signaling have been tested in clinical trials, some have gone one to be approved for cancer treatment (10, 50) [as reviewed in (10)]. However, although blockade of VEGFR3 has no noticeable effect on established lymphatics, VEGF-C signaling has been described to promote homeostasis of intestinal and brain meningeal LVs (51, 52). Therefore, it would be crucial to develop treatment that specifically target pro-tumorigenic LEC functions in order to exclude any potential intestinal or neurological side effect.

In mice, LECs from tumors present a distinct molecular profile compared to dermal LECs. Altered pathways include chemokines, extracellular matrix, cell adhesion, and inflammatory responses (6). These observations reflect significant levels of LEC plasticity that is highly regulated by the tissue microenvironment.

Pro-Lymphangiogenic Factors and Tumor Cell Spreading

The TME is composed of cancer cells, the extracellular matrix (ECM), stromal cells, and various immune cell types, impacting both tumor cell development and anti-tumor immunity. All these cells produce many factors that lead to the establishment of an intratumoral environment characterized by chronic inflammation, immunosuppression, angiogenesis, and lymphangiogenesis, the latter being the focus of this review **(Figure 1.1)**.

The proliferation, migration and survival of LECs depend mainly on VEGFR2/3 signaling axis, which is driven by VEGF-C and VEGF-D (Vascular Endothelial Growth Factors –C and –D) (53, 54) produced by many different cell types, including tumor cells and immune cells. VEGF-C and VEGF-D, considered to be major drivers of tumor lymphangiogenesis, are associated with LN and/or distant organ metastasis (38, 46–48, 55–57) **(Figure 1.1)**. Using orthotopic spontaneous metastasis models in nude mice, it has been shown that VEGF-C expression by tumor cells favors metastatic propagation in distal organs (57). Moreover, a recent study indicated that, in a transgenic mouse model with increased lymphangiogenesis in the lung, TA-LVs contribute to the dissemination of metastases to distant organs (38). *In vitro* studies have deciphered the molecular mechanisms implicated in the activation of VEGF-C/D signaling pathway. Following VEGFR-3 engagement, the protein kinase C is activated, leading to the phosphorylation of AKT, and subsequent LEC migration, survival and proliferation (53). Neuropilin 2 (Nrp-2), an additional receptor for VEGF-C, is also expressed by LECs, and contributes to lymphatic sprouting (58, 59).

In several human cancer, VEGF-C and COX-2 (cyclo-oxygenase 2, an enzyme implicated in prostaglandin pathway) expression are associated with LV density and LN metastasis (60–63). Interestingly, preclinical and clinical trials using different Non-Steroidal Anti-Inflammatory Drugs (NSAIDs), blocking COX-2 and subsequent prostaglandin production, have reported a decrease in cancer incidence, tumor cell dissemination, and finally global cancer morbidity. These observations suggest that NSAIDs could be applied for the treatment of metastasis (64–66). In mice, beside a direct effect on LECs, VEGF-C/D increases the levels of prostaglandins in the TME, further promoting TA-lymphangiogenesis. VEGF-D indeed inhibits the enzyme 15-hydroxyprostaglandin dehydrogenase (15-PGDH), therefore enhancing LEC exposure to prostaglandins in collecting lymphatic vessels (67). The engagement of EP3 signaling (prostaglandin E2 receptor 3) on tumor-associated stromal cells promotes lymphangiogenesis (68). Moreover, increased amounts of prostaglandins amplify the production of VEGF-C by tumor and immune cells, contributing to lymphangiogenesis and tumor cell dissemination (62, 63).

TNF-α interaction with its TNF receptor 1 (TNFR-1) triggers VEGF-C secretion by tumor-associated macrophages (TAM), amplifying LV expansion and metastasis (69). On the other hand, TNF-α signaling in LECs directly favors their proliferation and their migration, without however being sufficient to constitute a fully competent lymphatic network (69). Indeed, TNF-α induced lymphangiogenesis completely depends on the VEGF-C/VEGFR3-induced LEC tip formation. Similarly, VEGF-C/VEGFR3-induced LEC tip formation is required to trigger fibroblast growth factor (FGF2) induced lymphangiogenesis and foster tumor metastasis in mice (70). In contrast, proangiogenic factors such as platelet derived growth factor B (PDGF-BB) (71) and angiopoietins (ANGPTs) (72) can act as direct lymphaniogenic factors by binding, respectively, PDGF-BB

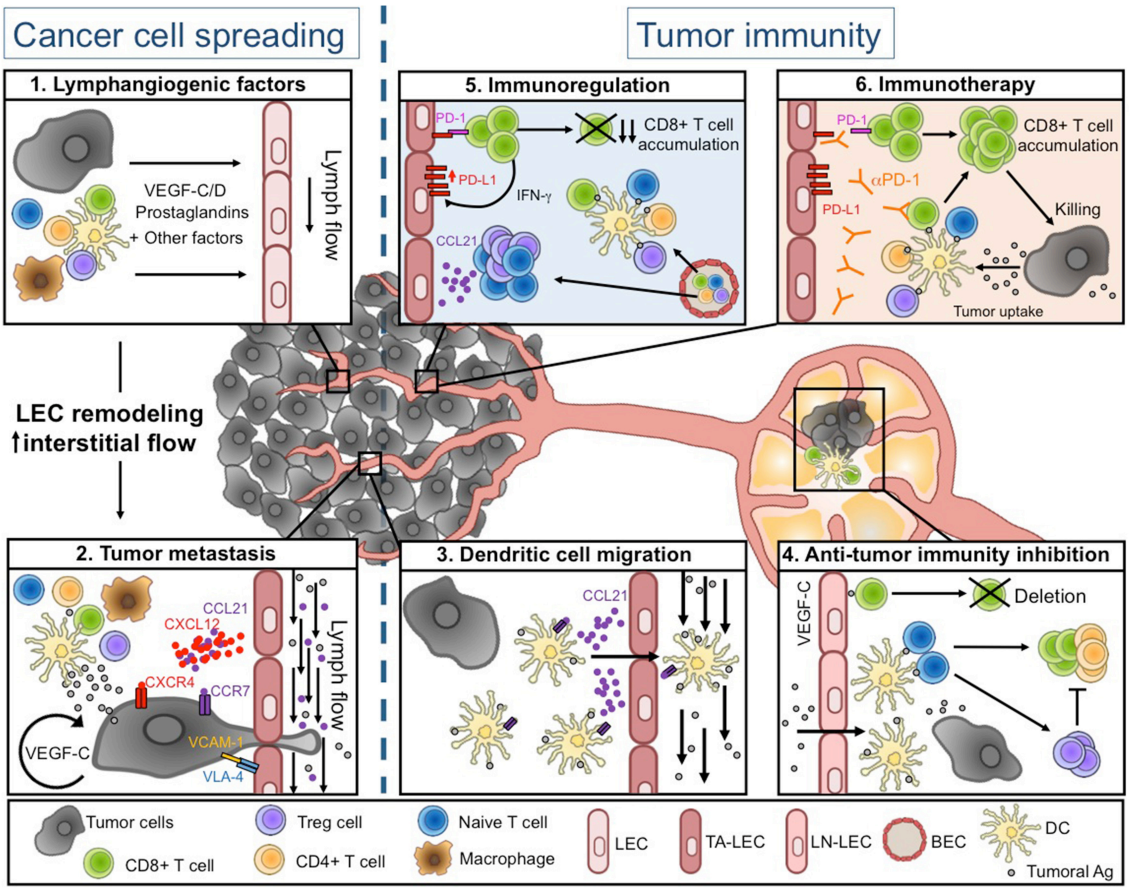

FIGURE 1 | Lymphatic vessel functions during tumor progression. **(Left)** *Tumor-associated lymphatics facilitate tumor cell spreading*. Soluble factors produced in the tumor microenvironment (TME) induce LEC remodeling and interstitial flow increase **(1)**, resulting in enhanced tumor cell migration into lymphatic vessels (LVs) **(2)**. **(Right)** *Tumor-associated lymphatics regulate anti-tumor immunity.* Tumor associated (TA-) LECs actively promote DC migration toward draining lymph nodes (LNs) **(3)**. DCs further present tumor-antigens to naïve T cells, leading to initiation of adaptive anti-tumor immunity. In tumor-draining LNs, VEGF-C exposed LEC cross-present tumor-antigens (Ags) and induce the deletion of anti-tumor $CD8^+$ T cells **(4)**. Intratumorally, naïve and activated T cells are weakly restimulated by local DCs, due to the immunosuppressive TME. The TME favors in particular the infiltration of Treg and naïve T cells through a CCL21-dependent pathway. TA-LECs also express high levels of PD-L1 in response to IFN-γ produced by effector T cells **(5)**. Upon anti-PD-1 immunotherapy, TA-LEC mediated immunosuppression might be abrogated, contributing to enhanced T cell activation and tumor cell elimination **(6)**. Drawing designed by Rémi Jeandenand.

receptors and receptors Tie1/2 expressed by LECs. Whether TGF-β enhances (73, 74) or inhibits (75) lymphangiogenesis depends on tumor models, rendering difficult the targeting of this cytokine for the regulation of LEC remodeling in tumors.

Mechanism of Metastasis Dissemination

Emerging evidence suggest that lymphatic vessels undergo several changes in response to lymphangiogenic factors during the course of metastasis. In addition to promoting tumoral cell transportation, LVs deliver lymphangiogenic factors produced by the primary tumor to condition sentinel LNs prior to the arrival and seeding of cancer cells (56, 76–78). In the mouse B16F10 melanoma model, LVs from distant metastatic regions, such as LNs and lungs, attract chemoresistant $CD133^+CXCR4^+$ melanoma cells by secreting CXCL12 (79).

TA-LVs were for long described as passive conduits for tumoral cell spread toward sentinel LNs and distant organs. However, several studies highlighted that the tumor microenvironment actively modifies LV features of primary tumor and draining LNs to further promote metastasis. VEGF-C acts in an autocrine manner to improve metastasis dissemination by favoring proteolytic activity and motility of tumor cells (80). Besides its direct effect on tumoral cells, VEGF-C modulates the expression of integrins and chemokines by LECs to facilitate tumor invasiveness. The integrin α4β1 (or VLA-4), which is considered as a marker of activated and proliferating LECs in human and murine tumors (81), is activated by the VEGF-C/PI3Kα pathway in LECs to promote lymphangiogenesis and tumor metastasis in LNs. Therefore, the blockade or the genetic deletion of this integrin on LVs prevents LEC migration and invasion, and inhibits VCAM-1 mediated adhesion of tumoral cell to LECs (82). The secretion of CCL21 by LECs, which drives CCR7-dependent tumor migration through LVs, is also enhanced in response to VEGF-C (80). Moreover,

CCL21-dependent recruitment of innate lymphoid cells results in the production of CXCL13 by tumoral stromal cells, which in turn induces metastasis through RANK/RANKL signaling (83). Transmural flow modulates LEC function by promoting the expression of CCL21 and by downregulating VE-cadherin and PECAM-1, two adhesion molecules crucial for cellular junctions (84). Modification of interstitial flow influences CCR7 ligand secretion by tumoral cells, providing an autologous chemotactic gradient (85). In human, CCR7 expression by tumor cells is associated with LN metastasis in several cancers (86–88) **(Figure 1.2)**.

CCL1 secretion by LECs located in the subcapsular sinuses of LNs is crucial to control tumor cell invasion into LNs. Indeed, blocking the CCL1 receptor (CCR8) inhibits metastasis by preventing tumor cell egress from collecting lymphatics into LNs without affecting their entry into intratumoral lymphatics (89). Recently, the screening of 810 mutant mouse strains allowed the identification of 23 genes that, when disrupted, alter the establishment of metastatic foci (90). Notably, they demonstrated that the deletion of the sphingosine-1-phosphate transporter SPNS2 in LECs decreases pulmonary metastasis and promotes effector T cell and natural killer cell infiltration in lungs (90).

Recently, Black et al., have shown that the pro-lymphangiogenic factor COX-2 enhances the expression of semaphorin 7a (sema7a) in breast tumoral cells. This leads in turn to the activation of β1-integrin receptors on adjacent tumoral cells and LECs, to finally increase lymphangiogenesis and cancer cell dissemination (91). Moreover, sema7a induces gp38 upregulation by tumor-infiltrating macrophages, therefore promoting their adhesion to LVs and consequently boosting lymphangiogenesis and metastasis in breast cancer (92). In agreement, Sema7a gene expression is observed in a high frequency in human breast cancer and correlates with metastasis and poor prognosis (91).

Apart from their implication in metastasis dissemination, accumulating studies indicate that LECs modulate anti-tumor immunity. The roles of LECs in tumor spreading and anti-tumoral immune responses are discussed below.

DUAL ROLE OF TUMOR-ASSOCIATED LYMPHATIC VESSELS IN ANTI-TUMOR IMMUNITY

Growing evidence highlight that, in addition to acting as drains for soluble factors and tumoral cell transport, TA-LVs further play important roles in shaping antitumor immunity. Therefore, the modulation of lymphangiogenesis could impact not only metastasis dissemination but also anti-tumor immunity and primary tumor growth. In the context of solid tumors, lymph flow from tumors is increased, driving intense interstitial flow in the tumor stroma, and enhancing lymphatic drainage to the draining LNs (93). TA-LVs are primarily required for the recruitment of immune cells and adaptive immune response initiation (39, 94). However, immunosuppressive features of LECs in TME will subsequently dampen ongoing anti-tumor immunity (5). Therefore, LVs play a dual role on tumor immunity that might be temporally regulated. Finally, immunotherapy approaches can be potentialized by TA-lymphangiogenesis in melanoma tumors (9), further highlighting the relevance of modulating LV functions during tumor development.

Lymphatic Vessels Are Necessary for the Initiation of Anti-Tumoral Responses

T cell activation and infiltration in tumors are key steps of antitumor immunity. Indeed, while Treg infiltration is associated with a poor outcome in patients, intratumoral cytotoxic T lymphocytes are beneficial for clinical outcome (95, 96). Although some studies have suggested that naïve T cell could infiltrate tumors and be locally activated (97–99), antigen transport by dendritic cells (DCs) through LVs toward draining LNs is nevertheless crucial for the initiation of tumor-specific T cell responses, at least in melanomas (39, 100). Indeed, tumor drainage, DC trafficking and subsequent induction of anti-tumor adaptive immune responses are drastically impaired in transgenic mice lacking or with disturbed local LVs (39, 94). Upon inflammation, LECs in afferent LVs produce CCL21 that is necessary to DC egress from the tissue toward lymphatics (101, 102). Moreover, the expression of CLEC-2 by DCs is essential for their migration into LNs. The activation of CLEC-2 by GP38, which is highly expressed by LECs and FRCs, induces actin polymerization and motility of DCs (103). In a tumoral context, CCR7 expression by DCs is primordial for their migration into tumor draining LNs and subsequent T cell activation (100) **(Figure 1.3)**.

In agreement with a role for lymphatic vasculature in the initiation of anti-tumor immunity, lymphatic vessel density (LVD) or lymphatic gene expression in primary tumors of colorectal or melanomas patients positively correlates with inflammation and immune cell infiltration (9, 39, 40, 104).

Lymphatic Vessels Suppress Effector T Cells During Tumor Progression

The lymphangiogenic factor VEGF-C produced in the tumor favors immunological tolerance in murine melanoma, including the induction of tumor-specific $CD8^+$ T cell deletion (5) **(Figure 1.4)** (5, 26). This is consistent with studies in human melanoma, where active CTLs can be found in the circulation, while they exhibit an exhausted phenotype when localized in tumors (105). In addition, LECs in tumor draining LNs cross-present tumor antigens through MHCI complexes, and further drove the apoptosis of tumor-specific $CD8^+$ T cells. The expression of the immunosuppressive molecule PD-L1 is enhanced at the surface of LECs after antigen specific interaction with $CD8^+$ T cells *in vitro* (7, 26). Moreover, blockade of PD-L1 on antigen pulsed immortalized LECs *in vitro* increases $CD8^+$ T cell activation (7). *In vivo*, in several tumor mouse models, TA-LECs express higher levels of PD-L1 compared to naïve skin LECs (7, 8), the highest PD-L1 expression being observed in immunogenic tumors (8). Recently, Lane et al. demonstrated that PD-L1 expression by non-hematopoietic cells prevents $CD8^+$ T

cells accumulation in melanoma. IFN-γ production by antigen-specific $CD8^+$ T cells is primarily necessary to induce PD-L1 expression on LECs. Using mice with LECs deficient for IFN-γ receptor, they established that the specific loss of IFN-γ sensitivity in LVs improves $CD8^+$ T cell-dependent control of melanoma tumor growth and mouse survival (8). Thus, during tumor development, a negative feedback loop is set up between LECs and T cells. LECs up-regulate PD-L1 expression in response to IFN-γ produced by tumor specific $CD8^+$ T cells, and subsequently inhibit T cell accumulation in tumors **(Figure 1.5)**.

In human metastatic melanoma, VEGF-C expression positively correlates with T cell infiltration and CCL21 expression (9). CCL21 plays a crucial role in the establishment of a tolerogenic tumor microenvironment by recruiting $CCR7^+$ regulatory T cells in primary tumors and by promoting the formation of lymphoid like stromal structures with immunosuppressive features (106). CCL21 further attracts naïve T cells that can be locally activated in response to immune blockade or vaccination (9).

CONCLUDING REMARKS

Recent studies indicate that tumor-associated LECs significantly contribute to shaping the immunosuppressive TME, therefore helping tumors hijack the immune system from an efficient to an incompetent anti-tumor response. Altogether, several observations highlight a new role for lymphatics in promoting tumor development, suggesting that lymphatic endothelium in the local microenvironment may be a novel target for immunomodulation. In agreement with these hypotheses, a recent publication demonstrated that following exposure to tumor derived factors, FRCs of the tumor draining LNs undergo multiple changes to convert into a immunosuppressive phenotype, such as decreased production of IL-7 and CCL19/21 (107). Whether a similar profound reprogramming occurs to LECs in tumor draining LNs remains to be determined.

Whereas, VEGFC driven TA-lymphangiogenesis correlates with increased intratumoral inflammation (39) and immune suppression in progressing tumors (5), it seems also to be necessary for the response of the tumor microenvironment to immunotherapeutic intervention, as demonstrated for PD-1 blocking antibodies (9) **(Figure 1.6)**. This suggests that TA-LECs potentiate immunotherapy by attracting naive T cells through a CCL21 dependent mechanism. Accordingly, LVs and immune cell infiltrates positively correlated in metastatic cutaneous melanoma and colorectal cancer patients (39, 40). Once in the tumor, naive T cells can be locally primed upon PD-1 blockade, which reverts the immunosuppressive T cell imprinting and induces long-lasting anti-tumor immunity. Therefore, it is tempting to speculate that LV density in tumors could be used as a predictor for positive response to immune checkpoint blockade. Additional research will determine how to selectively target LEC immunosuppressive functions in tumors, which could, combined to immunotherapeutic approaches, lead to the conversion of a "cold" into "hot" immunogenic TME and potentiate anti-tumor T cell responses.

AUTHOR CONTRIBUTIONS

LG, A-OG, and SH wrote the manuscript. LG conceptualized **Figure 1**.

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REFERENCES

1. Zitvogel L, Tesniere A, Kroemer G. Cancer despite immunosurveillance: immunoselection and immunosubversion. *Nat Rev Immunol.* (2006) 6:715–27. doi: 10.1038/nri1936
2. Motz GT, Coukos G. Deciphering and reversing tumor immune suppression. *Immunity.* (2013) 39:61–73. doi: 10.1016/j.immuni.2013.07.005
3. Chen DS, Mellman I. Oncology meets immunology: the cancer-immunity cycle. *Immunity.* (2013) 39:1–10. doi: 10.1016/j.immuni.2013.07.012
4. Malhotra D, Fletcher AL, Astarita J, Lukacs-Kornek V, Tayalia P, Gonzalez SF, et al. Transcriptional profiling of stroma from inflamed and resting lymph nodes defines immunological hallmarks. *Nat Immunol.* (2012) 13:499–510. doi: 10.1038/ni.2262
5. Lund AW, Duraes FV, Hirosue S, Raghavan VR, Nembrini C, Thomas SN, et al. VEGF-C promotes immune tolerance in B16 melanomas and cross-presentation of tumor antigen by lymph node lymphatics. *Cell Rep.* (2012) 1:191–9. doi: 10.1016/j.celrep.2012.01.005
6. Clasper S, Royston D, Baban D, Cao Y, Ewers S, Butz S, et al. A novel gene expression profile in lymphatics associated with tumor growth and nodal metastasis. *Cancer Res.* (2008) 68:7293–303. doi: 10.1158/0008-5472.CAN-07-6506
7. Dieterich LC, Ikenberg K, Cetintas T, Kapaklikaya K, Hutmacher C, Detmar M. Tumor-associated lymphatic vessels upregulate PDL1 to inhibit T-cell activation. *Front Immunol.* (2017) 8:66. doi: 10.3389/fimmu.2017.00066
8. Lane RS, Femel J, Breazeale AP, Loo CP, Thibault G, Kaempf A, et al. IFNgamma-activated dermal lymphatic vessels inhibit cytotoxic T cells in melanoma and inflamed skin. *J Exp Med.* (2018) 215:3057–74. doi: 10.1084/jem.20180654
9. Fankhauser M, M.Broggi AS, Potin L, Bordry N, Jeanbart L, Lund AW, et al. Tumor lymphangiogenesis promotes T cell infiltration and potentiates immunotherapy in melanoma. *Sci Transl Med.* (2017) 9:eaal4712. doi: 10.1126/scitranslmed.aal4712
10. Stacker SA, Williams SP, Karnezis T, Shayan R, Fox SB, Achen MG. Lymphangiogenesis and lymphatic vessel remodelling in cancer. Nature reviews. *Cancer.* (2014) 14:159–72. doi: 10.1038/nrc3677
11. Zheng W, Aspelund A, Alitalo K. Lymphangiogenic factors, mechanisms, and applications. *J Clin Invest.* (2014) 124:878–87. doi: 10.1172/JCI71603
12. Alitalo K, Tammela T, Petrova TV. Lymphangiogenesis in development and human disease. *Nature.* (2005) 438:946–53. doi: 10.1038/nature04480
13. Baluk P, Fuxe J, Hashizume H, Romano T, Lashnits E, Butz S, et al. Functionally specialized junctions between endothelial cells of lymphatic vessels. *J Exp Med.* (2007) 204:2349–62. doi: 10.1084/jem.20062596

14. Wigle JT, Harvey N, Detmar M, Lagutina I, Grosveld G, Gunn MD, et al. An essential role for Prox1 in the induction of the lymphatic endothelial cell phenotype. *EMBO J.* (2002) 21:1505–13. doi: 10.1093/emboj/21.7.1505
15. Wigle JT, Oliver G. Prox1 function is required for the development of the murine lymphatic system. *Cell.* (1999) 98:769–78. doi: 10.1016/S0092-8674(00)81511-1
16. Johnson NC, Dillard ME, Baluk P, McDonald DM, Harvey NL, Frase SL, et al. Lymphatic endothelial cell identity is reversible and its maintenance requires Prox1 activity. *Genes Dev.* (2008) 22:3282–91. doi: 10.1101/gad.1727208
17. Johnson LA, Banerji S, Lawrance W, Gileadi U, Prota G, Holder KA, et al. Dendritic cells enter lymph vessels by hyaluronan-mediated docking to the endothelial receptor LYVE-1. *Nat Immunol.* (2017) 18:762–70. doi: 10.1038/ni.3750
18. Olszewski WL. The innate reaction of the human skin lymphatic system to foreign and self-antigens. *Lymphat Res Biol.* (2005) 3:50–7. doi: 10.1089/lrb.2005.3.50
19. Pugh CW, MacPherson GG, Steer HW. Characterization of nonlymphoid cells derived from rat peripheral lymph. *J Exp Med.* (1983) 157:1758–79. doi: 10.1084/jem.157.6.1758
20. Mackay CR, Marston WL, Dudler L. Naive and memory T cells show distinct pathways of lymphocyte recirculation. *J Exp Med.* (1990) 171:801–17. doi: 10.1084/jem.171.3.801
21. Randolph GJ, Angeli V, Swartz MA. Dendritic-cell trafficking to lymph nodes through lymphatic vessels. *Nat Rev Immunol.* (2005) 5:617–28. doi: 10.1038/nri1670
22. Card CM, Yu SS, Swartz MA. Emerging roles of lymphatic endothelium in regulating adaptive immunity. *J Clin Invest.* (2014) 124:943–52. doi: 10.1172/JCI73316
23. Cohen JN, Guidi CJ, Tewalt EF, Qiao H, Rouhani SJ, Ruddell A, et al. Lymph node-resident lymphatic endothelial cells mediate peripheral tolerance via Aire-independent direct antigen presentation. *J Exp Med.* (2010) 207:681–8. doi: 10.1084/jem.20092465
24. Fletcher AL, Lukacs-Kornek V, Reynoso ED, Pinner SE, Bellemare-Pelletier A, Curry MS, et al. Lymph node fibroblastic reticular cells directly present peripheral tissue antigen under steady-state and inflammatory conditions. *J Exp Med.* (2010) 207:689–97. doi: 10.1084/jem.20092642
25. Tewalt EF, Cohen JN, Rouhani SJ, Guidi CJ, Qiao H, Fahl SP, et al. Lymphatic endothelial cells induce tolerance via PD-L1 and lack of costimulation leading to high-level PD-1 expression on CD8 T cells. *Blood.* (2012) 120:4772–82. doi: 10.1182/blood-2012-04-427013
26. Hirosue S, Vokali E, Raghavan VR, Rincon-Restrepo M, Lund AW, Corthesy-Henrioud P, et al. Steady-state antigen scavenging, cross-presentation, and $CD8^+$ T cell priming: a new role for lymphatic endothelial cells. *J Immunol.* (2014) 192:5002–11. doi: 10.4049/jimmunol.1302492
27. Rouhani SJ, Eccles JD, Riccardi P, Peske JD, Tewalt EF, Cohen JN, et al. Roles of lymphatic endothelial cells expressing peripheral tissue antigens in CD4 T-cell tolerance induction. *Nat Commun.* (2015) 6:6771. doi: 10.1038/ncomms7771
28. Dubrot J, Duraes FV, Potin L, Capotosti F, Brighouse D, Suter T, et al. Lymph node stromal cells acquire peptide-MHCII complexes from dendritic cells and induce antigen-specific CD4(+) T cell tolerance. *J Exp Med.* (2014) 211:1153–66. doi: 10.1084/jem.20132000
29. Reith W, LeibundGut-Landmann S, Waldburger JM. Regulation of MHC class II gene expression by the class II transactivator. Nature reviews. *Immunology.* (2005) 5:793–806. doi: 10.1038/nri1708
30. Dubrot J, Duraes FV, Harle G, Schlaeppi A, Brighouse D, Madelon N, et al. Absence of MHC-II expression by lymph node stromal cells results in autoimmunity. *Life Sci Alliance.* (2018) 1:e201800164. doi: 10.26508/lsa.201800164
31. Grigorova IL, Schwab SR, Phan TG, Pham TH, Okada T, Cyster JG. Cortical sinus probing, S1P1-dependent entry and flow-based capture of egressing T cells. *Nat Immunol.* (2009) 10:58–65. doi: 10.1038/ni.1682
32. Pham TH, Baluk P, Xu Y, Grigorova I, Bankovich AJ, Pappu R, et al. Lymphatic endothelial cell sphingosine kinase activity is required for lymphocyte egress and lymphatic patterning. *J Exp Med.* (2010) 207:17–27. doi: 10.1084/jem.20091619
33. Mendoza A, Fang V, Chen C, Serasinghe M, Verma A, Muller J, et al. Lymphatic endothelial S1P promotes mitochondrial function and survival in naive T cells. *Nature.* (2017) 546:158–61. doi: 10.1038/nature22352
34. Lukacs-Kornek V, Malhotra D, Fletcher AL, Acton SE, Elpek KG, Tayalia P, et al. Regulated release of nitric oxide by nonhematopoietic stroma controls expansion of the activated T cell pool in lymph nodes. *Nat Immunol.* (2011) 12:1096–104. doi: 10.1038/ni.2112
35. Podgrabinska S, Kamalu O, Mayer L, Shimaoka M, Snoeck H, Randolph GJ, et al. Inflamed lymphatic endothelium suppresses dendritic cell maturation and function via Mac-1/ICAM-1-dependent mechanism. *J Immunol.* (2009) 183:1767–79. doi: 10.4049/jimmunol.0802167
36. Christiansen AJ, Dieterich LC, Ohs I, Bachmann SB, Bianchi R, Proulx ST, et al. Lymphatic endothelial cells attenuate inflammation via suppression of dendritic cell maturation. *Oncotarget.* (2016) 7:39421–35. doi: 10.18632/oncotarget.9820
37. Liotta LA. Cancer cell invasion and metastasis. *Sci Am.* (1992) 266:54–9:62–3.
38. Ma Q, Dieterich LC, Ikenberg K, Bachmann SB, Mangana J, Proulx ST, et al. Unexpected contribution of lymphatic vessels to promotion of distant metastatic tumor spread. *Sci Adv.* (2018) 4:eaat4758. doi: 10.1126/sciadv.aat4758
39. Lund AW, Wagner M, Fankhauser M, Steinskog ES, Broggi MA, Spranger S, et al. Lymphatic vessels regulate immune microenvironments in human and murine melanoma. *J Clin Invest.* (2016) 126:3389–402. doi: 10.1172/JCI79434
40. Mlecnik B, Bindea G, Kirilovsky A, Angell HK, Obenauf AC, Tosolini M, et al. The tumor microenvironment and Immunoscore are critical determinants of dissemination to distant metastasis. *Sci Transl Med.* (2016) 8:327ra26. doi: 10.1126/scitranslmed.aad6352
41. Dieterich LC, Detmar M. Tumor lymphangiogenesis and new drug development. *Adv Drug Delivery Rev.* (2016) 99:148–60. doi: 10.1016/j.addr.2015.12.011
42. Gogineni A, Caunt M, Crow A, Lee CV, Fuh G, van Bruggen N, et al. Inhibition of VEGF-C modulates distal lymphatic remodeling and secondary metastasis. *PLoS ONE.* (2013) 8:e68755. doi: 10.1371/journal.pone.0068755
43. Burton JB, Priceman SJ, Sung JL, Brakenhielm E, An DS, Pytowski B, et al. Suppression of prostate cancer nodal and systemic metastasis by blockade of the lymphangiogenic axis. *Cancer Res.* (2008) 68:7828–37. doi: 10.1158/0008-5472.CAN-08-1488
44. Lin J, Lalani AS, Harding TC, Gonzalez M, Wu WW, Luan B, et al. Inhibition of lymphogenous metastasis using adeno-associated virus-mediated gene transfer of a soluble VEGFR-3 decoy receptor. *Cancer Res.* (2005) 65:6901–9. doi: 10.1158/0008-5472.CAN-05-0408
45. Roberts N, Kloos B, Cassella M, Podgrabinska S, Persaud K, Wu Y, et al. Inhibition of VEGFR-3 activation with the antagonistic antibody more potently suppresses lymph node and distant metastases than inactivation of VEGFR-2. *Cancer Res.* (2006) 66:2650–7. doi: 10.1158/0008-5472.CAN-05-1843
46. Stacker SA, Caesar C, Baldwin ME, Thornton GE, Williams RA, Prevo R, et al. VEGF-D promotes the metastatic spread of tumor cells via the lymphatics. *Nat Med.* (2001) 7:186–91. doi: 10.1038/84635
47. Skobe M, Hawighorst T, Jackson DG, Prevo R, Janes L, Velasco P, et al. Induction of tumor lymphangiogenesis by VEGF-C promotes breast cancer metastasis. *Nat Med.* (2001) 7:192–8. doi: 10.1038/84643
48. Mandriota SJ, Jussila L, Jeltsch M, Compagni A, Baetens D, Prevo R, et al. Vascular endothelial growth factor-C-mediated lymphangiogenesis promotes tumour metastasis. *EMBO J.* (2001) 20:672–82. doi: 10.1093/emboj/20.4.672
49. Hoshida T, Isaka N, Hagendoorn J, di Tomaso E, Chen YL, Pytowski B, et al. Imaging steps of lymphatic metastasis reveals that vascular endothelial growth factor-C increases metastasis by increasing delivery of cancer cells to lymph nodes: therapeutic implications. *Cancer Res.* (2006) 66:8065–75. doi: 10.1158/0008-5472.CAN-06-1392
50. Saif MW, Knost JA, Chiorean EG, Kambhampati SR, Yu D, Pytowski B, et al. Phase 1 study of the anti-vascular endothelial growth factor receptor 3 monoclonal antibody LY3022856/IMC-3C5 in patients with advanced and refractory solid tumors and advanced colorectal cancer. *Cancer Chemother Pharmacol.* (2016) 78:815–24. doi: 10.1007/s00280-016-3134-3

51. Antila S, Karaman S, Nurmi H, Airavaara M, Voutilainen MH, Mathivet T, et al. Development and plasticity of meningeal lymphatic vessels. *J Exp Med*. (2017) 214:3645–67. doi: 10.1084/jem.20170391
52. Nurmi H, Saharinen P, Zarkada G, Zheng W, Robciuc MR, Alitalo K. VEGF-C is required for intestinal lymphatic vessel maintenance and lipid absorption. *EMBO Mol Med*. (2015) 7:1418–25. doi: 10.15252/emmm.201505731
53. Makinen T, Veikkola T, Mustjoki S, Karpanen T, Catimel B, Nice EC, et al. Isolated lymphatic endothelial cells transduce growth, survival and migratory signals via the VEGF-C/D receptor VEGFR-3. *EMBO J*. (2001) 20:4762–73. doi: 10.1093/emboj/20.17.4762
54. Veikkola T, Jussila L, Makinen T, Karpanen T, Jeltsch M, Petrova TV, et al. Signalling via vascular endothelial growth factor receptor-3 is sufficient for lymphangiogenesis in transgenic mice. *EMBO J*. (2001) 20:1223–31. doi: 10.1093/emboj/20.6.1223
55. Tammela T, Alitalo K. Lymphangiogenesis: molecular mechanisms and future promise. *Cell*. (2010) 140:460–76. doi: 10.1016/j.cell.2010.01.045
56. Hirakawa S, Brown LF, Kodama S, Paavonen K, Alitalo K, Detmar M. VEGF-C-induced lymphangiogenesis in sentinel lymph nodes promotes tumor metastasis to distant sites. *Blood*. (2007) 109:1010–7. doi: 10.1182/blood-2006-05-021758
57. Das S, Ladell DS, Podgrabinska S, Ponomarev V, Nagi C, Fallon JT, et al. Vascular endothelial growth factor-C induces lymphangitic carcinomatosis, an extremely aggressive form of lung metastases. *Cancer Res*. (2010) 70:1814–24. doi: 10.1158/0008-5472.CAN-09-3675
58. Bagri A, Tessier-Lavigne M, Watts RJ. Neuropilins in tumor biology. *Clin Cancer Res*. (2009) 15:1860–4. doi: 10.1158/1078-0432.CCR-08-0563
59. Xu Y, Yuan L, Mak J, Pardanaud L, Caunt M, Kasman I, et al. Neuropilin-2 mediates VEGF-C-induced lymphatic sprouting together with VEGFR3. *J Cell Biol*. (2010) 188:115–30. doi: 10.1083/jcb.200903137
60. Siironen P, Ristimaki A, Narko K, Nordling S, Louhimo J, Andersson S, et al. VEGF-C and COX-2 expression in papillary thyroid cancer. *Endocr Relat Cancer*. (2006) 13:465–73. doi: 10.1677/erc.1.01114
61. Lyons TR, Borges VF, Betts CB, Guo Q, Kapoor P, Martinson HA, et al. Cyclooxygenase-2-dependent lymphangiogenesis promotes nodal metastasis of postpartum breast cancer. *J Clin Invest*. (2014) 124:3901–12. doi: 10.1172/JCI73777
62. Timoshenko AV, Chakraborty C, Wagner GF, Lala PK. COX-2-mediated stimulation of the lymphangiogenic factor VEGF-C in human breast cancer. *Br J Cancer*. (2006) 94:1154–63. doi: 10.1038/sj.bjc.6603067
63. Soumaoro LT, Uetake H, Takagi Y, Iida S, Higuchi T, Yasuno M, et al. Coexpression of VEGF-C and Cox-2 in human colorectal cancer and its association with lymph node metastasis. *Dis Colon Rectum*. (2006) 49:392–8. doi: 10.1007/s10350-005-0247-x
64. Giovannucci E. The prevention of colorectal cancer by aspirin use. *Biomed Pharmacother*. (1999) 53:303–8. doi: 10.1016/S0753-3322(00)88500-5
65. Rothwell PM, Fowkes FG, Belch JF, Ogawa H, Warlow CP, Meade TW. Effect of daily aspirin on long-term risk of death due to cancer: analysis of individual patient data from randomised trials. *Lancet*. (2011) 377:31–41. doi: 10.1016/S0140-6736(10)62110-1
66. Rothwell PM, Wilson M, Price JF, Belch JF, Meade TW, Mehta Z. Effect of daily aspirin on risk of cancer metastasis: a study of incident cancers during randomised controlled trials. *Lancet*. (2012) 379:1591–601. doi: 10.1016/S0140-6736(12)60209-8
67. Karnezis T, Shayan R, Caesar C, Roufail S, Harris NC, Ardipradja K, et al. VEGF-D promotes tumor metastasis by regulating prostaglandins produced by the collecting lymphatic endothelium. *Cancer Cell*. (2012) 21:181–95. doi: 10.1016/j.ccr.2011.12.026
68. Kubo H, Hosono K, Suzuki T, Ogawa Y, Kato H, Kamata H, et al. Host prostaglandin EP3 receptor signaling relevant to tumor-associated lymphangiogenesis. *Biomed Pharmacother*. (2010) 64:101–6. doi: 10.1016/j.biopha.2009.04.039
69. Ji H, Cao R, Yang Y, Zhang Y, Iwamoto H, Lim S, et al. TNFR1 mediates TNF-alpha-induced tumour lymphangiogenesis and metastasis by modulating VEGF-C-VEGFR3 signalling. *Nat Commun*. (2014) 5:4944. doi: 10.1038/ncomms5944
70. Cao R, Ji H, Feng N, Zhang Y, Yang X, Andersson P, et al. Collaborative interplay between FGF-2 and VEGF-C promotes lymphangiogenesis and metastasis. *Proc Natl Acad Sci USA*. (2012) 109:15894–9. doi: 10.1073/pnas.1208324109
71. Cao R, Bjorndahl MA, Religa P, Clasper S, Garvin S, Galter D, et al. PDGF-BB induces intratumoral lymphangiogenesis and promotes lymphatic metastasis. *Cancer Cell*. (2004) 6:333–45. doi: 10.1016/j.ccr.2004.08.034
72. Fagiani E, Lorentz P, Kopfstein L, Christofori G. Angiopoietin-1 and−2 exert antagonistic functions in tumor angiogenesis, yet both induce lymphangiogenesis. *Cancer Res*. (2011) 71:5717–27. doi: 10.1158/0008-5472.CAN-10-4635
73. Gore J, Imasuen-Williams IE, Conteh AM, Craven KE, Cheng M, Korc M. Combined targeting of TGF-beta, EGFR and HER2 suppresses lymphangiogenesis and metastasis in a pancreatic cancer model. *Cancer Lett*. (2016) 379:143–53. doi: 10.1016/j.canlet.2016.05.037
74. Liu D, Li L, Zhang XX, Wan DY, Xi BX, Hu Z, et al. SIX1 promotes tumor lymphangiogenesis by coordinating TGFbeta signals that increase expression of VEGF-C. *Cancer Res*. (2014) 74:5597–607. doi: 10.1158/0008-5472.CAN-13-3598
75. Oka M, Iwata C, Suzuki HI, Kiyono K, Morishita Y, Watabe T, et al. Inhibition of endogenous TGF-beta signaling enhances lymphangiogenesis. *Blood*. (2008) 111:4571–9. doi: 10.1182/blood-2007-10-120337
76. Harrell MI, Iritani BM, Ruddell A. Tumor-induced sentinel lymph node lymphangiogenesis and increased lymph flow precede melanoma metastasis. *Am J Pathol*. (2007) 170:774–86. doi: 10.2353/ajpath.2007.060761
77. Hirakawa S, Kodama S, Kunstfeld R, Kajiya K, Brown LF, Detmar M. VEGF-A induces tumor and sentinel lymph node lymphangiogenesis and promotes lymphatic metastasis. *J Exp Med*. (2005) 201:1089–99. doi: 10.1084/jem.20041896
78. Qian CN, Berghuis B, Tsarfaty G, Bruch M, Kort EJ, Ditlev J, et al. Preparing the "soil": the primary tumor induces vasculature reorganization in the sentinel lymph node before the arrival of metastatic cancer cells. *Cancer Res*. (2006) 66:10365–76. doi: 10.1158/0008-5472.CAN-06-2977
79. Kim M, Koh YJ, Kim KE, Koh BI, Nam DH, Alitalo K, et al. CXCR4 signaling regulates metastasis of chemoresistant melanoma cells by a lymphatic metastatic niche. *Cancer Res*. (2010) 70:10411–21. doi: 10.1158/0008-5472.CAN-10-2591
80. Issa A, Le TX, Shoushtari AN, Shields JD, Swartz MA. Vascular endothelial growth factor-C and C-C chemokine receptor 7 in tumor cell-lymphatic cross-talk promote invasive phenotype. *Cancer Res*. (2009) 69:349–57. doi: 10.1158/0008-5472.CAN-08-1875
81. Garmy-Susini B, Avraamides CJ, Schmid MC, Foubert P, Ellies LG, Barnes L, et al. Integrin alpha4beta1 signaling is required for lymphangiogenesis and tumor metastasis. *Cancer Res*. (2010) 70:3042–51. doi: 10.1158/0008-5472.CAN-09-3761
82. Garmy-Susini B, Avraamides CJ, Desgrosellier JS, Schmid MC, Foubert P, Ellies LG, et al. PI3Kalpha activates integrin alpha4beta1 to establish a metastatic niche in lymph nodes. *Proc Natl Acad Sci USA*. (2013) 110:9042–7. doi: 10.1073/pnas.1219603110
83. Irshad S, Flores-Borja F, Lawler K, Monypenny J, Evans R, Male V, et al. RORgammat(+) innate lymphoid cells promote lymph node metastasis of breast cancers. *Cancer Res*. (2017) 77:1083–96. doi: 10.1158/0008-5472.CAN-16-0598
84. Miteva DO, Rutkowski JM, Dixon JB, Kilarski W, Shields JD, Swartz MA. Transmural flow modulates cell and fluid transport functions of lymphatic endothelium. *Circ Res*. (2010) 106:920–31. doi: 10.1161/CIRCRESAHA.109.207274
85. Shields JD, Fleury ME, Yong C, Tomei AA, Randolph GJ, Swartz MA. Autologous chemotaxis as a mechanism of tumor cell homing to lymphatics via interstitial flow and autocrine CCR7 signaling. *Cancer Cell*. (2007) 11:526–38. doi: 10.1016/j.ccr.2007.04.020
86. Gunther K, Leier J, Henning G, Dimmler A, Weissbach R, Hohenberger W, et al. Prediction of lymph node metastasis in colorectal carcinoma by expressionof chemokine receptor CCR7. *Int J Cancer*. (2005) 116:726–33. doi: 10.1002/ijc.21123
87. Cabioglu N, Yazici MS, Arun B, Broglio KR, Hortobagyi GN, Price JE, et al. CCR7 and CXCR4 as novel biomarkers predicting axillary lymph node metastasis in T1 breast cancer. *Clin Cancer Res*. (2005) 11:5686–93. doi: 10.1158/1078-0432.CCR-05-0014

88. Mashino K, Sadanaga N, Yamaguchi H, Tanaka F, Ohta M, Shibuta K, et al. Expression of chemokine receptor CCR7 is associated with lymph node metastasis of gastric carcinoma. *Cancer Res*. (2002) 62:2937–41.
89. Das S, Sarrou E, Podgrabinska S, Cassella M, Mungamuri SK, Feirt N, et al. Tumor cell entry into the lymph node is controlled by CCL1 chemokine expressed by lymph node lymphatic sinuses. *J Exp Med*. (2013) 210:1509–28. doi: 10.1084/jem.20111627
90. van der Weyden L, Arends MJ, Campbell AD, Bald T, Wardle-Jones H, Griggs N, et al. Genome-wide *in vivo* screen identifies novel host regulators of metastatic colonization. *Nature*. (2017) 541:233–6. doi: 10.1038/nature20792
91. Black SA, Nelson AC, Gurule NJ, Futscher BW, Lyons TR. Semaphorin 7a exerts pleiotropic effects to promote breast tumor progression. *Oncogene*. (2016) 35:5170–8. doi: 10.1038/onc.2016.49
92. Elder AM, Tamburini BAJ, Crump LS, Black SA, Wessells VM, Schedin PJ, et al. Semaphorin 7A promotes macrophage-mediated lymphatic remodeling during postpartum mammary gland involution and in breast cancer. *Cancer Res*. (2018) 78:6473–85. doi: 10.1158/0008-5472.CAN-18-1642
93. Swartz MA, Lund AW. Lymphatic and interstitial flow in the tumour microenvironment: linking mechanobiology with immunity. Nature reviews. *Cancer*. (2012) 12:210–9. doi: 10.1038/nrc3186
94. Kimura T, Sugaya M, Oka T, Blauvelt A, Okochi H, Sato S. Lymphatic dysfunction attenuates tumor immunity through impaired antigen presentation. *Oncotarget*. (2015) 6:18081–93. doi: 10.18632/oncotarget.4018
95. Galon J, Costes A, Sanchez-Cabo F, Kirilovsky A, Mlecnik B, Lagorce-Pages C, et al. Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. *Science*. (2006) 313:1960–4. doi: 10.1126/science.1129139
96. Fridman WH, Pages F, Sautes-Fridman C, Galon J. The immune contexture in human tumours: impact on clinical outcome. Nature reviews. *Cancer*. (2012) 12:298–306. doi: 10.1038/nrc3245
97. Peske JD, Thompson ED, Gemta L, Baylis RA, Fu YX, Engelhard VH. Effector lymphocyte-induced lymph node-like vasculature enables naive T-cell entry into tumours and enhanced anti-tumour immunity. *Nat Commun*. (2015) 6:7114. doi: 10.1038/ncomms8114
98. Thompson ED, Enriquez HL, Fu YX, Engelhard VH. Tumor masses support naive T cell infiltration, activation, and differentiation into effectors. *J Exp Med*. (2010) 207:1791–804. doi: 10.1084/jem.20092454
99. Yu P, Lee Y, Liu W, Chin RK, Wang J, Wang Y, et al. Priming of naive T cells inside tumors leads to eradication of established tumors. *Nat Immunol*. (2004) 5:141–9. doi: 10.1038/ni1029
100. Roberts EW, Broz ML, Binnewies M, Headley MB, Nelson AE, Wolf DM, et al. Critical role for CD103(+)/CD141(+) dendritic cells bearing CCR7 for tumor antigen trafficking and priming of T cell immunity in melanoma. *Cancer Cell*. (2016) 30:324–36. doi: 10.1016/j.ccell.2016.06.003
101. Russo E, Teijeira A, Vaahtomeri K, Willrodt AH, Bloch JS, Nitschke M, et al. Intralymphatic CCL21 promotes tissue egress of dendritic cells through afferent lymphatic vessels. *Cell Rep*. (2016) 14:1723–34. doi: 10.1016/j.celrep.2016.01.048
102. Teijeira A, Rouzaut A, Melero I. Initial afferent lymphatic vessels controlling outbound leukocyte traffic from skin to lymph nodes. *Front Immunol*. (2013) 4:433. doi: 10.3389/fimmu.2013.00433
103. Acton SE, Astarita JL, Malhotra D, Lukacs-Kornek V, Franz B, Hess PR, et al. Podoplanin-rich stromal networks induce dendritic cell motility via activation of the C-type lectin receptor CLEC-2. *Immunity*. (2012) 37:276–89. doi: 10.1016/j.immuni.2012.05.022
104. Bordry N, Broggi MAS, de Jonge K, Schaeuble K, Gannon PO, Foukas PG, et al. Lymphatic vessel density is associated with CD8(+) T cell infiltration and immunosuppressive factors in human melanoma. *Oncoimmunology*. (2018) 7:e1462878. doi: 10.1080/2162402X.2018.1462878
105. Baitsch L, Baumgaertner P, Devevre E, Raghav SK, Legat A, Barba L, et al. Exhaustion of tumor-specific CD8(+) T cells in metastases from melanoma patients. *J Clin Invest*. (2011) 121:2350–60. doi: 10.1172/JCI46102
106. Shields JD, Kourtis IC, Tomei AA, Roberts JM, Swartz MA. Induction of lymphoidlike stroma and immune escape by tumors that express the chemokine CCL21. *Science*. (2010) 328:749–52. doi: 10.1126/science.1185837
107. Riedel A, Shorthouse D, Haas L, Hall BA, Shields J. Tumor-induced stromal reprogramming drives lymph node transformation. *Nat Immunol*. (2016) 17:1118–27. doi: 10.1038/ni.3492

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REVIEW
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The Interplay Between Lymphatic Vessels and Chemokines

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Chemokines are a family of small protein cytokines that act as chemoattractants to migrating cells, in particular those of the immune system. They are categorized functionally as either homeostatic, constitutively produced by tissues for basal levels of cell migration, or inflammatory, where they are generated in association with a pathological inflammatory response. While the extravasation of leukocytes via blood vessels is a key step in cells entering the tissues, the lymphatic vessels also serve as a conduit for cells that are recruited and localized through chemoattractant gradients. Furthermore, the growth and remodeling of lymphatic vessels in pathologies is influenced by chemokines and their receptors expressed by lymphatic endothelial cells (LECs) in and around the pathological tissue. In this review we summarize the diverse role played by specific chemokines and their receptors in shaping the interaction of lymphatic vessels, immune cells, and other pathological cell types in physiology and disease.

Keywords: chemokine, lymphatics, endothelial, chemokine receptor, lymphangiogenesis, lymphatic remodeling

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INTRODUCTION

Cells in complex vertebrates receive signals from extracellular environment which coordinate a raft of important cellular programs and functions (1). These signals can be through direct cell-to-cell contact or by the use of soluble molecules synthesized and secreted by neighboring or distant cells. Growth factors and cytokines are examples of soluble proteins that have potent cellular effects through designated cell surface receptors, such as growth and differentiation (2). A subset of the cytokine proteins that act to induce the movement of cells are the chemokines (*-kinos* from the Greek for movement) (3). Chemokines are small, highly conserved polypeptides of 70–100 amino acids. While having a conserved three-stranded β-sheet/α-helix tertiary structure they are divided into several subfamilies (CXC, CC, XC, and CX3C) based on variations in their quaternary structure and critical cysteine residues (4, 5). They exert their effects through cell surface G-protein coupled receptors on target cells (4) that can act as homo- or heterodimers depending on the context. This family has now expanded to include at least 51 chemokines and 20 receptors, plus (presently) four atypical or decoy receptors which typically dampen chemokine activity by binding and internalizing chemokines without initiating G-protein-dependent signaling (5–7).

Chemokines act by establishing gradients to direct random or directed migration of cells bearing cognate receptors from lower to higher concentrations of ligands. These gradients are often formed through the interaction with proteoglycans attached to the cell surface or extracellular matrix. Diversity within the chemokine system is generated both structurally and functionally through an array of different receptors and ligands with precise or promiscuous binding affinity, where splice variants, post-translational modifications including nitrosylation, citullination, and many forms of proteolytic cleavage (8) can all diversify signaling leading to events that are either chemoattractive or chemorepulsive (5, 9, 10). The biological effects of the chemokine family are broad-ranging as they can be used to move individual cells, subsets of cells or large groups of cells in order to achieve the outcomes of significant processes such as immune cell development, embryogenesis, angiogenesis, phagocytosis and survival/apoptosis (5). Expand this to controlling these cell population during infection, immunity, inflammation, and other pathologies and the extensive roles of chemokines in the mammal is clear.

The movement of cells in normal and pathological situations is highly dependent on the circulatory system, which allows long and short range transport, and exit and entry from all tissues. Previous studies have shown the critical role of blood vessels in chemokine action, in particular directing key cellular effectors of the immune response (11). Blood and lymphatic vessels work together to control fluid and cells in the circulation and tissues, yet the blood vessels have often received the most attention. However, the important and independent roles the lymphatics play in cellular interactions in normal physiology, development, and pathology are becoming evident through studies in a number of areas highlighting the organ- and subtype- specific activity of lymphatic vessels (12–14).

Lymphatic vessels have gained a greater prominence in our thinking over the past two decades as molecular tools have facilitated clear discrimination from blood vessels (15–17). Further, the characterization of factors required for growth and differentiation of lymphatic endothelial cells (LECs) *in vitro* has provided a more in-depth understanding of their unique biological function and differences to blood vascular endothelium (18). Extensive *in vivo* studies using promoters with specificity to the lymphatic compartment has also identified key functional roles for the lymphatics and LECs in development and disease (14, 19), and other functional screens have highlighted the unique features of LECs (20, 21). These unique responses of lymphatic vessels are often regulated through the interaction of cells and signaling molecules with the LECs lining the lumens of lymphatic vessels.

The paradigm of chemokine action involving the lymphatics is potentially complex. The lymphatics can both be the source of the chemokines, express the receptors, or both (**Figure 1**; **Table 1**). As a vessel for the passage of many circulating cells lymphatics also act as a conduit allowing the flow of chemokines or cells to other targets; for instance to lymph nodes (LNs). Akin to the action of chemokines in blood vessel function the lymphatics provide a surface for the attraction and interaction of immune cells in pathological contexts (3, 63, 64). This review aims to highlight the interplay between lymphatic vessels and chemokines in a range of biological contexts from embryonic development through to regulation of immunity and a range of human pathologies.

Abbreviations: BEC, Blood vascular endothelial cell; DC, Dendritic cell; LEC, Lymphatic endothelial cell; LN, Lymph node; LTi, Lymphoid tissue initiator; LTo, Lymphoid tissue organizer; SCS, Subcapsular sinus (of lymph node).

DEVELOPMENT

Regulation of Lymph Node Organogenesis

In accordance with their function in providing immune surveillance for particular organs or regions of tissue, LNs develop at strategic locations along the vasculature, typically at the branch points of large veins (25, 65). Although the mechanisms controlling the precise location and subsequent assembly of LNs are incompletely understood, recent evidence suggests multiple roles for lymphatics as well as the venous vasculature.

Chemokines are already known to be critical for initiating the development of LNs and other secondary lymphoid organs such as the Peyer's patches in the gut (25). Mice lacking CXCL13 or its receptor CXCR5 fail to develop particular subsets of peripheral LNs and also exhibit impaired Peyer's patch formation (66, 67). Combined deficiency of CXCR5 and CCR7 completely ablated the formation of peripheral LNs (68), although interestingly deficiency of CCR7 alone had only a mild impact (68, 69). In the prevailing model of LN development, lymphoid tissue organizer (LTo) cells—cells of mesenchymal origin induced by neuronally-derived retinoic acid signaling (70) at putative sites of LN development—secrete the chemokines CXCL13, CCL19, and CCL21 which in turn recruit CXCR5- and CCR7-expressing haematopoietic lymphoid tissue initiating (LTi) cells into the growing LN anlage (25, 65, 68). LTi cells extravasate from veins at junctions where smooth muscle coverage is sparse (71) and make contact with LTo cells, whereupon a positive feedback loop ensues: activation of IL-7Rα on LTi cells by LTo-expressed IL-7 upregulates lymphotoxin expression by LTi cells, which in turn promotes further chemokine secretion by LTo cells (66, 72). These reciprocal interactions lead to the expansion of both cell populations, growth of the LN anlage and subsequent differentiation of lymphoid organ subcompartments (25, 65). However, the persistence of LN formation when LTβR signaling was specifically ablated in CXCL13 or CXCL19-expressing mesenchymal cells (73, 74) suggested that additional LTo cell types may exist.

The contribution of lymphatics to LN organogenesis was previously unclear—LN development is still initiated in mice that lack lymphatics due to global or Tie2 promoter-restricted *Prox1* knockout, although the anlagen in these animals show defects in the differentiation and organization of mesenchymal LTo cells, and are often reduced in size (75). Recent studies utilizing cell type-specific gene knockout approaches have now elaborated multiple roles for lymphatics in LN formation. Lymphatic vessels contribute to early LN initiation by delivering recirculating LTi cells from peripheral tissue to the LN anlage

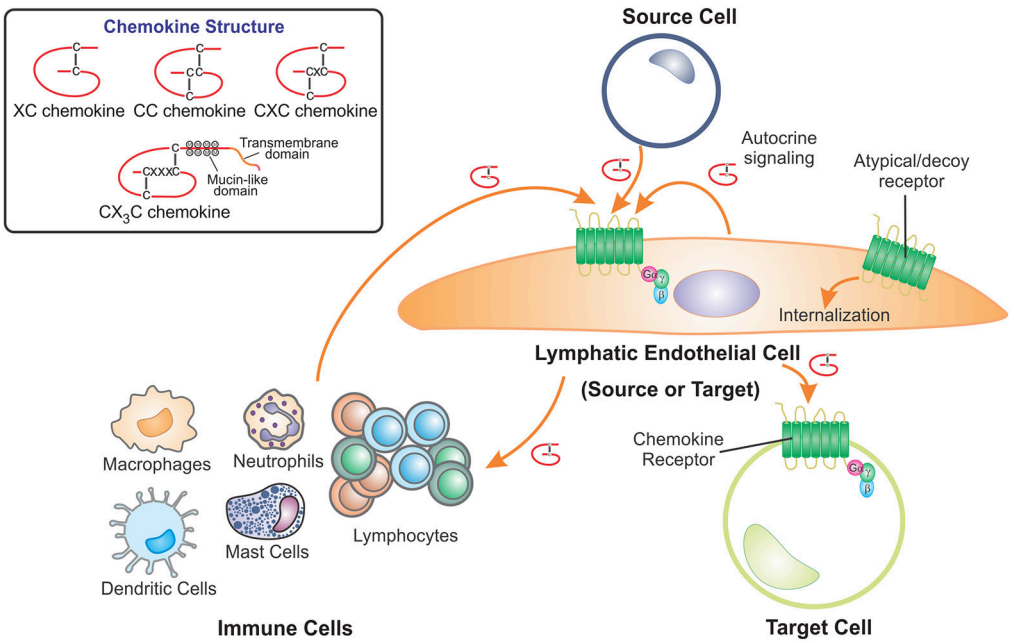

FIGURE 1 | LECs contribute to the role of chemokines by acting as either the source or target cell. Chemokines are a structurally related family of cytokines that direct cell movement and are classified as XC, CC, CXC, or CX_3C chemokines depending on the positions of conserved cysteine residues which form disulfide bridges. Lymphatic endothelial cells (LECs) receive chemokine signals, using cell surface G protein-coupled receptors, from source cells which include cells of the immune system, other LECs, fibroblasts and pathological cells types such as cancer cells. LECs also act as the source of chemokines, secreting these proteins to act on chemokine receptors present on other target cells. Furthermore, LECs can regulate chemokine availability by scavenging and internalizing secreted chemokines via atypical or decoy chemokine receptors.

through CCL21/CCR7-mediated chemotaxis (71, 74). The same sphingosine-1-phosphate (S1P) signaling that regulates lymphocyte egress in adult LNs retains LTi cells at the LN anlage, potentiating the molecular crosstalk between LTo and LTi cells that results in further chemokine-mediated LTi recruitment and subsequent LN maturation (25, 74). Peripheral LN anlagen typically form near major venous junctions which run parallel with collecting lymphatic vessels. LECs within the collecting vessel adjacent to the accumulation of LTi and LTo cells subsequently proliferate in a VEGF-C/VEGFR-3-dependent manner to form a disc which eventually expands to envelop the growing LN (71, 76). Functional lymph flow also appears to be essential for complete LN formation as it generates interstitial fluid force which likely stimulates CXCL13 expression by fibroblastic LTo cells (71). Notably, many of the cellular mechanisms and signaling pathways (including chemokines) involved in LN organogenesis are recapitulated in the development of tertiary lymphoid organs in response to pathological insult (25). This suggests that enhanced lymphangiogenesis and lymph flow may contribute to the *de novo* development of lymphoid organs in order to strengthen local immune responses, and that this mechanism may be therapeutically manipulable (25).

Regulation of Lymphatic Vascular Patterning

Where chemokines mediate interactions between the blood and lymphatic vasculature and other cell types, the endothelial cells lining these vessels are commonly characterized as the source of the chemokine ligand, or the surface to which it binds. However, endothelial cells themselves also express chemokine receptors and can respond to chemokine gradients generated by other cell types. As such, a growing list of chemokines and their receptors have been implicated in directing the growth and patterning of blood and lymphatic vasculature.

CXCR4 and its ligand CXCL12 have well-described roles in promoting angiogenesis and patterning the embryonic vasculature (77–81). Recently their role in patterning the lymphatic vasculature has also been described (45). In zebrafish, expression of *cxcr4a* and *cxcr4b* was detected in lymphatic progenitors sprouting from the posterior cardinal vein, as well as in the developing parachordal line, intersegmental lymphatic vessels, and other large trunk lymphatics such as the thoracic duct (45). Loss- and gain- of function experiments confirmed that these receptors were required for the development of the large trunk lymphatics. Accordingly, dynamically regulated expression of ligand-encoding genes *cxcl12a* and *cxcl12b* in the dorsal aorta and arterial intersomitic vessels directed the parallel migration of the growing lymphatic vessels along these paths (45). Interestingly, another group has shown that upregulation of *cxcl12a* was mediated by the microRNA miR-126, which also synergises with Flt4 (VEGFR-3) signaling (82).

Atypical or decoy chemokine receptors also play important roles in shaping developmental lymphangiogenesis. Mice deficient in ACKR3 (formerly known as CXCR7) exhibit defects in lymphatic development, typified by precocious development

TABLE 1 | Chemokine-mediated cellular interactions with LECs.

CHEMOKINE LIGANDS EXPRESSED BY LECs				
Ligand	**Receptor**	**Chemokine target cells**	**Biological context**	**References**
CCL21	CCR7	Activated DCs, T lymphocytes, neutrophils	Immune cell trafficking into initial lymphatics and to/within LNs	(22–24)
		LTi cells	Lymphoid organogenesis	(25)
		Tumor cells (various)	Lymphogenous tumor metastasis	(11, 26)
CCL27	CCR10	Skin-homing T cells, LEC subset	T cell trafficking in precollecting lymphatics	(27)
CXCL10	CXCR3	Macrophages	Upregulated in type 2 diabetes	(28)
		Tumor cells (Colorectal, melanoma)	LN metastasis	(29, 30)
CCL20	CCR6	DCs, T and B cell subsets	Immune cell trafficking to LN	(31, 32)
CXCL12	CXCR4	DCs	Immune cell trafficking to LN	(33)
		Tumor cells (various)	LN metastasis	(34–37)
CXCL1	CXCR2	Tumor cells (Gastric)	Invasion and metastasis	(38, 39)
CCL2	CCR2	Macrophages	Developmental lymphangiogenesis	(40)
CX3CL1	CX3CR1	DCs	Immune cell trafficking to LN	(41)
CCL5	CCR5	Tumor cells (Breast)	Metastatic niche formation, metastasis	(42)
CCL1	CCR8	Tumor cells (Melanoma)	LN metastasis	(43)
CHEMOKINE RECEPTORS EXPRESSED BY LECs				
Receptor	**Ligand/s**	**Chemokine source cells**	**Biological context**	**References**
CCR10	CCL27	Keratinocytes, tumor cells	LEC migration in tumor lymphangiogenesis, lymphatic patterning	(44)
	CCL28	Mucosal epithelia, tumor cells	LEC migration *in vitro* and *in vivo*	(44)
CXCR4	CXCL12	Embryonic arteries	Developmental lymphatic patterning	(45)
		Tumor cells and stroma	Tumor lymphangiogenesis, lymphogenous metastasis	(46)
CXCR2	CXCL5	Melanoma cells	Tumor lymphangiogenesis, lymphogenous metastasis	(47)
	CXCL1	Gastric cancer LECs	Tumor lymphangiogenesis, lymphogenous metastasis	(38)
	CXCL8	Overexpressed	Experimental lymphedema	(48)
ATYPICAL CHEMOKINE RECEPTORS EXPRESSED BY LECs				
Receptor	**Ligand/s[a]**	**Chemokine source cells**	**Biological context of receptor**	**References**
ACKR1 (DARC)	CCL2, CCL5, CCL7, CCL11, CCL13, CCL14, CCL17, CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, CXCL8, CXCL11	Various	Precollecting LECs	(27)
ACKR2 (D6, CCBP2)	CCL2, CCL3, CCL3L1, CCL4, CCL4L1, CCL5, CCL7, CCL8, CCL11, CCL12, CCL13, CCL14, CCL17, CCL22, CCL23, CCL24	Various	Afferent lymphatics in various tissues	(49–51)
			Developmental lymphatic patterning	(40)
			Immune cell trafficking in inflammation/immunity	(52, 53)
			Tumor lymphatics	(51, 54–57)
			Vascular tumors	(51)
			Kaposi sarcoma	(58)
ACKR3 (CXCR7)	CXCL11, CXCL12	Various	Increased expression during renal allograft rejection	(59)
	Adrenomedullin		Developmental lymphatic patterning	(60)
ACKR4 (CCRL1)	CCL19	Dermal stromal cells	Immune cell trafficking to LN	(61)
	CCL21	Fibroblastic reticular cells, LECs	DC trafficking into LN parenchyma	(62)
	CCL25, CXCL13	Lymphoid stromal cells	Immune cell trafficking; direct interaction with LEC-expressed ACKR4 not studied	(49)

[a] *Compiled from references (5) and (49).*

of lymph sacs and hyperplasia (60). This phenotype was found to be caused by excessive pro-proliferative signaling from adrenomedullin, a non-chemokine ligand for ACKR3 which is a positive regulator of lymphangiogenesis. Deficiency of ACKR2 (formerly D6; CCBP2, chemokine-binding protein 2) in mice results in hyper-branched lymphatics (40). ACKR2 scavenges LEC-expressed CCL2, which is chemotactic for monocytes via CCR2 signaling, thereby reducing the accumulation of macrophages in proximity to developing lymphatics (40). These macrophages deliver lymphangiogenic growth factors and play important roles in shaping developmental lymphangiogenesis (83, 84). These studies highlight the complex mechanisms by which chemokines orchestrate multiple cellular interactions within the developing embryo.

LEUKOCYTE TRAFFICKING IN INFLAMMATION AND IMMUNITY

Arguably the best-characterized chemokine-mediated functions involving the lymphatics are those that regulate trafficking of leukocytes in physiological homeostasis and during inflammation and immune responses. Leukocytes in the peripheral interstitium typically enter initial lymphatics in the first instance, and subsequently migrate through the local plexus of pre-collecting lymphatics before entering the large collecting lymphatic vessels that pump lymph and cells over long distances to LNs, where encounters between antigen-presenting cells and cognate T and B lymphocytes are coordinated (49, 85, 86).

Entry of Leukocytes Into Peripheral Lymphatic Vessels

CCL21, constitutively expressed by peripheral LECs, has a prominent role in trafficking CCR7-expressing dendritic cells (DCs) through afferent lymphatic vessels to LNs along with other CCR7-expressing cells such as T cell subsets and neutrophils (22–24). Notably, in the peripheral vasculature CCL21 expression is relatively specific to the endothelial cells of initial lymphatics; it is generally absent from blood vascular endothelial cells (BECs) with the exception of high endothelial venules in the LN (23). The elongated, positively-charged C terminus of CCL21 mediates strong binding affinity to diverse proteoglycans as well as collagen IV, allowing it to form chemotactic gradients on the surface of LECs and adjacent extracellular matrix (87). The requirement of CCL21-CCR7 interactions for trafficking immune cells through afferent lymphatics has been long recognized and has been reviewed extensively elsewhere (23, 49), but recent studies continue to shed light on the precise mechanisms and additional chemokines that are involved.

Although low levels of cellular trafficking occur under homeostatic conditions, this increases dramatically during immune responses (49, 86). Accordingly, constitutive expression of homeostatic chemokines in LECs is supplemented during inflammation by increased expression of these chemokines, along with additional "inflammatory" chemokines that shape the immune response. CCR7 is upregulated in DCs by inflammatory stimuli such as TNF-α, while the same stimuli increase CCL21 release by LECs by upregulating transcription and by releasing intracellular stores of the chemokine (88, 89). ACKR4 (previously known as CCRL1) expressed in dermal LECs and keratinocytes plays an essential role in properly directed egress of DCs from skin during inflammation by scavenging the more soluble CCR7 ligand CCL19, which would otherwise retain DCs in skin (61). LEC-expressed ACKR2 also regulates DC egress during inflammation by scavenging inflammatory chemokines to ensure preferential presentation of CCL21 on the cell surface of LECs. This in turn supports adhesion of mature CCR7+ DCs to LECs and their transport to LNs, in preference to immature DCs and inflammatory myeloid cells (52, 53). In mice lacking ACKR2, elevated presentation of inflammatory chemokines such as CCL2 on peripheral and LN LECs leads to congestion of lymphatics by myelomonocytic cells, with downstream impairment of lymphatic transport and consequently dampened antigen-specific immune responses (52). Similar roles for ACKR2 in orchestrating cell migration and resolving the inflammatory response have been described in a range of pathological contexts (50).

DC migration toward lymphatic vessels is also mediated by expression of CXCR4 in activated DCs and its ligand CXCL12 in LECs, although DCs seem to preferentially migrate toward CCL21 when both chemokines are present, indicating a coordinated rather than additive function (33). CX3CL1, an atypical chemokine possessing a transmembrane domain, is upregulated in LECs by TNF-α and mediates basolateral-to-apical migration of CX3CR1-expressing DCs through lymphatic endothelium and DC trafficking to LNs during dermal contact hypersensitivity responses *in vivo* (41). Interestingly, this chemokine is predominately shed from the basolateral surface of LECs by ADAM10 and ADAM17 metalloproteases, in contrast to remaining membrane-bound and behaving as a leukocyte adhesion molecule in BECs (41).

DCs commonly enter lymphatics through binding to immobilized CCL21 puncta specifically localized between the button-like intercellular junctions characteristic of initial lymphatics (90, 91). Direct contact between DCs and LECs also dynamically triggers localized release of CCL21 from within the trans-Golgi network of LECs, further potentiating transendothelial migration (92). Once inside lymphatic vessels, DCs crawl in a semi-directed manner within the flattened lumen, moving in multiple directions but ultimately following an intralymphatic gradient of CCL21 that is generated by lymphatic flow (91, 93). In this context, CCL21 immobilized on the LEC surface mediates not only chemotaxis but also adhesion. Although most DCs and other leukocytes exiting the periphery are thought to enter the initial lymphatics, specific chemokines may regulate entry of particular cell types into other segments of the lymphatic vasculature. CCL27 was found to be specifically expressed in pre-collecting lymphatics, where it promoted the attraction of CCR10+ T lymphocytes *in vitro* and *in vivo* (27). Pre-collecting LECs were also found to overexpress CCL27, CXCL12, CXCL14, and the promiscuous CC chemokine decoy receptor ACKR1 (formerly DARC, Duffy antigen receptor for chemokines) compared to initial LECs, whereas CCL21 was more abundantly expressed in initial LECs

(27). The same study found that pre-collecting and initial LECs in the adult human dermis could be discriminated by flow cytometry and immunofluorescence according to their expression levels of Podoplanin—pre-collecting LECs being designated as Podoplanin^{low} and initial LECs Podoplanin^{high} (27). The correspondence between Podoplanin and CCL21 expression levels between LEC subtypes may relate to the ability of the glycoprotein Podoplanin to bind and present CCL21 on the LEC cell surface (94). More remains to be understood about the specific immune cell types which are selectively recruited to initial vs. pre-collecting lymphatics, and the functional importance of these differences.

Chemokine Signaling Within Lymph Nodes

Once cells pass into collecting lymphatics the lymph flow rate accelerates, and cells are transported passively to LNs where they are delivered into the subcapsular sinus (SCS) (91, 93). Here chemokine gradients also are important in regulating migration and localization of different cell types in the LN parenchyma (49, 85). It has recently been demonstrated that the LECs comprising the LN SCS and medullary lymphatic sinuses have distinct expression profiles, including differential expression of several chemokines and receptors (95). ACKR4 is specifically expressed in LECs of the SCS "ceiling" where it plays an important role in scavenging and internalizing CCL21 to create a gradient that directs DC migration toward and ultimately through the SCS floor into the LN parenchyma (62). CCR7+ T cells arriving through afferent lymphatics have been observed to enter the LN parenchyma preferentially through medullary sinuses, but will transmigrate through the SCS floor only in conjunction with local changes induced by DCs (96). CCL21 is also produced abundantly by fibroblastic reticular cells (FRCs), which constitute the majority of the LN stroma and guide interactions between DCs and naïve T cells both structurally and chemically (97). Notably, lymphatic flow upregulates CCL21 expression by FRCs (98), reiterating the importance of the lymphatics for maintaining proper immune function in the LN microenvironment. Some DCs require additional signals to CCL21/CCR7 to access the LN parenchyma: in cutaneous allergic responses, CD301b+ DCs were found to require CCR8 signaling in response to CCL8 from interfollicular CD169+SIGN-R1+ macrophages (99). Within the LN parenchyma, chemokines from a variety of cellular sources exquisitely regulate localization of specific leukocyte subsets to coordinate effective immune responses, reviewed in detail elsewhere (49, 85).

In response to infection and inflammation, LN LECs respond robustly and dynamically. Proliferation of LECs supports the expansion of LNs during immune responses and coincides with increased expression of chemokines including CXCL9, CXCL10, CCL2, CCL5, and CCL20 (31, 32, 100) (**Table 2**). In the latter stages of inflammatory remodeling of the LN, the cortical and medullary sinuses expand substantially (31, 103). During certain infections, such as persistent infection with the helminth *Heligmosomoides polygyrus*, lymphangiogenesis driven by VEGF-A and VEGF-C from B lymphocytes results in a sustained expansion of LN LECs (104). Such changes potentially support the egress of leukocytes from the inflamed LN and the restoration of homeostasis, however may also influence lymphoid tissue functions in response to subsequent infections.

Egress of leukocytes from LNs occurs predominately via the medullary lymphatic sinuses, which channel cells into efferent collecting lymphatics (49, 86). Leukocyte retention in or egress from a LN is regulated by a balance of directional signals and changes in receptor expression (49). Prolonged signaling through CCR7 in T cells or CXCR5 in B cells leads to reduced expression or responsiveness of these receptors to their ligands expressed by LN LECs or other stromal cells, and leukocytes instead upregulate S1P receptor 1 (S1PR1), the main receptor promoting leukocyte egress (105–107). Notably, LECs have been defined to be the key cellular source of S1P regulating lymphocyte egress, as determined by conditional gene deletion of the two enzymes responsible for S1P generation (Sphk1 and Sphk2) by LYVE-1-directed expression of Cre recombinase (108).

While many of the major LEC-expressed chemokines and receptors that regulate leukocyte trafficking have been defined, many more questions remain. It is evident that different pathological stimuli elicit expression of different suites of chemokines and receptors in LECs (31, 101). This indicates a role for LECs in trafficking context-specific subsets of leukocytes to LNs, as well as interacting with other cells within the tissue microenvironment, which remains to be explored.

WOUND HEALING

Chemokine signaling plays an integral part in the healing process of various wounds including lacerations, surgical incisions, burns and skin grafts, as well as chronic diabetic and aging wounds. Wound healing is a dynamic and highly coordinated process with three distinct stages—inflammation, tissue formation, and tissue remodeling, with each stage having a distinct chemokine profile (109, 110). Inflammation is a crucial part of wound healing and despite the variety of tissue injuries that can occur, the subsequent events share a similar course (111). The aim of the initial inflammatory phase is to prevent further blood/fluid loss, protect against infection, and initiate the clearance of dead or dying cells and tissue debris (111). It begins immediately after tissue damage and is characterized by the formation of a platelet plug, the deposition of a fibrin matrix and the recruitment of neutrophils, which are the predominant inflammatory effector cells in the first 24–48 h (112). Monocytes enter the wound area 48–72 h after injury, differentiate into macrophages and play an important role in coordinating subsequent events of wound repair (111). The second stage, tissue formation, spans 2–10 days after injury, and aims to restore the barrier function of the epithelium (110, 111). Angiogenesis occurs from blood vessels at the wound edge and the newly formed capillaries, along with macrophages and fibroblasts, replace the fibrin matrix with granulation tissue, and allow for the proliferation and migration of keratinocytes across the wound surface (111, 113). In addition to angiogenesis, an adequate growth of lymphatic vessels in and around the wound zone is critical for the normal healing. Lymphangiogenesis follows angiogenesis via

TABLE 2 | Chemokines and receptors differentially expressed in physiological and disease-associated LECs.

Disease/tissue setting	Species	Ligands	Receptors	Atypical receptors	Reference
Pre-collecting LEC (podoplanin^{low}) vs. initial LEC (podoplanin^{high})	Human	↑ CCL27, CXCL12, CXCL14 ↓ CCL21	↑ ND ↓ ND	↑ ACKR1 (DARC) ↓ ND	(27)
LN subcapsular sinus vs. medullary lymphatic sinus LECs	Mouse	↑ CCL12, CXCL16, CCL25 ↓ CCL19, CCL21a, CCL21b, CXCL4 (PF4)	↑ CCR8, CXCR6 ↓ ND	↑ ACKR4 (CCRL1) ↓ ACKR1 (DARC)	(95)
Contact hypersensitivity inflamed ear skin vs. normal ear skin LECs	Mouse	↑ CXCL9, CXCL5, CXCL10, CXCL2, CCL12, CXCL14, CCL8, CCL2, CCL7, CCL9, CCL19, CXCL1, CXCL12 ↓ ND	↑ ND ↓ ND	↑ ND ↓ ND	(101)
T241/VEGF-C sarcoma vs. normal skin LECs	Mouse	↑ ND ↓ CXCL1, CXCL5	↑ ND ↓ ND	↑ ND ↓ ND	(102)
Herpes simplex virus-1 draining LN (day 6) vs. normal LN LECs	Mouse	↑ CCL21a, CCL7, CCL2, CCL5, CCL7, CCL20, CXCL9, CXCL10, CXCL13 ↓ CXCL1	↑ ND ↓ ND	↑ ACKR2 (D6, CCBP2) ↓ ND	(31)
Type 2 Diabetes vs. normal dermal LECs	Human	↑ CXCL10 ↓ CCL27, CXCL14	↑ ND ↓ ND	↑ ND ↓ ND	(28)
Afferent sentinel LN collecting lymphatic of footpad gastric tumor vs. normal	Rat	↑ CXCL14, CXCL1, CCL7 ↓ ND	↑ ND ↓ ND	↑ ND ↓ ACKR2 (D6, CCBP2)	(38)

Results derived from microarray studies of LECs isolated from in vivo settings. ↑ relatively higher, ↓ relatively lower mRNA expression in first vs. second disease/tissue setting. ND, none detected/none described.

sprouting from existing lymphatic vessels at the wound edge and is primarily stimulated by VEGF-C or VEGF-D secreted by macrophages located in the microenvironment (114–116). This facilitates the drainage of tissue edema and transport of DCs from the wound zone (114, 117–119). Macrophages also stimulate some fibroblasts to differentiate into myofibroblasts and working together with fibroblasts, a predominately type III collagen extracellular matrix is deposited and the edges of the wound are brought together over time (120, 121). The final stage of tissue remodeling begins 2–3 weeks after injury and can take over a year to complete (111). It is characterized by the progressive cessation of the inflammatory response and the remodeling of the type III collagen matrix to type I collagen (122).

Although the three phases of wound healing are distinct, the inflammatory reaction continues until tissue remodeling, albeit with changing cellular mediators of inflammation (113). Leukocytes have the dual role of acting as immunological effector cells as well as modulators of inflammation. In the acute phase, the production of proteases and reactive oxygen species aids with tissue degradation, while the secretion of growth factors in the later stages promotes tissue formation (113). Chemokines are integral in activating and recruiting leukocytes to specific microanatomical sites of the wound as well as stimulating angiogenesis (63, 109). Neutrophils, the initial responders of the acute inflammatory response, are recruited by CXCL1, CXCL5, CXCL7, and CXCL8 (formerly IL-8), secreted by activated platelets, BECs, pericytes and resident monocytes within the injured tissue (123–126). Monocyte and macrophage recruitment follows closely behind and is mediated by CCL2 secretion (63). CCL2 is also chemotactic for lymphocytes but after day 4 post-injury, CXCL9, CXCL10, and CCL22 secreted by monocytes and macrophages take over (127). The role of the CXC family of chemokines in angiogenesis is well-established (63) and the concentration of CXCL1, CXCL8, and CXCL12 in the healing wound is greatest during days 1–4 post-injury and correlates with an increasing number of blood vessels within the wound (113). The high levels of CXCL1 and CXCL8 within the wound also stimulate keratinocytes via CXCR2 to increase proliferation and migration, which enhances re-epithelialization (128, 129).

While the mechanisms of wound repair (111) and the chemokines involved (109, 113) have been extensively reviewed, the effect of these chemokines on the lymphatic vasculature and the role of chemokines secreted by the lymphatic endothelium on the healing wound are not well-established. The extent to which lymphatics will respond to chemokines secreted during the various stages of wound healing will largely depend on their expression of chemokine receptors. Like BECs, LECs express receptors CXCR1 and CXCR2, both of which are upregulated during inflammation (48, 130). As such, LECs have the potential to interact with the CXCL1, CXCL5, CXCL7, and CXCL8 chemokines that are expressed in the healing wound. Of these, CXCL1 and CXCL8 have been shown to promote lymphangiogenesis via increased LEC migration and tube formation, and additionally increased LEC proliferation in the case of CXCL8 (38, 48, 131). Interestingly, CXCR2 expressed on the surface of lymphatics acts as a scavenging receptor, capable of binding various inflammatory chemokines that can shape chemokine gradients. LEC-expressed CXCR2 is thus likely to influence the inflammatory response, and has also been shown to be important in lymphatic vessel remodeling, two key components of wound healing. For example, CXCR2 ligands, CXCL1 and CXCL2, have been shown to be elevated

during the inflammation stage of wound healing and in skin graft wounds (109, 132). LECs also have the ability to differentially secrete CXCL1 and CXCL8 depending on the local environment, which is thought to act in an autocrine and/or paracrine manner to increase lymphangiogenesis (133). However, in the context of a healing wound, LEC secretion of these chemokines may also have an endocrine effect in creating a chemokine gradient to recruit distant neutrophils. Another lymphangiogenic chemokine present in the healing wound is CXCL12, which binds CXCR4 expressed by LECs to induce migration and tube formation in a novel pro-lymphangiogenic pathway that is distinct from the classical VEGFR-3 pathway (46). Furthermore, increased secretion of CCL21 by LECs in the inflammatory wound environment may increase migration of DCs and other antigen-presenting cells to help activate an immune response, which may assist in healing of infected wounds (88, 101, 134).

Complications in wound healing impair the ability of lymphatic vessels to regenerate and repair, leading to impaired lymphatic drainage which results in lymphedema, with the risk for recurrent infection. Therefore, there is a clinical need to better understand the regulation of lymphatic vessel function during wound healing. Chemokines have been a focus for therapeutic approaches to promote wound healing, in particular targeting the CXCL12/CXCR4 signaling axis, a key pathway regulating the recruitment of bone-marrow derived stem cells with regenerative capacity (135). Greater understanding of the specific chemokine pathways involved in wound healing will present additional therapeutic opportunities.

CANCER

While cancer is a genetic disease initiated through the acquisition of specific mutations in key genes, the resulting changes to the cell biology within a host drives the important clinical manifestations of the disease. The progression of cancer, from its evasion of the immune system to its ultimate spread to critical organs systems in the body, has a reliance on altered chemokine signaling resulting from the presence of mutated tumor cells. The lymphatics play a key role in both controlling access to and interaction with the immune system, and also provide an initial means of escape for primary tumor cells, while chemokines also influence immune responses and the pattern of metastatic spread through directed migration of tumor cells in a tissue-specific manner (11, 17, 34, 136).

Leukocyte Recruitment and Egress

Multiple chemokines and receptors have been implicated in the recruitment of specific immune cell subsets to tumors and in influencing cancer immunotherapy responses (26, 137). However, the involvement of lymphatics in anti-tumor immune responses is only beginning to be understood. Powerful lymphangiogenic growth factors that drive the formation and remodeling of lymphatic vessels have been shown to upregulate chemokines in the context of cancer. VEGF-C has been shown to upregulate CCL21 expression by LECs, driving CCR7-dependent tumor chemoinvasion toward lymphatic vessels (138). CCL21 has also been shown to promote lymphoid-like stromal components and immune escape in melanoma tumors in mice, raising the concept that CCL21-secreting tumors can alter the host immune response from immunogenic to tolerogenic which then impacts on tumor progression (139). Recent extension of these observations has shown a role for VEGF-C-induced CCL21 in the tumor infiltration of naive T cells prior to immunotherapy via CCR7-dependent chemotaxis (140). The authors of this study propose that VEGF-C, through VEGFR-3 signaling, can potentiate immunotherapy by attracting abundant CCR7+ naive T cells, which are then locally activated by the immunotherapy. These studies point to a role for VEGF-C and potentially other lymphangiogenic factors as predictive biomarkers for immunotherapy, with a chemokine providing a key link in the signaling chain (140). Meanwhile, other studies are beginning to unravel the complex mechanisms by which lymphatics influence the tumor immune microenvironment (141, 142). Other groups have speculated that "key driver chemokines"—for example CXCL10, which is expressed by LECs in several pathological contexts and implicated in metastasis to LNs (28–30, 143)—may be valid targets in diseases including cancer because of their ability to enhance T-cell-dependent anti-cancer immunity (144).

Tissue-Specific Patterns of Metastasis

A common manifestation of chemokine involvement in directing patterns of metastasis is that tumor cells express chemokine receptors that respond to chemokines secreted by cells of a given tissue or organ, often co-opting the chemokine signaling used for tissue-specific homing of leukocytes (11, 26, 34, 35). This is true for lymphatic vessels and LECs present in the primary tumor, regional LNs or distant organs that are targets of metastatic spread. Studies in a variety of tumor types have shown that CCR7 present on the tumor cells mediates their migration toward CCL21-expressing initial lymphatics (in preference to blood vessels) and/or LNs, thereby promoting spread via the lymphogenous route (145) [recently reviewed in (11, 26)]. A similar mechanism is mediated by tumor-expressed CXCR4 and CXCL12 expressed in LECs and LNs (11, 26). In particular, CXCL12 secreted by LECs in the LN SCS contributes to an attractive and supportive metastatic niche for CXCR4+ tumor cells (36, 37).

The list of chemokines that similarly promote LN metastasis is expanding. Expression of CXCR3 in colorectal cancer was linked to increased metastasis to LNs, likely in response to expression of ligands CXCL9, CXCL10, and CCL21 in the lymphatic sinuses and paracortex of LNs (29). As the rate of spread of CXCR3 -expressing or -deficient cell lines was similar, potential growth effects were also considered. A similar effect was also seen in melanoma (30). In gastric cancer, LECs upregulated expression of CXCL1, which in turn increased tumor cell invasiveness via CXCR2 and stimulated lymphangiogenesis. Expression of both ligand and receptor in patient gastric cancer specimens was associated with LN metastasis and poor survival (38, 39). Soler-Cardona et al. characterized a mechanism in melanoma where neutrophils recruited to melanomas by CXCL5 appeared to facilitate transmigration of tumor cells through lymphatic endothelium (47). Expression of CCL1 in the LN SCS was also shown to regulate entry of CCR8+ melanoma cells into the

LN (43). It is noteworthy that recent studies in mouse models have shown that initial lymphogenous spread can transfer to the blood vascular system via high endothelial venules within regional LNs (146–148).

With the exception of their arrival to LNs via afferent lymphatics, tumor cells are presumed to home to specific organs via the blood vessels, guided by chemokines produced by BECs or transcytosed to the vessel lumen (11, 34). Nonetheless, LECs in distant organs can also participate in distant organ metastasis and metastatic niche formation. Lee et al. showed in mouse models that circulating IL-6 secreted by orthotopic breast cancer cells could influence LECs in LNs and lung. These distant LECs were induced to express CCL5, which was chemotactic for the tumor cells, and VEGF-A, which increased angiogenesis and vascular permeability at metastatic sites (42). Another study used a *Vegfr3*-luciferase reporter mouse and melanoma models to demonstrate pre-metastatic lymphangiogenesis in distant organs, and to identify midkine as a regulator of metastatic niche formation with prognostic significance (149). These studies open up new avenues of investigation into how lymphatics at distant metastatic sites can also influence metastasis.

Atypical Chemokine Receptors in Cancer

Atypical or decoy chemokine receptors have in a number of contexts been involved in modulating cancer progression through shaping the inflammatory response (7, 150). The atypical chemokine receptor ACKR2 has been shown to internalize and sequester an array of pro-inflammatory chemokines of the CC family (5, 49, 50). Mice deficient in ACKR2 had an increased susceptibility to the development of cutaneous tumors that was linked to the recruitment of immune cells (e.g., T cells and mast cells) to support their development (54). In this study ACKR2 was predominately expressed in LECs of human oral squamous cell carcinomas (OSCC) and not tumor cells or epithelial cells, and the levels in tumor LECs were upregulated compared to normal LECs (54). This same group had previously shown that ACKR2 is expressed by lymphatic endothelium and may influence the recirculation of leukocytes via a chemokine driven mechanism, as ACKR2 was expressed on the afferent lymphatics (51). Antigen-experienced T cell subsets express multiple CCR receptors, with CCR4 specifically implicated in cutaneous T cell homing (151). Mast cells also express CCL3 receptors, and these cells generally play a role in promoting tumor angiogenesis and recruiting other pro-tumorigenic leukocyte subsets. CCL3 further directly contributes to mast cell degranulation via CCR1 (152). Expression of ACKR2 therefore limited inflammation by restricting availability of chemokines that attracted these pro-inflammatory leukocytes (54). Other studies of ACKR2 have confirmed its role in the lymphatic system in other organs. ACKR2-deficient mice are more susceptible to inflammation-induced colon carcinogenesis, an effect that was attributed to lymphatic expression of ACKR2 using bone marrow transplantation experiments (55). ACKR2 was also found to be upregulated on lymphatics of inflamed and cancerous colon specimens (55). However, other groups have reported contrasting results (56, 57), potentially suggesting dynamic and context-specific roles for ACKR2 during inflammatory colon carcinogenesis. ACKR2 is also highly expressed in vascular tumors of lymphatic origin (51) and the spindle cells of Kaposi's sarcoma (58). In more aggressive tumors this receptor is down-regulated through the KRAS/BRAF/ERK pathway, leading to chemokine-mediated macrophage recruitment and increased angiogenesis and tumor growth (58).

In breast cancer the absence of a number of members of the atypical chemokine receptor subset predict involvement of axillary LN metastasis, a key clinicopathological indictor of disease progression (153). These observations were further validated by the characterization of genetic variants of two chemokine decoy receptors, ACKR1 and ACKR2, that associated with the metastatic potential of breast cancer (154, 155). Yu et al. found that the expression of the atypical chemokine receptors also predicted relapse-free survival in breast cancer where co-expression and co-genotype (two major alleles of DARC-rs12075 and D6-rs2228468) of the chemokine decoy receptors ACKR1 (DARC) and ACKR2 (D6) had significant associations. This data shows that host factors such as polymorphisms of major chemokine receptor genes and the expression of the protein receptors in cancers, including in lymphatic or blood vessels, could help predict prognosis (155).

Tumor Lymphangiogenesis

Lymphangiogenesis and lymphatic remodeling in tumors, commonly driven by VEGF-C and VEGF-D, are strongly associated with metastasis to LNs and distant organs (17). We recently identified a cooperative role for CCL27, CCL28, and their receptor CCR10 in VEGF-D driven tumor lymphangiogenesis (44). Here, CCR10 was expressed by LECs and upregulated by VEGF-D and the pro-inflammatory cytokine TNF-α. LECs were attracted to both CCL27 and CCL28 in a CCR10-dependent fashion. Further examination of CCR10-deficient mice confirmed a role for this receptor in lymphatic patterning. While CCL27 alone was not sufficient to drive metastasis, both chemokines enhanced LEC migration and worked in combination with VEGF-D to recruit LECs and form coherent vessels (44). The study suggests a cooperative action of chemokines, inflammatory mediators, and lymphangiogenic growth factors during cancer progression. Interestingly, VEGF-D was also shown to upregulate expression of ACKR2 in LECs *in vitro* (53). Other studies have shown a link between chemokine signaling and VEGFR-3-driven lymphangiogenesis in cancer where VEGF-C can upregulate CXCR4 and thereby cooperate with CXCL12 in driving lymphangiogenesis and metastasis (46). Notably CXCR4 has a well-established role in tumor angiogenesis as well, and is being actively pursued as a therapeutic target (64, 156). Tumor-expressed CXCL5 in melanoma has also been found to drive tumor lymphangiogenesis and lymphogenous metastasis through CXCR2 expressed on LECs (47). Gastric cancer cells induce expression of CXCL1 in LECs, which subseqently drives tumor lymphangiogenesis and lymphogenous metastasis (38). These studies illustrate that as well as being a source of chemokine ligands in cancer, lymphatic vessels can also be guided by chemokine receptor signaling.

Lymphangiogenesis is also coupled with chemokine signaling by fluid mechanics (157). Lymphatic flow is important in

stimulating chemokine secretion by LECs and other cells, as well as for generating gradients of chemokines that can be followed by migrating tumor cells (98, 157, 158). Under conditions of interstitial flow, tumor cells co-expressing a chemokine and its receptor can thereby exhibit "autologous chemotaxis," following a self-generated chemokine gradient toward lymphatics (159).

OTHER PATHOLOGIES

Lymphatic vessels have been observed to intersect with chemokine-mediated movement of important effector cells in a variety of diverse human pathologies. In type 2 diabetes patients a range of chemokines and related genes were differentially expressed in dermal LECs compared to non-diabetic patient LECs (28) (**Table 2**). Enhanced lymphatic density was observed in skin, along with upregulation of CXCL10 and downregulation of CCL27 and CXCL14 in response to pro-inflammatory conditions. TNF-α upregulated CXCL10 in LECs, and LEC-derived CXCL10 was able to mediate macrophage adhesion to LEC monolayers and invasion into agarose plugs (28). The study identified paracrine cross-talk allowing macrophage recruitment toward LECs via a chemokine-mediated mechanism.

Studies of the mechanisms of human kidney transplant rejection show that inflammatory infiltrates rich in lymphocytes attack both cortical tubules and endothelial cells. This is accompanied by significant increases in local lymphatic vessel density due to "lymphatic neoangiogenesis" (94). LECs from these vessels express and secrete CCL21 which attracts CCR7+ cells (94). A later study showed that ACKR3 (CXCR7) was also expressed by LECs during kidney rejection with nearly 1/3 of adult dermal lymphatics expressing ACKR3, and both ACKR3+ blood and lymphatic vessels increasing in number during allograft rejection (59).

A role for ACKR2 on lymphatic endothelium in autoimmune disease is implied from studies of the ACKR2-deficient mice during experimental autoimmune encephalomyelitis (EAE) (160) where encephalitogenic responses, including DC migration and T cell priming, were impaired (160). Interestingly other studies have shown that ACKR2-deficient mice develop enhanced symptoms of EAE (as well as collagen-induced arthritis) due to enhanced Th17 responses (161). These differences could be due to control of IL-17 production by ACKR2 (50).

FUTURE DIRECTIONS AND CONCLUSIONS

Lymphatic vessels, like blood vessels, are a highly interactive surface for cells of the immune system, and through the use of chemokines and their receptors can coordinate key interactions. These pathways can control the entry and function of particular immune subsets in a number of pathological conditions. Nonetheless LECs have distinct patterns of chemokine secretion and expression of chemokine receptors that distinguish them from the blood vessel system and mediate distinct roles and responses. The abundance and diversity of the chemokine family point to the likelihood that a plethora of novel chemokine functions and interactions remain to be discovered. Of note, several recent studies have undertaken differential expression profiling of LECs by microarray in a range of different pathologies, revealing multiple chemokines with as-yet undefined roles in disease (**Table 2**). These studies are complemented by *in vitro* analyses examining chemokines and receptors upregulated in LECs by specific stimuli (46, 60, 88). The emerging data suggests that chemokines and their receptors play a complex role in helping coordinate the movement of LECs and interactive circulatory cells in both normal development and a range of pathological conditions.

AUTHOR CONTRIBUTIONS

RF and SS conceived the review. RF, TK, SiM, ScM, and SS wrote and edited the manuscript.

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REFERENCES

1. Lim WA, Pawson T. Phosphotyrosine signaling: evolving a new cellular communication system. *Cell*. (2010) 142:661–7. doi: 10.1016/j.cell.2010.08.023
2. Lemmon MA and Schlessinger J. Cell signaling by receptor tyrosine kinases. *Cell*. (2010) 141:1117–34. doi: 10.1016/j.cell.2010.06.011
3. Mackay CR. Chemokines: immunology's high impact factors. *Nat. Immunol*. (2001) 2:95–101. doi: 10.1038/84298
4. Miller MC, Mayo KH. Chemokines from a structural perspective. *Int J Mol Sci*. (2017) 18:E2088. doi: 10.3390/ijms18102088
5. Hughes CE, Nibbs RJB. A guide to chemokines and their receptors. *FEBS J*. (2018) 285:2944–71. doi: 10.1111/febs.14466
6. Bachelerie F, Ben-Baruch A, Burkhardt AM, Combadiere C, Farber JM, Graham GJ, et al. International Union of Pharmacology. LXXXIX. Update on the extended family of chemokine receptors and introducing a new nomenclature for atypical chemokine receptors. *Pharmacol Rev*. (2014) 66:1–79. doi: 10.1124/pr.113.007724
7. Bonecchi R, Graham GJ. Atypical chemokine receptors and their roles in the resolution of the inflammatory response. *Front Immunol*. (2016) 7:224. doi: 10.3389/fimmu.2016.00224
8. Robledo MM, Bartolome RA, Longo N, Rodriguez-Frade JM, Mellado M, Longo I, et al. Expression of functional chemokine receptors CXCR3 and CXCR4 on human melanoma cells. *J Biol Chem*. (2001) 276:45098–105. doi: 10.1074/jbc.M106912200
9. Christopherson KW II, Hood AF, Travers JB, Ramsey H, Hromas RA. Endothelial induction of the T-cell chemokine CCL21 in T-cell autoimmune diseases. *Blood*. (2003) 101:801–6. doi: 10.1182/blood-2002-05-1586
10. Ogilvie P, Bardi G, Clark-Lewis I, Baggiolini M, Uguccioni M. Eotaxin is a natural antagonist for CCR2 and an agonist for CCR5. *Blood*. (2001) 97:1920–4. doi: 10.1182/blood.V97.7.1920
11. Zlotnik A, Burkhardt AM, Homey B. Homeostatic chemokine receptors and organ-specific metastasis. *Nat Rev Immunol*. (2011) 11:597–606. doi: 10.1038/nri3049
12. Karnezis T, Shayan R, Caesar C, Roufail S, Harris NC, Ardipradja K, et al. VEGF-D promotes tumor metastasis by regulating prostaglandins produced

by the collecting lymphatic endothelium. *Cancer Cell*. (2012) 21:181–95. doi: 10.1016/j.ccr.2011.12.026
13. Farnsworth RH, Karnezis T, Shayan R, Matsumoto M, Nowell CJ, Achen MG, et al. A role for bone morphogenetic protein-4 in lymph node vascular remodeling and primary tumor growth. *Cancer Res*. (2011) 71:6547–57. doi: 10.1158/0008-5472.CAN-11-0200
14. Petrova TV, Koh GY. Organ-specific lymphatic vasculature: from development to pathophysiology. *J Exp Med*. (2018) 215:35–49. doi: 10.1084/jem.20171868
15. Alitalo K, Tammela T, Petrova TV. Lymphangiogenesis in development and human disease. *Nature*. (2005) 438:946–53. doi: 10.1038/nature04480
16. Alitalo K. The lymphatic vasculature in disease. *Nature Med*. (2011) 17:1371–80. doi: 10.1038/nm.2545
17. Stacker SA, Williams SP, Karnezis T, Shayan R, Fox SB, Achen MG. Lymphangiogenesis and lymphatic vessel remodelling in cancer. *Nat Rev Cancer*. (2014) 14:159–72. doi: 10.1038/nrc3677
18. Makinen T, Veikkola T, Mustjoki S, Karpanen T, Catimel B, Nice EC, et al. Isolated lymphatic endothelial cells transduce growth, survival and migratory signals via the VEGF-C/D receptor VEGFR-3. *EMBO J*. (2001) 20:4762–73. doi: 10.1093/emboj/20.17.4762
19. Okuda KS, Baek S, Hogan BM. Visualization and tools for analysis of zebrafish lymphatic development. *Methods Mol Biol*. (2018) 1846:55–70. doi: 10.1007/978-1-4939-8712-2_4
20. Williams SP, Odell AF, Karnezis T, Farnsworth RH, Gould CM, Li J, et al. Genome-wide functional analysis reveals central signaling regulators of lymphatic endothelial cell migration and remodeling. *Sci Signal*. (2017) 10:eaal2987. doi: 10.1126/scisignal.aal2987
21. Williams SP, Gould CM, Nowell CJ, Karnezis T, Achen MG, Simpson KJ, et al. Systematic high-content genome-wide RNAi screens of endothelial cell migration and morphology. *Sci Data*. (2017) 4:170009. doi: 10.1038/sdata.2017.9
22. Johnson LA, Jackson DG. Control of dendritic cell trafficking in lymphatics by chemokines. *Angiogenesis*. (2014) 17:335–45. doi: 10.1007/s10456-013-9407-0
23. Comerford I, Harata-Lee Y, Bunting MD, Gregor C, Kara EE, McColl SR. A myriad of functions and complex regulation of the CCR7/CCL19/CCL21 chemokine axis in the adaptive immune system. *Cytokine Growth Factor Rev*. (2013) 24:269–83. doi: 10.1016/j.cytogfr.2013.03.001
24. Beauvillain C, Cunin P, Doni A, Scotet M, Jaillon S, Loiry ML, et al. CCR7 is involved in the migration of neutrophils to lymph nodes. *Blood*. (2011) 117:1196–204. doi: 10.1182/blood-2009-11-254490
25. Onder L, Ludewig B. A fresh view on lymph node organogenesis. *Trends Immunol*. (2018) 39:775–87. doi: 10.1016/j.it.2018.08.003
26. Zhou J, Xiang Y, Yoshimura T, Chen K, Gong W, Huang J, et al. The role of chemoattractant receptors in shaping the tumor microenvironment. *Biomed Res Int*. (2014) 2014:751392. doi: 10.1155/2014/751392
27. Wick N, Haluza D, Gurnhofer E, Raab I, Kasimir MT, Prinz M, et al. Lymphatic precollectors contain a novel, specialized subpopulation of podoplanin low, CCL27-expressing lymphatic endothelial cells. *Am J Pathol*. (2008) 173:1202–9. doi: 10.2353/ajpath.2008.080101
28. Haemmerle M, Keller T, Egger G, Schachner H, Steiner CW, Stokic D, et al. Enhanced lymph vessel density, remodeling, and inflammation are reflected by gene expression signatures in dermal lymphatic endothelial cells in type 2 diabetes. *Diabetes*. (2013) 62:2509–29. doi: 10.2337/db12-0844
29. Kawada K, Hosogi H, Sonoshita M, Sakashita H, Manabe T, Shimahara Y, et al. Chemokine receptor CXCR3 promotes colon cancer metastasis to lymph nodes. *Oncogene*. (2007) 26:4679–88. doi: 10.1038/sj.onc.1210267
30. Kawada K, Sonoshita M, Sakashita H, Takabayashi A, Yamaoka Y, Manabe T, et al. Pivotal role of CXCR3 in melanoma cell metastasis to lymph nodes. *Cancer Res*. (2004) 64:4010–7. doi: 10.1158/0008-5472.CAN-03-1757
31. Gregory JL, Walter A, Alexandre YO, Hor JL, Liu R, Ma JZ, et al. Infection programs sustained lymphoid stromal cell responses and shapes lymph node remodeling upon secondary challenge. *Cell Rep*. (2017) 18:406–18. doi: 10.1016/j.celrep.2016.12.038
32. Pegu A, Flynn JL, Reinhart TA. Afferent and efferent interfaces of lymph nodes are distinguished by expression of lymphatic endothelial markers and chemokines. *Lymphat Res Biol*. (2007) 5:91–103. doi: 10.1089/lrb.2007.1006
33. Kabashima K, Shiraishi N, Sugita K, Mori T, Onoue A, Kobayashi M, et al. CXCL12-CXCR4 engagement is required for migration of cutaneous dendritic cells. *Am J Pathol*. (2007) 171:1249–57. doi: 10.2353/ajpath.2007.070225
34. Rezaeeyan H, Shirzad R, McKee TD, Saki N. Role of chemokines in metastatic niche: new insights along with a diagnostic and prognostic approach. *APMIS*. (2018) 126:359–70. doi: 10.1111/apm.12818
35. Muller A, Homey B, Soto H, Ge N, Catron D, Buchanan ME, et al. Involvement of chemokine receptors in breast cancer metastasis. *Nature*. (2001) 410:50–6. doi: 10.1038/35065016
36. Kim M, Koh YJ, Kim KE, Koh BI, Nam DH, Alitalo K, et al. CXCR4 signaling regulates metastasis of chemoresistant melanoma cells by a lymphatic metastatic niche. *Cancer Res*. (2010) 70:10411–21. doi: 10.1158/0008-5472.CAN-10-2591
37. Hirakawa S, Detmar M, Kerjaschki D, Nagamatsu S, Matsuo K, Tanemura A, et al. Nodal lymphangiogenesis and metastasis: role of tumor-induced lymphatic vessel activation in extramammary Paget's disease. *Am J Pathol*. (2009) 175:2235–48. doi: 10.2353/ajpath.2009.090420
38. Xu J, Zhang C, He Y, Wu H, Wang Z, Song W, et al. Lymphatic endothelial cell-secreted CXCL1 stimulates lymphangiogenesis and metastasis of gastric cancer. *Int J Cancer*. (2012) 130:787–97. doi: 10.1002/ijc.26035
39. Wang Z, Wang Z, Li G, Wu H, Sun K, Chen J, et al. CXCL1 from tumor-associated lymphatic endothelial cells drives gastric cancer cell into lymphatic system via activating integrin beta1/FAK/AKT signaling. *Cancer Lett*. (2017) 385:28–38. doi: 10.1016/j.canlet.2016.10.043
40. Lee KM, Danuser R, Stein JV, Graham D, Nibbs RJ, Graham GJ. The chemokine receptors ACKR2 and CCR2 reciprocally regulate lymphatic vessel density. *EMBO J*. (2014) 33:2564–80. doi: 10.15252/embj.201488887
41. Johnson LA, Jackson DG. The chemokine CX3CL1 promotes trafficking of dendritic cells through inflamed lymphatics. *J Cell Sci*. (2013) 126(Pt 22):5259–70. doi: 10.1242/jcs.135343
42. Lee E, Fertig EJ, Jin K, Sukumar S, Pandey NB, Popel AS. Breast cancer cells condition lymphatic endothelial cells within pre-metastatic niches to promote metastasis. *Nat Commun*. (2014) 5:4715. doi: 10.1038/ncomms5715
43. Das S, Sarrou E, Podgrabinska S, Cassella M, Mungamuri SK, Feirt N, et al. Tumor cell entry into the lymph node is controlled by CCL1 chemokine expressed by lymph node lymphatic sinuses. *J. Exp. Med*. (2013) 210:1509–28. doi: 10.1084/jem.20111627
44. Karnezis T, Farnsworth RH, Harris NC, Williams SP, Caesar C, Byrne D, et al. CCL27/CCL28-CCR10 chemokine signaling mediates migration of lymphatic endothelial cells. *Cancer Res*. (2019). doi: 10.1158/0008-5472.CAN-18-1858. [Epub ahead of print].
45. Cha YR, Fujita M, Butler M, Isogai S, Kochhan E, Siekmann AF, et al. Chemokine signaling directs trunk lymphatic network formation along the preexisting blood vasculature. *Dev Cell*. (2012) 22:824–36. doi: 10.1016/j.devcel.2012.01.011
46. Zhuo W, Jia L, Song N, Lu XA, Ding Y, Wang X, et al. The CXCL12-CXCR4 chemokine pathway: a novel axis regulates lymphangiogenesis. *Clin Cancer Res*. (2012) 18:5387–98. doi: 10.1158/1078-0432.CCR-12-0708
47. Soler-Cardona A, Forsthuber A, Lipp K, Ebersberger S, Heinz M, Schossleitner K, et al. CXCL5 Facilitates melanoma cell-neutrophil interaction and lymph node metastasis. *J Invest Dermatol*. (2018) 138:1627–35. doi: 10.1016/j.jid.2018.01.035
48. Choi I, Lee YS, Chung HK, Choi D, Ecoiffier T, Lee HN, et al. Interleukin-8 reduces post-surgical lymphedema formation by promoting lymphatic vessel regeneration. *Angiogenesis*. (2013) 16:29–44. doi: 10.1007/s10456-012-9297-6
49. Schulz O, Hammerschmidt SI, Moschovakis GL, Forster R. Chemokines and chemokine receptors in lymphoid tissue dynamics. *Annu Rev Immunol*. (2016) 34:203–42. doi: 10.1146/annurev-immunol-041015-055649
50. Bonavita O, Mollica Poeta V, Setten E, Massara M, Bonecchi R. ACKR2: an atypical chemokine receptor regulating lymphatic biology. *Front Immunol*. (2016) 7:691. doi: 10.3389/fimmu.2016.00691
51. Nibbs RJ, Kriehuber E, Ponath PD, Parent D, Qin S, Campbell JD, et al. The β-chemokine receptor D6 is expressed by lymphatic endothelium and a subset of vascular tumors. *Am J Pathol*. (2001) 158:867–77. doi: 10.1016/S0002-9440(10)64035-7

52. Lee KM, McKimmie CS, Gilchrist DS, Pallas KJ, Nibbs RJ, Garside P, et al. D6 facilitates cellular migration and fluid flow to lymph nodes by suppressing lymphatic congestion. *Blood*. (2011) 118:6220–9. doi: 10.1182/blood-2011-03-344044
53. McKimmie CS, Singh MD, Hewit K, Lopez-Franco O, Le Brocq M, Rose-John S, et al. An analysis of the function and expression of D6 on lymphatic endothelial cells. *Blood*. (2013) 121:3768–77. doi: 10.1182/blood-2012-04-425314
54. Nibbs RJ, Gilchrist DS, King V, Ferra A, Forrow S, Hunter KD, et al. The atypical chemokine receptor D6 suppresses the development of chemically induced skin tumors. *J Clin Invest*. (2007) 117:1884–92. doi: 10.1172/JCI30068
55. Vetrano S, Borroni EM, Sarukhan A, Savino B, Bonecchi R, Correale C, et al. The lymphatic system controls intestinal inflammation and inflammation-associated Colon Cancer through the chemokine decoy receptor D6. *Gut*. (2010) 59:197–206. doi: 10.1136/gut.2009.183772
56. Langenes V, Svensson H, Borjesson L, Gustavsson B, Bemark M, Sjoling A, et al. Expression of the chemokine decoy receptor D6 is decreased in colon adenocarcinomas. *Cancer Immunol Immunother*. (2013) 62:1687–95. doi: 10.1007/s00262-013-1472-0
57. Bordon Y, Hansell CA, Sester DP, Clarke M, Mowat AM, Nibbs RJ. The atypical chemokine receptor D6 contributes to the development of experimental colitis. *J Immunol*. (2009) 182:5032–40. doi: 10.4049/jimmunol.0802802
58. Savino B, Caronni N, Anselmo A, Pasqualini F, Borroni EM, Basso G, et al. ERK-dependent downregulation of the atypical chemokine receptor D6 drives tumor aggressiveness in Kaposi sarcoma. *Cancer Immunol Res*. (2014) 2:679–89. doi: 10.1158/2326-6066.CIR-13-0202
59. Neusser MA, Kraus AK, Regele H, Cohen CD, Fehr T, Kerjaschki D, et al. The chemokine receptor CXCR7 is expressed on lymphatic endothelial cells during renal allograft rejection. *Kidney Int*. (2010) 77:801–8. doi: 10.1038/ki.2010.6
60. Klein KR, Karpinich NO, Espenschied ST, Willcockson HH, Dunworth WP, Hoopes SL, et al. Decoy receptor CXCR7 modulates adrenomedullin-mediated cardiac and lymphatic vascular development. *Dev Cell*. (2014) 30:528–40. doi: 10.1016/j.devcel.2014.07.012
61. Bryce SA, Wilson RA, Tiplady EM, Asquith DL, Bromley SK, Luster AD, et al. ACKR4 on stromal cells scavenges CCL19 to enable CCR7-dependent trafficking of APCs from inflamed skin to lymph nodes. *J Immunol*. (2016) 196:3341–53. doi: 10.4049/jimmunol.1501542
62. Ulvmar MH, Werth K, Braun A, Kelay P, Hub E, Eller K, et al. The atypical chemokine receptor CCRL1 shapes functional CCL21 gradients in lymph nodes. *Nat Immunol*. (2014) 15:623–30. doi: 10.1038/ni.2889
63. Belperio JA, Keane MP, Arenberg DA, Addison CL, Ehlert JE, Burdick MD, et al. CXC chemokines in angiogenesis. *J Leukoc Biol*. (2000) 68:1–8. doi: 10.1189/jlb.68.1.1
64. Romagnani P, Lasagni L, Annunziato F, Serio M, Romagnani S. CXC chemokines: the regulatory link between inflammation and angiogenesis. *Trends Immunol*. (2004) 25:201–9. doi: 10.1016/j.it.2004.02.006
65. van de Pavert SA, Mebius RE. New insights into the development of lymphoid tissues. *Nat Rev Immunol*. (2010) 10:664–74. doi: 10.1038/nri2832
66. Ansel KM, Ngo VN, Hyman PL, Luther SA, Forster R, Sedgwick JD, et al. A chemokine-driven positive feedback loop organizes lymphoid follicles. *Nature*. (2000) 406:309–14. doi: 10.1038/35018581
67. Forster R, Mattis AE, Kremmer E, Wolf E, Brem G, Lipp M. A putative chemokine receptor, BLR1, directs B cell migration to defined lymphoid organs and specific anatomic compartments of the spleen. *Cell*. (1996) 87:1037–47. doi: 10.1016/S0092-8674(00)81798-5
68. Ohl L, Henning G, Krautwald S, Lipp M, Hardtke S, Bernhardt G, et al. Cooperating mechanisms of CXCR5 and CCR7 in development and organization of secondary lymphoid organs. *J Exp Med*. (2003) 197:1199–204. doi: 10.1084/jem.20030169
69. Forster R, Schubel A, Breitfeld D, Kremmer E, Renner-Muller I, Wolf E, et al. CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs. *Cell*. (1999) 99:23–33. doi: 10.1016/S0092-8674(00)80059-8
70. van de Pavert SA, Olivier BJ, Goverse G, Vondenhoff MF, Greuter M, Beke P, et al. Chemokine CXCL13 is essential for lymph node initiation and is induced by retinoic acid and neuronal stimulation. *Nat Immunol*. (2009) 10:1193–9. doi: 10.1038/ni.1789
71. Bovay E, Sabine A, Prat-Luri B, Kim S, Son K, Willrodt AH, et al. Multiple roles of lymphatic vessels in peripheral lymph node development. *J Exp Med*. (2018) 215:2760–77. doi: 10.1084/jem.20180217
72. Luther SA, Ansel KM, Cyster JG. Overlapping roles of CXCL13, interleukin 7 receptor alpha, and CCR7 ligands in lymph node development. *J Exp Med*. (2003) 197:1191–8. doi: 10.1084/jem.20021294
73. Chai Q, Onder L, Scandella E, Gil-Cruz C, Perez-Shibayama C, Cupovic J, et al. Maturation of lymph node fibroblastic reticular cells from myofibroblastic precursors is critical for antiviral immunity. *Immunity*. (2013) 38:1013–24. doi: 10.1016/j.immuni.2013.03.012
74. Onder L, Morbe U, Pikor N, Novkovic M, Cheng HW, Hehlgans T, et al. Lymphatic endothelial cells control initiation of lymph node organogenesis. *Immunity*. (2017) 47:80–92 e84. doi: 10.1016/j.immuni.2017.05.008
75. Vondenhoff MF, van de Pavert SA, Dillard ME, Greuter M, Goverse G, Oliver G, et al. Lymph sacs are not required for the initiation of lymph node formation. *Development*. (2009) 136:29-34. doi: 10.1242/dev.028456
76. Lee YG, Koh GY. Coordinated lymphangiogenesis is critical in lymph node development and maturation. *Dev Dyn*. (2016) 245:1189–97. doi: 10.1002/dvdy.24456
77. Tachibana K, Hirota S, Iizasa H, Yoshida H, Kawabata K, Kataoka Y, et al. The chemokine receptor CXCR4 is essential for vascularization of the gastrointestinal tract. *Nature*. (1998) 393:591–4. doi: 10.1038/31261
78. Gupta SK, Lysko PG, Pillarisetti K, Ohlstein E, Stadel JM. Chemokine receptors in human endothelial cells. Functional expression of CXCR4 and its transcriptional regulation by inflammatory cytokines. *J Biol Chem*. (1998) 273:4282–7. doi: 10.1074/jbc.273.7.4282
79. Mirshahi F, Pourtau J, Li H, Muraine M, Trochon V, Legrand E, et al. SDF-1 activity on microvascular endothelial cells: consequences on angiogenesis in *in vitro* and *in vivo* models. *Thromb Res*. (2000) 99:587–94. doi: 10.1016/S0049-3848(00)00292-9
80. Pitulescu ME, Schmidt I, Giaimo BD, Antoine T, Berkenfeld F, Ferrante F, et al. Dll4 and Notch signalling couples sprouting angiogenesis and artery formation. *Nat Cell Biol*. (2017) 19:915–27. doi: 10.1038/ncb3555
81. Hasan SS, Tsaryk R, Lange M, Wisniewski L, Moore JC, Lawson ND, et al. Endothelial Notch signalling limits angiogenesis via control of artery formation. *Nat Cell Biol*. (2017) 19:928–40. doi: 10.1038/ncb3574
82. Chen J, Zhu RF, Li FF, Liang YL, Wang C, Qin YW, et al. MicroRNA-126a directs lymphangiogenesis through interacting with chemokine and Flt4 signaling in zebrafish. *Arterioscler Thromb Vasc Biol*. (2016) 36:2381–93. doi: 10.1161/ATVBAHA.116.308120
83. Harvey NL and Gordon EJ. Deciphering the roles of macrophages in developmental and inflammation stimulated lymphangiogenesis. *Vasc Cell*. (2012) 4:15. doi: 10.1186/2045-824X-4-15
84. Gordon EJ, Rao S, Pollard JW, Nutt SL, Lang RA, Harvey NL. Macrophages define dermal lymphatic vessel calibre during development by regulating lymphatic endothelial cell proliferation. *Development*. (2010) 137:3899–910. doi: 10.1242/dev.050021
85. Lian J, Luster AD. Chemokine-guided cell positioning in the lymph node orchestrates the generation of adaptive immune responses. *Curr Opin Cell Biol*. (2015) 36:1–6. doi: 10.1016/j.ceb.2015.05.003
86. Forster R, Braun A, Worbs T. Lymph node homing of T cells and dendritic cells via afferent lymphatics. *Trends Immunol*. (2012) 33:271–80. doi: 10.1016/j.it.2012.02.007
87. Weber M, Hauschild R, Schwarz J, Moussion C, de Vries I, Legler DF, et al. Interstitial dendritic cell guidance by haptotactic chemokine gradients. *Science*. (2013) 339:328–32. doi: 10.1126/science.1228456
88. Johnson LA, Jackson DG. Inflammation-induced secretion of CCL21 in lymphatic endothelium is a key regulator of integrin-mediated dendritic cell transmigration. *Int Immunol*. (2010) 22:839–49. doi: 10.1093/intimm/dxq435
89. Aebischer D, Iolyeva M, Halin C. The inflammatory response of lymphatic endothelium. *Angiogenesis*. (2014) 17:383–93. doi: 10.1007/s10456-013-9404-3
90. Baluk P, Fuxe J, Hashizume H, Romano T, Lashnits E, Butz S, et al. Functionally specialized junctions between endothelial cells of lymphatic vessels. *J Exp Med*. (2007) 204:2349–62. doi: 10.1084/jem.20062596

91. Tal O, Lim HY, Gurevich I, Milo I, Shipony Z, Ng LG, et al. DC mobilization from the skin requires docking to immobilized CCL21 on lymphatic endothelium and intralymphatic crawling. *J Exp Med*. (2011) 208:2141–53. doi: 10.1084/jem.20102392
92. Vaahtomeri K, Brown M, Hauschild R, De Vries I, Leithner AF, Mehling M, et al. Locally triggered release of the chemokine CCL21 promotes dendritic cell transmigration across lymphatic endothelia. *Cell Rep*. (2017) 19:902–9. doi: 10.1016/j.celrep.2017.04.027
93. Russo E, Teijeira A, Vaahtomeri K, Willrodt AH, Bloch JS, Nitschke M, et al. Intralymphatic CCL21 promotes tissue egress of dendritic cells through afferent lymphatic vessels. *Cell Rep*. (2016) 14:1723–34. doi: 10.1016/j.celrep.2016.01.048
94. Kerjaschki D, Regele HM, Moosberger I, Nagy-Bojarski K, Watschinger B, Soleiman A, et al. Lymphatic neoangiogenesis in human kidney transplants is associated with immunologically active lymphocytic infiltrates. *J Am Soc Nephrol*. (2004) 15:603–12. doi: 10.1097/01.ASN.0000113316.52371.2E
95. Iftakhar EKI, Fair-Makela R, Kukkonen-Macchi A, Elima K, Karikoski M, Rantakari P, et al. Gene-expression profiling of different arms of lymphatic vasculature identifies candidates for manipulation of cell traffic. *Proc Natl Acad Sci USA*. (2016) 113:10643–8. doi: 10.1073/pnas.1602357113
96. Braun A, Worbs T, Moschovakis GL, Halle S, Hoffmann K, Bolter J, et al. Afferent lymph-derived T cells and DCs use different chemokine receptor CCR7-dependent routes for entry into the lymph node and intranodal migration. *Nat Immunol*. (2011) 12:879–87. doi: 10.1038/ni.2085
97. Fletcher AL, Acton SE, Knoblich K. Lymph node fibroblastic reticular cells in health and disease. *Nat Rev Immunol*. (2015) 15:350–61. doi: 10.1038/nri3846
98. Tomei AA, Siegert S, Britschgi MR, Luther SA, Swartz MA. Fluid flow regulates stromal cell organization and CCL21 expression in a tissue-engineered lymph node microenvironment. *J Immunol*. (2009) 183:4273–83. doi: 10.4049/jimmunol.0900835
99. Sokol CL, Camire RB, Jones MC, Luster AD. The chemokine receptor CCR8 promotes the migration of dendritic cells into the lymph node parenchyma to initiate the allergic immune response. *Immunity*. (2018) 49:449–63 e446. doi: 10.1016/j.immuni.2018.07.012
100. Malhotra D, Fletcher AL, Astarita J, Lukacs-Kornek V, Tayalia P, Gonzalez SF, et al. Transcriptional profiling of stroma from inflamed and resting lymph nodes defines immunological hallmarks. *Nat Immunol*. (2012) 13:499–510. doi: 10.1038/ni.2262
101. Vigl B, Aebischer D, Nitschke M, Iolyeva M, Rothlin T, Antsiferova O, et al. Tissue inflammation modulates gene expression of lymphatic endothelial cells and dendritic cell migration in a stimulus-dependent manner. *Blood*. (2011) 118:205–15. doi: 10.1182/blood-2010-12-326447
102. Clasper S, Royston D, Baban D, Cao Y, Ewers S, Butz S, et al. A novel gene expression profile in lymphatics associated with tumor growth and nodal metastasis. *Cancer Res*. (2008) 68:7293–303. doi: 10.1158/0008-5472.CAN-07-6506
103. Tan KW, Yeo KP, Wong FH, Lim HY, Khoo KL, Abastado JP, et al. Expansion of cortical and medullary sinuses restrains lymph node hypertrophy during prolonged inflammation. *J Immunol*. (2012) 188:4065–80. doi: 10.4049/jimmunol.1101854
104. Dubey LK, Karempudi P, Luther SA, Ludewig B, Harris NL. Interactions between fibroblastic reticular cells and B cells promote mesenteric lymph node lymphangiogenesis. *Nat Commun*. (2017) 8:367. doi: 10.1038/s41467-017-00504-9
105. Park C, Hwang IY, Sinha RK, Kamenyeva O, Davis MD, Kehrl JH. Lymph node B lymphocyte trafficking is constrained by anatomy and highly dependent upon chemoattractant desensitization. *Blood*. (2012) 119:978–89. doi: 10.1182/blood-2011-06-364273
106. Pham TH, Okada T, Matloubian M, Lo CG, Cyster JG. S1P1 receptor signaling overrides retention mediated by G alpha i-coupled receptors to promote T cell egress. *Immunity*. (2008) 28:122–33. doi: 10.1016/j.immuni.2007.11.017
107. Lo CG, Xu Y, Proia RL, Cyster JG. Cyclical modulation of sphingosine-1-phosphate receptor 1 surface expression during lymphocyte recirculation and relationship to lymphoid organ transit. *J Exp Med*. (2005) 201:291–301. doi: 10.1084/jem.20041509
108. Pham TH, Baluk P, Xu Y, Grigorova I, Bankovich AJ, Pappu R, et al. Lymphatic endothelial cell sphingosine kinase activity is required for lymphocyte egress and lymphatic patterning. *J Exp Med*. (2010) 207:17–27. doi: 10.1084/jem.20091619
109. Ridiandries A, Tan JTM, Bursill CA. The role of chemokines in wound healing. *Int J Mol Sci*. (2018) 19:3217. doi: 10.3390/ijms19103217
110. Singer AJ, Clark RA. Cutaneous wound healing. *N Engl J Med*. (1999) 341:738–46. doi: 10.1056/NEJM199909023411006
111. Gurtner GC, Werner S, Barrandon Y, Longaker MT. Wound repair and regeneration. *Nature*. (2008) 453:314–21. doi: 10.1038/nature07039
112. Grose R, Werner S. Wound-healing studies in transgenic and knockout mice. *Mol Biotechnol*. (2004) 28:147–66. doi: 10.1385/MB:28:2:147
113. Gillitzer R, Goebeler M. Chemokines in cutaneous wound healing. *J Leukoc Biol*. (2001) 69:513–21.
114. Karpanen T, Alitalo K. Molecular biology and pathology of lymphangiogenesis. *Annu Rev Pathol*. (2008) 3:367–97. doi: 10.1146/annurev.pathmechdis.3.121806.151515
115. Skobe M, Hamberg LM, Hawighorst T, Schirner M, Wolf GL, Alitalo K, et al. Concurrent induction of lymphangiogenesis, angiogenesis, and macrophage recruitment by vascular endothelial growth factor-C in melanoma. *Am J Pathol*. (2001) 159:893–903. doi: 10.1016/S0002-9440(10)61765-8
116. Schoppmann SF, Birner P, Stockl J, Kalt R, Ullrich R, Caucig C, et al. Tumor-associated macrophages express lymphatic endothelial growth factors and are related to peritumoral lymphangiogenesis. *Am J Pathol*. (2002) 161:947–56. doi: 10.1016/S0002-9440(10)64255-1
117. Martinez-Corral I, Olmeda D, Dieguez-Hurtado R, Tammela T, Alitalo K, Ortega S. *In vivo* imaging of lymphatic vessels in development, wound healing, inflammation, and tumor metastasis. *Proc. Natl. Acad. Sci. USA*. (2012) 109:6223–8. doi: 10.1073/pnas.1115542109
118. Baluk P, Tammela T, Ator E, Lyubynska N, Achen MG, Hicklin DJ, et al. Pathogenesis of persistent lymphatic vessel hyperplasia in chronic airway inflammation. *J. Clin. Invest*. (2005) 115:247–57. doi: 10.1172/JCI200522037
119. Kerjaschki D. The crucial role of macrophages in lymphangiogenesis. *J Clin Invest*. (2005) 115:2316–9. doi: 10.1172/JCI26354
120. Opalenik SR, Davidson JM. Fibroblast differentiation of bone marrow-derived cells during wound repair. *FASEB J*. (2005) 19:1561–3. doi: 10.1096/fj.04-2978fje
121. Werner S, Krieg T, Smola H. Keratinocyte-fibroblast interactions in wound healing. *J Invest Dermatol*. (2007) 127:998–1008. doi: 10.1038/sj.jid.5700786
122. Lovvorn HN III, Cheung DT, Nimni ME, Perelman N, Estes JM, Adzick NS. Relative distribution and crosslinking of collagen distinguish fetal from adult sheep wound repair. *J Pediatr Surg*. (1999) 34:218–23. doi: 10.1016/S0022-3468(99)90261-0
123. Brandt E, Petersen F, Ludwig A, Ehlert JE, Bock L, Flad HD. The beta-thromboglobulins and platelet factor 4: blood platelet-derived CXC chemokines with divergent roles in early neutrophil regulation. *J Leukoc Biol*. (2000) 67:471–8. doi: 10.1002/jlb.67.4.471
124. Goebeler M, Yoshimura T, Toksoy A, Ritter U, Brocker EB, Gillitzer R. The chemokine repertoire of human dermal microvascular endothelial cells and its regulation by inflammatory cytokines. *J Invest Dermatol*. (1997) 108:445–51. doi: 10.1111/1523-1747.ep12289711
125. Rossi D, Zlotnik A. The biology of chemokines and their receptors. *Annu Rev Immunol*. (2000) 18:217–42. doi: 10.1146/annurev.immunol.18.1.217
126. Murdoch C, Finn A. Chemokine receptors and their role in inflammation and infectious diseases. *Blood*. (2000) 95:3032–43. Available online at: http://www.bloodjournal.org/content/95/10/3032.long?sso-checked=true (accessed March 27, 2019).
127. Engelhardt E, Toksoy A, Goebeler M, Debus S, Brocker EB, Gillitzer R. Chemokines IL-8, GROalpha, MCP-1, IP-10, and Mig are sequentially and differentially expressed during phase-specific infiltration of leukocyte subsets in human wound healing. *Am J Pathol*. (1998) 153:1849–60. doi: 10.1016/S0002-9440(10)65699-4
128. Michel G, Kemeny L, Peter RU, Beetz A, Ried C, Arenberger P, et al. Interleukin-8 receptor-mediated chemotaxis of normal human epidermal cells. *FEBS Lett*. (1992) 305:241–3. doi: 10.1016/0014-5793(92)80677-9
129. Tuschil A, Lam C, Haslberger A, Lindley I. Interleukin-8 stimulates calcium transients and promotes epidermal cell proliferation. *J Invest Dermatol*. (1992) 99:294–8. doi: 10.1111/1523-1747.ep12616634
130. Murdoch C, Monk PN, Finn A. Cxc chemokine receptor expression on human endothelial cells. *Cytokine*. (1999) 11:704–12. doi: 10.1006/cyto.1998.0465

131. Schraufstatter IU, Chung J, Burger M. IL-8 activates endothelial cell CXCR1 and CXCR2 through Rho and Rac signaling pathways. *Am J Physiol Lung Cell Mol Physiol*. (2001) 280:L1094–103. doi: 10.1152/ajplung.2001.280.6.L1094
132. Kondo T, Novick AC, Toma H, Fairchild RL. Induction of chemokine gene expression during allogeneic skin graft rejection. *Transplantation*. (1996) 61:1750–7. doi: 10.1097/00007890-199606270-00015
133. Miyake M, Goodison S, Urquidi V, Gomes Giacoia E, Rosser CJ. Expression of CXCL1 in human endothelial cells induces angiogenesis through the CXCR2 receptor and the ERK1/2 and EGF pathways. *Lab Invest*. (2013) 93:768–78. doi: 10.1038/labinvest.2013.71
134. MartIn-Fontecha A, Sebastiani S, Hopken UE, Uguccioni M, Lipp M, Lanzavecchia A, et al. Regulation of dendritic cell migration to the draining lymph node: impact on T lymphocyte traffic and priming. *J Exp Med*. (2003) 198:615–21. doi: 10.1084/jem.20030448
135. Nishimura Y, Ii M, Qin G, Hamada H, Asai J, Takenaka H, et al. CXCR4 antagonist AMD3100 accelerates impaired wound healing in diabetic mice. *J Invest Dermatol*. (2012) 132(3 Pt 1):711–20. doi: 10.1038/jid.2011.356
136. Farnsworth RH, Achen MG, Stacker SA. The evolving role of lymphatics in cancer metastasis. *Curr Opin Immunol*. (2018) 53:64–73. doi: 10.1016/j.coi.2018.04.008
137. Nagarsheth N, Wicha MS, Zou W. Chemokines in the cancer microenvironment and their relevance in cancer immunotherapy. *Nat Rev Immunol*. (2017) 17:559–72. doi: 10.1038/nri.2017.49
138. Issa A, Le TX, Shoushtari AN, Shields JD, Swartz MA. Vascular endothelial growth factor-C and C-C chemokine receptor 7 in tumor cell-lymphatic cross-talk promote invasive phenotype. *Cancer Res*. (2009) 69:349–57. doi: 10.1158/0008-5472.CAN-08-1875
139. Shields JD, Kourtis IC, Tomei AA, Roberts JM, Swartz MA. Induction of lymphoidlike stroma and immune escape by tumors that express the chemokine CCL21. *Science*. (2010) 328:749–52. doi: 10.1126/science.1185837
140. Fankhauser M, Broggi MAS, Potin L, Bordry N, Jeanbart L, Lund AW, et al. Tumor lymphangiogenesis promotes T cell infiltration and potentiates immunotherapy in melanoma. *Sci Transl Med*. (2017) 9:eaal4712. doi: 10.1126/scitranslmed.aal4712
141. Lund AW, Medler TR, Leachman SA, Coussens LM. Lymphatic vessels, inflammation, and immunity in skin cancer. *Cancer Discov*. (2016) 6:22–35. doi: 10.1158/2159-8290.CD-15-0023
142. Lund AW, Wagner M, Fankhauser M, Steinskog ES, Broggi MA, Spranger S, et al. Lymphatic vessels regulate immune microenvironments in human and murine melanoma. *J Clin Invest*. (2016) 126:3389–402. doi: 10.1172/JCI79434
143. Mancardi S, Vecile E, Dusetti N, Calvo E, Stanta G, Burrone OR, et al. Evidence of CXC, CC and C chemokine production by lymphatic endothelial cells. *Immunology*. (2003) 108:523–30. doi: 10.1046/j.1365-2567.2003.01613.x
144. Karin N, Razon H. Chemokines beyond chemo-attraction: CXCL10 and its significant role in cancer and autoimmunity. *Cytokine*. (2018) 109:24–8. doi: 10.1016/j.cyto.2018.02.012
145. Irino T, Takeuchi H, Matsuda S, Saikawa Y, Kawakubo H, Wada N, et al. CC-Chemokine receptor CCR7: a key molecule for lymph node metastasis in esophageal squamous cell carcinoma. *BMC Cancer*. (2014) 14:291. doi: 10.1186/1471-2407-14-291
146. Achen MG and Stacker SA. Exit stage left: a tumor cell's journey from lymph node to beyond. *Trends Cancer*. (2018) 4:519–22. doi: 10.1016/j.trecan.2018.05.007
147. Pereira ER, Kedrin D, Seano G, Gautier O, Meijer EFJ, Jones D, et al. Lymph node metastases can invade local blood vessels, exit the node, and colonize distant organs in mice. *Science*. (2018) 359:1403–7. doi: 10.1126/science.aal3622
148. Brown M, Assen FP, Leithner A, Abe J, Schachner H, Asfour G, et al. Lymph node blood vessels provide exit routes for metastatic tumor cell dissemination in mice. *Science*. (2018) 359:1408–11. doi: 10.1126/science.aal3662
149. Olmeda D, Cerezo-Wallis D, Riveiro-Falkenbach E, Pennacchi PC, Contreras-Alcalde M, Ibarz N, et al. Whole-body imaging of lymphovascular niches identifies pre-metastatic roles of midkine. *Nature*. (2017) 546:676–80. doi: 10.1038/nature22977
150. Massara M, Bonavita O, Mantovani A, Locati M and Bonecchi R. Atypical chemokine receptors in cancer: friends or foes? *J Leukoc Biol*. (2016) 99:927-933. doi: 10.1189/jlb.3MR0915-431RR
151. Reiss Y, Proudfoot AE, Power CA, Campbell JJ and Butcher EC. CC chemokine receptor (CCR)4 and the CCR10 ligand cutaneous T cell-attracting chemokine (CTACK) in lymphocyte trafficking to inflamed skin. *J Exp Med*. (2001) 194:1541–7. doi: 10.1084/jem.194.10.1541
152. Miyazaki D, Nakamura T, Toda M, Cheung-Chau KW, Richardson RM, Ono SJ. Macrophage inflammatory protein-1alpha as a costimulatory signal for mast cell-mediated immediate hypersensitivity reactions. *J Clin Invest*. (2005) 115:434–42. doi: 10.1172/JCI18452
153. Zeng XH, Ou ZL, Yu KD, Feng LY, Yin WJ, Li J, et al. Absence of multiple atypical chemokine binders (ACBs) and the presence of VEGF and MMP-9 predict axillary lymph node metastasis in early breast carcinomas. *Med Oncol*. (2014) 31:145. doi: 10.1007/s12032-014-0145-y
154. Yang C, Yu KD, Xu WH, Chen AX, Fan L, Ou ZL, et al. Effect of genetic variants in two chemokine decoy receptor genes, DARC and CCBP2, on metastatic potential of breast cancer. *PLoS ONE*. (2013) 8:e78901. doi: 10.1371/journal.pone.0078901
155. Yu KD, Wang X, Yang C, Zeng XH, Shao ZM. Host genotype and tumor phenotype of chemokine decoy receptors integrally affect breast cancer relapse. *Oncotarget*. (2015) 6:26519–27. doi: 10.18632/oncotarget.4470
156. Chatterjee S, Behnam Azad B, Nimmagadda S. The intricate role of CXCR4 in cancer. *Adv Cancer Res*. (2014) 124:31–82. doi: 10.1016/B978-0-12-411638-2.00002-1
157. Swartz MA, Lund AW. Lymphatic and interstitial flow in the tumour microenvironment: linking mechanobiology with immunity. *Nat Rev Cancer*. (2012) 12:210–9. doi: 10.1038/nrc3186
158. Miteva DO, Rutkowski JM, Dixon JB, Kilarski W, Shields JD, Swartz MA. Transmural flow modulates cell and fluid transport functions of lymphatic endothelium. *Circ Res*. (2010) 106:920–31. doi: 10.1161/CIRCRESAHA.109.207274
159. Shields JD, Fleury ME, Yong C, Tomei AA, Randolph GJ, Swartz MA. Autologous chemotaxis as a mechanism of tumor cell homing to lymphatics via interstitial flow and autocrine CCR7 signaling. *Cancer Cell*. (2007) 11:526–38. doi: 10.1016/j.ccr.2007.04.020
160. Liu L, Graham GJ, Damodaran A, Hu T, Lira SA, Sasse M, et al. Cutting edge: the silent chemokine receptor D6 is required for generating T cell responses that mediate experimental autoimmune encephalomyelitis. *J Immunol*. (2006) 177:17–21. doi: 10.4049/jimmunol.177.1.17
161. Hansell CA, MacLellan LM, Oldham RS, Doonan J, Chapple KJ, Anderson EJ, et al. The atypical chemokine receptor ACKR2 suppresses Th17 responses to protein autoantigens. *Immunol Cell Biol*. (2015) 93:167–76. doi: 10.1038/icb.2014.90

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Comparative Transcriptomic Analysis Identifies a Range of Immunologically Related Functional Elaborations of Lymph Node Associated Lymphatic and Blood Endothelial Cells

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Lymphatic and blood vessels are formed by specialized lymphatic endothelial cells (LEC) and blood endothelial cells (BEC), respectively. These endothelial populations not only form peripheral tissue vessels, but also critical supporting structures in secondary lymphoid organs, particularly the lymph node (LN). Lymph node LEC (LN-LEC) also have been shown to have important immunological functions that are not observed in LEC from tissue lymphatics. LN-LEC can maintain peripheral tolerance through direct presentation of self-antigen via MHC-I, leading to CD8 T cell deletion; and through transfer of self-antigen to dendritic cells for presentation via MHC-II, resulting in CD4 T cell anergy. LN-LEC also can capture and archive foreign antigens, transferring them to dendritic cells for maintenance of memory CD8 T cells. The molecular basis for these functional elaborations in LN-LEC remain largely unexplored, and it is also unclear whether blood endothelial cells in LN (LN-BEC) might express similar enhanced immunologic functionality. Here, we used RNA-Seq to compare the transcriptomic profiles of freshly isolated murine LEC and BEC from LN with one another and with freshly isolated LEC from the periphery (diaphragm). We show that LN-LEC, LN-BEC, and diaphragm LEC (D-LEC) are transcriptionally distinct from one another, demonstrating both lineage and tissue-specific functional specializations. Surprisingly, tissue microenvironment differences in gene expression profiles were more numerous than those determined by endothelial cell lineage specification. In this regard, both LN-localized endothelial cell populations show a variety of functional elaborations that suggest how they may function as antigen presenting cells, and also point to as yet unexplored roles in both positive and negative regulation of innate and adaptive immune responses. The present work

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has defined in depth gene expression differences that point to functional specializations of endothelial cell populations in different anatomical locations, but especially the LN. Beyond the analyses provided here, these data are a resource for future work to uncover mechanisms of endothelial cell functionality.

Keywords: endothelial cell, lymph node, lymphatic, RNA-Seq, antigen presentation, scavenger receptors, chemokines, cytokines and receptors

INTRODUCTION

Lymphatic and blood vessels are formed by specialized endothelial cells that are closely related but distinct (1). These endothelial populations form vessels in peripheral tissue, but also supporting structures in secondary lymphoid organs, particularly lymph node (LN). Blood endothelial cells (BEC) form high endothelial venules, which control the entry of lymphocytes from the bloodstream, while lymphatic endothelial cells (LEC) form lymphatic sinuses that control entry of tissue-localized immune cells, and organization and exit of all immune cells, in addition to the flow of lymph. To determine the basis for these functional attributes, several studies have evaluated transcriptomes of LEC and BEC, primarily from peripheral tissue vessels. Most of these have used microarray approaches and often relied on endothelial cells cultured *in vitro* (1–11), (see also EndoDB (12) for a comprehensive listing of prior studies, associated databases, and analysis tools). While they have revealed differences in LEC and BEC in genes implicated in vascular tube formation, transport of solutes, and immune cell trafficking, microarray hybridization-based approaches posed several limitations, including high background levels and limited range of detection. Furthermore, these studies also concluded that even short-term primary cultures of LEC and BEC *ex vivo* resulted in some level of de-differentiation. Additionally, these studies used cells isolated from the skin and did not compare LEC and BEC from different anatomical sites. Analysis of transcriptional programs to understand the functionality and diversity of LEC and BEC in different anatomical locations remains to be done.

Recent studies have demonstrated that LN-associated LEC (LN-LEC) also actively participate in controlling innate and adaptive immune responses. We previously demonstrated that LN-LEC, but not LEC in tissue lymphatics, adventitiously expressed transcripts for proteins otherwise restricted to a small number of peripheral tissues. We showed that a peptide epitope from one of these, the melanocyte protein tyrosinase (Tyr), was presented on LN-LEC associated MHC-I molecules to Tyr-specific CD8 T cells (13–15). Although this induced activation and proliferation, LN-LEC also expressed high levels of PD-L1 that resulted in deletion of Tyr-specific CD8 T cells (15). LEC from tissue lymphatics express negligible levels of PD-L1 (14). In a separate study, we established that LN-LEC could induce Lag3 dependent CD8 T cell deletion via expression of MHC-II molecules, and that LEC from tissue lymphatics express negligible levels of MHC-II (16). While LN-LEC were incapable of presenting acquired Ag via these MHC-II molecules, they nonetheless transferred endogenous antigens to dendritic cells (DC) for presentation to CD4 T cells, resulting in anergy (16). These results point to an important role for LN-LEC in establishing systemic peripheral T cell tolerance. Conversely, others have shown that LN-LEC capture and archive exogenous antigens that induce antigen-specific memory CD8 T cell persistence (17). This occurs via transfer of LEC-archived antigens to migratory DC as a result of LEC apoptosis during LN contraction and also via direct exchange of archived antigens by the two cell types (18). The molecular mechanisms involved in these different processes of antigen acquisition, expression, and transfer by LN-LEC remain unclear, and the specific microenvironmental influences that control the phenotypic as well as functional distinctions between LEC in the LN and in the periphery remain to be fully understood.

In this study, we address these issues, as well as the technical limitations of previous studies, by using RNA-Seq analysis to compare the transcriptomes of freshly isolated murine LN-associated LEC and BEC (LN-BEC) as well as freshly isolated LEC from the diaphragm (D-LEC) as representative of peripheral tissue lymphatics. RNA-Seq has greatly improved the analysis of whole transcriptomes with higher sensitivity and dynamic range coupled to lower technical variations compared to microarrays and quantitative PCR (19, 20). Our work provides an important resource for further exploration of endothelial cell functionality in different anatomical locations.

RESULTS AND DISCUSSION

LN-LEC, LN-BEC, and D-LEC Are Transcriptionally Distinct

LEC and BEC populations were purified from relevant tissues using magnetic bead enrichment and electronic cell sorting from 10 to 33 C57BL/6 mice for each replicate sample, and subjected to RNA-Seq (**Figures S1A,B**). This yielded 48–98 million reads per replicate, with an average length of 180 nucleotides, and an average of 85.7% uniquely mapped reads. These reads mapped a total of 23,284 genes. One previous study estimated that one transcript copy per liver cell corresponds to 3 FPKM (21), while another estimated that genes expressed at FPKM > 1 were reproducibly and accurately detected in bulk RNA-Seq experiments (22). Based on this, we identified

Abbreviations: BEC, blood endothelial cells; LEC, lymphatic endothelial cells; LN, lymph node; LN-BEC, lymph node-associated blood endothelial cells; LN-LEC, lymph node-associated lymphatic endothelial cells; D-LEC, lymphatic endothelial cells from diaphragm; DC, dendritic cells; DEG, genes differentially expressed between any two cell types; 5X-DEG, genes whose differential expression in pairwise comparisons was greater than 5-fold; GO, Gene Ontology; ECM, extracellular matrix.

genes with FPKM $\geq$ 1 and p-adjusted $<$ 0.05 in all replicate comparisons, which ensures that low level FPKM values are consistent. This gave a total of 15,331 genes considered to be expressed in at least one cell population (**Table S1**). Similar gene numbers were expressed in LN-LEC, LN-BEC, and D-LEC, respectively (**Figure S1C**). Principal component analysis revealed that the transcriptional profiles of replicates clustered tightly, and LN-LEC, LN-BEC, and D-LEC differed from each other (**Figure S2A**). The pan-endothelial marker (CD31) was strongly expressed in all endothelial cell populations (**Table 1**). Established markers of LEC (LYVE-1, PDPN, PROX-1, and RELN) were strongly expressed in LN-LEC and D-LEC with minimal (1.0–3.4%) cross expression by LN-BEC (**Table 1**). Established markers of BEC (NRP-1, VEGFR-1, VWF, and NOTCH-4) were strongly expressed in LN-BEC with minimal cross expression in LN-LEC and D-LEC (0.2–4.7%). The low levels of cross expression of these genes are consistent with very low cross-contamination or genuine low-level expression. Known markers of fibroblast reticular cells and hematopoietic subpopulations were evident only at very low to negligible levels (**Table 1**). Consistent with our previous findings (13, 14), Tyr was expressed by LN-LEC but not LN-BEC and D-LEC, while (PD-L1) was expressed at high levels in LN-LEC and LN-BEC but not D-LEC. These data established a high level of confidence in further analyzing gene expression patterns of that differ among LN-LEC, LN-BEC, and D-LEC.

TABLE 1 | RNA-seq validation of stromal cell-specific markers and hematopoietic cell-lineage markers based on normalized gene expression levels (FPKM).

Lineage	Gene	Average LN-LEC	Average D-LEC	Average LN-BEC
Endothelial	**CD31/Pecam1**	30662	31820	98007
Lymphatic endothelial	Pdpn	**18631**	**63207**	632
	Lyve1	**60105**	**165926**	1730
	Prox1	**8005**	**7925**	228
	Reln	**2994**	**85960**	78
Blood endothelial	Vegfr1 (Flt1)	195	61	**14897**
	Vwf	125	164	**6455**
	Notch4	92	198	**4188**
	Nrp1	666	106	**46414**
Fibroblastic reticular	Pdgfra	184	98	227
	Pdgfrb	90	35	345
	Des	96	49	161
Hematopoietic	Cd45	40	15	289
T cell	Cd3 (d,e,g)	6	3	168
	Cd8 (a,b1)	8	3	62
	Cd4	13	8	26
B cell	Cd19	18	4	183
	Cd20	17	6	125
Dendritic cell	Cd11c	3	2	4
Macrophage	Cd11b	1	3	7
Tolerogenic profile	Tyr	**346**	9	19
	Cd274	**11612**	866	**9798**
Housekeeping genes	Actb	1,052,300	1,561,806	1,328,475
	Hprt	3837	5214	4941

Genes and FPKM values highlighted in bold represent previously identified lineage and phenotypic markers associated with the cell types.

Differential Gene Analyses Reveal Subsets of Genes Specific Only to LN-LEC, LN-BEC, or D-LEC, and Subsets of Genes Shared by at Least Two Cell Populations

Genes that were differentially expressed between any two cell types (DEG) were identified based on an adjusted $p <$ 0.05. Comparisons of LN-LEC vs. D-LEC, LN-LEC vs. LN-BEC, and D-LEC vs. LN-BEC identified 7210, 6109, and 6994 DEG, respectively (**Figure 1A**). We next identified genes whose differential expression in these pairwise comparisons was greater than 5-fold (5X-DEG). A total of 1512, 1634, and 937 5X-DEG were overexpressed in LN-LEC, LN-BEC, and D-LEC respectively, accounting for a total of 3137 unique 5X-DEG (**Figures 1A,B**). Since the total expressed genes in these populations were similar (**Figure S1C**), the substantially higher numbers of 5X-DEG in LN-BEC and LN-LEC relative to D-LEC suggests that the two LN populations have more elaborated functionalities.

Hierarchical clustering identified subsets of 5X-DEG distinct to only LN-LEC, LN-BEC, and D-LEC, and subsets shared by two cell types: LN-LEC+LN-BEC, LN-LEC+D-LEC, and D-LEC+LN-BEC (**Figure 1C**; **Table S2**). There were relatively few 5X-DEG in the D-LEC+LN-BEC shared subset, consistent with the distinct developmental origins and anatomical locations of these two populations. Intriguingly, the LN-LEC+LN-BEC shared subset contained 3.4 times more 5X-DEG than the LN-LEC+D-LEC shared subset. Thus, despite their different developmental origins, the two LN-localized endothelial populations are more transcriptionally related to one another than the two LEC populations that occupy distinct anatomical niches.

We used GOrilla software to identify biological process and molecular function Gene Ontology (GO) terms in each 5X-DEG subset that were highly ranked based on enrichment score (see Methods), which emphasizes co-expression of multiple genes associated with a term, rather than overexpression of individual genes. We identified GO terms with significant (p-adjusted $<$ 0.001) enrichment scores in all 5X-DEG subsets except D-LEC+LN-BEC (**Figure S2B**), and this subset was thus excluded from the analyses below. These GO terms were often interrelated and were further grouped into clusters based on visual inspection (**Table S3**; **Figure S2C**). These clusters, and the overexpressed genes that they contained, are discussed in more details in sections below.

Differential Expression of Extracellular Matrix Components and Cell Adhesion Molecules Suggest Specialized Structural and Functional Attributes of LEC and BEC in Distinct Tissue Microenvironments

GO terms related to extracellular matrix (ECM) were highly ranked in all 5 5X-DEG subsets and identified overexpressed

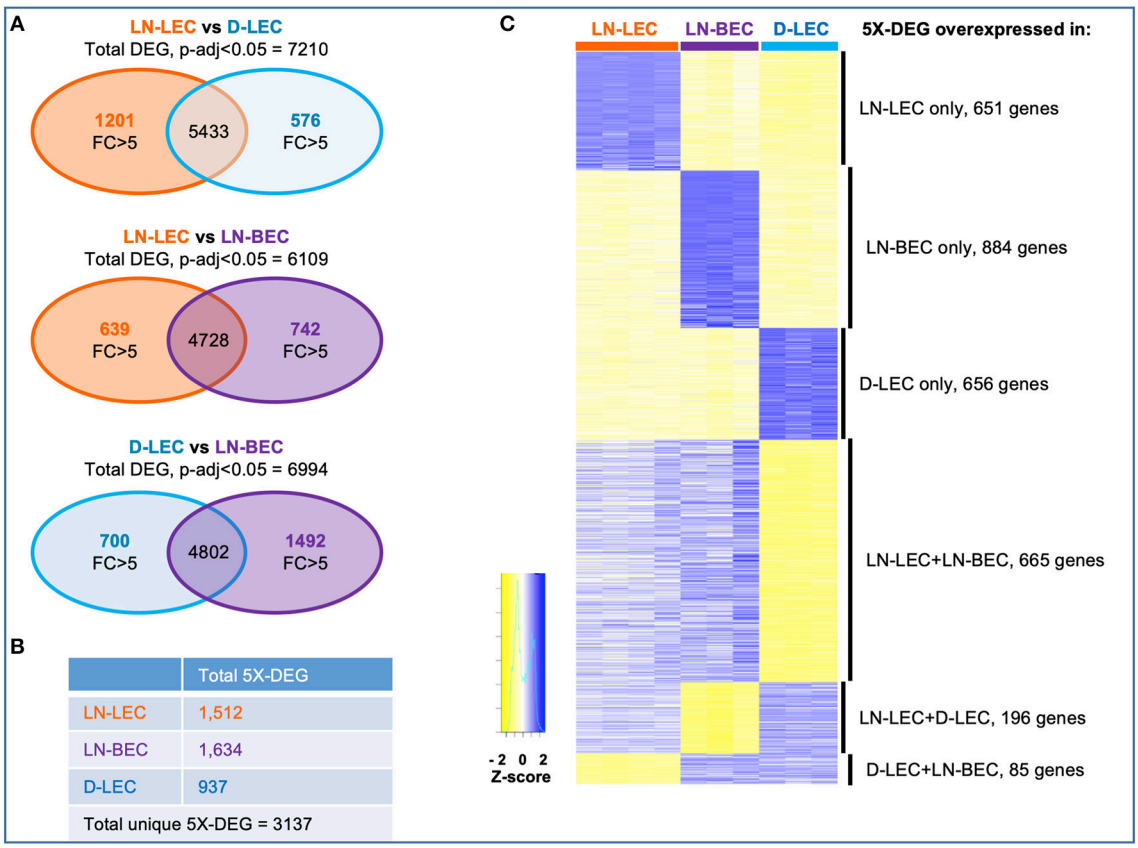

FIGURE 1 | Differential gene analysis and hierarchical clustering of differentially expressed genes in LN-LEC, LN-BEC, and D-LEC revealed distinct and shared subsets by at least two populations. **(A)** Venn diagrams showing pairwise comparisons of LN-LEC, LN-BEC, and D-LEC. **(B)** Total number of unique and cell type specific 5X-DEG. **(C)** Hierarchical clustering of distinct and shared subsets of 5X-DEG. Complete lists of 5X-DEGs in each subset are listed in **Table S2**.

genes in several different processes and functions. Each subset overexpressed different collagen molecules while 3 laminin family members were overexpressed in one of the two LN endothelial cell populations, and 4 fibronectin family members were overexpressed in one of the two LEC populations (**Figure 2**). Members of the tenascin, thrombospondin, elastin, and proteoglycan families were overexpressed in one or both of the two LEC populations, but none were overexpressed in LN-BEC. Similarly, ECM remodeling enzymes of the MMP, ADAM, and LOXL families were widely overexpressed in the LEC subpopulations, but minimally in LN-BEC. These data point to an elaboration of ECM components in LEC compared to BEC, and also suggest that LEC in different anatomical locations create distinct ECM microenvironments through both synthesis and remodeling activities. These may contribute to distinct structural and functional attributes of adjacent lumenal and ablumenal compartments.

GO terms related to cell adhesion were also highly ranked in all 5 5X-DEG subsets. Each of the 5 subsets overexpressed different integrin molecules (**Figure 2**). The LN-LEC, LN-BEC, D-LEC, and LN-LEC+LN-BEC also overexpressed different cadherin and cadherin-like family members and cell adhesion molecules (CAMs), known to mediate homophilic adhesion of endothelial population of common lineage and origin. No integrins or cadherin family members were overexpressed in the LN-LEC+D-LEC subset, suggesting a significant distinction in the patterns of cell engagement by these two LEC subpopulations. The leukocyte CAMs, all of which play well-established roles in mediating the extravasation of cells into lymphoid and peripheral tissues, were overexpressed in the LN-BEC only, but also LN-LEC+LN-BEC 5X-DEG subsets. Their function in LN-LEC remains to be established. Claudin and catenin family members were also overexpressed almost exclusively in the LN endothelial populations, with only a single catenin gene overexpressed in D-LEC associated subset. Taken together, these data suggest that LN endothelial subpopulations are endowed with an enriched capacity for interactions with a diversity of other cells relative to D-LEC.

Conversely, GO terms related to cytoskeleton were highly ranked in the D-LEC only 5X-DEG subset, and to a lesser extent, the LN-LEC+D-LEC subset. These subsets contained a variety of overexpressed genes encoding cytoskeletal proteins and binding molecules (**Figure 3**). The enhanced expression of these cytoskeletal proteins and binding molecules are likely attributes of LEC in peripheral tissue lymphatics that allow them to maintain shape in the face of ECM-mediated displacement during body movement.

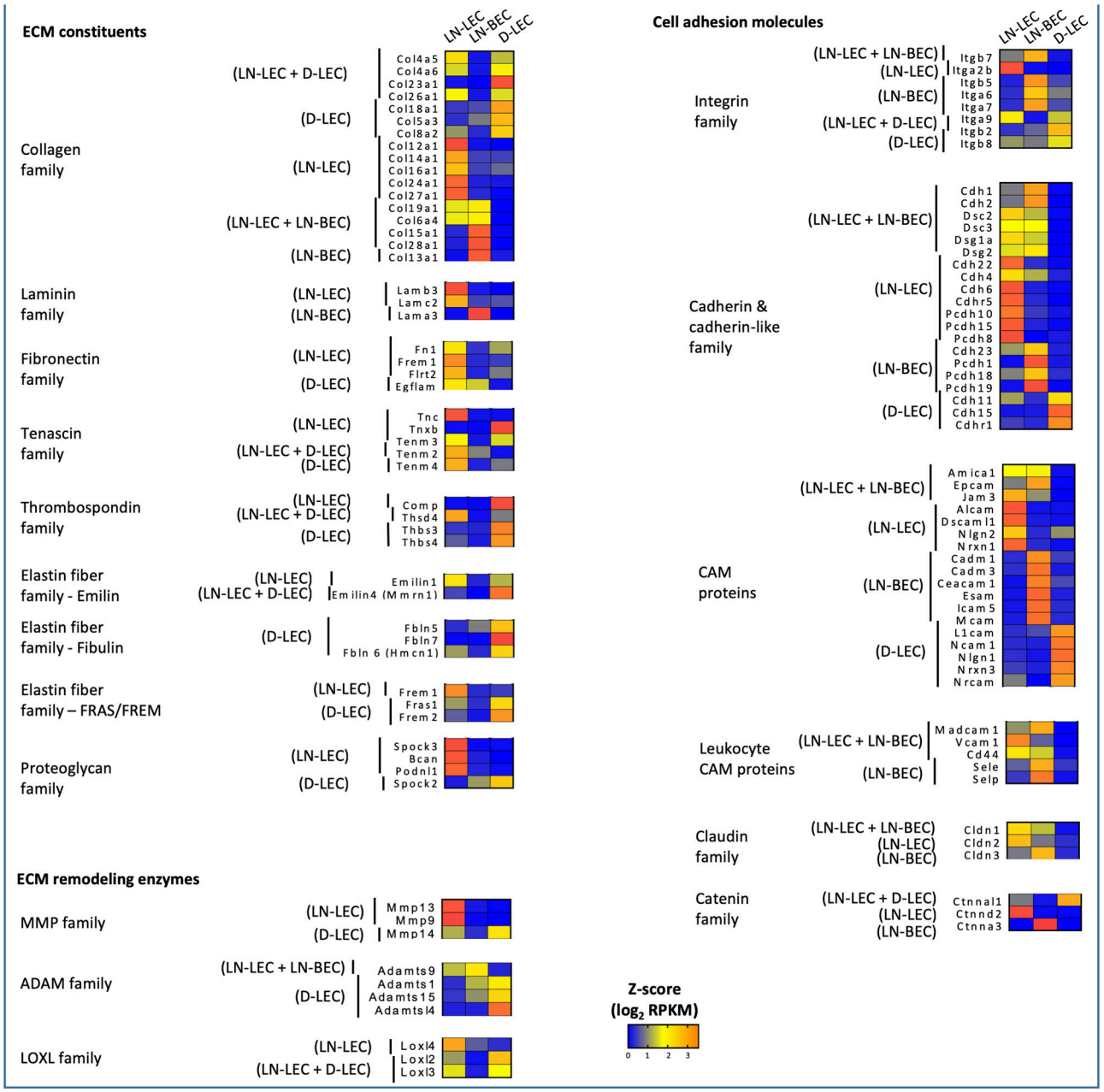

FIGURE 2 | 5X-DEG in all five subsets expressed distinct and shared members of the ECM constituents, remodeling enzymes, cell adhesion molecules. Heatmap analysis based on Z-score values of average log2 FPKM for replicates in each cell type (details in Methods).

Chemokine Expression Patterns Suggest a Collaborative Division of Labor Between LEC and BEC in Maintaining Spatial Organization and Compartmentalization of Cells in LN

GO terms related to chemokines were highly ranked in the LN-associated 5X-DEG subsets, but not those associated with D-LEC. Nonetheless, the patterns of chemokine expression in the LN-associated subsets revealed a surprising degree of complexity. The homeostatic chemokines CCL19 and CCL21 are two CCR7 ligands that have been implicated in homing of multiple immune cell subsets to LN via blood and lymph and organizing the T-cell zone of secondary lymphoid organs. CCL19 was overexpressed in the LN-LEC+LN-BEC subset (**Figure 4**), while CCL21 was not expressed at all in any analyzed EC population (<1 FPKM).

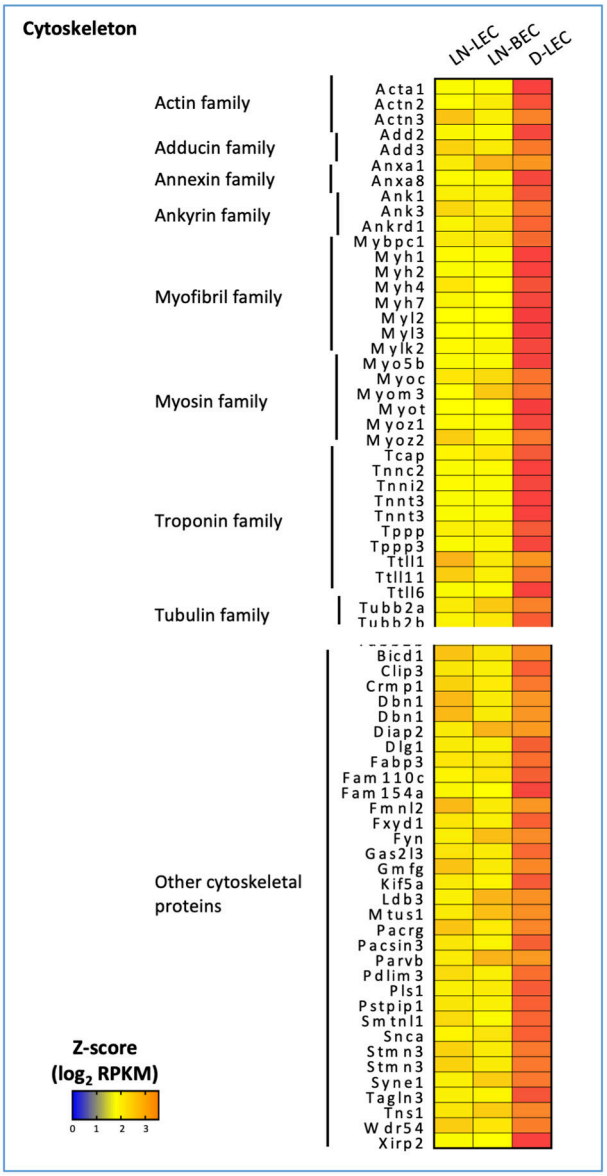

FIGURE 3 | 5X-DEG in D-LEC only subset showed enriched representation of different cytoskeletal protein groups compared to other 5X-DEG subsets. Heatmap analysis based on Z-score values of average log2 FPKM for replicates in each cell type (details in Methods).

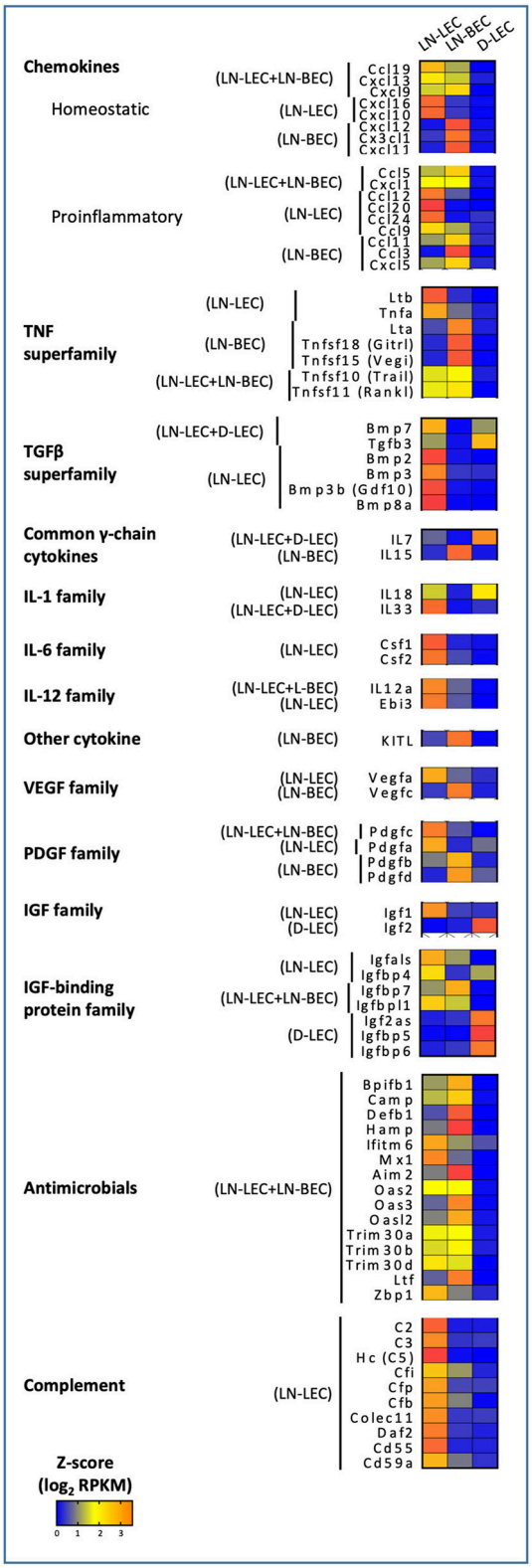

FIGURE 4 | 5X-DEG revealed immunomodulatory role of LEC and BEC via cytokines and innate effector molecules production. Heatmap analysis based on Z-score values of average log2 FPKM for replicates in each cell type (details in Methods).

While this is at odds with previous studies (10), we also confirmed using Q-PCR that CCL21 expression in LN-LEC was negligible compared to expression in bulk LN (**Figure S3**). Previous studies demonstrated the preferential ability of CCL19 to recruit $CCR7^+$ cells compared to CCL21 (23–26) and that CCL19 signaling blocks directed migration of $CCR7^+$ cells toward weak CCL21 signal (27). Our results suggest that autocrine secretion of CCL19 by LEC and BEC may play a role in organizing $CCR7^+$ cells in the face of distinct gradients of CCL21.

The homeostatic chemokine CXCL13, a ligand for CXCR5 that organizes the B-cell zone of secondary lymphoid organs, was

also overexpressed in the LN-LEC+LN-BEC subset (**Figure 4**). A previous study reported CXCL13 expression in LEC but not BEC isolated from peripheral LN (28), while another reported that it is highly expressed on HEV from Peyer's Patches (29). Because our LN samples were pooled from both peripheral and mesenteric LN, it is possible that LN-BEC in mesenteric LN may be similar to those in Peyer's Patches due to their close anatomical proximity. Nonetheless, insofar as CXCL13 appears to be essential for organization of LN but not B-cell entry (30, 31), our results suggest that both LN-LEC and LN-BEC have the potential to influence this process.

CXCL12, a chemokine that plays multiple roles but is particularly involved in homing of both T- and B-cells into LN (29, 32–34), was overexpressed only in LN-BEC (**Figure 4**). This is in keeping with its principle function in homing, as opposed to organization, of LN. LN-BEC also selectively expressed CX3CL1, a membrane associated chemokine that binds to $CX3CR1^+$ cells. These include memory CD8 T-cells that reside in the LN-subcapsular cortex (35), and efferocytic T-zone macrophages (TZM) (36). There is no current evidence demonstrating that CX3CL1 mediates recruitment or localization of these cells in LN. To the contrary, CX3CR1 deficient TZM localize in normal numbers, but are deficient in clearance of apoptotic cells (36) because CX3CL1 also functions as an "eat me" signal (37) Nonetheless, the selective expression of CX3CL1 in LN-BEC is not entirely consistent with this role, and suggests that it may play a role in recruitment of cells to LN.

In contrast, LN-LEC overexpressed another membrane-bound chemokine CXCL16, which has activities as a chemoattractant and a scavenger receptor (**Figure 4**). As a chemoattractant it binds to CXCR6, which is expressed on activated CD8 and CD4 T cells (38–42). CXCL16 expressed in LN fibroblastic reticular cells was shown to mediate migration and mild adhesion of $CXCR6^+$ CD8 and CD4 T cells (43). CXCL16 expression by LN-LEC could function similarly, and could mediate immune regulation of activated T cells along with other molecules such as PD-L1 and Lag3 (15, 16).

Perhaps surprisingly, the LN-LEC+LN-BEC, LN-LEC, and LN-BEC subsets each overexpressed several proinflammatory chemokines, which collectively support recruitment of a variety of immune cells, many of which are not resident in resting SLO (**Figure 4**). CXCL1 is essential for neutrophil migration and neutrophil extracellular trap formation (44), while CCL5 mediates recruitment of leukocytes expressing its cognate receptor, CCR5 (45).However, D-LEC do not overexpress any similar chemokines. Given the pervasiveness of blood and lymphatic vessels in the LN, the shared expression of these chemoattractant molecules seems consistent with a role in recruitment of T-cells and DC on the one hand and B-cells on the other. However, it is also conceivable that only well-localized subpopulations of each endothelial cell type express either chemokine, enabling them to participate in organizational processes.

CXCR3 is a chemokine receptor that is widely expressed on activated and memory type I CD4 and CD8 T cells. Interestingly, LN-LEC and LN-BEC both overexpress one CXCR3 ligand, CXCL9, while the two others, CXCL10 and CXCL11, are overexpressed only in LN-LEC or LN-BEC, respectively, although CXCL11 is a pseudogene in C57Bl/6 mice (**Figure 4**). CCR5 is a chemokine receptor with a similar expression pattern on T cells, and additionally on macrophages and dendritic cells, and two of its ligands, CCL5 and CCL3, are overexpressed by LN-LEC+LN-BEC and LN-LEC subsets, respectively (**Figure 4**). This suggests a subtle interplay between LN-LEC and LN-BEC in fine-tuning organization and movement of different antigen experienced and antigen presenting cells in the LN.

In keeping with this idea, we also found overexpression of atypical chemokine receptors in LN-LEC, LN-BEC, D-LEC, and LN-LEC+LN-BEC subsets, but not the LN-LEC+D-LEC subset (**Figure 5**). These molecules typically function as decoy receptors to create chemoattractant gradients through chemokine sequestration. The LN-LEC+LN-BEC subset overexpressed ACKR5, which binds to CCL19. Since this same subset overexpresses CCL19, this may suggest that expression of the chemokine and the decoy receptor differs based on precise location within the LN. LN-LEC and D-LEC respectively overexpressed the atypical chemokine receptors, ACKR4 and ACKR3, which bind to CCL21, and CXCL11 and CXCL12, respectively. LN-BEC overexpressed ACKR1, which binds to CCL2, CCL5, CXCL1, CXCL4, CXCL5, CXCL7, and CXCL8. These data suggest that the expression of these chemokine decoy receptors may further augment cooperative interplay of LEC and BEC in controlling chemokine gradients in LN to promote directional sensing, migration, and activation of immune cells.

TNF and TNFR Superfamily Expression Patterns Suggest Overlapping but Distinct Involvement of LEC and BEC in Maintenance of LN Microarchitecture and Involvement in Autocrine and Paracrine Signaling Mechanisms

GO terms related to TNF and TNFR superfamily members were also highly ranked in the LN-associated 5X-DEG subsets, but not those associated with D-LEC. The LN-BEC 5X-DEG subset overexpressed TNFR2 (TNFRSF1B) (**Figure 5**). TNFR1 (TNFRSF1A) and LTβR were overexpressed at similar levels in LN-LEC and LN-BEC relative to D-LEC, but the fold change was <5 (GSE119499). However, LN-LEC overexpressed TNFα and LTβ, while LN-BEC overexpressed LTα (**Figure 4**). Since LTα can form either a homotrimer that binds TNFRs or a heterotrimer with LTβ that binds LTβR, this suggests that these two populations differ in expression of these alternative forms, possibly leading to differences in autocrine or paracrine signaling that could influence lymphoid tissue microarchitecture. It is well-established that LTβR signaling is required form homeostatic maintenance of HEV phenotype, including expression of PNAd and MadCAM-1 (46) and one major source of $LT\alpha1\beta2$ is dendritic cells (47). Conversely, LTβR signaling plays a role in expression of homeostatic chemokines (48) and dendritic cell maturation (49, 50). Our data suggest that dendritic cells influence LN-LEC and LN-BEC phenotypes via the LTβR signaling pathway, and vice versa. Additionally,

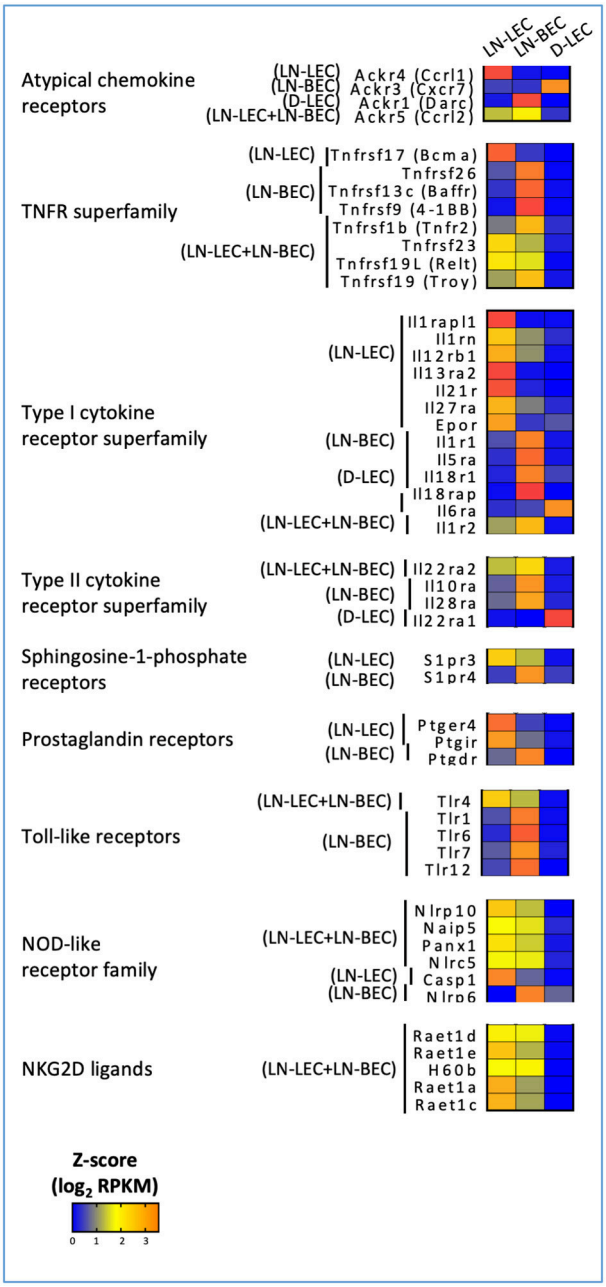

FIGURE 5 | 5X-DEG revealed immunosensory role LEC and BEC via cytokines and pathogen sensing. Heatmap analysis based on Z-score values of average log2 FPKM for replicates in each cell type (details in Methods).

the LN-LEC+LN-BEC 5X-DEG subset overexpressed RankL (**Figure 4**). The roles of these molecules in LN development and maintenance are well-established (51–56), but the specific involvement of endothelial cells as either initiators or recipients of TNF-related signaling has not been well-described.

Other overexpressed TNF and TNFR superfamily members have been associated with induction of cell death. The LN-LEC+LN-BEC subset overexpressed TRAIL (TNSFSF10), a TNF-superfamily ligand known to induce death of activated cells that express TRAIL receptors (57–60) (**Figure 4**). However, we did not detect pro-apoptotic TNFRSF10B (TRAIL-R2) expression in either LN endothelial subset. Instead, they expressed a decoy receptor for the ligand, TNFRSF23 (mDCTRAILR1) (61) (**Figure 5**). These data suggest that LEC and BEC in the LN induce TRAIL-mediated apoptosis of other cell types while protecting themselves. Conversely, the LN-LEC+LN-BEC 5X-DEG subset overexpressed TNFRSF19L (RELT). RELT has been shown to induce cellular cell death in multiple cell types via a mechanism distinct from TNFR1 (62). A ligand for RELT has not been identified (63). It is intriguing to consider whether RELT represents a mechanism by which inflammation driven LN angiogenesis and lymphangiogenesis might be downregulated after resolution.

Several overexpressed TNF and TNFR superfamily members have costimulatory or survival promoting functions. LN-LEC+LN-BEC overexpress TNFSF15 (VEGI, TL1A) (**Figure 4**), which acts as a T cell co-stimulator to induce a variety of distinct T cell subsets and immunopathologies (64–69) and promotes DC maturation (70). It also inhibits expression of VEGFR1 and induces endothelial apoptosis to inhibit vasculogenesis, but promotes lymphangiogenesis (71–74). LN-BEC selectively overexpress TNFSF18 (GITRL) (**Figure 4**), which co-stimulates both effector and regulatory T cells (75–77). Interestingly, LN-BEC also selectively overexpress TNFRSF9 (4-1BB), which could render them susceptible to signals delivered by 4-1BBL^{+} cells such as DC, and TNFRSF13C (BAFFR), which promotes B-cell survival and isotype switching (78, 79) (**Figure 5**). Another receptor for BAFF, TNFRSF17 (BCMA), which promotes survival of long-lived plasma cells (80) was overexpressed in LN-LEC (**Figure 5**). The impact of signals delivered by these receptors on endothelial function is unknown.

Selective Expression of Multiple TGFβ-Superfamily Members Suggests That LN-LEC Contribute to Immunosuppressive Functions in Homeostasis

GO terms related to the TGFβ-superfamily were highly ranked in the LN-LEC+D-LEC and LN-LEC 5X-DEG subsets, but not in the D-LEC, LN-BEC, and LN-LEC+LN-BEC subsets. LN-LEC+D-LEC overexpressed TGFβ3 and BMP7, while LN-LEC overexpressed BMP2, BMP3, BMP3B, and BMP8A (**Figure 4**). TGFβ3 is highly homologous to TGFβ1 and TGFβ2, which were comparably expressed in all three endothelial populations. However, TGFβ3 binds more potently to TGFβ receptors I (TGFBR1/ALK-5) and II (TGFBR2) (81–83). TGFβ3 plays similar roles in immunosuppression and stimulation as TGFβ1 (84, 85), TGFβ3, BMP7, and BMP2 have been shown to suppress survival, proliferation, differentiation of *in vitro* grown human B cells into antibody-secreting cells (86–88). BMP2 inhibits T cell proliferation (89) and promotes Foxp3^{+} Treg generation in the context of TGFβ treatment (90). Little is known about the immunological activity of the other BMPs remaining molecules expressed in LN-LEC. Nonetheless, expression of

these molecules may be associated with previously described tolerogenic properties of LN-LEC (13, 15, 16, 91).

Expression of Cytokines and Innate Effector Molecules Suggests Additional Immunomodulatory Roles of LN-Localized LEC and BEC

GO terms for several additional cytokines and growth factors were highly ranked, almost exclusively in LN-associated 5X-DEG subsets. These included the common γ-chain cytokines, IL-7 and IL-15, which were overexpressed LN-LEC+D-LEC and LN-BEC, respectively (**Figure 4**). These observations corroborate earlier work (7), and suggest a division of labor between LEC and BEC in maintaining IL-7Rα$^+$ and IL-15Rα$^+$ cells in LN. They also point to D-LEC as a source of IL-7 for homeostatic T cell maintenance. LN-LEC+D-LEC also overexpressed IL-18, while LN-LEC expressed IL-33, both members of the IL-1 family. IL-18 synergizes with IL-7 in activation and priming of naïve CD8 T cells (92), in that IL-7 upregulates IL-18R. Finally, LN-LEC+LN-BEC and LN-LEC subsets overexpressed IL-12a (p35) and EBI3, respectively. IL-12a (p35) pairs with EBI3 to form IL-35 (93, 94), which has been demonstrated to induce inducible regulatory T cells (iTregs) (95), and suppress T cell proliferation (96). The LN-LEC+LN-BEC subset overexpressed KITL, a ligand for the cell surface tyrosine kinase KIT found on lymphocytes and hematopoietic stem cells, while LN-LEC overexpressed CSF1 and CSF2, ligands for CSFR1 and CSFR2 receptors expressed on macrophages. Collectively, these patterns of expression reinforce the expansive roles played by both LN endothelial populations, and particularly LN-LEC, in promoting the survival of different immune subpopulations, and in providing a context for their differentiation.

Consistent with earlier work (7), LN-LEC expressed the proangiogenic factor, VEGFA, while LN-BEC expressed the lymphangiogenic factor, VEGFC (**Figure 4**). This creates the possibility that these two cell types could cross-regulate one another. LEC and BEC also expressed several members of the PDGF family (**Figure 4**), which could support fibroblastic reticular cells. These data point to a dynamic co-dependence among different stromal cell types that may regulate the balance between cell populations under steady state and inflammatory conditions.

LN-LEC and D-LEC subsets, respectively overexpressed IGF1 and IGF2, distinct members of the insulin-like growth factor (IGF) family (**Figure 4**), while transcripts for IGF-family receptors (IGFR1 and IGFR2) were detected but not differentially expressed in all 3 endothelial populations. These 3 populations collectively overexpressed most of the IGF binding proteins (IGFBP), which bind to IGFs and modulate their activity in distinct ways (97–100). Together, these data demonstrated LEC in different anatomical niches control the local tissue milieus to support cellular growth, differentiation, and function via intricate networks of cellular IGF1- and IGF2-signaling and counter balance mechanisms to maintain tissue homeostasis.

GO terms associated with innate host defense mechanisms were also highly ranked in the LN-LEC+LN-BEC 5X-DEG subset, corresponding to a range of genes with antimicrobial activities to viral, bacterial, and fungal organisms (**Figure 4**). This suggests an as yet unappreciated role for LN endothelial populations to prevent pathogen dissemination. In addition, the LN-LEC 5X-DEG subset overexpressed a number of molecules associated with classical and non-classical complement cascades. These included C2, C3, and C5, which participate directly in the proteolytic cascade. The resulting products include the anaphylatoxins C3a and C5a, which serve as chemoattractant for neutrophils, monocytes, and macrophages (101–103), and modulate the functions of APCs and T cells (104–106), and C3b, which binds to pathogens, immune complexes, and apoptotic cells to promote phagocytosis (107). These data suggest that LN-LEC may collaborate with subcapsular sinus macrophages, follicular dendritic cells, and B cells to promote both innate and adaptive immune responses through complement component secretion. LN-LEC also overexpressed of CD55 (DAF), DAF2, and CD59a, all of which prevent formation of the membrane attack complex and enable LN-LEC to protect themselves from the actions of the products they secrete.

Expression of Cytokine Receptors and Pathogen Sensing Molecules Suggests Additional Immunosensory Roles of LN-Localized LEC and BEC

GO terms for several cytokine receptors and pathogen sensing molecules were highly ranked, again almost exclusively in LN-associated 5X-DEG subsets. LN-LEC+LN-BEC, LN-BEC, and most prominently, LN-LEC, overexpressed members of the type I cytokine receptor superfamily, including components of the IL-1, IL12, IL18, IL27 receptors, and antagonists and decoys (**Figure 5**). LN-LEC and LN-BEC also overexpressed receptors for several other immune relevant molecules, including IL10, sphingosine-1-phosphate, and prostaglandins. These data indicate that the LN endothelial cells are poised to sense and respond to a variety of cytokine cues in their local milieu, although the consequences of signaling by any given receptor remain to be established.

LN-LEC, LN-BEC, and LN-LEC+LN-BEC overexpressed several toll-like receptors (TLR) and NOD-like receptors (NLR). LN-LEC+LN-BEC overexpressed TLR4, while several other TLR were selectively overexpressed by LN-BEC. The patterns of NLR overexpression were more complex. As with the immune receptors above the consequences of signaling by any given TLR or NLR remain to be established. Interestingly, however, LN-BEC overexpressed NLRP6, which inhibits inflammasome formation (108, 109). The LN-LEC+LN-BEC 5X-DEG subset overexpressed several ligands for the NKG2D receptor that is expressed on NK, NKT, γδ T cells, and activated CD8 T cells (**Figure 5**). While these ligands are generally associated with promoting effector activity via NKG2D signaling, RAET1E expressed on endothelial cells was demonstrated to inhibit NK cell activation by inducing NKG2D internalization (110). This raises the question of whether the responses of LN-LEC and LN-BEC to prototypical pro-immune receptor signaling may be counter-regulatory.

LN-LEC and LN-BEC Overexpress Molecules Involved in MHC-I and MHC-II Antigen Processing and Presentation

GO terms for MHC-I and MHC-II antigen processing and presentation pathways were highly ranked exclusively in the LN-LEC+LN-BEC 5X-DEG subset. Overexpressed MHC-I pathway components included H-2K^b, β2 microglobulin, several Qa and Cd1d molecules, TAP1 and 2 components of the immunoproteasome (**Figure 6**). Overexpressed MHC-II pathway components included H-2Aβ, invariant chain, peptide editors (H-2Oα, H-2DMβ2, H-2DMβ1), and cathepsins S and G. While not achieving 5X differential expression, the MHC-I H-2D^b molecule, and the MHC-II components H-2Aα and H-2DMα were also overexpressed in the two LN cell populations relative to D-LEC (GSE119499). Cathepsin L was also overexpressed by 5X in LN-LEC relative to D-LEC, but this did not give rise to an enriched GO term score (**Table S1**). These data reinforce previous studies from our lab demonstrating that LN-LEC efficiently present endogenous antigens via H-2K^b to CD8 T cells (14–16), and suggest that LN-LEC have elevated capacity to present antigens via additional classical and non-classical MHC-I molecules. They also suggest that LN-BEC have a similar capacity. We have also reported that LN-LEC are unable to present endogenous or exogenous antigens to CD4 T cells despite expressing MHC-II molecules, and have suggested that this is due to a deficiency in H-2DM expression (16). While both H-2DMα and H-2DMβ were overexpressed in LN-LEC, their level of expression was still low (<100 FPKM), compared with FPKM values >1000 for H-2Aα, H-2Aβ, invariant chain, and cathepsins S and L, consistent with this earlier conclusion. We have concluded that the MHC-II molecules expressed on LN-LEC are primarily involved in engaging LAG-3 on T cells to induce peripheral tolerance (16). Given the similar expression of MHC-II components in LN-LEC and LN-BEC, we suggest that the latter cells serve a similar function.

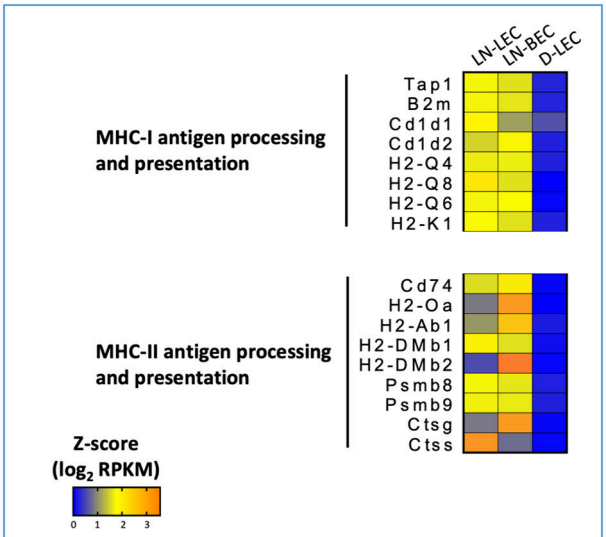

FIGURE 6 | 5X-DEG in LN-LEC+LN-LBEC shared subset revealed overrepresentation of molecules involved in antigen processing and presentation via MHC-I and MHC-II pathways. Heatmap analysis based on Z-score values of average log2 FPKM for replicates in each cell type (details in Methods).

LN-LEC Express Elevated Number of Molecules Involved in Exogenous Material Acquisition That Potentially Contribute to Their Functions in Antigen Archival and Peripheral Tolerance

GO terms for receptor mediated endocytosis were highly ranked almost exclusively in the LN-LEC 5X-DEG subset. Overexpressed molecules included C-type lectin receptors, scavenger receptors, and Fc receptors (**Figure 7A**). C-type lectin receptors have been categorized as binding to either carbohydrate, non-carbohydrate structures, or both. LN-LEC overexpressed some C-type lectins that bind carbohydrates (CLEC4A3, CLEC4D, CLEC4E, CLEC4G, CD209D) and others that bind non-carbohydrates (CLEC1A, CLEC1B, CLEC9A). We found a single C-type lectin (CLEC4G) in the LN-LEC+LN-BEC, although this subset was not enriched for GO terms associated with receptor mediated endocytosis. C-type lectin receptors also can signal via immune tyrosine activation or inhibitory motifs, or through non-canonical structural features that mediate positive or negative immune stimuli. C-type lectin receptors in the LN-LEC include all 3 of these signaling categories.

The scavenger receptors MSR-1 (SR-A1), MARCO (SR-A6), STAB-2 (SR-H2), and CXCL16 (SR-G1) were also overexpressed in LN-LEC (**Figure 7A**). Previous studies demonstrated that the class A scavenger receptors, MSR-1 and MARCO, are expressed primarily on macrophage subpopulations, and are associated with recognition of surface molecules of Gram-positive and -negative bacteria (111, 112), modified and oxidized LDL (113, 114), hepatitis C virus (115), β-amyloid (116), and heat shock proteins (117). The expression of MSR-1 and MARCO under steady state condition was reported to be restricted to macrophages in the LN and the marginal zones of the spleen (118). Our data extends this. In addition, we demonstrated by immunofluorescence and flow cytometry that subpopulations of LN-LEC express MARCO and MSR-1, while LN-BEC, fibroblastic reticular cells (FRC), and other CD45^{neg} LN stromal cell populations do not (**Figures 7B,C**). Membrane-bound CXCL16 and STAB-2 bind phosphatidylserine and oxidized lipids (119–123), and membrane-bound CXCL16 also mediates phagocytosis of bacteria (124). LN-LEC also overexpressed the Fc receptor, FCGR2B, which has been shown to be essential for internalization of immune complexes by DC (125–127).

Again, while the subset did not have high GO enrichment scores, LN-LEC+LN-BEC also expressed two scavenger receptors, CD36 (SR-B2) and SCARF-1 (SR-F1). SCARF-1 and CD36 have been previously reported as pattern recognition molecules for fungal pathogens in innate immunity (128). More recently SCARF-1 expressed by splenic DC, macrophages, and

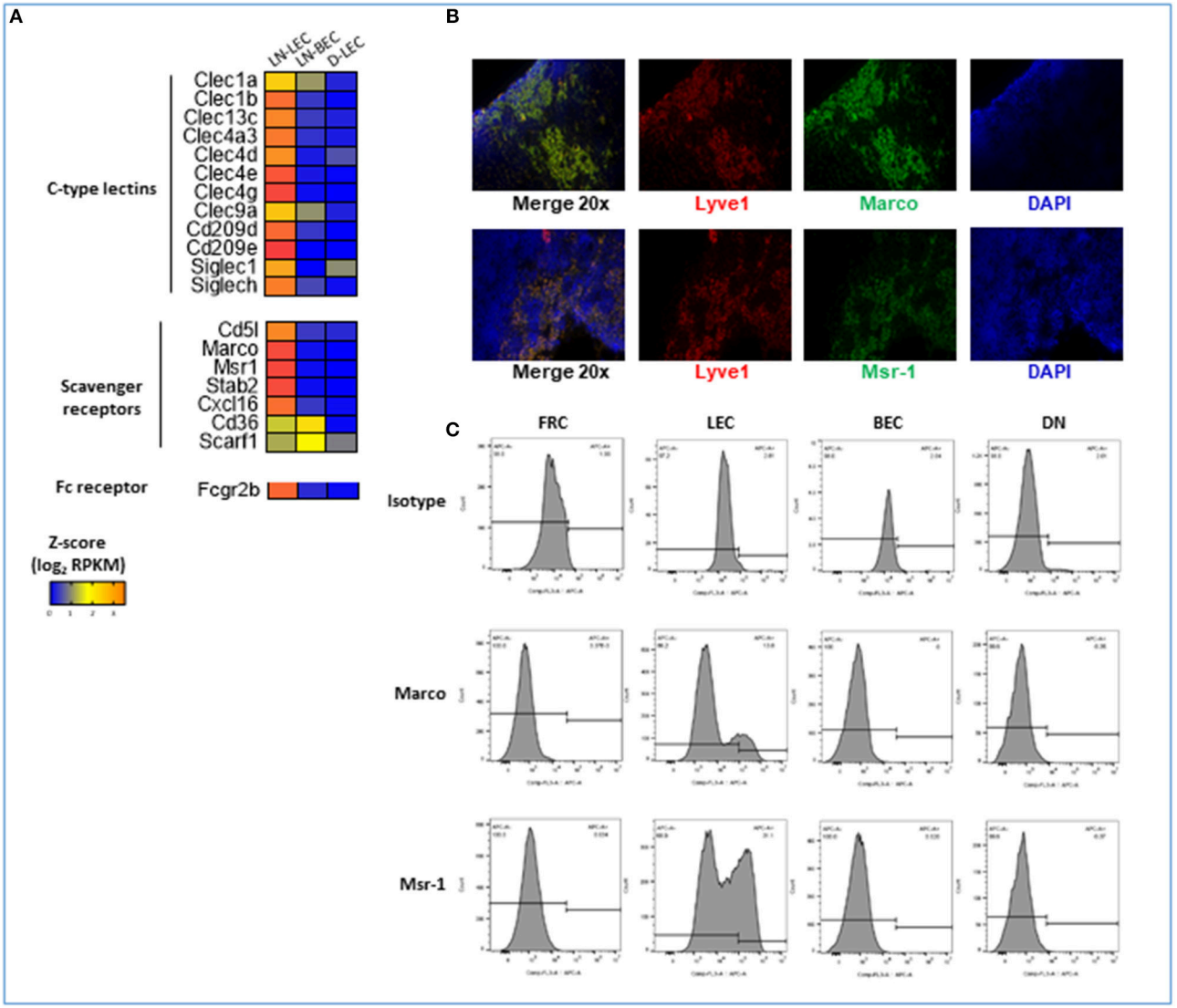

FIGURE 7 | 5X-DEG in LN-LEC only subset revealed enriched overrepresentation of molecules involved in receptor-mediated endocytosis and scavenger receptor activity. **(A)** Heatmap analysis based on Z-score values of average log2 FPKM for replicates in each cell type (details in Methods). **(B)** Flow cytometry analyses of LN stromal cell populations for expression of Marco and Msr-1. **(C)** Immunofluorescent (IF) staining in adjacent tissue sections of LN for detection of Marco and Msr-1 co-expression with Lyve-1 (LEC marker).

endothelial cell was shown to bind the complement molecule, C1q to mediate apoptotic cell clearance, thus preventing generation of autoantibodies to DNA-containing antigens that lead to lupus-like disease and autoimmunity (129). CD36 was reported to facilitate transfer of surface antigen between CD8α$^+$ DC and mTEC to promote tolerance to self-antigens during T cell development (130).

Collectively, these data point to a previously undescribed but comprehensive capability of LN-LEC to internalize a broad array of extracellular materials using a variety of pattern recognition elements, either alone or in conjunction with other immune recognition molecules. We suggest that many of these material may be delivered into antigen processing and presentation pathways, at least for MHC-I molecules (13, 16). Our previous work indicates that the MHC-II pathway is non-functional in LN-LEC in the steady state (16), but it remains possible that this changes under conditions of pathogen exposure and inflammation. An important question is whether this is primarily a means of generating tolerance to exogenous self-antigens, or whether LN-LEC may sometimes also serve as accessory antigen presenting cells during an active immune response. In addition, this internalization capability is likely to be important in the antigen acquisition and archiving functions of LN-LEC (17, 18).

Attempting to Identify Peripheral Tissue Antigens Expressed by LN-LEC

We previously demonstrated that LN-LEC adventitiously expressed transcripts for proteins otherwise restricted to

a small number of non-hematopoietic, non-endothelial peripheral tissues, typified by Tyr (13, 14). Because expression of these molecules is not dependent on Aire, as is the case for medullary thymic epithelial cells (131), it has not been possible to characterize the full range of peripheral tissue antigens potentially displayed by LN-LEC. With the expansion of available data on tissue specific expression, it has also become more difficult to unambiguously identify genes whose expression is rigorously limited to only a small number of tissues. Using the characteristics of Tyr expression (FPKM<343), we hypothesized that there would be an elevated number of transcripts at or below this expression level in LN-LEC compared to D-LEC. However, we found that these two subsets contained equal number of 5X-DEG that met this criterion (**Table S4**). Thus, although the cut-off criteria based on Tyr expression is a good first step, a more comprehensive approach is needed to identify the candidate peripheral tissues antigens expressed by LN-LEC.

Conclusions

This study provides comprehensive comparative transcriptomic analyses of LN-LEC, LN-BEC, and D-LEC and has defined in detail gene expression differences that point to functional specializations of EC in different anatomical locations. Our goal was to provide a broad compendium of gene expression differences based on anatomic location and endothelial lineage, with a focus on genes of immunological interest. We believe that this information will be a significant and extremely useful resource for many workers in this field. Our data identify significantly expanded cohorts of immunologically significant genes either shared by LN-localized ECs, or expressed distinctly by one or the other LN-localized subset. These genes extend the understanding of both populations as regulators, not only of hematopoetic cell trafficking, but also cellular differentiation. They also point to an emerging understanding that LN-localized EC express a variety of receptors that enable them to sense immunologically relevant changes in their environment. Further exploration of the consequences of this sensing on EC proliferation, differentiation, and function in immunoregulation is an important area for further work. This study clarifies that both LN-localized EC function as antigen presenting cells, and this issue explored in somewhat greater detail elsewhere (Santambrogio et al., Manuscript Submitted[1]). Finally, this study highlights the surprising number of molecules involved in uptake of exogenous materials expressed distinctly by LN-LEC. The involvement of these molecules in enabling antigen archiving and peripheral tolerance to exogenous self- antigens is another rich area for further exploration.

While our comparative analysis identifies profound differences in bulk populations that are based on anatomical location, it does not address the almost certain heterogeneity that exists at the single cell level in each location, some of which has been pointed to in our own previous work (14). However, given the limited depth of single cell coverage, many of the differences we have identified might not have been immediately evident with that approach. Nonetheless, the results presented here provide a springboard for further work to establish the existence of heterogeneity in expression within LN anatomical niches using a variety of technical approaches. Taken together, the comparative gene expression profiles provided here would be useful resources for future work to uncover novel mechanisms of endothelial functionality and specialization in peripheral tissues and LN.

[1]Santambrogio L, Berendam SJ, Engelhard VH. The antigen processing and presentation machinery in lymphatic endothelial cells. *Front Immunol.* In Review.

MATERIALS AND METHODS

Mice

C57BL/6 mice (6–8 weeks of age) were purchased from NCI and were housed at pathogen-free facilities at the University of Virginia. All procedures were carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and were approved by the University of Virginia Animal Care and Use Committee.

LEC and BEC Isolation and Cell Sorting

Inguinal, axillary, brachial, cervical, and mesenteric LN were harvested, pooled, mechanically disrupted, and enzymatically digested for 15 min, followed by MACS bead depletion of $CD45^+$ cells as previously described (16). Diaphragm tissues were treated in the same way. $CD45^{neg}$ cells were electronically sorted based on absence of expression of CD45 (eBioscience, clone 30-F11), expression of pan-endothelial marker, CD31 (eBioscience, clone 390), and presence or absence of PDPN (Biolegend, clone 8.1.1) to distinguish LEC from BEC. Cells were collected in RNA Protect (Qiagen).

RNA Extraction, cDNA Library Construction, and Sequencing

Total RNA was purified using RNAeasy mini kit (Qiagen) per manufacturer's instructions. cDNA library preparation and sequencing were performed by the Genomic Services Laboratory at Hudson Alpha, USA. Briefly, purified total RNAs (RIN score of 7.0 or higher) were prepared for sequencing using the Ovation RNA-Seq System V2 kit (Nugen) followed by RNA-Seq of 100 paired-end reads using the Illumina HiSeq 2500 v4 platform.

Mapping, Quantification, and Differential Gene Analysis

Raw RNA-Seq read quality was assessed using FastQC (132) and low-quality regions were trimmed using Fastx-trimmer (http://hannonlab.cshl.edu/fastx_toolkit/index.html). Cleaned reads were aligned to the mouse reference genome (build mm9) using STAR (133) and read counts on known mouse genes were calculated using featureCounts, part of the Subread package (134). Next, uniquely aligned reads were analyzed using the DEseq2 package in the R statistical computing environment (R Development Core Team, 2011, http://www.R-project.org/) to obtain normalized counts, estimate dispersion, and determine a negative binomial model for each gene. Principal

Component Analysis was performed on rlog-transformed counts for quality assessment. Differentially expressed genes (DEG) were determined using DESeq2 and the Benjamini-Hochberg False Discovery Rate procedure was used to re-estimate the adjusted *p*-values. In our analyses, DEG were identified as those with an average FPKM of 1 or greater and replicate comparisons of *p*-adjusted < 0.05 in all cell types. 5X-DEG were identified as those with fold-change of 5 or greater. Hierarchical gene clustering analysis was performed using complete linkage and Euclidean distance as measure of similarity to display the DEG expression patterns.

Gene Ontology Analysis of 5X-DEG Subsets

Gene ontology analysis was performed with GOrilla software using the two ranked lists method (135). We used the 5X-DEG subsets as the target set and the all annotated genes from mouse reference genome (build mm9) as the background set. Briefly, GOrilla generates an enrichment score, which is the number of genes in the intersection of genes in the GO term (designated as B) and the number of genes in the target set (designated as b) for each associated GO term. We used the list of 5X-DEG for each subset as target. Enrichment of GO terms is then tested for statistical significance using a hypergeometric test and *p*-adjusted < 0.001 was considered as significant. Analyses were performed against gene ontologies: biological process and molecular function. We then identified the relationship between GO terms using hierarchical directed acyclic graph generated by GOrilla.

Immunofluorescence Staining and Flow Cytometry Analyses of LN-LEC

Immunofluorescence staining of adjacent LN tissue sections of C57BL/6 mice (purchased from NCI) were performed using rat anti-mouse LYVE-1 (R&D Systems, MAB2125), goat anti-mouse MARCO (R&D Systems, AF2956), goat anti-mouse MSR1 (R&D Systems, AF1797), normal goat IgG (R&D Systems, AB-108-C), and rat IgG2a (R&D Systems, MAB006) antibodies at final concentrations of 5 μg, respectively. Subsequent detection were performed using donkey anti-goat IgG-FITC conjugated (R&D Systems, F0109) and mouse anti-rat IgG2a (eBioscience, cat# 11-4817-82) at manufacturers' recommended concentrations. Flow cytometry detection of MARCO and MSR1 were performed using cells gated on CD31 and gp38 expressions as described above using anti-mouse MARCO-APC conjugated (R&D Systems, FAB 2956A), anti-mouse MSR1-APC conjugated (R&D Systems, FAB1797A), and isotype control antibodies (R&D Systems, IC005A and IC006A).

DATA AVAILABILITY

The RNA-Seq datasets generated from this study have been deposited in GEO archives under the accession number GSE119499.

ETHICS STATEMENT

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Procedures were approved by the University of Virginia Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

SR, AW, JP, and KC purified the cells and extracted the RNA. AR performed the qRT-PCR. SB performed immunofluorescence staining and flow cytometry analyses. AK and ST performed bioinformatics and statistical analyses. SB and NG analyzed the data. SB, AW, and VE designed the figures. SB and VE wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.00816/full#supplementary-material

REFERENCES

1. Amatschek S, Kriehuber E, Bauer W, Reininger B, Meraner P, Wolpl A, et al. Blood and lymphatic endothelial cell-specific differentiation programs are stringently controlled by the tissue environment. *Blood*. (2007) 109:4777–85. doi: 10.1182/blood-2006-10-053280
2. Kriehuber E, Breiteneder-Geleff S, Groeger M, Soleiman A, Schoppmann SF, Stingl G, et al. Isolation and characterization of dermal lymphatic and blood endothelial cells reveal stable and functionally specialized cell lineages. *J Exp Med*. (2001) 194:797–808. doi: 10.1084/jem.194.6.797
3. Podgrabinska S, Braun P, Velasco P, Kloos B, Pepper MS, Skobe M. Molecular characterization of lymphatic endothelial cells. *Proc Natl Acad Sci USA*. (2002) 99:16069–74. doi: 10.1073/pnas.242401399
4. Hirakawa S, Hong YK, Harvey N, Schacht V, Matsuda K, Libermann T, et al. Identification of vascular lineage-specific genes by transcriptional profiling of isolated blood vascular and lymphatic endothelial cells. *Am J Pathol*. (2003) 162:575–586. doi: 10.1016/S0002-9440(10)63851-5
5. Lacorre DA, Baekkevold ES, Garrido I, Brandtzaeg P, Haraldsen G, Amalric F, et al. Plasticity of endothelial cells: rapid dedifferentiation of freshly isolated high endothelial venule endothelial cells outside

the lymphoid tissue microenvironment. *Blood*. (2004) 103:4164–72. doi: 10.1182/blood-2003-10-3537
6. Wick N, Saharinen P, Saharinen J, Gurnhofer E, Steiner CW, Raab I, et al. Transcriptomal comparison of human dermal lymphatic endothelial cells *ex vivo* and *in vitro*. *Physiol Genomics*. (2007) 28:179–92. doi: 10.1152/physiolgenomics.00037.2006
7. Malhotra D, Fletcher AL, Astarita J, Lukacs-Kornek V, Tayalia P, Gonzalez SF, et al. Transcriptional profiling of stroma from inflamed and resting lymph nodes defines immunological hallmarks. *Nat Immunol*. (2012) 13:499–510. doi: 10.1038/ni.2262
8. Nolan DJ, Ginsberg M, Israely E, Palikuqi B, Poulos MG, James D, et al. Molecular signatures of tissue-specific microvascular endothelial cell heterogeneity in organ maintenance and regeneration. *Dev Cell*. (2013) 26:204–19. doi: 10.1016/j.devcel.2013.06.017
9. Keuschnigg J, Karinen S, Auvinen K, Irjala H, Mpindi JP, Kallioniemi O, et al. Plasticity of blood- and lymphatic endothelial cells and marker identification. *PLoS ONE*. (2013) 8:e74293. doi: 10.1371/journal.pone.0074293
10. Lee M, Kiefel H, LaJevic MD, Macauley MS, Kawashima H, O'Hara E, et al. Transcriptional programs of lymphoid tissue capillary and high endothelium reveal control mechanisms for lymphocyte homing. *Nat Immunol*. (2014) 15:982–95. doi: 10.1038/ni.2983
11. Iftakhar-E-Khuda I, Fair-Mäkelä R, Kukkonen-Macchi A, Elima K, Karikoski M, Rantakari P, et al. Gene-expression profiling of different arms of lymphatic vasculature identifies candidates for manipulation of cell traffic. *Proc Natl Acad Sci USA*. (2016) 113:10643–8. doi: 10.1073/pnas.1602357113
12. Khan S, Taverna F, Rohlenova K, Treps L, Geldhof V, de Rooij L, et al. EndoDB: a database of endothelial cell transcriptomics data. *Nucleic Acids Res*. (2018) 47:D736–44. doi: 10.1093/nar/gky997
13. Cohen JN, Guidi CJ, Tewalt EF, Qiao H, Rouhani SJ, Ruddell A, et al. Lymph node-resident lymphatic endothelial cells mediate peripheral tolerance via Aire-independent direct antigen presentation. *J Exp Med*. (2010) 207:681–8. doi: 10.1084/jem.20092465
14. Cohen JN, Tewalt EF, Rouhani SJ, Buonomo EL, Bruce AN, Xu X, et al. Tolerogenic properties of lymphatic endothelial cells are controlled by the lymph node microenvironment. *PloS ONE*. (2014) 9:e87740. doi: 10.1371/journal.pone.0087740
15. Tewalt EF, Cohen JN, Rouhani SJ, Guidi CJ, Qiao H, Fahl SP, et al. Lymphatic endothelial cells induce tolerance via PD-L1 and lack of costimulation leading to high-level PD-1 expression on CD8 T cells. *Blood*. (2012) 120:4772–82. doi: 10.1182/blood-2012-04-427013
16. Rouhani SJ, Eccles JD, Riccardi P, Peske JD, Tewalt EF, Cohen JN, et al. Roles of lymphatic endothelial cells expressing peripheral tissue antigens in CD4 T-cell tolerance induction. *Nat Commun*. (2015) 6:6771. doi: 10.1038/ncomms7771
17. Tamburini BA, Burchill MA, Kedl RM. Antigen capture and archiving by lymphatic endothelial cells following vaccination or viral infection. *Nat Commun*. (2014) 5:3989. doi: 10.1038/ncomms4989
18. Kedl RM, Lindsay RS, Finlon JM, Lucas ED, Friedman RS, Tamburini BAJ. Migratory dendritic cells acquire and present lymphatic endothelial cell-archived antigens during lymph node contraction. *Nat Commun*. (2017) 8:2034. doi: 10.1038/s41467-017-02247-z
19. Oshlack A, Robinson MD, Young MD. From RNA-seq reads to differential expression results. *Genome Biol*. (2010) 11:220. doi: 10.1186/gb-2010-11-12-220
20. Wilhelm BT, Briau M, Austin P, Faubert A, Boucher G, Chagnon P, et al. RNA-seq analysis of 2 closely related leukemia clones that differ in their self-renewal capacity. *Blood*. (2011) 117:e27–38. doi: 10.1182/blood-2010-07-293332
21. Mortazavi A, Williams BA, McCue K, Schaeffer L, Wold B. Mapping and quantifying mammalian transcriptomes by RNA-Seq. *Nat Methods*. (2008) 5:621–8. doi: 10.1038/nmeth.1226
22. Marinov GK, Williams BA, McCue K, Schroth GP, Gertz J, Myers RM, et al. From single-cell to cell-pool transcriptomes: stochasticity in gene expression and RNA splicing. *Genome Res*. (2014) 24:496–510. doi: 10.1101/gr.161034.113
23. Corbisier J, Galès C, Huszagh A, Parmentier M, Springael JY. Biased signaling at chemokine receptors. *J Biol Chem*. (2015) 290:9542–54. doi: 10.1074/jbc.M114.596098
24. Raju R, Gadakh S, Gopal P, George B, Advani J, Soman S, et al. Differential ligand-signaling network of CCL19/CCL21-CCR7 system. *Database*. (2015) 2015: bav106. doi: 10.1093/database/bav106
25. Zidar DA, Violin JD, Whalen EJ, Lefkowitz RJ. Selective engagement of G protein coupled receptor kinases (GRKs) encodes distinct functions of biased ligands. *Proc Natl Acad Sci USA*. (2009) 106:9649–54. doi: 10.1073/pnas.0904361106
26. Byers MA, Calloway PA, Shannon L, Cunningham HD, Smith S, Li F, et al. Arrestin 3 mediates endocytosis of CCR7 following ligation of CCL19 but not CCL21. *J Immunol*. (2008) 181:4723–4732. doi: 10.4049/jimmunol.181.7.4723
27. Hansen M, Met Ö, Larsen NB, Rosenkilde MM, Andersen MH, Svane IM, et al. Autocrine CCL19 blocks dendritic cell migration toward weak gradients of CCL21. *Cytotherapy*. (2016) 18:1187–196. doi: 10.1016/j.jcyt.2016.06.010
28. Yang J, Zhang S, Zhang L, Xie X, Wang H, Jie Z, et al. Lymphatic endothelial cells regulate B-cell homing to lymph nodes via a NIK-dependent mechanism. *Cell Mol Immunol*. (2018) 16:165–77. doi: 10.1038/cmi.2017.167
29. Okada T, Ngo VN, Ekland EH, Förster R, Lipp M, Littman DR, et al. Chemokine Requirements for B Cell Entry to Lymph Nodes and Peyer's Patches. *J Exp Med*. (2002) 196:65–75. doi: 10.1084/jem.20020201
30. Ansel KM, Harris RBS, Cyster JG. CXCL13 is required for B1 cell homing, natural antibody production, and body cavity immunity. *Immunity*. (2002) 16:67–76. doi: 10.1016/S1074-7613(01)00257-6
31. Ansel KM, Ngo VN, Hyman PL, Luther SA, Förster R, Sedgwick JD, et al. A chemokine-driven positive feedback loop organizes lymphoid follicles. *Nature*. (2000) 406:309–14. doi: 10.1038/35018581
32. Miyasaka M, Tanaka T. Lymphocyte trafficking across high endothelial venules: dogmas and enigmas. *Nat Rev Immunol*. (2004) 4:360–70. doi: 10.1038/nri1354
33. Bai Z, Hayasaka H, Kobayashi M, Li W, Guo Z, Jang MH, et al. CXC chemokine ligand 12 promotes CCR7-dependent naive T cell trafficking to lymph nodes and Peyer's patches. *J Immunol*. (2009) 182:1287–1295. doi: 10.4049/jimmunol.182.3.1287
34. Scimone ML, Felbinger TW, Mazo IB, Stein JV, Von Andrian UH, Weninger W. CXCL12 mediates CCR7-independent homing of central memory cells, but not naive T cells, in peripheral lymph nodes. *J Exp Med*. (2004) 199:1113–20. doi: 10.1084/jem.20031645
35. Böttcher JP, Beyer M, Meissner F, Abdullah Z, Sander J, Höchst B, et al. Functional classification of memory $CD8^+$ T cells by CX_3CR1 expression. *Nat Commun*. (2015) 6:8306. doi: 10.1038/ncomms9306
36. Baratin M, Simon L, Jorquera A, Ghigo C, Dembele D, Nowak J, et al. T cell zone resident macrophages silently dispose of apoptotic cells in the lymph node. *Immunity*. (2017) 47:349–62.e5. doi: 10.1016/j.immuni.2017.07.019
37. Truman LA, Ford CA, Pasikowska M, Pound JD, Wilkinson SJ, Dumitriu IE, et al. CX3CL1/fractalkine is released from apoptotic lymphocytes to stimulate macrophage chemotaxis. *Blood*. (2008) 112:5026–5036. doi: 10.1182/blood-2008-06-162404
38. Kim CH, Kunkel EJ, Boisvert J, Johnston B, Campbell JJ, Genovese MC, et al. Bonzo/CXCR6 expression defines type 1-polarized T-cell subsets with extralymphoid tissue homing potential. *J Clin Invest*. (2001) 107:595–601. doi: 10.1172/JCI11902
39. Unutmaz D, Xiang W, Sunshine MJ, Campbell J, Butcher E, Littman DR. The primate lentiviral receptor Bonzo/STRL33 is coordinately regulated with CCR5 and its expression pattern is conserved between human and mouse. *J Immunol Baltim Md*. (2000) 165:3284–3292. doi: 10.4049/jimmunol.165.6.3284
40. Matloubian M, David A, Engel S, Ryan JE, Cyster JG. A transmembrane CXC chemokine is a ligand for HIV-coreceptor Bonzo. *Nat Immunol*. (2000) 1:298–304. doi: 10.1038/79738
41. Sato T, Thorlacius H, Johnston B, Staton TL, Xiang W, Littman DR, et al. Role for CXCR6 in recruitment of activated CD8+ lymphocytes to inflamed liver. *J Immunol Baltim Md*. (2005) 174:277–83. doi: 10.4049/jimmunol.174.1.277
42. Matsumura S, Wang B, Kawashima N, Braunstein S, Badura M, Cameron TO, et al. Radiation-induced CXCL16 release by breast cancer cells attracts effector T cells. *J Immunol*. (2008) 181:3099–107. doi: 10.4049/jimmunol.181.5.3099
43. Hara T, Katakai T, Lee J-H, Nambu Y, Nakajima-Nagata N, Gonda H, et al. A transmembrane chemokine, CXC chemokine ligand 16, expressed by lymph

node fibroblastic reticular cells has the potential to regulate T cell migration and adhesion. *Int Immunol*. (2006) 18:301–311. doi: 10.1093/intimm/dxh369
44. Jin L, Batra S, Douda DN, Palaniyar N, Jeyaseelan S. CXCL1 contributes to host defense in polymicrobial sepsis via modulating T cell and neutrophil functions. *J Immunol Baltim Md*. (2014) 193:3549–58. doi: 10.4049/jimmunol.1401138
45. Sakthivel SK, Singh UP, Singh S, Taub DD, Igietseme JU, Lillard JW. CCL5 regulation of mucosal chlamydial immunity and infection. *BMC Microbiol*. (2008) 8:136. doi: 10.1186/1471-2180-8-136
46. Browning JL, Allaire N, Ngam-Ek A, Notidis E, Hunt J, Perrin S, et al. Lymphotoxin-beta receptor signaling is required for the homeostatic control of HEV differentiation and function. *Immunity*. (2005) 23:539–550. doi: 10.1016/j.immuni.2005.10.002
47. Moussion C, Girard J-P. Dendritic cells control lymphocyte entry to lymph nodes through high endothelial venules. *Nature*. (2011) 479:542–6. doi: 10.1038/nature10540
48. Ngo VN, Korner H, Gunn MD, Schmidt KN, Riminton DS, Cooper MD, et al. Lymphotoxin alpha/beta and tumor necrosis factor are required for stromal cell expression of homing chemokines in B and T cell areas of the spleen. *J Exp Med*. (1999) 189:403–412. doi: 10.1084/jem.189.2.403
49. Kabashima K, Banks TA, Ansel KM, Lu TT, Ware CF, Cyster JG. Intrinsic lymphotoxin-beta receptor requirement for homeostasis of lymphoid tissue dendritic cells. *Immunity*. (2005) 22:439–50. doi: 10.1016/j.immuni.2005.02.007
50. Upadhyay V, Fu Y-X. Lymphotoxin signalling in immune homeostasis and the control of microorganisms. *Nat Rev Immunol*. (2013) 13:270–9. doi: 10.1038/nri3406
51. De Togni P, Goellner J, Ruddle NH, Streeter PR, Fick A, Mariathasan S, et al. Abnormal development of peripheral lymphoid organs in mice deficient in lymphotoxin. *Science*. (1994) 264:703–707.
52. Alimzhanov MB, Kuprash DV, Kosco-Vilbois MH, Luz A, Turetskaya RL, Tarakhovsky A, et al. Abnormal development of secondary lymphoid tissues in lymphotoxin β-deficient mice. *Proc Natl Acad Sci USA*. (1997) 94:9302–9307.
53. Kuprash DV, Alimzhanov MB, Tumanov AV, Anderson AO, Pfeffer K, Nedospasov SA. TNF and Lymphotoxin β cooperate in the maintenance of secondary lymphoid tissue microarchitecture but not in the development of lymph nodes. *J Immunol*. (1999) 163:6575–80.
54. Fütterer A, Mink K, Luz A, Kosco-Vilbois MH, Pfeffer K. The lymphotoxin β receptor controls organogenesis and affinity maturation in peripheral lymphoid tissues. *Immunity*. (1998) 9:59–70. doi: 10.1016/S1074-7613(00)80588-9
55. Tumanov AV, Grivennikov SI, Kruglov AA, Shebzukhov YV, Koroleva EP, Piao Y, et al. Cellular source and molecular form of TNF specify its distinct functions in organization of secondary lymphoid organs. *Blood*. (2010) 116:3456–64. doi: 10.1182/blood-2009-10-249177
56. Kim D, Mebius RE, MacMicking JD, Jung S, Cupedo T, Castellanos Y, et al. Regulation of peripheral lymph node genesis by the tumor necrosis factor family member TRANCE. *J Exp Med*. (2000) 192:1467–1478.doi: 10.1084/jem.192.10.1467
57. Pan G, O'Rourke K, Chinnaiyan AM, Gentz R, Ebner R, Ni J, et al. The receptor for the cytotoxic ligand TRAIL. *Science*. (1997) 276:111–113.
58. Schneider P, Thome M, Burns K, Bodmer JL, Hofmann K, Kataoka T, et al. TRAIL receptors 1 (DR4) and 2 (DR5) signal FADD-dependent apoptosis and activate NF-kappaB. *Immunity*. (1997) 7:831–836.
59. Bertsch U, Röder C, Kalthoff H, Trauzold A. Compartmentalization of TNF-related apoptosis-inducing ligand (TRAIL) death receptor functions: emerging role of nuclear TRAIL-R2. *Cell Death Dis*. (2014) 5:e1390. doi: 10.1038/cddis.2014.351
60. Aktas O, Schulze-Topphoff U, Zipp F. The role of TRAIL/TRAIL receptors in central nervous system pathology. *Front Biosci J Virtual Libr*. (2007) 12:2912–2921. doi: 10.2741/2281
61. Schneider P, Olson D, Tardivel A, Browning B, Lugovskoy A, Gong D, et al. Identification of a new murine tumor necrosis factor receptor locus that contains two novel murine receptors for tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). *J Biol Chem*. (2003) 278:5444–54. doi: 10.1074/jbc.M210783200
62. Moua P, Checketts M, Xu L-G, Shu H-B, Reyland ME, Cusick JK. RELT family members activate p38 and induce apoptosis by a mechanism distinct from TNFR1. *Biochem Biophys Res Commun*. (2017) 491:25–32. doi: 10.1016/j.bbrc.2017.07.022
63. Bossen C, Ingold K, Tardivel A, Bodmer JL, Gaide O, Hertig S, et al. Interactions of tumor necrosis factor (TNF) and TNF receptor family members in the mouse and human. *J Biol Chem*. (2006) 281:13964–71. doi: 10.1074/jbc.M601553200
64. Migone TS, Zhang J, Luo X, Zhuang L, Chen C, Hu B, et al. TL1A is a TNF-like ligand for DR3 and TR6/DcR3 and functions as a T cell costimulator. *Immunity*. (2002) 16:479–492. doi: 10.1016/S1074-7613(02)00283-2
65. Pappu BP, Borodovsky A, Zheng TS, Yang X, Wu P, Dong X, et al. TL1A-DR3 interaction regulates Th17 cell function and Th17-mediated autoimmune disease. *J Exp Med*. (2008) 205:1049–62. doi: 10.1084/jem.20071364
66. Takedatsu H, Michelsen KS, Wei B, Landers CJ, Thomas LS, Dhall D, et al. TL1A (TNFSF15) regulates the development of chronic colitis by modulating both T-helper 1 and T-helper 17 activation. *Gastroenterology*. (2008) 135:552–67. doi: 10.1053/j.gastro.2008.04.037
67. Meylan F, Song YJ, Fuss I, Villarreal S, Kahle E, Malm IJ, et al. The TNF-family cytokine TL1A drives IL-13-dependent small intestinal inflammation. *Mucosal Immunol*. (2011) 4:172–85. doi: 10.1038/mi.2010.67
68. Yu X, Pappu R, Ramirez-Carrozzi V, Ota N, Caplazi P, Zhang J, et al. TNF superfamily member TL1A elicits type 2 innate lymphoid cells at mucosal barriers. *Mucosal Immunol*. (2014) 7:730–40. doi: 10.1038/mi.2013.92
69. Richard AC, Tan C, Hawley ET, Gomez-Rodriguez J, Goswami R, Yang XP, et al. The TNF-family ligand TL1A and its receptor DR3 promote T cell-mediated allergic immunopathology by enhancing differentiation and pathogenicity of IL-9-producing T cells. *J Immunol Baltim Md*. (2015) 194:3567–82. doi: 10.4049/jimmunol.1401220
70. Tian F, Grimaldo S, Fujita M, Fugita M, Cutts J, Vujanovic NL, et al. The endothelial cell-produced antiangiogenic cytokine vascular endothelial growth inhibitor induces dendritic cell maturation. *J Immunol Baltim Md*. (2007) 179:3742–3751. doi: 10.4049/jimmunol.179.9.6369
71. Qi JW, Qin TT, Xu LX, Zhang K, Yang GL, Li J, et al. TNFSF15 inhibits vasculogenesis by regulating relative levels of membrane-bound and soluble isoforms of VEGF receptor 1. *Proc Natl Acad Sci USA*. (2013) 110:13863–68. doi: 10.1073/pnas.1304529110
72. Qin TT, Xu GC, Qi JW, Yang GL, Zhang K, Liu HL, et al. Tumour necrosis factor superfamily member 15 (Tnfsf15) facilitates lymphangiogenesis via up-regulation of Vegfr3 gene expression in lymphatic endothelial cells. *J Pathol*. (2015) 237:307–318. doi: 10.1002/path.4577
73. Zhai Y, Ni J, Jiang GW, Lu J, Xing L, Lincoln C, et al. VEGI, a novel cytokine of the tumor necrosis factor family, is an angiogenesis inhibitor that suppresses the growth of colon carcinomas *in vivo*. *FASEB J*. (1999) 13:181–9.
74. Yu J, Tian S, Metheny-Barlow L, Chew LJ, Hayes AJ, Pan H, et al. Modulation of endothelial cell growth arrest and apoptosis by vascular endothelial growth inhibitor. *Circ Res*. (2001) 89:1161–1167. doi: 10.1161/hh2401.101909
75. Stephens GL, McHugh RS, Whitters MJ, Young DA, Luxenberg D, Carreno BM, et al. Engagement of glucocorticoid-induced TNFR family-related receptor on effector T cells by its ligand mediates resistance to suppression by CD4+CD25+ T cells. *J Immunol Baltim Md*. (2004) 173:5008–5020. doi: 10.4049/jimmunol.173.8.5008
76. Ji H, Liao G, Faubion WA, Abadía-Molina AC, Cozzo C, Laroux FS, et al. Cutting edge: the natural ligand for glucocorticoid-induced TNF receptor-related protein abrogates regulatory T cell suppression. *J Immunol Baltim Md*. (2004) 172:5823–5827. doi: 10.4049/jimmunol.172.10.5823
77. Igarashi H, Cao Y, Iwai H, Piao J, Kamimura Y, Hashiguchi M, et al. GITR ligand-costimulation activates effector and regulatory functions of CD4+ T cells. *Biochem Biophys Res Commun*. (2008) 369:1134–8. doi: 10.1016/j.bbrc.2008.03.024
78. Schiemann B, Gommerman JL, Vora K, Cachero TG, Shulga-Morskaya S, Dobles M, et al. An essential role for BAFF in the normal development of B cells through a BCMA-independent pathway. *Science*. (2001) 293:2111–4. doi: 10.1126/science.1061964
79. Bossen C, Cachero TG, Tardivel A, Ingold K, Willen L, Dobles M, et al. TACI, unlike BAFF-R, is solely activated by oligomeric BAFF and APRIL to support survival of activated B cells and plasmablasts. *Blood*. (2008) 111:1004–12. doi: 10.1182/blood-2007-09-110874

80. O'Connor BP, Raman VS, Erickson LD, Cook WJ, Weaver LK, Ahonen C, et al. BCMA is essential for the survival of long-lived bone marrow plasma cells. *J Exp Med*. (2004) 199:91–8. doi: 10.1084/jem.20031330
81. Jacobsen SE, Ruscetti FW, Roberts AB, Keller JR. TGF-beta is a bidirectional modulator of cytokine receptor expression on murine bone marrow cells. Differential effects of TGF-beta 1 and TGF-beta 3. *J Immunol*. (1993) 151:4534–44.
82. Jacobsen SE, Keller JR, Ruscetti FW, Kondaiah P, Roberts AB, Falk LA. Bidirectional effects of transforming growth factor beta (TGF-beta) on colony-stimulating factor-induced human myelopoiesis *in vitro*: differential effects of distinct TGF-beta isoforms. *Blood*. (1991) 78:2239–47.
83. ten Dijke P, Iwata KK, Goddard C, Pieler C, Canalis E, McCarthy TL, et al. Recombinant transforming growth factor type beta 3: biological activities and receptor-binding properties in isolated bone cells. *Mol Cell Biol*. (1990) 10:4473–4479.
84. Okamura T, Morita K, Iwasaki Y, Inoue M, Komai T, Fujio K, et al. Role of TGF-β3 in the regulation of immune responses. *Clin Exp Rheumatol*. (2015) 33:S63-69.
85. Komai T, Okamura T, Inoue M, Yamamoto K, Fujio K. Reevaluation of pluripotent cytokine TGF-β3 in immunity. *Int J Mol Sci*. (2018) 19:2261. doi: 10.3390/ijms19082261
86. Huse K, Bakkebø M, Oksvold MP, Forfang L, Hilden VI, Stokke T, et al. Bone morphogenetic proteins inhibit CD40L/IL-21-induced Ig production in human B cells: differential effects of BMP-6 and BMP-7. *Eur J Immunol*. (2011) 41:3135–45. doi: 10.1002/eji.201141558
87. Tsuchida Y, Sumitomo S, Ishigaki K, Suzuki A, Kochi Y, Tsuchiya H, et al. TGF-β3 Inhibits antibody production by human B Cells. *PLoS ONE*. (2017) 12:e0169646. doi: 10.1371/journal.pone.0169646
88. Bollum LK, Huse K, Oksvold MP, Bai B, Hilden VI, Forfang L, et al. BMP-7 induces apoptosis in human germinal center B cells and is influenced by TGF-β receptor type I ALK5. *PLoS ONE*. (2017) 12:e0177188. doi: 10.1371/journal.pone.0177188
89. Varas A, Sacedón R, Hidalgo L, Martínez VG, Valencia J, Cejalvo T, et al. Interplay between BMP4 and IL-7 in human intrathymic precursor cells. *Cell Cycle Georget Tex*. (2009) 8:4119–26. doi: 10.4161/cc.8.24.10149
90. Lu L, Ma J, Wang X, Wang J, Zhang F, Yu J, et al. Synergistic effect of TGF-beta superfamily members on the induction of Foxp3+ Treg. *Eur J Immunol*. (2010) 40:142–52. doi: 10.1002/eji.200939618
91. Lukacs-Kornek V, Malhotra D, Fletcher AL, Acton SE, Elpek KG, Tayalia P, et al. Regulated release of nitric oxide by nonhematopoietic stroma controls expansion of the activated T cell pool in lymph nodes. *Nat Immunol*. (2011) 12:1096–104. doi: 10.1038/ni.2112
92. Walsh MC, Pearce EL, Cejas PJ, Lee J, Wang LS, Choi Y. IL-18 synergizes with IL-7 to drive slow proliferation of naive CD8 T cells by costimulating self-peptide-mediated TCR signals. *J Immunol*. (2014) 193:3992–4001. doi: 10.4049/jimmunol.1400396
93. Collison LW, Chaturvedi V, Henderson AL, Giacomin PR, Guy C, Bankoti J, et al. IL-35-mediated induction of a potent regulatory T cell population. *Nat Immunol*. (2010) 11:1093–101. doi: 10.1038/ni.1952
94. Niedbala W, Wei XQ, Cai B, Hueber AJ, Leung BP, McInnes IB, et al. IL-35 is a novel cytokine with therapeutic effects against collagen-induced arthritis through the expansion of regulatory T cells and suppression of Th17 cells. *Eur J Immunol*. (2007) 37:3021–29. doi: 10.1002/eji.200737810
95. Chaturvedi V, Collison LW, Guy CS, Workman CJ, Vignali DAA. Cutting edge: human regulatory T cells require IL-35 to mediate suppression and infectious tolerance. *J Immunol*. (2011) 186:6661–6. doi: 10.4049/jimmunol.1100315
96. Bettini M, Castellaw AH, Lennon GP, Burton AR, Vignali DAA. Prevention of autoimmune diabetes by ectopic pancreatic β-cell expression of interleukin-35. *Diabetes*. (2012) 61:1519–26. doi: 10.2337/db11-0784
97. Baxter RC. Insulin-like growth factor (IGF)-binding proteins: interactions with IGFs and intrinsic bioactivities. *Am J Physiol Endocrinol Metab*. (2000) 278:E967–76. doi: 10.1152/ajpendo.2000.278.6.E967
98. Tamura K, Yoshie M, Hashimoto K, Tachikawa E. Inhibitory effect of insulin-like growth factor-binding protein-7 (IGFBP7) on *in vitro* angiogenesis of vascular endothelial cells in the rat corpus luteum. *J Reprod Dev*. (2014) 60:447–53. doi: 10.1262/jrd.2014-069
99. Bach LA. IGFBP-6 five years on; not so "forgotten"? *Growth Horm IGF Res*. (2005) 15:185–92. doi: 10.1016/j.ghir.2005.04.001
100. Zhao Z, Liu B, Li B, Song C, Diao H, Guo Z, et al. Inhibition of long noncoding RNA IGF2AS promotes angiogenesis in type 2 diabetes. *Biomed Pharmacother Biomedecine Pharmacother*. (2017) 92:445–50. doi: 10.1016/j.biopha.2017.05.039
101. Gutzmer R, Lisewski M, Zwirner J, Mommert S, Diesel C, Wittmann M, et al. Human monocyte-derived dendritic cells are chemoattracted to C3a after up-regulation of the C3a receptor with interferons. *Immunology*. (2004) 111:435–43. doi: 10.1111/j.1365-2567.2004.01829.x
102. Gutzmer R, Köther B, Zwirner J, Dijkstra D, Purwar R, Wittmann M, et al. Human plasmacytoid dendritic cells express receptors for anaphylatoxins C3a and C5a and are chemoattracted to C3a and C5a. *J Invest Dermatol*. (2006) 126:2422–29. doi: 10.1038/sj.jid.5700416
103. Ehrengruber MU, Geiser T, Deranleau DA. Activation of human neutrophils by C3a and C5A. Comparison of the effects on shape changes, chemotaxis, secretion, and respiratory burst. *FEBS Lett*. (1994) 346:181–4.
104. Clarke EV, Tenner AJ. Complement modulation of T cell immune responses during homeostasis and disease. *J Leukoc Biol*. (2014) 96:745–56. doi: 10.1189/jlb.3MR0214-109R
105. Kwan W-H, Hashimoto D, Paz-Artal E, Ostrow K, Greter M, Raedler H, et al. Antigen-presenting cell-derived complement modulates graft-versus-host disease. *J Clin Invest*. (2012) 122:2234–38. doi: 10.1172/JCI61019
106. Strainic MG, Liu J, Huang D, An F, Lalli PN, Muqim N, et al. Locally produced complement fragments C5a and C3a provide both costimulatory and survival signals to naive CD4+ T cells. *Immunity*. (2008) 28:425–5. doi: 10.1016/j.immuni.2008.02.001
107. Brock SR, Parmely MJ. Francisella tularensis confronts the complement system. *Front Cell Infect Microbiol*. (2017) 7:523. doi: 10.3389/fcimb.2017.00523
108. Chen GY, Liu M, Wang F, Bertin J, Núñez G. A functional role for Nlrp6 in intestinal inflammation and tumorigenesis. *J Immunol*. (2011) 186:7187–94. doi: 10.4049/jimmunol.1100412
109. Elinav E, Strowig T, Kau AL, Henao-Mejia J, Thaiss CA, Booth CJ, et al. NLRP6 inflammasome regulates colonic microbial ecology and risk for colitis. *Cell*. (2011) 145:745–57. doi: 10.1016/j.cell.2011.04.022
110. Thompson TW, Kim AB, Li PJ, Wang J, Jackson BT, Huang KTH, et al. Endothelial cells express NKG2D ligands and desensitize antitumor NK responses. *eLife*. (2017) 6:e30881. doi: 10.7554/eLife.30881
111. Dunne DW, Resnick D, Greenberg J, Krieger M, Joiner KA. The type I macrophage scavenger receptor binds to gram-positive bacteria and recognizes lipoteichoic acid. *Proc Natl Acad Sci USA*. (1994) 91:1863–67.
112. Hampton RY, Golenbock DT, Penman M, Krieger M, Raetz CR. Recognition and plasma clearance of endotoxin by scavenger receptors. *Nature*. (1991) 352:342–44. doi: 10.1038/352342a0
113. Kodama T, Freeman M, Rohrer L, Zabrecky J, Matsudaira P, Krieger M. Type I macrophage scavenger receptor contains alpha-helical and collagen-like coiled coils. *Nature*. (1990) 343:531–5. doi: 10.1038/343531a0
114. Rohrer L, Freeman M, Kodama T, Penman M, Krieger M. Coiled-coil fibrous domains mediate ligand binding by macrophage scavenger receptor type II. *Nature*. (1990) 343:570–2. doi: 10.1038/343570a0
115. Plüddemann A, Neyen C, Gordon S. Macrophage scavenger receptors and host-derived ligands. *Methods San Diego Calif*. (2007) 43:207–17. doi: 10.1016/j.ymeth.2007.06.004
116. Frenkel D, Wilkinson K, Zhao L, Hickman SE, Means TK, Puckett L, et al. Scara1 deficiency impairs clearance of soluble amyloid-β by mononuclear phagocytes and accelerates Alzheimer's-like disease progression. *Nat Commun*. (2013) 4:2030. doi: 10.1038/ncomms3030
117. Berwin B, Hart JP, Rice S, Gass C, Pizzo SV, Post SR, et al. Scavenger receptor-A mediates gp96/GRP94 and calreticulin internalization by antigen-presenting cells. *EMBO J*. (2003) 22:6127–36. doi: 10.1093/emboj/cdg572
118. Prabhudas M, Bowdish D, Drickamer K, Febbraio M, Herz J, Kobzik L, et al. Standardizing scavenger receptor nomenclature. *J Immunol*. (2014) 192:1997–2006. doi: 10.4049/jimmunol.1490003
119. Fukumoto N, Shimaoka T, Fujimura H, Sakoda S, Tanaka M, Kita T, et al. Critical roles of CXC chemokine ligand 16/scavenger receptor that binds phosphatidylserine and oxidized lipoprotein in the pathogenesis of both

acute and adoptive transfer experimental autoimmune encephalomyelitis. *J Immunol*. (2004) 173:1620–1627. doi: 10.4049/jimmunol.173.3.1620
120. Shimaoka T, Nakayama T, Hieshima K, Kume N, Fukumoto N, Minami M, et al. Chemokines generally exhibit scavenger receptor activity through their receptor-binding domain. *J Biol Chem*. (2004) 279:26807–10. doi: 10.1074/jbc.C400163200
121. Tabata S, Kadowaki N, Kitawaki T, Shimaoka T, Yonehara S, Yoshie O, et al. Distribution and kinetics of SR-PSOX/CXCL16 and CXCR6 expression on human dendritic cell subsets and CD4+ T cells. *J Leukoc Biol*. (2005) 77:777–86. doi: 10.1189/jlb.1204733
122. Penberthy KK, Ravichandran KS. Apoptotic cell recognition receptors and scavenger receptors. *Immunol Rev*. (2016) 269:44–59. doi: 10.1111/imr.12376
123. Park SY, Yun Y, Lim JS, Kim MJ, Kim SY, Kim JE, et al. Stabilin-2 modulates the efficiency of myoblast fusion during myogenic differentiation and muscle regeneration. *Nat Commun*. (2016) 7:10871. doi: 10.1038/ncomms10871
124. Shimaoka T, Nakayama T, Kume N, Takahashi S, Yamaguchi J, Minami M, et al. Cutting edge: SR-PSOX/CXC chemokine ligand 16 mediates bacterial phagocytosis by APCs through its chemokine domain. *J Immunol*. (2003) 171:1647–51. doi: 10.4049/jimmunol.171.4.1647
125. Li F, Smith P, Ravetch JV. Inhibitory Fcγ receptor is required for the maintenance of tolerance through distinct mechanisms. *J Immunol*. (2014) 192:3021–8. doi: 10.4049/jimmunol.1302934
126. Fukuyama H, Nimmerjahn F, Ravetch JV. The inhibitory Fcgamma receptor modulates autoimmunity by limiting the accumulation of immunoglobulin G+ anti-DNA plasma cells. *Nat Immunol*. (2005) 6:99–106. doi: 10.1038/ni1151
127. McGaha TL, Karlsson MCI, Ravetch JV. FcgammaRIIB deficiency leads to autoimmunity and a defective response to apoptosis in Mrl-MpJ mice. *J Immunol*. (2008) 180:5670–5679. doi: 10.4049/jimmunol.180.8.5670
128. Means TK, Mylonakis E, Tampakakis E, Colvin RA, Seung E, Puckett L, et al. Evolutionarily conserved recognition and innate immunity to fungal pathogens by the scavenger receptors SCARF1 and CD36. *J Exp Med*. (2009) 206:637–53. doi: 10.1084/jem.20082109
129. Ramirez-Ortiz ZG, Pendergraft WF, Prasad A, Byrne MH, Iram T, Blanchette CJ, et al. The scavenger receptor SCARF1 mediates the clearance of apoptotic cells and prevents autoimmunity. *Nat Immunol*. (2013) 14:917–26. doi: 10.1038/ni.2670
130. Perry JSA, Russler-Germain EV, Zhou YW, Purtha W, Cooper ML, Choi J, et al. CD36 mediates cell-surface antigens to promote thymic development of the regulatory T Cell receptor repertoire and Allo-tolerance. *Immunity*. (2018) 48:923–36.e4. doi: 10.1016/j.immuni.2018.04.007
131. Anderson MS, Venanzi ES, Chen Z, Berzins SP, Benoist C, Mathis D. The cellular mechanism of Aire control of T cell tolerance. *Immunity*. (2005) 23:227–39. doi: 10.1016/j.immuni.2005.07.005
132. Andrews S. *FastQC: A Quality Control Tool for High Throughput Sequence Data*. (2010) Available online at: http://www.bioinformatics.babraham.ac.uk/projects/fastqc
133. Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, et al. STAR: ultrafast universal RNA-seq aligner. *Bioinforma Oxf Engl*. (2013) 29:15–21. doi: 10.1093/bioinformatics/bts635
134. Liao Y, Smyth GK, Shi W. featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. *Bioinforma Oxf Engl*. (2014) 30:923–30. doi: 10.1093/bioinformatics/btt656
135. Eden E, Navon R, Steinfeld I, Lipson D, Yakhini Z. GOrilla: a tool for discovery and visualization of enriched GO terms in ranked gene lists. *BMC Bioinformatics*. (2009) 10:48. doi: 10.1186/1471-2105-10-48

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The Antigen Processing and Presentation Machinery in Lymphatic Endothelial Cells

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Until a few years ago, lymphatic vessels and lymphatic endothelial cells (LEC) were viewed as part of a passive conduit for lymph and immune cells to reach lymph nodes (LN). However, recent work has shown that LEC are active immunological players whose interaction with dendritic cells and T cells is of important immunomodulatory relevance. While the immunological interaction between LEC and other immune cells has taken a center stage, molecular analysis of LEC antigen processing and presentation machinery is still lagging. Herein we review the current knowledge of LEC MHC I and MHC II antigen processing and presentation pathways, Including the role of LEC in antigen phagocytosis, classical, and non-classical MHC II presentation, proteasome processing and MHC I presentation, and cross-presentation. The ultimate goal is to provide an overview of the LEC antigen processing and presentation machinery that constitutes the molecular basis for their role in MHC I and MHC II-restricted immune responses.

Keywords: lymphatic endothelial cells, MHC class I, MHC class II, antigen processing and presentation, lymph

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MHC I AND MHC II ANTIGEN PROCESSING MACHINERY

MHC I and MHC II Molecules

Under basal physiological conditions both human and murine lymphatic endothelial cells (LEC) express both MHC class I and MHC class II molecules (1). However, as previously reported for blood endothelial cells (BEC) (2) the level of MHC II expression differs according to the anatomical location from which the cells are isolated (1, 3). LEC from LN (LN-LEC) express a high number of MHC II molecules while LEC from diaphragm express a much lower number (1). The MHC II surface expression in LN-LEC is similar to what observed in BEC but less than fibroblastic reticular cells from LN (1). LEC MHC II molecules are both endogenously synthesized or acquired from hematopoietic cells, as determined by chimera experiments in MHC $II^{-/-}$ mice (1, 4, 5). At the transcription level, MHC II expression is regulated by CIITA, which is not a DNA binding factor but instead a transactivator that regulates quantitative aspects of MHC-II expression by binding the MHC-II enhanceosome (6). CIITA expression is under the control of 4 different promoters (I, II, III, IV) and, in non-professional APC, MHC II expression is mostly regulated by CIITA IV (6). This promoter is responsive to IFNγ and other pro-inflammatory cytokines, which induce MHC II expression/up-regulation in fibroblasts and BEC (6). Similarly, in LEC it has been shown that endogenous MHC II expression is controlled by CIITA IV (4, 5). However, it is interesting to notice that, in contrast to other non-professional APC where pro-inflammatory stimuli greatly

up-regulate surface MHC II molecules, pro-inflammatory stimuli induce less robust MHC II up-regulation in LEC (3, 5, 7). In the future, it would be of interest to analyze why, even though LEC express the type IV INFγ-inducible CIITA, they do not strongly up-regulate MHCII during pro-inflammatory conditions (5).

The Proteasome and TAP

Every cell expresses the constitutive 26S proteasome (8). This large barrel-shaped protein complex is formed in part by the catalytic 20S core, which consists of two pairs of outer α rings organized in seven α (α1–α7) subunits and two pairs of inner β rings organized in seven β subunits (β1–β7). The outer α subunits function as docking domains that regulate access of substrates to the catalytic chamber. Three of the β subunits (β1, β2, and β5) have proteolytic activities, including caspase-like activity (β1), trypsin-like activity (β2), and chymotrypsin-like activity (β5) (9). In the 26S proteasome, this 20S core is capped at both ends by the 19S regulatory complex (9). Ubiquitinated proteins are recognized by the 19S regulatory elements, which transfer them to the 20S for proteolysis (10). Peptides will then be transported in the ER by the transporter associated with antigen processing (TAP) and trimmed by the ER aminopeptidase I (ERAPI). In the ER the MHC class I heavy chain and β_2m will transiently associates with TAP to load the peptides into the binding groove (11).

Following IFNγ or TNFα stimulation, new proteasome subunits are incorporated to generate the immunoproteasome (4, 12). β1 is exchanged with the large multifunctional peptidase 2 (LMP2) (also known as iβ1 or psmb9). β2 is exchanged with the multicatalytic endopeptidase complex-like-1 (MECL-1) (also known as iβ2 or psmb10). β5 is exchanged with the large multifunctional peptidase 7 (LMP7) (also known as iβ5 or psmb8). The 19S regulatory complex is exchanged with the Proteasome Activator α (PA28α) and PA28β, known as 11S regulator (2). The proteolytic functions of the immunoproteasome are greatly enhanced compared to those of the constitutive proteasome, as the immunoproteasome is more efficient in degrading ubiquitinated proteins and viral proteins, and in generating peptides for MHC-I presentation (13).

Until a few years ago, the presence of the proteasome in LEC was only indirectly analyzed by determining that TAP deficient mice were much less efficient in presenting MHC-I restricted OVA-derived peptides (7). However, a recent paper reported proteasome transcripts in LEC and BEC from different anatomical locations [**Table 1** and (14)]. All of these endothelial populations expressed comparable transcript levels for constitutive proteasome subunits and 19S regulatory subunits. However, LEC and BEC from LN expressed 5-8-fold higher levels of transcripts for psmb8, psmb9, and psmb10, and twice as much PA28α and β. This suggests that LN-localized LEC preferentially express immunoproteasomes. Similarly, LN-localized LEC and BEC express 2–6 fold higher levels of TAP1 and TAP2, and twice as much ERAP1 and tapasin. Although these cells were isolated from resting LN, this suggests that their MHC-I processing and presentation capability is elevated.

Other non-proteasomal proteases have been implicated in MHC-I presentation (15). These additional peptidases can trim the proteasome-generated N-extended precursors or even destroy epitopes, by trimming below the size needed for presentation. Among these, LEC from both LN and lymphatic vessels express significant and comparable transcript levels of tripeptidyl peptidases I and II and nardilysin, but negligible levels of thimet [**Table 1** and (14)]. The functional implications of these additional LEC proteases, in generating the LEC MHC immunopeptidome, are currently unknown.

Endosomes and Lysosomes

Late endosomes (LE) and lysosomes (Lyso) are sub-cellular compartments, present in all cell types, specialized for the degradation of endogenous and exogenous materials for maintenance of cellular proteostasis and, in immune cells, for immunosurveillance (16). These organelles characteristically exhibit a low acidic pH, high concentrations of proteases, and expression of lysosome-associated membrane protein (Lamp) protein family members (16). In professional antigen presenting cells, LE and Lyso are also enriched in MHC class II proteins and molecules that regulate peptide loading (Invariant Chain, DM and DO) (17–21) and are referred as MHC class II compartments (MIIC) (22). Ultrastructurally these compartments can appear with different morphologies: multivesicular, multilamellar, or a combination of both (16).

Multivesicular bodies (MVB) are late endosomal compartments with a diameter of between 400 and 500 nm and a limiting membrane that encloses several internal vesicles with diameters of between 40 and 90 nm (16). MVB receive bio-synthetic cargo from the trans-Golgi, cytosolic cargo through autophagy, and exogenous proteins through phagocytosis. MVB are ubiquitously distributed and ultrastructural analysis has shown their presence in LEC (LS, unpublished observation) (23). However, it is currently unknown whether all/or a fraction of these compartments are MHC-II positive and whether there are differences in MHC-II expression in MVBs under steady state and inflammatory conditions. On the other hand the multilamellar bodies (MLB), which are lysosomal-like compartment formed by concentric lamellae and particularly enriched in MHC class II molecules (16) are more specifically expressed in professional APCs, such as DCs, B cells and macrophages, and they have not been found in LEC (LS unpublished observation).

Invariant Chain, DM, and DO

The MHC II molecules in association with their chaperone Invariant Chain, traffic from the trans-Golgi network to the plasma membrane before internalization to the endosomal MIIC. Sorting signals on the cytosolic tail of the Invariant chain are recognized by the clathrin-coated vesicle machinery for transport to LE/Lyso, where the Invariant chain will be processed

Abbreviations: BEC, blood endothelial cells; LEC, lymphatic endothelial cells; LN, lymph node; LN-BEC, lymph node-associated blood endothelial cells; LN-LEC, lymph node-associated lymphatic endothelial cells; DC, dendritic cells; LE, late endosomes; Lyso, lysosomes; MIIC, MHC class II compartments; MVB, multivesicular bodies; MLB, multilamellar bodies; DM, HLA-DM; Ii, invariant chain; DO, HLA-DO.

TABLE 1 | Comparative transcriptome profiling of antigen processing and presentation pathway genes from mouse lymphatic and blood endothelial cells.

Gene	Description	Lymph node LEC	Lymph node blood EC	Diaphragm LEC
MHC-I AND RELATED				
B2m	Beta-2 microglobulin	477796[a]	450780	69620
H2-K1	Histocompatibility 2, K1, K region	221754	201711	28827
H2-D1	Histocompatibility 2, D region locus 1	107074	86751	24764
H2-T23	Histocompatibility 2, T region locus 23	10686	9464	6609
H2-M3	Histocompatibility 2, M region locus 3	2375	1656	1029
H2-K2	Histocompatibility 2, K region locus 2	1770	1203	904
Mr1	Major histocompatibility complex, class I-related	1430	722	1134
H2-Ke6	H2-K region expressed gene 6	1373	1642	1671
H2-T10	Histocompatibility 2, T region locus 10	949	635	166
H2-T24	Histocompatibility 2, T region locus 24	771	591	244
Cd1d1	CD1d1 antigen	639	386	197
H2-Q4	Histocompatibility 2, Q region locus 4	443	447	62
H2-Q6	Histocompatibility 2, Q region locus 6	436	347	3
H2-Ke2	H2-K region expressed gene 2	258	321	258
H2-Q8	NA	194	206	4
H2-T3	Histocompatibility 2, T region locus 3	21	2	11
Cd1d2	CD1d2 antigen	20	25	3
H2-M2	Histocompatibility 2, M region locus 2	19	194	4
H2-M5	Histocompatibility 2, M region locus 5	7	14	6
H2-Q1	histocompatibility 2, Q region locus 1	3	3	1
H2-Q10	Histocompatibility 2, Q region locus 10	2	11	6
H2-Bl	Histocompatibility 2, blastocyst	1	2	1
MHC-II AND RELATED				
Cd74	CD74 antigen (invariant chain)	1999	2517	64
H2-Ab1	Histocompatibility 2, class II antigen A, beta 1	1063	2305	187
H2-Aa	Histocompatibility 2, class II antigen A, alpha	380	1584	100
H2-Eb1	Histocompatibility 2, class II antigen E beta	304	762	79
H2-DMb1	Histocompatibility 2, class II, locus Mb1	103	85	5
Ciita	Class II transactivator	36	92	13
H2-Ob	Histocompatibility 2, O region beta locus	33	494	8
H2-DMa	Histocompatibility 2, class II, locus DMa	33	83	10
H2-Oa	Histocompatibility 2, O region alpha locus	11	36	0
H2-DMb2	Histocompatibility 2, class II, locus Mb2	9	51	1
H2-Eb2	Histocompatibility 2, class II antigen E beta2	2	26	0
PROTEASOME				
Psma1	Proteasome subunit, alpha 1	1804	1990	2194
Psma2	Proteasome subunit, alpha 2	2436	2472	2091
Psma3	Proteasome subunit, alpha 3	1313	1320	1313
Psma4	Proteasome subunit, alpha 4	2137	1939	1765
Psma5	Proteasome subunit, alpha 5	690	680	635
Psma6	Proteasome subunit, alpha 6	4479	4501	4096
Psma7	Proteasome subunit, alpha 7	3964	3890	3738
Psma8	Proteasome subunit, alpha 8	11	52	16
Psmb1	Proteasome subunit, beta 1	3600	3617	3368
Psmb2	Proteasome subunit, beta 2	3303	2471	2984
Psmb3	Proteasome subunit, beta 3	1751	2020	2107
Psmb4	Proteasome subunit, beta 4	2504	2775	2663
Psmb5	Proteasome subunit, beta 5	1405	1376	1159
Psmb6	Proteasome subunit, beta 6	2893	2753	2380
Psmb7	Proteasome subunit, beta 7	4851	5900	2203

(Continued)

TABLE 1 | Continued

Gene	Description	Lymph node LEC	Lymph node blood EC	Diaphragm LEC
Psmb8	Proteasome subunit, beta 8 (LMP7)	4679	4348	564
Psmb9	Proteasome subunit, beta 9 (LMP2)	4288	4159	563
Psmb10	Proteasome subunit, beta 10	5571	6015	1179
Psmb11	Proteasome subunit, beta 11	1	6	3
Psmc1	Proteasome 26S subunit, ATPase 1	1714	1902	1824
Psmc2	Proteasome 26S subunit, ATPase 2	2290	2252	2852
Psmc3	Proteasome 26S subunit, ATPase 3	2431	2573	2450
Psmc3ip	Proteasome 26S subunit, ATPase 3, interacting protein	41	30	58
Psmc4	Proteasome 26S subunit, ATPase, 4	2271	2575	2753
Psmc5	Protease 26S subunit, ATPase 5	1763	1666	1815
Psmc6	Proteasome 26S subunit, ATPase, 6	2164	2496	2319
Psmd1	Proteasome 26S subunit, non-ATPase, 1	2038	2012	2786
Psmd10	Proteasome 26S subunit, non-ATPase, 10	982	698	499
Psmd11	Proteasome 26S subunit, non-ATPase, 11	482	502	450
Psmd12	Proteasome 26S subunit, non-ATPase, 12	2264	2639	2378
Psmd13	Proteasome 26S subunit, non-ATPase, 13	267	272	258
Psmd14	Proteasome 26S subunit, non-ATPase, 14	1289	1302	1376
Psmd2	Proteasome 26S subunit, non-ATPase, 2	2695	2767	3100
Psmd3	Proteasome 26S subunit, non-ATPase, 3	1064	1201	1117
Psmd4	Proteasome 26S subunit, non-ATPase, 4	1057	1213	1307
Psmd5	Proteasome 26S subunit, non-ATPase, 5	726	827	896
Psmd6	Proteasome 26S subunit, non-ATPase, 6	2893	2460	3000
Psmd7	Proteasome 26S subunit, non-ATPase, 7	2678	2492	2579
Psmd8	Proteasome 26S subunit, non-ATPase, 8	2601	2684	2594
Psmd9	Proteasome 26S subunit, non-ATPase, 9	1228	916	1206
Psme1	Proteasome activator subunit 1 (PA28 alpha)	4330	4983	2175
Psme2	Proteasome activator subunit 2 (PA28 beta)	769	903	391
Psme3	Proteasome activator subunit 3 (PA28 gamma, Ki)	2495	2173	1951
Psme4	Proteasome activator subunit 4	2923	2697	2175
Psmf1	Proteasome inhibitor subunit 1	950	918	926
Psmg1	Proteasome assembly chaperone 1	442	414	384
Psmg2	Proteasome assembly chaperone 2	1435	1081	1677
Psmg3	Proteasome assembly chaperone 3	283	232	243
Psmg4	Proteasome assembly chaperone 4	667	591	494
OTHER PEPTIDASES FOR MHC-I PROCESSING				
Tpp1	Tripeptidyl peptidase I	11374	9235	6824
Tpp2	Tripeptidyl peptidase II	2910	3005	2429
Nrd1	Nardilysin	2694	2582	3040
Thop1	Thimet oligopeptidase 1	62	66	82
TAP, TAPASIN, AND ERAP1				
Tapbp	TAP binding protein	24961	29085	11373
Tap1	Transporter 1, ATP-binding cassette, sub-family B (MDR/TAP)	3796	3489	565
Tap2	Transporter 2, ATP-binding cassette, sub-family B (MDR/TAP)	2398	2223	630
Erap1	Endoplasmic reticulum aminopeptidase 1	1421	1467	655
Tapbpl	TAP binding protein-like	502	589	222
CATHEPSINS				
Ctsd	Cathepsin D	32104	8139	14906
Ctsb	Cathepsin B	15143	10660	39555
Ctsl	Cathepsin L	13726	7431	2687
Ctsh	Cathepsin H	3836	1095	1823
Ctss	Cathepsin S	3670	1013	46

(Continued)

TABLE 1 | Continued

Gene	Description	Lymph node LEC	Lymph node blood EC	Diaphragm LEC
Ctsz	Cathepsin Z	3352	3482	2129
Ctso	Cathepsin O	3005	3189	2627
Ctsa	Cathepsin A	2141	2659	1973
Ctsf	Cathepsin F	709	351	790
Ctsk	Cathepsin K	299	60	123
Ctsc	Cathepsin C	89	338	24
Ctsg	Cathepsin G	46	147	2
Ctsw	Cathepsin W	19	53	11
Ctse	Cathepsin E	2	7	0
CYSTATINS				
Cst3	Cystatin C	13094	28792	25352
Cstb	Cystatin B	9445	3702	3390
Cst10	Cystatin 10 (chondrocytes)	6582	16279	22
Cst6	Cystatin E/M	67	66	90
Cstad	CSA-conditional, T cell activation-dependent protein	34	74	12
Csta	Cystatin A	11	7	5
Cst7	Cystatin F (leukocystatin)	5	19	2
Cst9	Cystatin 9	0	0	7

[a]*Data are reported as normalized gene expression levels as Fragments Per Kilobase of transcript per Million mapped reads (FPKM). Data is from Berendam. (14).*

by Cathepsins to generate class II-associated invariant chain peptides (CLIP), which occupy the MHC II binding groove and will be exchanged with peptides derived from endosomal processing (24). MHC II/Invariant Chain complexes are present at high levels in LEC and confocal microscopy, performed on primary LEC indicates that MHC II is correctly targeted both at the cell surface and in endosomal compartments (1).

HLA-DM (H-2M in mice) is part of the endosomal antigen processing and presentation machinery and aids peptide loading onto MHC II molecules. HLA-DM (DM) was originally discovered following the analysis of B-cell lines that were inefficient at presenting peptides derived from the processing of phagocytosed proteins but easily presented peptides supplied exogenously (17–19, 25, 26). It was later determined that these cells were defective in the expression of either the HLA-DMA or HLA-DMB genes. Subsequent *in vitro* and *in vivo* experiments determined that the role of DM is to catalyze CLIP removal, stabilize empty MHC II molecules for peptide loading and skew the immunopeptidome repertoire toward high affinity peptides (17, 25, 26). Mice lacking H-2M expressed similar I-A^b MHC II cell surface levels as wild type mice. However, the I-A^b MHC II molecules were less compact/SDS-resistant and were predominantly associated with CLIP (27). In contrast, lack of DM led to decreased peptide capture by I-A^d molecules, but enhanced peptide loading by I-E^d MHC II molecules (28). Finally, lack of DM generated a substantial pool of empty or loosely occupied I-A^k MHC II conformers with increase peptide binding activity. Mass spectrometry profiles confirmed the presence of an MHC II-peptidome in absence of DM (28, 29). Additionally DM requirements are different for CLIP binding in different registers (30). These results demonstrate that DM has distinct roles depending on its specific class II partners.

Subsequently, an additional protein, DO, was discovered, whose role is to inhibit DM function (18, 31). Importantly, while DM expression is not greatly increased following pro-inflammatory stimuli (TLR activation) that induces dendritic cell maturation, DO is down-regulated (32). As such it was hypothesized that high DO expression in immature dendritic cells would inhibit DM activity and skew the MHC II peptidome toward a broader and less stably bound repertoire. Upon DC maturation, reduced DO expression would lead to high DM activity, shaping the peptide repertoire toward long-lived surface class II MHC complexes, thus promoting productive immune responses (18, 33, 34).

Transcript analysis has shown that Invariant chain, I-A alpha, and I-A beta are expressed significantly in LEC and BEC from LN, but not LEC from lymphatic vessels, but DM and DO expression is very low to negligible, albeit DM is up-regulated following inflammatory stimuli (1, 4) [**Table 1** and (14)]. Because removal of CLIP from I-A^b molecules is strongly DM dependent, this could explain the inefficient processing and presentation of I-A^b restricted antigens by LEC (1). However, the haplotype variation data described above indicate that general conclusions about the ability of LEC to present MHC II restricted antigens should await analysis of other mouse haplotypes.

Cathepsins

Cathepsins are a large family of serine, cysteine or aspartyl proteases that are present in endo-lysosomal compartments, and may be secreted at steady state or during pathological conditions (35). Cathepsins are most active at acid pH, can still function at neutral pH but are inactive at alkaline pH (36). Although these enzymes are present in most cells, certain cathepsins are enriched in particular antigen presenting cells. For example, Cathepsin S

is highly expressed in dendritic cells and B cells, Cathepsin F in macrophages, and Cathepsin L in thymocytes (36–41).

Transcriptome analysis indicated that LEC from vessels express relatively low levels of cathepsins L and F, and negligible levels of Cathepsin S, while the levels of Cathepsin S and L were significantly elevated in LEC from LN [**Table 1** and (14)]. However, measured Cathepsin L activity was variable among LN LEC and not evident in LEC from diaphragm (1). The activity of Cathepsin L indicates that at least some LEC could potentially cleave the Invariant Chain and generate CLIP peptides (1). Additionally, LEC could not efficiently process HA (an influenza membrane protein) and the IE-α protein as determined by either CD4 T cell recognition of the MHC II presented HA epitope or FACS analysis using the Y-Ae Ab that recognize I-Ab molecules loaded with the IE-α epitope, either under basal conditions or upon IFNγ stimulation (1). Furthermore, new evidence indicates that LEC express high levels of Cystatin C, B and 10 [**Table 1** and (14)], which function as natural inhibitors of cathepsins (42). Altogether, the data point to the possibility that CatL and S activity in LEC is diminished, which could affect the generation of LIP10 and CLIP, and might also diminish the processing of other endogenous antigens.

Exogenous Peptides Binding and Antigen Exchange

The MHC I and MHC II presented immunopeptidome not only derives from endosomally processed proteins but also from pre-processed peptides that can be directly acquired from the extracellular milieu. Recent proteomic analyses have indicated that processed peptides are present in every biological fluid, among which lymph and blood, have been best characterized (43–50). The Eisen and Raghavan groups demonstrated binding of extracellular peptides to MHC I molecules and their regulation of CD8 T cell function (51, 52). Our group, among others, characterized extracellular peptide binding to MHC II surface molecules (44, 53–57). We determined that peptides carried in lymph were present in the HLA-DR1 immunopeptidome of immature dendritic cells and some of these peptides were not generated by endosomal processing, pinpointing the physiological relevance of MHC II surface/early endosomes loading (44). As such, the peptides present in the lymph, which derive from the metabolic and catabolic process of different parenchymal organs could contribute to the LEC MHC II immunopeptidome, since it has already been shown that LEC can readily bind and present pre-processed peptides (1).

PHAGOCYTOSIS AND AUTOPHAGY

Only very recently LEC have been analyzed for their ability to capture exogenous and endogenous antigens through phagocytosis. *In vivo* experiments using fluorescently labeled OVA indicated that within 90 min the subcutaneously injected protein was identified in LYVE-1$^+$ cells, present in LN sub-capsular sinuses (7). Additionally, genes encoding several scavenger receptors, known to be involved in receptor-mediated endocytosis, are upregulated in LEC from lymph node (14). LEC efficiency in processing phagocytosed proteins through the MHC II pathway in steady state condition is low (1); nevertheless LEC can transfer Ags to dendritic cells, which are known to be present in close proximity with LEC in the lymphatic capillary and collectors, to induce CD4 T-cell anergy (1, 58). In addition, LEC efficiently present MHC-I peptides, and it has been reported that phagocytosis in early endosomes can route exogenous antigens (both self and non self) for cross-presentation on MHC class I in a proteasome and TAP-1-dependent manner (1, 3, 7, 58–61). It is interesting to consider that the acquisition of cross-presented material is mediated by these scavenger receptors. A second mechanism that can transfer endogenous proteins in the endosomes is autophagy. Although autophagy has been extensively characterized in BEC (62), there are no reports on the role of autophagy in antigen processing and presentation in LEC.

LEC AND PATHOGEN IMMUNITY

A growing body of evidences indicates that LEC are involved in immune response to pathogens. It has been recently reported that in extrapulmonary tuberculosis, the lymphatic system is the most common site of infection and LEC function as a niche for *Mycobacterium tuberculosis* (59). Indeed *M. tuberculosis* can replicate in the LEC cytosol and within autophagosomes suggesting that LEC are a previously unrecognized site for infection persistence. Similarly, Hantaviruses have been shown to have a tropism for lymphatic vessels and LEC infection with either Andes virus and Hantaan virus induces LEC hyperpermeabilization and pulmonary edema (63). The edema can be inhibited by $\alpha_v\beta_3$ integrin as well as VEGFR3 antibodies (63). A LEC role in HIV infection was also reported in promoting infection and latency formation in resting CD4+ T cells (64, 65). Recently an interesting role of LEC in antigen persistence, after resolution of the infection, has been shown (66). After viral challenge and vaccination, the antigen was captured by LEC under proliferative conditions and stored for extended periods of time. This "antigen archiving" mechanism positively influenced the degree of protective immunity provided by circulating memory CD8$^+$ T-cells (66, 67).

COSTIMULATORY AND CO-INHIBITORY MOLECULES

Effective activation of T-cells requires the display of MHC-I and MHC-II-peptide complexes as well as an antigen-independent signals provided by co-stimulatory molecules, among which CD40, CD80 (B7.1), and CD86 (B7.2) have been extensively analyzed in their requirements for naïve and memory T-cells activation (68, 69). LN LEC were shown to express very low levels of CD40 and negligible levels of CD80 and CD86 (3, 60). More recent transcriptome analysis has validated these observations, and extended them to include additional costimulatory molecules [**Table 1** and (14)]. Importantly these costimulatory molecules did not up-regulate following stimulation with an MHC-I cognate ligand as well as inflammatory signals (TLRs binders or IFNγ) (3, 60).

In contrast, LEC in LN, but not in peripheral tissue lymphatics, express multiple inhibitory receptors that engage counter-receptors on activated T-cells to dampen the immune response (69). These include PD-L1 (CD274) and PD-L2 (CD273), which are present on resting LEC and greatly up-regulated by inflammatory stimuli (1, 70). Interestingly, the ligand for LAG-3, another inhibitory receptor on T-cells, is MHC-II, and induction of CD8 T-cell tolerance by LEC depends on engagement of LAG-3 as well as PD-1 (1, 60). Consequently, it has been suggested that in the absence of functional Ag presentation, the expression of MHC-II molecules on LEC is concerned with inducing Lag-3 mediated tolerance. While the low expression of costimulatory molecules would suggest that LEC would be unable to activate T-cells, they stimulate profound proliferation of CD8 T-cells *in vivo* and *in vitro*, and after peptide pulsing and CD4 T-cell proliferation *in vitro* (58, 60). However, the expression of the co-inhibitory molecules leads to deletional tolerance of CD8 T-cells due to a failure to sustain upregulation of the IL-2 receptor. Thus, LEC represent an important mechanism for mediation of systemic peripheral tolerance (58, 60, 61).

EXOSOMES AND OTHER VESICLES

Most cells in the human body release vesicles of different sizes and content which can be classified as apoptotic bodies, micro and macrovesicles and exosomes (71). Exosomes are small (30–120 mm) vesicles generated from the multivesicular late endosomes upon fusion with the plasma membrane and release in the extracellular milieu. Exosomes from different sources have been shown to transport a protein cargo as well as mRNAs and microRNAs. Their physiological and pathological relevance has been established in several immune and cancer-related models (72). Although very little is known about LEC-released exosomes, recently it has been shown that LEC release a vesicular fraction, which includes exosomes, following an inflammatory signal (73). The LEC-derived exosomes are reportedly enriched with a motility-promoting protein signature, which act as a cue for the dendritic cells migratory response (73). In particular LEC released vesicles accumulate in the perivascular stroma of small lymphatic vessels, mostly in the presence of inflammatory cytokines and promote directional migration of CX3CR1-expressing cells (73).

CONCLUDING REMARKS

LEC cells are anatomically placed between parenchymal organs and draining lymph nodes, functioning as a conduit for the lymphatic fluid and are known to control DC and T cell migration in and out of the lymph node (74, 75). During the last few years their functionality in antigen processing and presentation and T cell immune responses has emerged. Under steady-state conditions LEC can present self-antigens to induce T cell tolerance either through expression of peripheral tissue antigens (76) or acquisition of extracellular antigens through phagocytosis or by acquisition of pre-loaded MHC II molecules from DC. Under inflammatory conditions LEC also play an immunosuppressive role by decreasing DC maturation (77) and by up-regulating surface PDL1 (76).

However, the advances in understanding the cross-talk between LEC and T cells has not been paralleled by a detailed mechanistic analysis of their antigen processing and presentation machinery. Characterization of LEC immunoproteasomes, endosomal processing compartments, and antigen acquisition from the lymphatic fluid still needs to be investigated. Nevertheless, the work to date points to an emerging picture of the role played by LEC in maintenance of self-tolerance.

AUTHOR CONTRIBUTIONS

LS and VE wrote the review. SB contributed the primary data presented in the table.

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REFERENCES

1. Rouhani SJ, Eccles JD, Riccardi P, Peske JD, Tewalt EF, Cohen JN, et al. Roles of lymphatic endothelial cells expressing peripheral tissue antigens in CD4 T-cell tolerance induction. *Nat Commun.* (2015) 6:6771. doi: 10.1038/ncomms7771
2. Pober JS, Merola J, Liu R, Manes TD. Antigen presentation by vascular cells. *Front Immunol.* (2017) 8:1907. doi: 10.3389/fimmu.2017.01907
3. Tewalt EF, Cohen JN, Rouhani SJ, Engelhard VH. Lymphatic endothelial cells - key players in regulation of tolerance and immunity. *Front Immunol.* (2012) 3:305. doi: 10.3389/fimmu.2012.00305
4. Dubrot J, Duraes FV, Potin L, Capotosti F, Brighouse D, Suter T, et al. Lymph node stromal cells acquire peptide-MHCII complexes from dendritic cells and induce antigen-specific CD4(+) T cell tolerance. *J Exp Med.* (2014) 211:1153–66. doi: 10.1084/jem.20132000
5. Dubrot J, Duraes FV, Harle G, Schlaeppi A, Brighouse D, Madelon N, et al. Absence of MHC-II expression by lymph node stromal cells results in autoimmunity. *Life Sci Alliance.* (2018) 1:e201800164. doi: 10.26508/lsa.201800164
6. LeibundGut-Landmann S, Waldburger JM, Krawczyk M, Otten LA, Suter T, Fontana A, et al. Mini-review: specificity and expression of CIITA, the master regulator of MHC class II genes. *Eur J Immunol.* (2004) 34:1513–25. doi: 10.1002/eji.200424964
7. Hirosue S, Vokali E, Raghavan VR, Rincon-Restrepo M, Lund AW, Corthesy-Henrioud P, et al. Steady-state antigen scavenging, cross-presentation, and CD8+ T cell priming:a new role for lymphatic endothelial cells. *J Immunol.* (2014) 192:5002–11. doi: 10.4049/jimmunol.1302492
8. Bard JAM, Goodall EA, Greene ER, Jonsson E, Dong KC, Martin, A. Structure and function of the 26S proteasome. *Annu Rev Biochem.* (2018) 87:697–724. doi: 10.1146/annurev-biochem-062917-011931
9. Rousseau A, Bertolotti A. Regulation of proteasome assembly and activity in health and disease. *Nat Rev Mol Cell Biol.* (2018) 19:697–712. doi: 10.1038/s41580-018-0040-z
10. Yu H, Matouschek A. Recognition of Client Proteins by the Proteasome. *Annu Rev Biophys.* (2017) 46:149–73. doi: 10.1146/annurev-biophys-070816-033719
11. Hansen TH, Bouvier M. MHC class I antigen presentation:learning from viral evasion strategies. *Nat Rev Immunol.* (2009) 9:503–13. doi: 10.1038/nri2575

12. Ferrington DA, Gregerson DS. Immunoproteasomes:structure, function, and antigen presentation. *Prog Mol Biol Transl Sci.* (2012) 109:75–112. doi: 10.1016/B978-0-12-397863-9.00003-1
13. Kimura H, Caturegli P, Takahashi M, Suzuki, K. New insights into the function of the immunoproteasome in immune and nonimmune cells. *J Immunol Res.* (2015) 2015:541984. doi: 10.1155/2015/541984
14. Berendam SJ, Koeppel AF, Godfrey NR, Rouhani SJ, Woods AN, Rodriguez AB, et al. Comparative transcriptomic analysis identify a range of immunologically related functional elaborations of lymph node associated lymphatic and blood endothelial cells. *Front Immunol.* (2019). doi: 10.3389/fimmu.2019.00816
15. Kloetzel PM, Ossendorp, F. Proteasome and peptidase function in MHC-class-I-mediated antigen presentation. *Curr Opin Immunol.* (2004) 16:76–81. doi: 10.1016/j.coi.2003.11.004
16. Stern LJ, Potolicchio I, Santambrogio, L. MHC class II compartment subtypes:structure and function. *Curr Opin Immunol.* (2006) 18:64–9. doi: 10.1016/j.coi.2005.11.005
17. Doebele RC, Busch R, Scott HM, Pashine A, Mellins ED. Determination of the HLA-DM interaction site on HLA-DR molecules. *Immunity.* (2000) 13:517–27. doi: 10.1016/S1074-7613(00)00051-0
18. Mellins ED, Stern LJ. HLA-DM and HLA-DO, key regulators of MHC-II processing and presentation. *Curr Opin Immunol.* (2014) 26:115–22. doi: 10.1016/j.coi.2013.11.005
19. Denzin LK, Cresswell, P. HLA-DM induces CLIP dissociation from MHC class II alpha beta dimers and facilitates peptide loading. *Cell.* (1995) 82:155–65. doi: 10.1016/0092-8674(95)90061-6
20. van Ham SM, Tjin EP, Lillemeier BF, Gruneberg U, van Meijgaarden KE, Pastoors L, et al. HLA-DO is a negative modulator of HLA-DM-mediated MHC class II peptide loading. *Curr Biol.* (1997) 7:950–7. doi: 10.1016/S0960-9822(06)00414-3
21. Roche PA, Furuta, K. The ins and outs of MHC class II-mediated antigen processing and presentation. *Nat Rev Immunol.* (2015) 15:203–16. doi: 10.1038/nri3818
22. Tulp A, Verwoerd D, Dobberstein B, Ploegh HL, Pieters, J. Isolation and characterization of the intracellular MHC class II compartment. *Nature.* (1994) 369:120–6. doi: 10.1038/369120a0
23. Zolla V, Nizamutdinova IT, Scharf B, Clement CC, Maejima D, Akl T, et al. Aging-related anatomical and biochemical changes in lymphatic collectors impair lymph transport, fluid homeostasis, and pathogen clearance. *Aging Cell.* (2015) 14:582–94. doi: 10.1111/acel.12330
24. Cresswell P, Roche PA. Invariant chain-MHC class II complexes:always odd and never invariant. *Immunol Cell Biol.* (2014) 92:471–2. doi: 10.1038/icb.2014.36
25. Morris P, Shaman J, Attaya M, Amaya M, Goodman S, Bergman C, et al. An essential role for HLA-DM in antigen presentation by class II major histocompatibility molecules. *Nature.* (1994) 368:551–4. doi: 10.1038/368551a0
26. Sloan VS, Cameron P, Porter G, Gammon M, Amaya M, Mellins E, et al. Mediation by HLA-DM of dissociation of peptides from HLA-DR. *Nature.* (1995) 375:802–6. doi: 10.1038/375802a0
27. Martin WD, Hicks GG, Mendiratta SK, Leva HI, Ruley HE, Van Kaer, L. H2-M mutant mice are defective in the peptide loading of class II molecules, antigen presentation, and T cell repertoire selection. *Cell.* (1996) 84:543–50. doi: 10.1016/S0092-8674(00)81030-2
28. Koonce CH, Wutz G, Robertson EJ, Vogt AB, Kropshofer H, Bikoff EK. DM loss in k haplotype mice reveals isotype-specific chaperone requirements. *J Immunol.* (2003) 170:3751–61. doi: 10.4049/jimmunol.170.7.3751
29. Bikoff EK, Wutz G, Kenty GA, Koonce CH, Robertson EJ. Relaxed DM requirements during class II peptide loading and CD4+ T cell maturation in BALB/c mice. *J Immunol.* (2001) 166:5087–98. doi: 10.4049/jimmunol.166.8.5087
30. Belmares MP, Busch R, Mellins ED, McConnell HM. Formation of two peptide/MHC II isomers is catalyzed differentially by HLA-DM. *Biochemistry.* (2003) 42:838–47. doi: 10.1021/bi020466p
31. Guce AI, Mortimer SE, Yoon T, Painter CA, Jiang W, Mellins ED, et al. HLA-DO acts as a substrate mimic to inhibit HLA-DM by a competitive mechanism. *Nat Struct Mol Biol.* (2013) 20:90–8. doi: 10.1038/nsmb.2460
32. Fallas JL, Tobin HM, Lou O, Guo D, Sant'Angelo DB, Denzin LK. Ectopic expression of HLA-DO in mouse dendritic cells diminishes MHC class II antigen presentation. *J Immunol.* (2004) 173:1549–60. doi: 10.4049/jimmunol.173.3.1549
33. Yi W, Seth NP, Martillotti T, Wucherpfennig KW, Sant'Angelo DB, Denzin LK. Targeted regulation of self-peptide presentation prevents type I diabetes in mice without disrupting general immunocompetence. *J Clin Invest.* (2010) 120:1324–36. doi: 10.1172/JCI40220
34. Santambrogio L, DiLorenzo TP. Autoimmunity. The benefit of self-control. *Immunol Cell Biol.* (2010) 88:513–4. doi: 10.1038/icb.2010.59
35. Kramer L, Turk D, Turk, B. The future of cysteine cathepsins in disease management. *Trends Pharmacol Sci.* (2017) 38:873–98. doi: 10.1016/j.tips.2017.06.003
36. Turk V, Stoka V, Vasiljeva O, Renko M, Sun T, Turk B, et al. Cysteine cathepsins:from structure, function and regulation to new frontiers. *Biochim Biophys Acta.* (2012) 1824:68–88. doi: 10.1016/j.bbapap.2011.10.002
37. Riese RJ, Chapman HA. Cathepsins and compartmentalization in antigen presentation. *Curr Opin Immunol.* (2000) 12:107–13. doi: 10.1016/S0952-7915(99)00058-8
38. Mason RW, Johnson DA, Barrett AJ, Chapman HA. Elastinolytic activity of human cathepsin L. *Biochem J.* (1986) 233:925–7. doi: 10.1042/bj2330925
39. Reilly JJ, Chen P, Sailor LZ, Mason RW, Chapman HA. Uptake of extracellular enzyme by a novel pathway is a major determinant of cathepsin L levels in human macrophages. *J Clin Invest.* (1990) 86:176–83. doi: 10.1172/JCI114682
40. Shi GP, Bryant RA, Riese R, Verhelst S, Driessen C, Li Z, et al. Role for cathepsin F in invariant chain processing and major histocompatibility complex class II peptide loading by macrophages. *J Exp Med.* (2000) 191:1177–86. doi: 10.1084/jem.191.7.1177
41. Shi GP, Villadangos JA, Dranoff G, Small C, Gu L, Haley KJ, et al. Cathepsin S required for normal MHC class II peptide loading and germinal center development. *Immunity.* (1999) 10:197–206. doi: 10.1016/S1074-7613(00)80020-5
42. Kopitar-Jerala, N. The role of cystatins in cells of the immune system. *FEBS Lett.* (2006) 580:6295–301. doi: 10.1016/j.febslet.2006.10.055
43. Clement CC, Aphkhazava D, Nieves E, Callaway M, Olszewski W, Rotzschke O, et al. Protein expression profiles of human lymph and plasma mapped by 2D-DIGE and 1D SDS-PAGE coupled with nanoLC-ESI-MS/MS bottom-up proteomics. *J Proteom.* (2013) 78:172–87. doi: 10.1016/j.jprot.2012.11.013
44. Clement CC, Becerra A, Yin L, Zolla V, Huang L, Merlin S, et al. The dendritic cell major histocompatibility complex II (MHC II) peptidome derives from a variety of processing pathways and includes peptides with a broad spectrum of HLA-DM sensitivity. *J Biol Chem.* (2016) 291:5576–95. doi: 10.1074/jbc.M115.655738
45. Clement CC, Cannizzo ES, Nastke MD, Sahu R, Olszewski W, Miller NE, et al. An expanded self-antigen peptidome is carried by the human lymph as compared to the plasma. *PLoS ONE.* (2010) 5:e9863. doi: 10.1371/journal.pone.0009863
46. Clement CC, Rotzschke O, Santambrogio, L. The lymph as a pool of self-antigens. *Trends Immunol.* (2011) 32:6–11. doi: 10.1016/j.it.2010.10.004
47. Clement CC, Santambrogio, L. The lymph self-antigen repertoire. *Front Immunol.* (2013) 4:424. doi: 10.3389/fimmu.2013.00424
48. Petricoin EF, Belluco C, Araujo RP, Liotta LA. The blood peptidome:a higher dimension of information content for cancer biomarker discovery. *Nat Rev Cancer.* (2006) 6:961–7. doi: 10.1038/nrc2011
49. Smith CR, Batruch I, Bauca JM, Kosanam H, Ridley J, Bernardini MQ, et al. Deciphering the peptidome of urine from ovarian cancer patients and healthy controls. *Clin Proteom.* (2014) 11:23. doi: 10.1186/1559-0275-11-23
50. Ling XB, Lau K, Deshpande C, Park JL, Milojevic D, Macaubas C, et al. Urine peptidomic and targeted plasma protein analyses in the diagnosis and monitoring of systemic juvenile idiopathic arthritis. *Clin Proteom.* (2010) 6:175–93. doi: 10.1007/s12014-010-9058-8
51. Eisen HN, Hou XH, Shen C, Wang K, Tanguturi VK, Smith C, et al. Promiscuous binding of extracellular peptides to cell surface class I MHC protein. *Proc Natl Acad Sci USA.* (2012) 109:4580–5. doi: 10.1073/pnas.1201586109
52. Geng J, Altman JD, Krishnakumar S, Raghavan, M. Empty conformers of HLA-B preferentially bind CD8 and regulate CD8(+) T cell function. *Elife.* (2018) 7:e36341. doi: 10.7554/eLife.36341

53. Potolicchio I, Chitta S, Xu X, Fonseca D, Crisi G, Horejsi V, et al. Conformational variation of surface class II MHC proteins during myeloid dendritic cell differentiation accompanies structural changes in lysosomal MIIC. *J Immunol.* (2005) 175:4935–47. doi: 10.4049/jimmunol.175.8.4935
54. Bosch B, Berger AC, Khandelwal S, Heipertz EL, Scharf B, Santambrogio L, et al. Disruption of multivesicular body vesicles does not affect major histocompatibility complex (MHC) class II-peptide complex formation and antigen presentation by dendritic cells. *J Biol Chem.* (2013) 288:24286–92. doi: 10.1074/jbc.M113.461996
55. Chou CL, Mirshahidi S, Su KW, Kim A, Narayan K, Khoruzhenko S, et al. Short peptide sequences mimic HLA-DM functions. *Mol Immunol.* (2008) 45:1935–43. doi: 10.1016/j.molimm.2007.10.033
56. Marin-Esteban V, Falk K, Rotzschke O. "Chemical analogues" of HLA-DM can induce a peptide-receptive state in HLA-DR molecules. *J Biol Chem.* (2004) 279:50684–90. doi: 10.1074/jbc.M407598200
57. Rupp B, Gunther S, Makhmoor T, Schlundt A, Dickhaut K, Gupta S, et al. Characterization of structural features controlling the receptiveness of empty class II MHC molecules. *PLoS ONE.* (2011) 6:e18662. doi: 10.1371/journal.pone.0018662
58. Rouhani SJ, Eccles JD, Tewalt EF, Engelhard VH. Regulation of T-cell tolerance by lymphatic endothelial cells. *J Clin Cell Immunol.* (2014) 5:242. doi: 10.4172/2155-9899.1000242
59. Lerner TR, de Souza Carvalho-Wodarz C, Repnik U, Russell MR, Borel S, Diedrich CR, et al. Lymphatic endothelial cells are a replicative niche for *Mycobacterium tuberculosis*. *J Clin Invest.* (2016) 126:1093–108. doi: 10.1172/JCI83379
60. Tewalt EF, Cohen JN, Rouhani SJ, Guidi CJ, Qiao H, Fahl SP, et al. Lymphatic endothelial cells induce tolerance via PD-L1 and lack of costimulation leading to high-level PD-1 expression on CD8 T cells. *Blood.* (2012) 120:4772–82. doi: 10.1182/blood-2012-04-427013
61. Cohen JN, Guidi CJ, Tewalt EF, Qiao H, Rouhani SJ, Ruddell A, et al. Lymph node-resident lymphatic endothelial cells mediate peripheral tolerance via Aire-independent direct antigen presentation. *J Exp Med.* (2010) 207:681–8. doi: 10.1084/jem.20092465
62. Gatica D, Chiong M, Lavandero S, Klionsky DJ. Molecular mechanisms of autophagy in the cardiovascular system. *Circ Res.* (2015) 116:456–67. doi: 10.1161/CIRCRESAHA.114.303788
63. Mackow ER, Gorbunova EE, Dalrymple NA, Gavrilovskaya IN. Role of vascular and lymphatic endothelial cells in hantavirus pulmonary syndrome suggests targeted therapeutic approaches. *Lymphat Res Biol.* (2013) 11:128–35. doi: 10.1089/lrb.2013.0006
64. Schilthuis M, Verkaik S, Walhof M, Philipose A, Harlow O, Kamp D, et al. Lymphatic endothelial cells promote productive and latent HIV infection in resting CD4+ T cells. *Virol J.* (2018) 15:152. doi: 10.1186/s12985-018-1068-6
65. Zhang X, Jiang S, Yu J, Kuzontkoski PM, Groopman JE. Cocaine enhances HIV-1 gp120-induced lymphatic endothelial dysfunction in the lung. *Physiol Rep.* (2015) 3:e12482. doi: 10.14814/phy2.12482
66. Tamburini BA, Burchill MA, Kedl RM. Antigen capture and archiving by lymphatic endothelial cells following vaccination or viral infection. *Nat Commun.* (2014) 5:3989. doi: 10.1038/ncomms4989
67. Kedl RM, Lindsay RS, Finlon JM, Lucas ED, Friedman RS, Tamburini BAJ. Migratory dendritic cells acquire and present lymphatic endothelial cell-archived antigens during lymph node contraction. *Nat Commun.* (2017) 8:2034. doi: 10.1038/s41467-017-02247-z
68. Schildberg FA, Klein SR, Freeman GJ, Sharpe AH. Coinhibitory pathways in the B7-CD28 ligand-receptor family. *Immunity.* (2016) 44:955–72. doi: 10.1016/j.immuni.2016.05.002
69. Zang X, Allison JP. The B7 family and cancer therapy:costimulation and coinhibition. *Clin Cancer Res.* (2007) 13:5271–9. doi: 10.1158/1078-0432.CCR-07-1030
70. Thomas SN, Rutkowski JM, Pasquier M, Kuan EL, Alitalo K, Randolph GJ, et al. Impaired humoral immunity and tolerance in K14-VEGFR-3-Ig mice that lack dermal lymphatic drainage. *J Immunol.* (2012) 189:2181–90. doi: 10.4049/jimmunol.1103545
71. Zhang H, Freitas D, Kim HS, Fabijanic K, Li Z, Chen H, et al. Identification of distinct nanoparticles and subsets of extracellular vesicles by asymmetric flow field-flow fractionation. *Nat Cell Biol.* (2018) 20:332–43. doi: 10.1038/s41556-018-0040-4
72. Meldolesi J. Exosomes and ectosomes in intercellular communication. *Curr Biol.* (2018) 28:R435–44. doi: 10.1016/j.cub.2018.01.059
73. Brown M, Johnson LA, Leone DA, Majek P, Vaahtomeri K, Senfter D, et al. Lymphatic exosomes promote dendritic cell migration along guidance cues. *J Cell Biol.* (2018) 217:2205–21. doi: 10.1083/jcb.201612051
74. Yeo KP, Angeli V. Bidirectional crosstalk between lymphatic endothelial cell and T cell and its implications in tumor immunity. *Front Immunol.* (2017) 8:83. doi: 10.3389/fimmu.2017.00083
75. Russo E, Teijeira A, Vaahtomeri K, Willrodt AH, Bloch JS, Nitschke M, et al. Intralymphatic CCL21 promotes tissue egress of dendritic cells through afferent lymphatic vessels. *Cell Rep.* (2016) 14:1723–34. doi: 10.1016/j.celrep.2016.01.048
76. Fletcher AL, Acton SE, Knoblich, K. Lymph node fibroblastic reticular cells in health and disease. *Nat Rev Immunol.* (2015) 15:350–61. doi: 10.1038/nri3846
77. Christiansen AJ, Dieterich LC, Ohs I, Bachmann SB, Bianchi R, Proulx ST, et al. Lymphatic endothelial cells attenuate inflammation via suppression of dendritic cell maturation. *Oncotarget.* (2016) 7:39421–35. doi: 10.18632/oncotarget.9820

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Chronic Liver Disease in Humans Causes Expansion and Differentiation of Liver Lymphatic Endothelial Cells

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Liver lymphatic vessels support liver function by draining interstitial fluid, cholesterol, fat, and immune cells for surveillance in the liver draining lymph node. Chronic liver disease is associated with increased inflammation and immune cell infiltrate. However, it is currently unknown if or how lymphatic vessels respond to increased inflammation and immune cell infiltrate in the liver during chronic disease. Here we demonstrate that lymphatic vessel abundance increases in patients with chronic liver disease and is associated with areas of fibrosis and immune cell infiltration. Using single-cell mRNA sequencing and multi-spectral immunofluorescence analysis we identified liver lymphatic endothelial cells and found that chronic liver disease results in lymphatic endothelial cells (LECs) that are in active cell cycle with increased expression of CCL21. Additionally, we found that LECs from patients with NASH adopt a transcriptional program associated with increased IL13 signaling. Moreover, we found that oxidized low density lipoprotein, associated with NASH pathogenesis, induced the transcription and protein production of IL13 in LECs both *in vitro* and in a mouse model. Finally, we show that oxidized low density lipoprotein reduced the transcription of *PROX1* and decreased lymphatic stability. Together these data indicate that LECs are active participants in the liver, expanding in an attempt to maintain tissue homeostasis. However, when inflammatory signals, such as oxidized low density lipoprotein are increased, as in NASH, lymphatic function declines and liver homeostasis is impeded.

Keywords: lymphatic endothelial cells, cirrhosis, fibrosis, non-alcoholic steatohepatitis, hepatitis C virus, alcoholic liver disease, interleukin-13, oxidized low density lipoprotein

INTRODUCTION

Deaths from chronic liver disease (CLD) have increased by 31% between the years 2000 and 2015 (1). CLD arises due to chronic inflammation in the liver as a result of a number of environmental insults including viral infection (hepatitis C or B virus-HCV/HBV), alcohol consumption (Alcohol associated liver disease-ALD) and diet-induced obesity (Non-alcoholic steatohepatitis-NASH).

Due to the regenerative capacity of the liver, the liver participates in a dynamic process that can result in several rounds of injury and repair. However, chronic injury eventually leads to severe fibrosis, cirrhosis, and the decline of liver function. While removal of the insult causing injury can be effective at reversing liver fibrosis (2), many patients with advanced disease do not improve or ultimately progress to cirrhosis (3, 4). As a result, these people remain at an elevated risk for development of hepatocellular carcinoma despite the removal of chronic insult (5). Limited therapeutic options exist for these patients causing the rates of morbidity and mortality to continue to climb (6).

The lymphatic system transports interstitial fluid (lymph) from the tissue to the circulatory system for removal from the body (7, 8). In addition, lymphatics participate in the acquisition of fat and the formation of chylomicrons in the gut (9), reverse cholesterol transport (10), and trafficking of dendritic cells (DCs) from the tissue to the lymph node (LN) (11, 12). During CLD, increased lymphatic permeability has been implicated in the formation of ascites, or fluid accumulation, in the peritoneal cavity (13). Furthermore, increased lymphatic vessel permeability has been demonstrated to increase inflammation and immune dysfunction in other tissues (14–17). Obesity and hypercholesterolemia are also associated with lymphatic permeability, hyperplasia, and inflammation at peripheral sites in humans (18, 19) and in animals (20).

Lymphatic vessels are comprised of lymphatic endothelial cells (LECs). LEC interactions with immune cells can guide trafficking of immune cells as well as promote self-tolerance and enhance protective immunity (21–25). Despite the multi-faceted role of LECs in programming immune responses in the lymph node and skin, the role of lymphatics in coordinating the immune response in the liver has not been addressed. Furthermore, with the advent of single cell sequencing, several reports have addressed different cell populations within the liver, including specific interrogation of liver endothelial cell populations (26–28). However, in none of these reports have lymphatic endothelial cells been identified. Thus, the transcriptional profile and function of liver lymphatic endothelial cells in homeostasis or disease is yet unknown. However, previous case reports from almost 20 years ago, using common endothelial markers and histology, did demonstrate that lymphatic vessels increase in diameter and abundance during chronic viral hepatitis (29, 30). Despite these observations, little to nothing is known about lymphatics in non-viral cases of CLD, or the cause and/or consequence of lymphatic expansion in the liver. As diet-induced CLD has surpassed chronic viral infection as the leading cause of liver transplantation (31), understanding the role of lymphatics in the liver in people with diet-induced CLD is an important functional process that needs to be addressed.

Here we demonstrate a significant increase in lymphatic vessel density in patients with CLD. We show that in addition to virally induced CLD, that non-virally induced CLD also results in a significant increase in lymphatic vessel density in the liver. To identify specific differences in LECs during disease we performed transcriptional profiling of LECs, using a single-cell platform, from non-diseased and diseased human livers. While other endothelial cell populations have been identified by single cell RNA sequencing in the liver, this is the first demonstration of isolation of LECs and subsequent transcriptional profiling of this rare cell population in the liver. We find that LECs from NASH or HCV infected livers engage a transcriptional program that results in more LECs in active cell cycle and more CCL21 expression. However, when comparing the transcriptional profile of LECs from patients with NASH to patients with HCV we find that NASH specifically induces the activation of the IL13 pathway. Furthermore, we demonstrate that not only is the IL13 pathway increased in patients with NASH, but also that oxidized LDL, commonly associated with inflammation in NASH, can induce the upregulation of *IL13* transcript and protein in LECs. Finally, we provide evidence that IL13 expression by LECs occurs specifically in the liver and that oxidized LDL results in the downregulation of the LEC transcription factor, *PROX1*, and reduced lymphatic stability.

Abbreviations: CLD, chronic liver disease; LEC, lymphatic endothelial cell; PEC, portal endothelial cell; HBV, hepatitis B virus; HCV, hepatitis C virus; PBC, primary biliary cirrhosis; NASH, non-alcoholic steatohepatitis; ALD, alcoholic liver disease; AIH, autoimmune hepatitis; PSC, primary sclerosing cholangitis; PDPN, podoplanin; IL13, Interleukin 13; MELD, model for end-stage liver disease.

MATERIALS AND METHODS

Patient Samples

For immunohistochemistry archived patient specimens were obtained from the University of Colorado Anschutz Medical Campus biorepository core facility **(Supplementary Table 1)**. For single cell sequencing, patients were selected from a biorepository of patients who had undergone liver transplantation and collected under the IRB protocol of HRR and MSK. Transplanted livers were harvested and non-parenchymal cells (NPCs) were isolated and frozen in a single cell suspension as described (32). Additionally, non-diseased NPCs were purchased from Triangle research laboratories (Lonza, Triangle research park, NC). For Non-diseased patients ($n = 6$) the age range was 37 to 58 with a mean age of 49 where five patients were male and one was female. For HCV samples ($n = 3$) the age range was 47–55 with a mean age of 51 and all patients were male. For NASH samples ($n = 2$) the age range was 49–55 with a mean age of 52 and all patients were females. For Ki67 analysis: the age range was 35–61 for diseased patients and included NASH, ALD, HCV, AIH, PBC, and AIH with NASH. The average age of all diseased patients was 49.4 with 62.5% of the patients being female. The same non-diseased patients from Lonza were used as described above. All patients provided written and informed consent and the study was approved by the institutional review boards at the University of Colorado—Anschutz.

Flow Sorting and Flow Cytometric Analysis

To enrich LECs from hepatic NPCs we thawed frozen samples in RPMI containing 10% Human Serum AB (Gemini Bio-products, West Sacramento, CA) and 1% DNASE (MP Biomedicals, Santa Ana, CA). Cells were washed 2x with PBS containing 2% FBS

(Atlas Biologicals, Fort Collins, CO) and stained with antibodies against CD45 (clone HI30), CD31 (PECAM1, clone WM59), Podoplanin (clone NC-08) and CD146 (clone P1H12) from Biolegend (San Diego, CA), and CD68 (clone KP1) from abcam (San Francisco, CA). Cells from either Non-diseased, NASH or HCV were sorted using an aria Fusion sorter (BD Biosciences, Franklin lakes, NJ) and enriched LECs were sorted into RPMI containing 50% human serum AB. Enriched LECs from each condition were pooled for single cell sequencing as described below. Flow cytometric analysis of human liver LECs: isolated liver NPCs were stained with Fixable viability dye 510 (BD Biosciences) and stained with the above surface antibodies. Following surface staining cells were fixed and permeabilized (Thermo Fischer, Waltham, MA) and stained for Ki67 (clone 11F6) (Biolegend). Flow cytometry was performed using a BD FACSCanto II instrument and was acquired with BD FACSDiva software (BD Biosciences). Analysis ws performed using FlowJo 10 (Treestar, Woodburn, OR).

Single Cell RNA Sequencing

Approximately 10,000 LEC-enriched hepatic NPCs were loaded onto a 10x genomics (San Francisco, CA) controller per manufacturer instructions to generate barcoded single cell GEMs using the 10x genomics 3′ kit. mRNA was converted to cDNA within each barcoded single cell GEM and libraries were generated as previously described. 10x libraries were sequenced using a NovaSeq 6000 (Illumina, San Diego, CA) to a predicted depth of 100,000 reads/cell. All cell preparation was performed at the University of Colorado Genomics Shared Resource Core. To control for sequencing batch effects, a minimum of three non-diseased samples were included with each diseased capture and sequencing run.

Quantification of Single Cell RNA Sequencing

scRNA-seq data was processed with the 10x Genomics Cell Ranger Suite for demultiplexing, alignment, assignment of reads to genes, and unique molecular identifier (UMI) deduplication to remove PCR duplicates. Further analysis, including cell clustering, cell type identification, marker gene identification, and differential expression analyses was performed using the R packages Seurat (33) and scran (34). For cell cycle analysis Seurat assigns each cell a score based on its expression of G2/M and S phase markers. These marker sets are anti-correlated in their expression levels, and cells expressing neither are assigned to G1 phase. Cells with fewer than 250 detectable genes or >20% of UMIs derived from mitochondrial genes were excluded from the analysis to eliminate cells with insufficient expression data for clustering and dead cells, respectively **(Supplementary Figure 1)**. Ingenuity pathway analysis (IPA) was performed using IPA software (Qiagen, Venlo, Netherlands). Raw data, count matrices, metadata including cluster assignment are available at the Gene Expression Omnibus under accession GSE129933 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE129933).

Multispectral Fluorescence Immunohistochemistry and Vectra Analysis

Five micron thick tissue sections were sequentially stained for human PDPN, CD3, CD19, CCL21, and CD68. Slides were dewaxed with xylene, heat treated in pH9 antigen retrieval buffer for 15 min in a pressure cooker, blocked in Antibody (Ab) Diluent (Perkin Elmer, Waltham, MA), incubated for 30 min with the primary Ab, 10 min with horseradish peroxidase (HRP)-conjugated secondary polymer (anti-mouse/anti-rabbit, Perkin Elmer, Waltham, MA), and 10 min with HRP-reactive OPAL fluorescent reagents (Perkin Elmer). Slides were washed between staining steps with PBS 0.01% tween 20 and stripped between each round of staining with heat treatment in antigen retrieval buffer. After the final staining round the slides were stained with spectral DAPI (Perkin Elmer), and coverslipped with Prolong Diamond mounting media (Thermo Fisher, Waltham, MA). Multispectral imaging was preformed using the Vectra 3.0 Automated Quantitative Pathology Imaging System (Perkin Elmer). Whole slide scans were collected using the 10x objective and 10 to 20 regions were selected for multispectral imaging with the 20x objective. The multispectral images were analyzed with inForm software (Perkin Elmer) to unmix adjacent fluorochromes, subtract autofluorescence, segment the tissue into lymphatic vessels and non-lymphatic vessels, segment the cells into nuclear, and membrane compartments, and to phenotype the cells according to morphology and cell marker expression. Lymphatic vessel density was quantified using the Nikon AR software where LVD = vessel area/total area × 100%. Lymphatic vessels with CCL21 staining less 0.036 were classified as $CCL21^{lo}$ or negative while lymphatic vessels with a value >0.036 were classified as $CCL21^{hi}$ or positive using inForm software. For CCL21 analysis three Non-diseased, 5 HCV, and three NASH patient samples were interrogated. For T, B, and macrophage cell analysis three or four non-diseased, 3–5 NASH, and 4 HCV patient samples were quantified. A student's *t*-test was performed where two asterisks represents a *p*-value less than 0.001. Patient data is included in **Supplementary Table 1**.

Branch Forming Assay

Branch forming assay was performed as previously described (35, 36). Briefly, a 4.2 mg/ml matrigel (Corning, Tewksbury, MA) pad with either 100 μg/ml oxidized LDL (Alfa Aeser, Ward Hill, MA), 0.25 mM Palmitic Acid (Caymen Chemicals, Ann Arbor, MI, or equivalent amount of DMSO was allowed to solidify for one and a half hours. Fifteen thousand Human Lymphatic Endothelial Cells (PromoCell, Heidelberg, Germany) mixed with 100 μg/ml oxidized LDL, 0.25 mM Palmitic Acid, or equivalent amount of DMSO in endothelial cell growth medium (PromoCell) were placed on top of the matrigel pad. Cells were incubated at 37°C for 21 h, then imaged using a Zeiss microscope, and Axiom camera. For RT-PCR the matrigel was dissolved using ice cold 5 mM EDTA rocking on ice for 1 h. HLECs were pelleted and lysed with RLT buffer, mRNA was extracted using the RNeasy micro kit per manufacturer instructions

and cDNA was synthesized using the QuantiTect RT Kit (Qiagen, Venlo, Netherlands) following standard protocols. Quantitative PCR was performed on an Applied Biosystems 7,300 Real-time PCR machine and fold changes in mRNA levels were calculated using the delta-delta CT method. For each gene, all samples were normalized to the average fold change of the vehicle treatment group (DMSO). The following Qiagen QuantiTect primers were used: *GUSB* (QT00046046), *IL13* (QT00000511), *PROX*1 (QT01006670), and *VEGFR3/FLT4* (QT00063637).

Animal Studies

Six to eight week old C56BL6/J mice were IV injected with 85 μg of unlabeled or DIL or DIO labeled human highly oxidized LDL (Kalen Biochemicals, Germantown, MD) or 100 μg PolyI:C (InvivoGen, San Diego, CA). Following indicated incubation time mice were administered a second dose of stimulant. For *in vivo* BFA experiments, 18 h following the second injection mice were administered 250 μg of Brefeldin A as previously described for analysis of *in vivo* cytokine production by T cells (25, 37). Ninety minutes after Brefeldin A injection mice were euthanized and livers and lymph nodes were processed as described (38) with Brefeldin A in each buffer. For flow cytometric analysis of LEC production of IL13, single cell suspensions of liver and lymph node cells were stained with CD45 (clone 30-F11), CD31 (clone 390), PDPN (clone 8.1.1) from biolegend and CD146 (clone P1H12) and IL13 (clone ebio13A) from ebioscience. Flow cytometry was performed using a BD FACSCanto II instrument and data were acquired with BD FACSDiva software (BD Biosciences) or Cyan ADP and acquired with summit software. Analysis was performed using FlowJo 10 (Treestar). All procedures were approved by the University

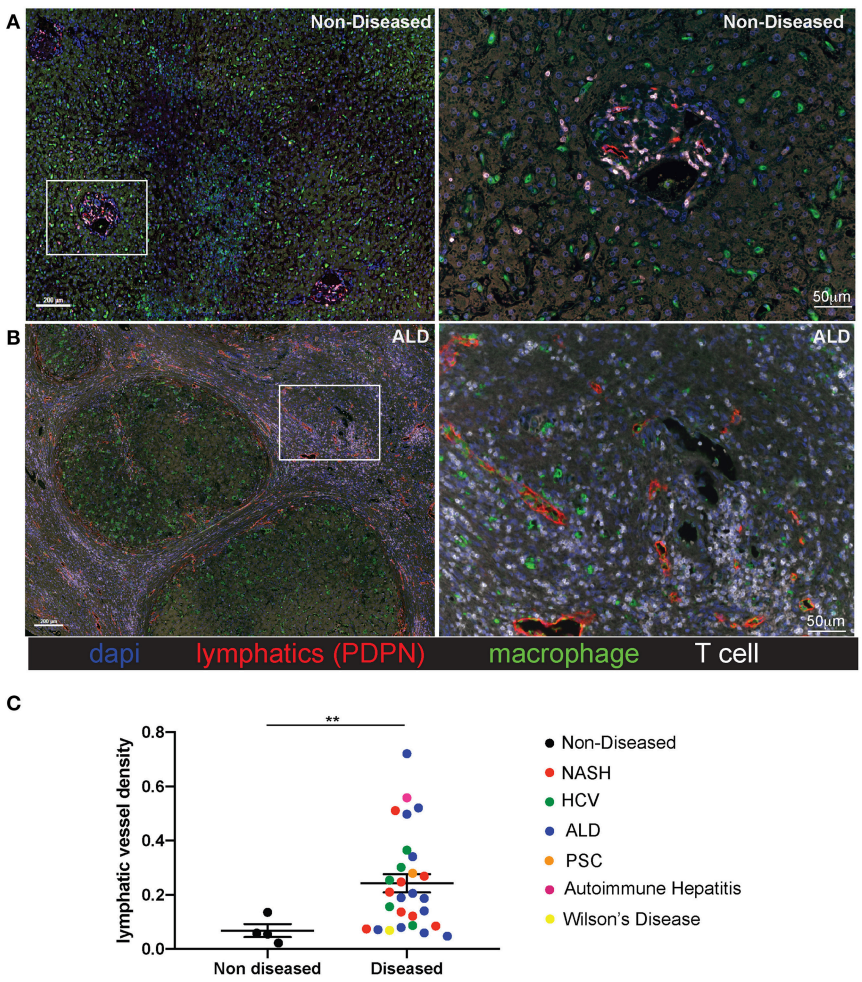

FIGURE 1 | Lymphatic vessels increase in fibrotic regions of cirrhotic livers independent of disease etiology. Liver explants were obtained from cirrhotic patients who received liver transplantation. Non-Alcoholic Steatohepatitis (NASH) ($n = 8$), Alcoholic liver disease (ALD) ($n = 12$), chronic Hepatitis C viral infection (HCV) ($n = 5$), Autoimmune hepatitis (AIH) ($n = 1$), Wilson's disease ($n = 1$), Primary sclerosing cholangitis (PSC) ($n = 1$) and four non-diseased livers. Representative images from non-diseased **(A)** or ALD **(B)** explants are shown. Five micrometer sections were stained with anti-podoplanin (lymphatic vessels D2/40-red), anti-CD3 (T cells-white), anti-CD68 (macrophages-green), and dapi (nuclei-blue) and imaged using the Perkin Elmer Vectra 3.0 imaging system and linear unmixed with inFORM software. **(C)** Lymphatic vessels density was determined using inFORM software and normalized to area in each disease listed and designated by color of dot. Statistical analysis was performed using a student's *t*-test. $^{**}P < 0.01$.

of Colorado School of Medicine Institutional Animal Care and Use Committee.

Statistical Analysis

For all graphs with statistical analysis an unpaired student's *t*-test was used evaluate statistical significance between two points using Prism software (GraphPad, San Diego, CA). One asterisk denotes a *p*-value of <0.05, two asterisks denotes a *p*-value <0.01 and three asterisks denotes a *p*-value <0.001. Statistical analysis for single cell RNA sequencing (differential expression testing) was performed using the Wilcoxon Rank-Sum Test implemented in Seurat.

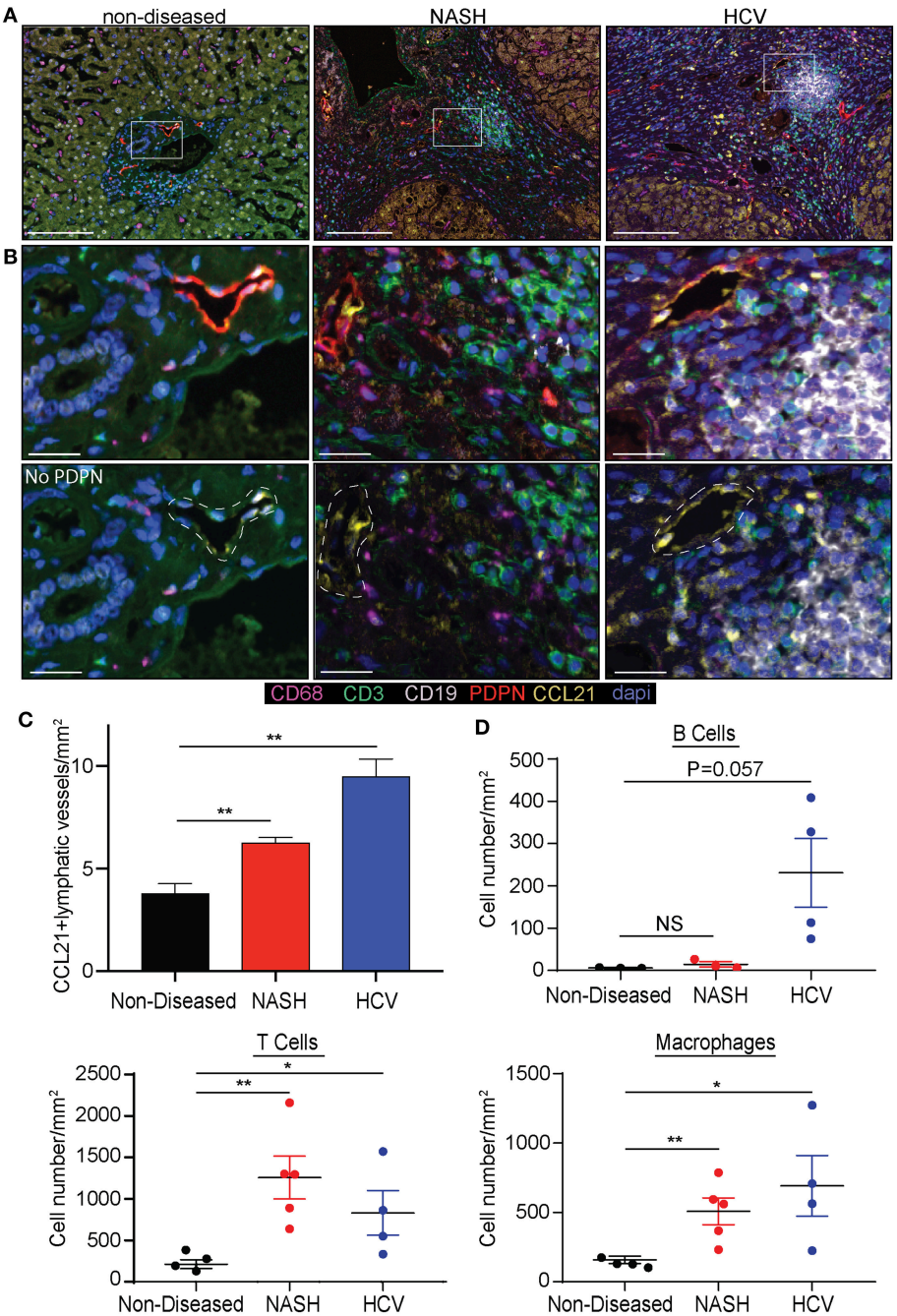

FIGURE 2 | Chronic liver disease induces the expansion of CCL21$^+$ lymphatic vessels and immune cell recruitment to the liver. **(A)** Lymphatic vessel (PDPN-Red) expression of CCL21 (Yellow), along with Macrophages (CD68-Magenta), T cells (CD3-Green) and B cells (CD19-White) in non-diseased, NASH, and HCV explanted livers. **(B)** Zoomed in representative examples from **(A)** shown with and without PDPN. White dotted line denotes where lymphatic vessel appears in the image. **(C)** Quantification of CCL21$^+$ lymphatic vessels in non-diseased ($n = 3$), HCV ($n = 5$), and NASH ($n = 3$) livers. **(D)** Quantification of B cells, T cells, and Macrophages in liver tissue from Non-diseased (black, $n = 3$), NASH (red, $n = 3$–5) or HCV (blue, $n = 4$). *$P < 0.05$, **$P < 0.01$.

RESULTS

Lymphatic Vessel Density Increases in End Stage Liver Disease

Liver tissue was obtained from 28 patients with end stage liver disease at the time of transplantation (Non-Alcoholic Steatohepatitis (NASH), Alcoholic liver disease (ALD), chronic Hepatitis C viral infection (HCV), Autoimmune hepatitis (AIH), Wilson's disease, Primary sclerosing cholangitis (PSC), and four patients with non-diseased livers **(Supplementary Table 1)**. Lymphatic vessels in the liver were assessed via immunofluorescence staining with the anti-podoplanin antibody (red) to mark lymphatic vessels in addition to anti-CD3 to mark T cells (white), anti-CD68 to mark macrophages (green), and dapi to label nuclei (blue). Shown are representative images from both a non-diseased liver **(Figure 1A)** and an ALD patient explant **(Figure 1B)**. In cirrhotic livers, the density of lymphatic vessels is significantly increased, regardless of disease etiology, when compared to liver sections from non-diseased controls (**Figure 1C**). Changes in LVD were independent of Model for End Stage Liver Disease (MELD) score at time of transplant, age, body mass index (BMI), race, or disease etiology (**Supplementary Figure 2**). This increased lymphatic vessel density is confined to areas of active inflammation. As such, the lymphatic vessels are in close proximity to regions of inflammation as determined by the frequency of T cells, macrophages, and fibrotic areas. Alternatively, neither lymphatic vessels, nor T cells were found in the regenerative nodules **(Figures 1A,B** and **Supplementary Figure 3)**. This supports lymphatic expansion as a universal marker and potential critical mechanism for chronic liver disease progression.

Increased CCL21 Expression and Immune Cell Infiltration Occurs in Cirrhotic Livers

As we determined lymphatic vessel density was increased during disease we asked if CCL21, T cell, B cell or macrophage frequency was changed. Others have reported expression of CCL21 by LECs in other tissues and CCL21 is a chemokine that can recruit CCR7+ dendritic cells and T cells (39). We first validated that liver LECs express CCL21 protein using a CCL21 specific antibody. In the imaging of non-diseased and diseased livers we observed that there were populations of LECs that express no or low levels of CCL21 **(Figure 2A)** while others express high levels of CCL21 protein (**Figure 2B**). To stratify lymphatic vessels based on CCL21 expression we calculated negative expression

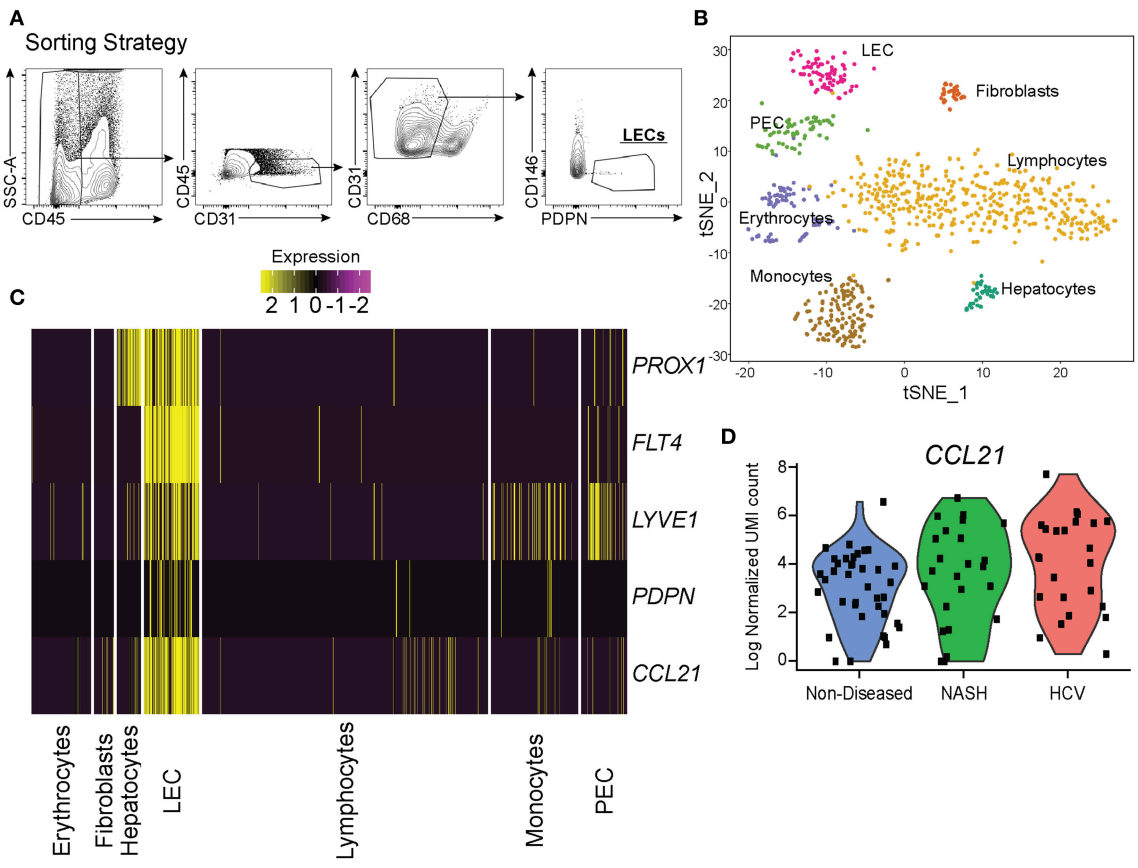

FIGURE 3 | Single cell analysis of liver lymphatic endothelial cells. **(A)** Sorting strategy used to identify LECs. **(B)** TSNE analysis from single cell mRNA sequencing of cells obtained from the sorting strategy in **(A)**. **(C)** Expression of LEC specific genes expressed by isolated cell subsets from the liver (yellow = high, purple = low). **(D)** Normalized CCL21 mRNA expression by LECs from non-diseased (blue), NASH (green), or HCV (red) livers.

TABLE 1 | LECs and PECs have similar but distinct transcriptional profiles.

Gene	avg_log2(LEC/PEC)	pct.LEC	pct.PEC	pval	Pval adj
CCL21	5.158128464	0.763	0.045	2.00E-11	2.26E-07
TFF3	3.305995867	0.789	0.045	1.17E-11	1.33E-07
NTS	2.140331515	0.316	0	6.73E-05	0.760564313
ADIRF	1.637091524	0.711	0.227	2.39E-06	0.027008311
S100A10	1.47389027	0.763	0.386	4.70E-06	0.053149996
S100A6	1.29723903	0.789	0.318	1.39E-06	0.015742032
FABP4	1.086755403	0.658	0.091	2.56E-07	0.002893748
JUNB	−1.508928712	0.158	0.636	7.56E-06	0.085490214
AC090498.1	−1.65001069	0.184	0.545	1.62E-04	1
ZFP36	−1.665549178	0.079	0.5	6.20E-05	0.700906334
NBEAL1	−1.699311818	0.105	0.455	2.87E-04	1
RDX	−1.836136132	0.053	0.409	1.89E-04	1
C11orf96	−1.881095457	0	0.273	5.79E-04	1
FCN3	−1.963822515	0.026	0.477	1.15E-05	0.129553478
SAT1	−2.283288374	0.026	0.5	3.07E-06	0.034715003
MTRNR2L12	−2.322539602	0.053	0.523	6.06E-06	0.068538598
PLPP3	−2.351071763	0	0.318	1.66E-04	1
HSPG2	−2.394084071	0.026	0.477	5.88E-06	0.066463691
CCL14	−2.467042053	0.053	0.477	1.60E-05	0.181344818
IFI27	−2.604129481	0.079	0.659	5.81E-08	6.57E-04
BST2	−2.622236874	0	0.432	5.77E-06	0.065268266
RNASE1	−2.767317205	0	0.523	2.99E-07	0.003384829

Shown is the average log2 gene expression fold change between LEC and PEC in non-diseased livers, the percent of cells in each subset that expresses a given gene, and the raw and adjusted P-values. Data was filtered to include only genes in which the p-value between LEC and PEC was <0.0006.

to be <0.01 counts, based on a no-CCL21 antibody control, $CCL21^{lo}$ expression to be between 0.01 and 0.036 counts and $CCL21^{hi}$ expression to be between 0.036 and 0.252 counts, as assessed by InFORM software **(Supplementary Figure 4)**. Using PDPN to label lymphatic vessels we found that the number of lymphatic vessels that had $CCL21^{hi/+}$ expression was about 4 vessels per mm^2 in non-diseased livers, while the frequency of lymphatic vessels with high expression of CCL21 was between 6 and 10 vessels per mm^2 in patients with HCV and NASH **(Figure 2C)**. These vessels were also often associated with infiltrating immune cells and thus we quantified the accumulation of B cells, T cells and macrophages in the liver. Similar to previous results, we found that end stage liver disease resulted in the significant accumulation of T cells and macrophages in the liver of patients with HCV and NASH while B cells were less frequent **(Figure 2D)**. These studies led us to ask if chronic liver disease induces LECs differentiation in the liver that results in modulation of the inflammatory state of the liver microenvironment.

Isolation and Single Cell Sequencing of Lymphatic Endothelial Cells in the Liver

To understand how LECs were transcriptionally regulated in the liver we isolated LECs and subjected them to single cell mRNA sequencing. While other endothelial cell populations within the liver have been evaluated transcriptionally, the transcriptome of liver lymphatic vessels has yet to be reported. This could be due to the LECs in the liver being a fragile, rare and difficult to identify population. Therefore, we used our expertise in lymphatic endothelial cell flow cytometry to isolate and acquire the transcriptional signature of lymphatic endothelial cells in the liver. Once LECs from explanted human livers were isolated by flow sorting, using our published liver LEC marker set (38) **(Figure 3A)**, we subjected the sorted cells to single cell mRNA sequencing using the 10x genomics 3′ platform. Individual groups of cells were clustered using TSNE clustering based on transcriptional profile **(Figure 3B)**. Interestingly, while we sorted our population of cells based on known phenotypic markers for LECs we still were able to visualize a number of contaminating cells based on their transcriptional profile. However, using this analysis we were also able to discern that in the non-diseased human liver there are two distinct populations of endothelial cells that are transcriptionally distinct from liver sinusoidal endothelial cells (LSECS) (26).

LECs were identified using the expression of prospero homeobox protein 1 (*PROX1*), lymphatic vessel endothelial hyaluronan receptor 1 (*LYVE-1*), podoplanin (*PDPN*), vascular endothelial growth factor receptor 3 (*FLT4/VEGFR3*), and *CCL21* **(Figure 3C)**. Based on the expression of these LEC-associated markers we were able to divide these two populations into a fully differentiated LEC population and an endothelial cell population that resembles the recently reported portal endothelial cells (PEC) (27). Specifically, these two populations are distinguished by their expression of several markers such as *PROX1*, *PDPN*,

CCL21, Neurotensin, and trefoil factor 3 (*TFF3*) by the LEC cluster; bone marrow stromal antigen two precursor (*BST2*), interferon alpha inducible protein 27 (*IFI27*) and ribonuclease 1 (*RNASE1*) by the other endothelial cluster similar to PECs **(Table 1).** These factors have been previously reported to be associated with either LECs or blood endothelial cells (BECs) in other model systems and in primary endothelial cell cultures confirming that these subsets are of lymphatic or blood origin, respectively (40, 41). As seen in **Figure 2**, liver LECs express CCL21 protein while LECs from diseased livers had more $CCL21^{hi/+}$ vessels. This was confirmed by our transcriptional analysis and similar to other reports demonstrating expression of the chemokine *CCL21* by LECs **(Figure 3D)** (42, 43). We also discovered that *TFF3*—a gene upregulated in hypoxia that induces expression of VEGF and protects barrier function is upregulated by the LEC population (44, 45). The liver PEC-like population has increased expression of genes such as *IFI27* which is also expressed by LECs in the lymph node (46–48); and *HSPG2* encoding Perlecan which is predominantly expressed by BECs, but whose expression can be increased during the final maturation of lymphatic vessels in the skin (49). These data suggest that the PEC-like population may be a progenitor cell for LECs with transcripts found in both cells from blood and lymphatic lineages. Finally, we confirm that the structures we visualized in the livers of cirrhotic patients **(Figure 1)** are the same LEC population we are evaluating transcriptionally based on the expression of PDPN in this specific endothelial population **(Figure 3C)**. Thus, based on transcriptional profiling we were able to distinguish LECs from other cells in the liver in order to evaluate changes in these cells during chronic liver disease.

Liver Disease Results in the Proliferation of Lymphatic Endothelial Cells

As stated above we identified lymphatic endothelial cells in the liver both by flow cytometry and transcriptional profiling.

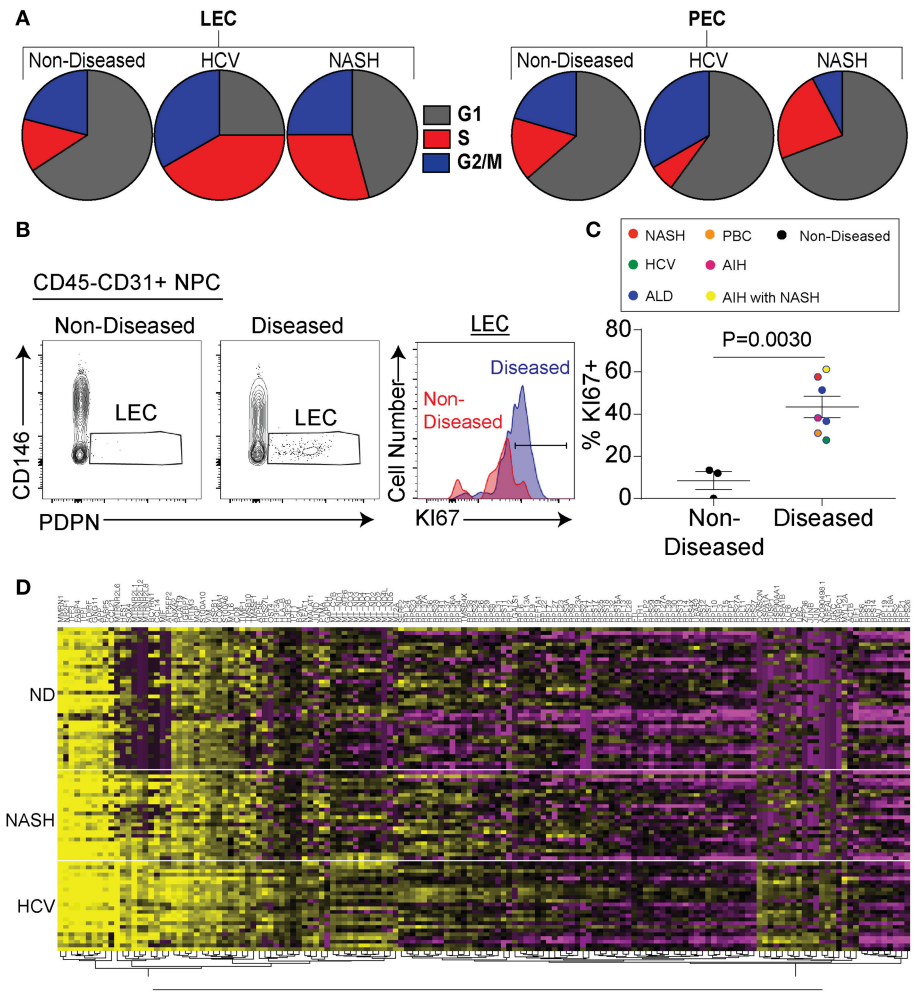

FIGURE 4 | Chronic liver disease results in increased LEC proliferation. **(A)** Frequency of LECs (left) and PECs (right) in each stage of cell cycle based on gene expression from single cell sequencing data. **(B)** Representative flow cytometric profiles of LECs from non-diseased (left and red) or diseased (right and blue). **(C)** Quantification of **(B)**. **(D)** Hierarchical clustering of differentially expressed genes from LECs sorted from non-diseased (ND), NASH, or HCV explanted livers.

TABLE 2 | Transcriptional differences between LECs from patients with NASH or chronic HCV infection.

Gene	avg_log2(NASH/HCV)	pct.NASH	pct.HCV	pval	pval_adj
SYTL2	2.154809339	0.5	0.042	2.91E-04	1
AC090498.1	1.266572393	0.875	0.875	1.24E-04	1
EIF1	−0.552823526	0.417	0.958	5.94E-04	1
MALAT1	−0.970590552	0.958	1	9.62E-05	1
DUSP1	−1.06840421	0.333	0.875	4.43E-04	1
IGLC2	−1.268154318	0.125	0.667	4.03E-04	1
MTRNR2L8	−1.483505058	0.125	0.708	2.74E-04	1
DONSON	−1.612257661	0.458	1	1.82E-07	0.002055027
ACVR2B	−1.80158542	0	0.5	9.98E-05	1
IGHG3	−2.106156828	0	0.542	4.18E-05	0.472913317
IGKC	−2.5839658	0.042	0.875	2.81E-08	3.18E-04
MTRNR2L6	−2.796201221	0	0.625	6.66E-06	0.075261418
MTRNR2L1	−3.042838611	0.083	0.875	1.81E-07	0.002050256

Shown is the average log_2 gene expression fold change differences between LECs isolated from NASH and HCV, the percent of cells in each subset that expresses a given gene, and the raw and adjusted P-values. Data was filtered to include only genes in which the p-value between HCV and NASH were <0.0006.

Being that we saw a substantial increase in the frequency of lymphatic vessels in disease **(Figure 1)** we evaluated if chronic liver disease induced the specific expansion and differentiation of LECs. We first compared liver LECs from non-diseased to diseased livers (both HCV and NASH) and found LECs from diseased livers downregulated pathways involved in apoptosis while upregulating pathways involved in free radical scavenging **(Supplementary Table 2)**. Similarly, upstream pathways activated in LECs from diseased livers included Jun N-terminal kinase (JNK), mitogen-activated protein kinase (MAPK), as well as tumor necrosis factor (TNF) **(Supplementary Table 3)**. We also observed a higher proportion of LECs from diseased patients that are in active cell cycle compared to LECs from non-diseased livers or PECs **(Figure 4A)**. To confirm this transcriptional data we utilized flow cytometry to measure Ki67 expression by LECs from non-diseased and diseased livers. Using this approach, we were able to confirm our transcriptional data demonstrating that a higher frequency of LECs from diseased livers have Ki67 expression compared to LECs from non-diseased livers **(Figure 4B)**. This difference was consistent across patients, suggesting the expansion of LECs is a common event during chronic liver disease **(Figure 4C)**. Finally, when comparing genes between liver LECs from non-diseased, HCV or NASH we found that each group clustered differently **(Figure 4D)**. Many of the same genes were differentially expressed between non-diseased and both NASH and HCV **(Figure 4D)** suggesting different inflammatory stimuli induce some of the same transcriptional programs. However, while there were many similarities in the transcriptional programs of LECs from HCV or NASH explanted livers there were also differences **(Figure 4D)**. Taken together, these data demonstrate that LECs and PECs, are differentially regulated during chronic liver disease and that chronic liver disease results in the preferential expansion of LECs. These data also suggest that different inflammatory insults may regulate different gene programs in LECs.

CLD Alters Signaling Pathways in Liver LECs

To examine differences in gene expression in LECs dependent on disease etiology we compared the gene expression of LECs from NASH patients to HCV patients **(Table 2)**. We also used IPA software to evaluate transcriptional pathways **(Supplementary Table 4)** induced by the different disease etiologies. Intriguingly, the IL13 signaling pathway was upregulated in LECs isolated from patients with NASH **(Supplementary Table 4)**. IL13 has been shown to be involved both in maintaining lymphatic vessel structure and permeability (35, 50) and in the conversion of hepatic stellate cells (HSC) to myofibroblasts (51, 52). While IL13 expression was not detected in any of the cell types we evaluated, suggesting a signal strength issue; CD36, FABP4 and TFF3, genes that are targets of IL13 signaling, were upregulated (53–55), and ATF3, which inhibits IL13 transcription (56), was downregulated in LECs from NASH livers (**Figure 5A**). These data not only gave us potential leads to follow, but also led us to the conclusion that while CLD uniformly induces the expansion of lymphatic vessels in the liver, NASH-associated liver disease elicited a unique transcriptional profile in liver LECs that involves IL13 signaling.

We next asked if these transcriptional differences were a result of direct stimulation of LECs by factors associated with the disease. Therefore, we asked if oxidized LDL (oxLDL) or palmitic acid (PA) affected the lymphatic branching and transcriptional profile of human LECs (hLECs) *in vitro* (**Figure 5B**). We used oxLDL as it accumulates in response to free radicals generated by inflammation, has been shown to affect other cell types such as macrophages and endothelial cells in atherosclerosis and is elevated in NASH (57–62). Further, oxLDL has a dramatic effect on macrophage activation, while LDL alone does not (63). We used PA as it is an important dietary fatty acid that is largely consumed in foods [reviewed in (64)]. We observed that when LECs were treated with vehicle (DMSO), PA or oxLDL and

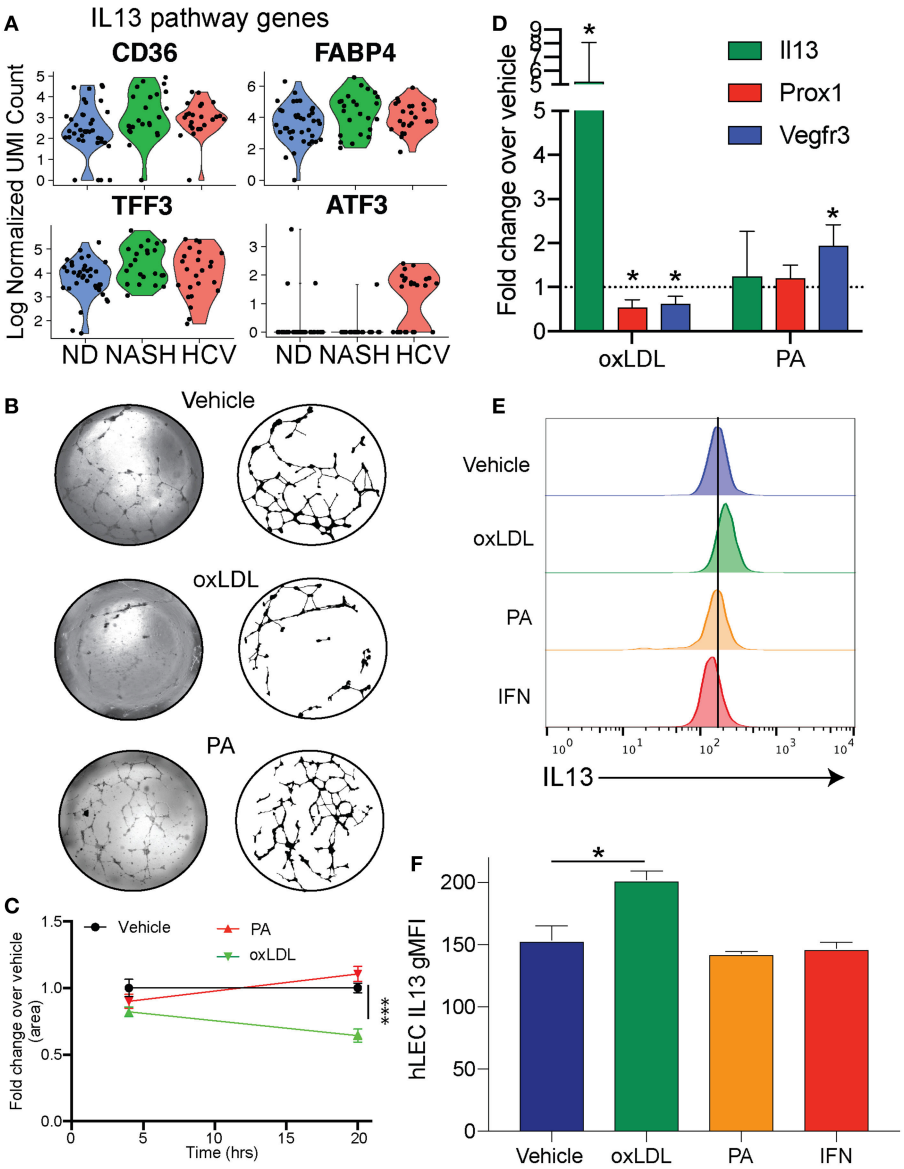

FIGURE 5 | Cholesterol regulates IL13 signaling in LECs. **(A)** Genes involved in IL13 signaling in LECs from Non-diseased (ND, blue), NASH (green), and HCV (red) **(B)**. Representative images of lymphatic branching from hLECs treated for 24 h with Vehicle (DMSO), Ox-LDL (100 μg/ml) or PA (0.25 mM). **(C)** Quantification of **(B)**. **(D)** Quantitative RT-PCR of hLEC treated with the indicated stimulus for 24 h. **(E)** Representative flow cytometric profiles of IL13 protein production by hLECs after 24 h with the indicated stimulus. **(F)** Quantification of **(E)**. $^{*}P < 0.05$. $^{***}P < 0.001$.

visualized at 4 and 24 h post-treatment that only the vehicle and PA treated LECs were able to maintain their branched structures over the 24-h period (**Figures 5B,C**). OxLDL treated LECs were able to form the branch structures, but by 24 h the structures had collapsed (**Figures 5B,C**). To determine if these dietary constituents were inducing transcriptional changes in the LECs we performed qRT-PCR on the hLECs and evaluated the transcript abundance of *IL13* (**Figure 5D**) because the IL13 pathway was upregulated in LECs from people with end stage NASH (**Figure 5A**) and IL13 has been shown to cause defects in lymphatic branching and inhibit *PROX1* expression (35). Interestingly, we found that *IL13* was upregulated only in LECs that were treated with oxLDL, but not PA (**Figure 5D**). Consistent with LEC structure collapse, oxLDL treatment resulted in the decreased expression of *PROX1* and the *PROX1*-dependent gene, *FLT4 (VEGFR3)* (**Figure 5D**). This is in contrast to PA which did not affect *PROX1* expression. We next asked if IL13 protein levels were increased after oxLDL, PA or interferon alpha. Interferon signaling is significantly increased in HCV while IL13 signaling is not. We found using flow cytometry that there is a significant increase in protein expression of IL13 in hLECs treated with oxLDL, but not PA or IFN (**Figures 5E,F**).

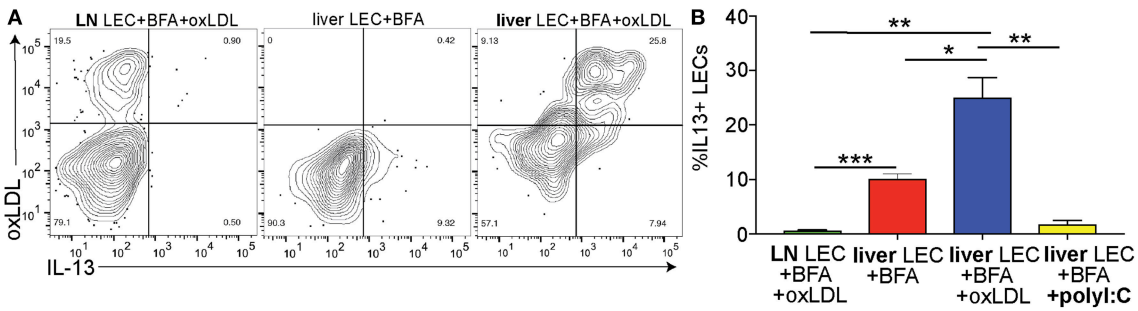

FIGURE 6 | Cholesterol results in IL13 production by liver LECs *in vivo*. **(A)** Representative flow plots of cholesterol uptake and IL13 production by liver and lymph node LECs from Brefeldin A treated mice. LECs were gated as in 3A. **(B)** Quantification of A. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

These data demonstrate that oxLDL can induce transcriptional and functional changes in LECs *in vitro* that are similar to the signaling pathways we observed in patients with NASH. Thus, IL13 signaling in LECs from patients with NASH could be caused by increased levels of oxLDL in the liver.

OxLDL Uniquely Induces IL13 Production by Liver LECs *in vivo*

Above we demonstrated that the IL13 signaling pathway is activated in LECs of people with NASH (**Figure 3**) and increased IL13 gene and protein expression *in vitro* when LECs were treated with oxLDL (**Figures 5D–F**). We next asked if LECs in the liver and LN of mice treated with oxLDL were able to produce IL13 protein *in vivo*. To answer this question, we intravenously injected C57BL/6 mice with fluorescently labeled, oxLDL or the interferon inducing toll like receptor agonist, polyI:C (as an inflammatory control that should not induce IL13). To determine if these stimuli resulted in the production of IL13 by liver and/or lymph node (LN) LECs we directly measured production of cytokines *in vivo* 6 days post-injection **(Figure 6A)**. Using an *in vivo* Brefeldin A assay (25, 37, 65), we found that acute stimulation with oxLDL, but not polyI:C elicited the production of IL13 by liver LECs **(Figure 6B)**. Interestingly, while the LECs in the skin draining LN were able to take up oxLDL, they did not produce IL13 (**Figure 6**). These findings were confirmed using Balb/c mice that have been engineered to express YFP under the control of the IL13 promoter (IL13-YFP) **(Supplementary Figure 5)**. These findings support our conclusions that liver LECs directly respond to oxLDL by producing IL13 and that liver LECs have a unique functional response to dietary stimulation compared to lymph node LECs.

DISCUSSION

The role of lymphatic vessels in normal and disrupted liver homeostasis has largely been ignored. Previous studies have demonstrated an increase in lymphatic vessel-like structures in the liver during chronic viral infection and in the setting of portal hypertension (29, 66). Others have proposed that the ascites associated with chronic liver disease may be a consequence of lymphatic dysfunction or increased lymphatic permeability (13). Furthermore, altered lymphatic function in sites peripheral to the liver has been documented in humans and animal models of obesity, infection, and hypercholesterolemia (18–20, 67). These findings seem to link liver function and lymphatic function, however even recent studies utilizing single cell RNA sequencing to evaluate liver cell populations or even specifically liver endothelial cell populations have failed to identify lymphatic endothelial cells (26, 27). This is likely due to the low frequency of lymphatic endothelial cells in normal human livers and the inability to maintain LEC viability or distinguish these populations for downstream transcriptional profiling. Thus, the precise identification of and transcriptional profile of liver lymphatic vessels in steady state and during chronic liver disease had yet to be achieved. In this study we aimed to understand the lymphatic system in the non-diseased human liver and in the setting of chronic liver disease.

We have previously developed methodology to evaluate liver LECs by flow cytometry (38). In this manuscript we demonstrate a strategy that utilized both flow cytometric sorting and single-cell mRNA sequencing to directly analyze the transcriptional profile of LECs from the liver during steady state and disease. As evident from our data **(Figure 2B)** our flow cytometric sorting strategy does not result in a pure population of LECs but rather an enrichment of these cells. However we do demonstrate that transcriptionally the markers *PDPN*, *PROX1*, *VEGFR3*, *CCL21,* and *LYVE-1*, when combined together, adequately label LECs and no other cell type in the liver expresses all of these markers. We were intrigued to find that the expansion of the LEC population in the liver was a result of active cell cycle and cell division suggesting that these cells are actively responding to accommodate the inflammation associated with disease. This expansion of LECs was a direct consequence of the increased expression of pro-proliferative and anti-apoptotic gene expression in LECs in the setting of chronic liver disease. Furthermore, LECs in diseased livers maintained high expression of the chemokine CCL21 suggesting that lymphatic vessels and lymphatic endothelial cells in particular may play an active role in immune cell recruitment, trafficking or programing during chronic liver disease.

When comparing different disease etiologies we found significant differences in the LEC transcriptional profile and pathways between NASH and HCV. Of the pathways that were differentially regulated we identified IL13 signaling as uniquely upregulated in LECs from individuals with end-stage NASH **(Figure 5** and **Supplementary Table 2)**. This pathway was interesting as IL13 has been identified as a pro-fibrogenic factor in the liver (52) as well as a factor involved in regulating lymphatic stability (35, 50). Indeed, our data points to a role for LECs in IL13 signaling through the direct stimulation of LECs with oxLDL. Our findings that IL13 is not produced by LECs either in livers from patients with HCV or in response to IFNα or polyI:C suggests that IL13 is not generally produced during inflammation. Instead, these findings suggest that IL13 production by LECs is a result of increased cholesterol, specifically oxLDL, found in the liver of patients with NASH. From these findings it is difficult to determine if release of IL13 from LECs results in either autocrine or paracrine signaling. However, our *in vitro* findings suggest that when IL13 is present, either by adding exogenous IL13 (35) or by inducing IL13 production by oxLDL, that LECs receive a signal to reduce *PROX1* expression. Loss of *PROX1* expression likely results in the decreased ability of LECs to maintain branched structures *in vitro*. Thus, we predict that while LECs receive signals to divide in order to accommodate the increased inflammation in the liver during disease, that in NASH the vessels become unstable and perhaps more permeable. These findings are intriguing as ascites is associated with chronic liver disease and may be due to increased permeability of the liver lymphatics (13). This is an important consideration when evaluating treatment options for patients with NASH compared to patients with HCV especially as NASH associated CLD is on the rise. Future studies will address whether IL13 production by LECs impacts liver specific cells such as hepatic stellate cells or is important for autocrine signaling within the LECs.

ETHICS STATEMENT

All patients provided written and informed consent and the study was approved by the institutional review boards at the University of Colorado—Anschutz.

AUTHOR CONTRIBUTIONS

BT and MB designed and executed experiments, analyzed results, and drafted the manuscript. JF performed experiments, analyzed the data, and critically reviewed the manuscript. AG, KR, RF, and JH designed experiments, analyzed single cell mRNA sequencing data and critically reviewed the manuscript. MK provided samples, designed experiments, and critically reviewed the manuscript. RS analyzed immunofluorescence data and critically reviewed the manuscript. HR provided samples and insightful discussion.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.01036/full#supplementary-material

REFERENCES

1. QuickStats: Death Rates* for Chronic Liver Disease and Cirrhosis,(dagger) by Sex and Age Group - National Vital Statistics System, United States, 2000 and 2015. *MMWR Morb Mortal Wkly Rep.* (2017) 66:1031. doi: 10.15585/mmwr.mm6638a9
2. Schuppan D, Surabattula R, Wang XY. Determinants of fibrosis progression and regression in NASH. *J Hepatol.* (2018) 68:238–50. doi: 10.1016/j.jhep.2017.11.012
3. Tachi Y, Hirai T, Miyata A, Ohara K, Iida T, Ishizu Y, et al. Progressive fibrosis significantly correlates with hepatocellular carcinoma in patients with a sustained virological response. *Hepatol Res.* (2015) 45:238–46. doi: 10.1111/hepr.12331
4. Poynard T, Moussalli J, Munteanu M, Thabut D, Lebray P, Rudler M, et al. FibroFrance, slow regression of liver fibrosis presumed by repeated biomarkers after virological cure in patients with chronic hepatitis. *J Hepatol C.* (2013) 59:675–83. doi: 10.1016/j.jhep.2013.05.015
5. Roche B, Coilly A, Duclos-Vallee JC, Samuel D. The impact of treatment of hepatitis C with DAAs on the occurrence of HCC. *Liver Int.* (2018) 38 (Suppl):139–145. doi: 10.1111/liv.13659
6. Tapper EB, Parikh ND. Mortality due to cirrhosis and liver cancer in the United States, 1999-2016: observational study. *BMJ.* (2018) 362:k2817. doi: 10.1136/bmj.k2817
7. Randolph GJ, Miller NE. Lymphatic transport of high-density lipoproteins and chylomicrons. *J Clin Invest.* (2014) 124:929–35. doi: 10.1172/JCI71610
8. Wiig H, Swartz MA. Interstitial fluid and lymph formation and transport: physiological regulation and roles in inflammation and cancer. *Physiol Rev.* (2012) 92:1005–60. doi: 10.1152/physrev.00037.2011

9. Dixon JB, Raghunathan S, Swartz MA. A tissue-engineered model of the intestinal lacteal for evaluating lipid transport by lymphatics. *Biotechnol Bioeng.* (2009) 103:1224–35. doi: 10.1002/bit.22337
10. Lim HY, Thiam CH, Yeo KP, Bisoendial R, Hii CS, McGrath KC, et al. Lymphatic vessels are essential for the removal of cholesterol from peripheral tissues by SR-BI-mediated transport of HDCell. *Metab L.* (2013) 17:671–84. doi: 10.1016/j.cmet.2013.04.002
11. Thomas SN, Rutkowski JM, Pasquier M, Kuan EL, Alitalo K, Randolph GJ, et al. Impaired humoral immunity and tolerance in K14-VEGFR-3-Ig mice that lack dermal lymphatic drainage. *J Immunol.* (2012) 189:2181–90. doi: 10.4049/jimmunol.1103545
12. Platt AM, Rutkowski JM, Martel C, Kuan EL, Ivanov S, Swartz MA, et al. Normal dendritic cell mobilization to lymph nodes under conditions of severe lymphatic hypoplasia. *J Immunol.* (2013) 190:4608–20. doi: 10.4049/jimmunol.1202600
13. Chung C, Iwakiri Y. The lymphatic vascular system in liver diseases: its role in ascites formation. *Clin Mol Hepatol.* (2013) 19:99–104. doi: 10.3350/cmh.2013.19.2.99
14. Kuan EL, Ivanov S, Bridenbaugh EA, Victora G, Wang W, Childs EW, et al. Collecting lymphatic vessel permeability facilitates adipose tissue inflammation and distribution of antigen to lymph node-homing adipose tissue dendritic cells. *J Immunol.* (2015) 194:5200–10. doi: 10.4049/jimmunol.1500221
15. Souza-Smith FM, Siggins RW, Molina PE. Mesenteric lymphatic-perilymphatic adipose crosstalk: role in alcohol-induced perilymphatic adipose tissue inflammation. *Alcohol Clin Exp Res.* (2015) 39:1380–7. doi: 10.1111/acer.12796
16. Savetsky IL, Albano NJ, Cuzzone DA, Gardenier JC, Torrisi JS, Garcia Nores GD, et al. Lymphatic function regulates contact hypersensitivity dermatitis in obesity. *J Invest Dermatol.* (2015) 135:2742–52. doi: 10.1038/jid.2015.283
17. Fonseca DM, Hand TW, Han SJ, Gerner MY, Glatman Zaretsky A, Byrd AL, et al. Microbiota-dependent sequelae of acute infection compromise tissue-specific immunity. *Cell.* (2015) 163:354–66. doi: 10.1016/j.cell.2015.08.030
18. Greene AK, Grant FD, Slavin SA. Lower-extremity lymphedema and elevated body-mass index. *N Engl J Med.* (2012) 366:2136–7. doi: 10.1056/NEJMc1201684
19. Nitti MD, Hespe GE, Kataru RP, Garcia Nores GD, Savetsky IL, Torrisi JS, et al. Obesity-induced lymphatic dysfunction is reversible with weight loss. *J Physiol.* (2016) 594:7073–87. doi: 10.1113/JP273061
20. Garcia Nores GD, Cuzzone DA, Albano NJ, Hespe GE, Kataru RP, Torrisi JS, et al. Obesity but not high-fat diet impairs lymphatic function. *Int J Obes.* (2016) 40:1582–90. doi: 10.1038/ijo.2016.96
21. Rouhani SJ, Eccles JD, Riccardi P, Peske JD, Tewalt EF, Cohen JN, et al. Roles of lymphatic endothelial cells expressing peripheral tissue antigens in CD4 T-cell tolerance induction. *Nat Commun.* (2015) 6:6771. doi: 10.1038/ncomms7771
22. Tewalt EF, Cohen JN, Rouhani SJ, Guidi CJ, Qiao H, Fahl SP, et al. Lymphatic endothelial cells induce tolerance via PD-L1 and lack of costimulation leading to high-level PD-1 expression on CD8 T cells. *Blood.* (2012) 120:4772–82. doi: 10.1182/blood-2012-04-427013
23. Bordry N, Broggi MAS, de Jonge K, Schaeuble K, Gannon PO, Foukas PG, et al. Lymphatic vessel density is associated with CD8(+) T cell infiltration and immunosuppressive factors in human melanoma. *Oncoimmunology.* (2018) 7:e1462878. doi: 10.1080/2162402X.2018.1462878
24. Dubrot J, Duraes FV, Potin L, Capotosti F, Brighouse D, Suter T, et al. Lymph node stromal cells acquire peptide-MHCII complexes from dendritic cells and induce antigen-specific CD4(+) T cell tolerance. *J Exp Med.* (2014) 211:1153–66. doi: 10.1084/jem.20132000
25. Tamburini BA, Burchill MA, Kedl RM. Antigen capture and archiving by lymphatic endothelial cells following vaccination or viral infection. *Nat Commun.* (2014) 5:3989. doi: 10.1038/ncomms4989
26. Halpern KB, Shenhav R, Massalha H, Toth B, Egozi A, Massasa EE, et al. Paired-cell sequencing enables spatial gene expression mapping of liver endothelial cells. *Nat Biotechnol.* (2018) 36:962. doi: 10.1038/nbt.4231
27. MacParland SA, Liu JC, Ma X-Z, Innes BT, Bartczak AM, Gage BK, et al. Single cell RNA sequencing of human liver reveals distinct intrahepatic macrophage populations. *Nat Commun.* (2018) 9:4383. doi: 10.1038/s41467-018-06318-7
28. Zheng C, Zheng L, Yoo JK, Guo H, Zhang Y, Guo X, et al. Landscape of infiltrating T cells in liver cancer revealed by single-cell sequencing. *Cell.* (2017) 169:1342–56 e16. doi: 10.1016/j.cell.2017.05.035
29. Yamauchi Y, Michitaka K, Onji M. Morphometric analysis of lymphatic and blood vessels in human chronic viral liver diseases. *Am J Pathol.* (1998) 153:1131–7. doi: 10.1016/S0002-9440(10)65657-X
30. Yokomori H, Oda M, Kaneko F, Kawachi S, Tanabe M, Yoshimura K, et al. Lymphatic marker podoplanin/D2-40 in human advanced cirrhotic liver–re-evaluations of microlymphatic abnormalities. *BMC Gastroenterol.* (2010) 10:131. doi: 10.1186/1471-230X-10-131
31. Cholankeril G, Ahmed A. Alcoholic liver disease replaces hepatitis C virus infection as the leading indication for liver transplantation in the United States. *Clin Gastroenterol Hepatol.* (2018) 16:1356–8. doi: 10.1016/j.cgh.2017.11.045
32. Tu Z, Bozorgzadeh A, Pierce RH, Kurtis J, Crispe IN, Orloff MS. TLR-dependent cross talk between human Kupffer cells and NK cells. *J Exp Med.* (2008) 205:233–44. doi: 10.1084/jem.20072195
33. Satija R, Farrell JA, Gennert D, Schier AF, Regev A. Spatial reconstruction of single-cell gene expression data. *Nat Biotechnol.* (2015) 33:495–502. doi: 10.1038/nbt.3192
34. Lun AT, McCarthy DJ, Marioni JC. A step-by-step workflow for low-level analysis of single-cell RNA-seq data with Bioconductor. *F1000Res.* (2016) 5:2122. doi: 10.12688/f1000research.9501.2
35. Shin K, Kataru RP, Park HJ, Kwon BI, Kim TW, Hong YK, et al. TH2 cells and their cytokines regulate formation and function of lymphatic vessels. *Nat Commun.* (2015) 6:6196. doi: 10.1038/ncomms7196
36. Elder AM, Tamburini BAJ, Crump LS, Black SA, Wessells VM, Schedin PJ, et al. Semaphorin 7A promotes macrophage-mediated lymphatic remodeling during postpartum mammary gland involution and in breast cancer. *Cancer Res.* (2018) 78:6473–85. doi: 10.1158/0008-5472.CAN-18-1642
37. White JT, Cross EW, Burchill MA, Danhorn T, McCarter MD, Rosen HR, et al. Virtual memory T cells develop and mediate bystander protective immunity in an IL-15-dependent manner. *Nat Commun.* (2016) 7:11291. doi: 10.1038/ncomms11291
38. Finlon JM, Burchill MA, Tamburini BA. Digestion of the murine liver for a flow cytometric analysis of lymphatic endothelial cells. *J Vis Exp.* (2019) 143:e58621. doi: 10.3791/58621
39. Nelson GM, Padera TP, Garkavtsev I, Shioda T, Jain RK. Differential gene expression of primary cultured lymphatic and blood vascular endothelial cells. *Neoplasia.* (2007) 9:1038–45. doi: 10.1593/neo.07643
40. Amatschek S, Kriehuber E, Bauer W, Reininger B, Meraner P, Wolpl A, et al. Blood and lymphatic endothelial cell-specific differentiation programs are stringently controlled by the tissue environment. *Blood.* (2007) 109:4777–85. doi: 10.1182/blood-2006-10-053280
41. Russo E, Teijeira A, Vaahtomeri K, Willrodt AH, Bloch JS, Nitschke M, et al. Intralymphatic CCL21 promotes tissue egress of dendritic cells through afferent lymphatic vessels. *Cell Rep.* (2016) 14:1723–34. doi: 10.1016/j.celrep.2016.01.048
42. Tal O, Lim HY, Gurevich I, Milo I, Shipony Z, Ng LG, et al. DC mobilization from the skin requires docking to immobilized CCL21 on lymphatic endothelium and intralymphatic crawling. *J Exp Med.* (2011) 208:2141–53. doi: 10.1084/jem.20102392
43. Furuta GT, Turner JR, Taylor CT, Hershberg RM, Comerford K, Narravula S, et al. Hypoxia-inducible factor 1-dependent induction of intestinal trefoil factor protects barrier function during hypoxia. *J Exp Med.* (2001) 193:1027–34. doi: 10.1084/jem.193.9.1027
44. Guleng B, Han J, Yang JQ, Huang QW, Huang JK, Yang XN, et al. TFF3 mediated induction of VEGF via hypoxia in human gastric cancer SGC-7901 cells. *Mol Biol Rep.* (2012) 39:4127–34. doi: 10.1007/s11033-011-1195-2
45. Jin W, Jin W, Pan D. Ifi27 is indispensable for mitochondrial function and browning in adipocytes. *Biochem Biophys Res Commun.* (2018) 501:273–9. doi: 10.1016/j.bbrc.2018.04.234
46. Wang H, Qiu X, Lin S, Chen X, Wang T, Liao T. Knockdown of IFI27 inhibits cell proliferation and invasion in oral squamous cell carcinoma. *World J Surg Oncol.* (2018) 16:64. doi: 10.1186/s12957-018-1371-0

47. Heng TS, Painter MW, Immunological Genome Project Consortium. The immunological genome project: networks of gene expression in immune cells. *Nat Immunol.* (2008) 9:1091–4. doi: 10.1038/ni1008-1091
48. Rutkowski JM, Boardman KC, Swartz MA. Characterization of lymphangiogenesis in a model of adult skin regeneration. *Am J Physiol Heart Circ Physiol.* (2006) 291:H1402–10. doi: 10.1152/ajpheart.00038.2006
49. Noureddin M, Vipani A, Bresee C, Todo T, Kim IK, Alkhouri N, et al. NASH leading cause of liver transplant in women: updated analysis of indications for liver transplant and ethnic and gender variances. *Am J Gastroenterol.* (2018) 113:1649–59. doi: 10.1038/s41395-018-0088-6
50. Savetsky IL, Ghanta S, Gardenier JC, Torrisi JS, Garcia Nores GD, Hespe GE, et al. Th2 cytokines inhibit lymphangiogenesis. *PLoS ONE.* (2015) 10:e0126908. doi: 10.1371/journal.pone.0126908
51. Sugimoto R, Enjoji M, Nakamuta M, Ohta S, Kohjima M, Fukushima M, et al. Effect of IL-4 and IL-13 on collagen production in cultured LI90 human hepatic stellate cells. *Liver Int.* (2005) 25:420–8. doi: 10.1111/j.1478-3231.2005.01087.x
52. Liu Y, Munker S, Mullenbach R, Weng HL. IL-13 signaling in liver fibrogenesis. *Front Immunol.* (2012) 3:116. doi: 10.3389/fimmu.2012.00116
53. Berry A, Balard P, Coste A, Olagnier D, Lagane C, Authier H, et al. IL-13 induces expression of CD36 in human monocytes through PPARgamma activation. *Eur J Immunol.* (2007) 37:1642–52. doi: 10.1002/eji.200636625
54. Blanchard, Durual S, Estienne M, Bouzakri K, Heim MH, Blin N, et al. IL-4 and IL-13 up-regulate intestinal trefoil factor expression: requirement for STAT6 and de novo protein synthesis. *J Immunol.* (2004) 172:3775–83. doi: 10.4049/jimmunol.172.6.3775
55. Ge XN, Bastan I, Dileepan M, Greenberg Y, Ha SG, Steen KA, et al. FABP4 regulates eosinophil recruitment and activation in allergic airway inflammation. *Am J Physiol Lung Cell Mol Physiol.* (2018) 315:L227–40. doi: 10.1152/ajplung.00429.2017
56. Jadhav K, Zhang Y. Activating transcription factor 3 in immune response and metabolic regulation. *Liver Res.* (2017) 1:96–102. doi: 10.1016/j.livres.2017.08.001
57. Siddiqui MS, Fuchs M, Idowu MO, Luketic VA, Boyett S, Sargeant C, et al. Severity of nonalcoholic fatty liver disease and progression to cirrhosis are associated with atherogenic lipoprotein profile. *Clin Gastroenterol Hepatol.* (2015) 13:1000-8 e3. doi: 10.1016/j.cgh.2014.10.008
58. Puri P, Baillie RA, Wiest MM, Mirshahi F, Choudhury J, Cheung O, et al. A lipidomic analysis of nonalcoholic fatty liver disease. *Hepatology.* (2007) 46:1081–90. doi: 10.1002/hep.21763
59. van Tits LJ, Stienstra R, van Lent PL, Netea MG, Joosten LA, Stalenhoef AF. Oxidized LDL enhances pro-inflammatory responses of alternatively activated M2 macrophages: a crucial role for Kruppel-like factor 2. *Atherosclerosis.* (2011) 214:345–9. doi: 10.1016/j.atherosclerosis.2010.11.018
60. Huangfu N, Wang Y, Cheng J, Xu Z, Wang S. Metformin protects against oxidized low density lipoprotein-induced macrophage apoptosis and inhibits lipid uptake. *Exp Ther Med.* (2018) 15:2485–91. doi: 10.3892/etm.2018.5704
61. Sata M, Walsh K. Oxidized LDL activates fas-mediated endothelial cell apoptosis. *J Clin Invest.* (1998) 102:1682–9. doi: 10.1172/JCI3531
62. Ampuero J, Ranchal I, Gallego-Duran R, Pareja MJ, Del Campo JA, Pastor-Ramirez H, et al. Oxidized low-density lipoprotein antibodies/high-density lipoprotein cholesterol ratio is linked to advanced non-alcoholic fatty liver disease lean patients. *J Gastroenterol Hepatol.* (2016) 31:1611–8. doi: 10.1111/jgh.13335
63. McGettigan B, McMahan R, Orlicky D, Burchill M, Danhorn T, Francis P, et al. Dietary lipids differentially shape NASH progression and the transcriptome of kupffer cells and infiltrating macrophages. *Hepatology.* (2018). doi: 10.1002/hep.30401. [Epub ahead of print].
64. Carta G, Murru E, Banni S, Manca C. Palmitic acid: physiological role, metabolism and nutritional implications. *Front Physiol.* (2017) 8:902. doi: 10.3389/fphys.2017.00902
65. Haluszczak C, Akue AD, Hamilton SE, Johnson LD, Pujanauski L, Teodorovic L, et al. The antigen-specific CD8+ T cell repertoire in unimmunized mice includes memory phenotype cells bearing markers of homeostatic expansion. *J Exp Med.* (2009) 206:435–48. doi: 10.1084/jem.20081829
66. Oikawa H, Masuda T, Sato S, Yashima A, Suzuki K, Sato S, et al. Changes in lymph vessels and portal veins in the portal tract of patients with idiopathic portal hypertension: a morphometric study. *Hepatology.* (1998) 27:1607–10. doi: 10.1002/hep.510270621
67. Lim HY, Rutkowski JM, Helft J, Reddy ST, Swartz MA, Randolph GJ, et al. Hypercholesterolemic mice exhibit lymphatic vessel dysfunction and degeneration. *Am J Pathol.* (2009) 175:1328–37. doi: 10.2353/ajpath.2009.080963

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Lymphatic Migration of Immune Cells

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Lymphatic vessels collect interstitial fluid that has extravasated from blood vessels and return it to the circulatory system. Another important function of the lymphatic network is to facilitate immune cell migration and antigen transport from the periphery to draining lymph nodes. This migration plays a crucial role in immune surveillance, initiation of immune responses and tolerance. Here we discuss the significance and mechanisms of lymphatic migration of innate and adaptive immune cells in homeostasis, inflammation and cancer.

Keywords: lymphatic, migration, neutrophils, T cells, dendritic cells

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INTRODUCTION

The lymphatic system transports fluids from the periphery back into the circulatory system (1) using a series of open-ended capillaries known as lymphatic vessels (2). This network can be exploited by pathogens to facilitate rapid spread throughout the host (3). To prevent pathogen dissemination and enable a fast targeted immune response, the lymphatic system possesses filter-like structures termed lymph nodes (LNs) (3), where innate immune cells, such as macrophages, neutrophils and dendritic cells (DCs) trap and kill pathogens (3) and activate the adaptive immune response (4).

There are two routes by which immune cells can enter LNs: leukocytes can arrive from the bloodstream by crossing high endothelial venules (HEVs) (5). Alternatively, tissue-resident immune cells can enter afferent lymphatic vessels and migrate to draining LNs (dLNs) (5–8). Cells of the innate immune system including DCs, neutrophils, monocytes as well as adaptive immune leukocytes, such as T and B cells use lymphatic vessels to migrate from tissues into LNs (6–11). Lymphocytes exit LNs *via* efferent lymphatic vessels, and eventually return to the circulatory system by the thoracic duct (12), however, in this review we will focus on the mechanisms and consequences of immune cell migration *via* the afferent lymphatic system.

In vitro and *ex vivo* models including adhesion and transmigration assays and analysis of immune cell migration in explanted skin provided important mechanistic insight into leukocyte entry and migration within lymphatic vessels (13–17), while *in vivo* approaches allowed to examine this complex biological process *in situ* (**Table 1**). Historically, *in vivo* analysis of immune cell migration in afferent lymphatics involved direct transfer of immune cells into the skin, lymphatic cannulation, as well application of fluorescent sensitizers to the skin to label cells and induce inflammation (18). More recently photoconvertible transgenic mice have been utilized to track immune cell migration from the skin and tumors (6, 9, 19–23), while intravital imaging approaches, such as *in vivo* two-photon microscopy enabled direct visualization of immune cell migration in lymphatic vessels (6, 16, 24, 25).

TABLE 1 | Methods for investigating immune cell migration *via* afferent lymphatic vessels.

Approach	Advantages	Limitations
Adhesion and transwell assays and explanted skin preparations	• Can be used for chemical and genetic manipulation of cells of interest • Allows to investigate molecular mechanisms of lymphatic migration	• *Ex vivo* or immortalized cells may differ phenotypically and functionally from the same cells *in vivo* • May not replicate all the biological conditions, such as temperatures, pressures, and solute concentrations found *in vivo* • Tissue preparation may alter cellular functions
Direct transfer of purified and labeled donor cells into the skin	• Technically straight forward • Donor cells can be manipulated *ex vivo* • Allows to investigate molecular mechanisms and kinetics of migration	• The isolation and *ex vivo* manipulation of cells may alter cellular phenotypes • Non-physiological cell numbers are used to detect migrating cells • Transferred cells are not native to tissues
Mobilization of tissue immune cells by application of fluorescent tracers/sensitizers	• Can be used to examine migration of endogenous cells in response to inflammation	• Relies on uptake of tracer by cells of interest • Fluorescent label can be taken up by lymph node cells • Induces inflammation
Lymphatic cannulation	• Provides direct insight into the cellular content of normal afferent lymph	• Difficult to perform on small animals • Cannulation may induce inflammation • Anesthesia may alter lymphatic migration
Photolabeling of cells in photoconvertible transgenic mice using UV or violet light to monitor migration of endogenous cells *in vivo*	• Cells can be labeled *in situ* by exposure to light • No *ex vivo* manipulation required • Steady-state and inflammation-induced migration can be accurately quantified	• Difficult to perform in internal organs, requires surgery • Anesthesia may alter lymphatic cell migration • UV light may induce an inflammatory response, however, this response can be reduced if violet light is used to photoconvert
Intravital microscopy to directly visualize immune cells migrating inside lymphatic vessels	• Can be used to directly visualize immune cell migration and interactions with lymphatic vessels in their native environment • Provides information about cellular dynamics	• Requires a dedicated imaging setup • Requires fluorescent reporter mice or adoptive transfer of labeled cells • Anesthesia may alter lymphatic cell migration • Surgery to expose internal organs may cause extensive inflammation

LYMPHATIC MIGRATION OF INNATE IMMUNE CELLS

Dendritic Cells

There are two distinct DC populations: plasmacytoid, which produce high amounts of type 1 interferon, and conventional DCs (cDCs) (26). Upon sensing inflammatory stimuli, cDCs enter lymphatic vessels and migrate to LNs (26, 27). They carry antigens (8) and pathogens including viruses (28, 29), spores (30) and bacteria (31–33) from the site of infection to LNs, while DC-mediated transport of innocuous antigens regulates tolerance (34). In dLNs DCs present antigen to $CD4^+$ T cells, or cross-present to $CD8^+$ T cells, thereby regulating adaptive immune responses (26). DC migration from the periphery has been discussed extensively in several recent reviews (27, 35). Here we provide a brief overview of the mechanisms of DC migration *via* lymphatic vessels (**Figure 1**).

The most important regulator of DC migration is the chemokine receptor CCR7. Consistent with this, CCR7-deficient DCs show a 90 percent reduction in migration from the periphery in response to inflammatory stimuli (10, 36). An elegant intravital microscopy study demonstrated that CCR7 is required for the LPS-induced directed migration of DCs toward lymphatic vessels and subsequent transmigration (25). DCs also use CCR7 for trafficking to dLNs from the lamina propria (37), lung (34), and skin (23) under homeostatic conditions. Interestingly, CCR7-dependent DC migration decreases lymphatic permeability (38), indicating bi-directional communication between the lymphatic network and immune cells.

Lymphatic vessels express CCR7 ligand, CCL21 (25, 39–41), which is required for DC trafficking from skin to LNs under homeostatic (41) and inflammatory (42) conditions. Imaging studies have provided important insight into the role of CCL21 in DC migration. Firstly, the size of the CCL21 gradient, and distribution of lymphatic vessels, indicates that most skin DCs are able to sense CCL21 gradients (41). Secondly, DCs enter lymphatic vessels at sites of high CCL21 expression (25), suggesting that CCL21 directly regulates entry into lymphatics. Finally, intravital microscopy has revealed that CCL21 also enhances DC migration within lymphatic vessels (40). Collectively, these observations suggest that CCL21 regulates multiple steps in the lymphatic migration of DCs. In contrast, the other CCR7 ligand, CCL19, appears to be dispensable for DC lymphatic trafficking (42).

Inflammatory mediators regulate DC lymphatic migration (26). The cytokines Interleukin-1β (IL-1β) and Tumor Necrosis Factor-α (TNF-α) promote inflammation-induced DC migration to LNs (43–45). Furthermore, the lipid prostaglandin E2 increased CCR7 expression on DCs, augmenting migration towards CCL19 and CCL21 *in vitro* (46). Additional regulators of CCR7-mediated migration include the cell surface molecules

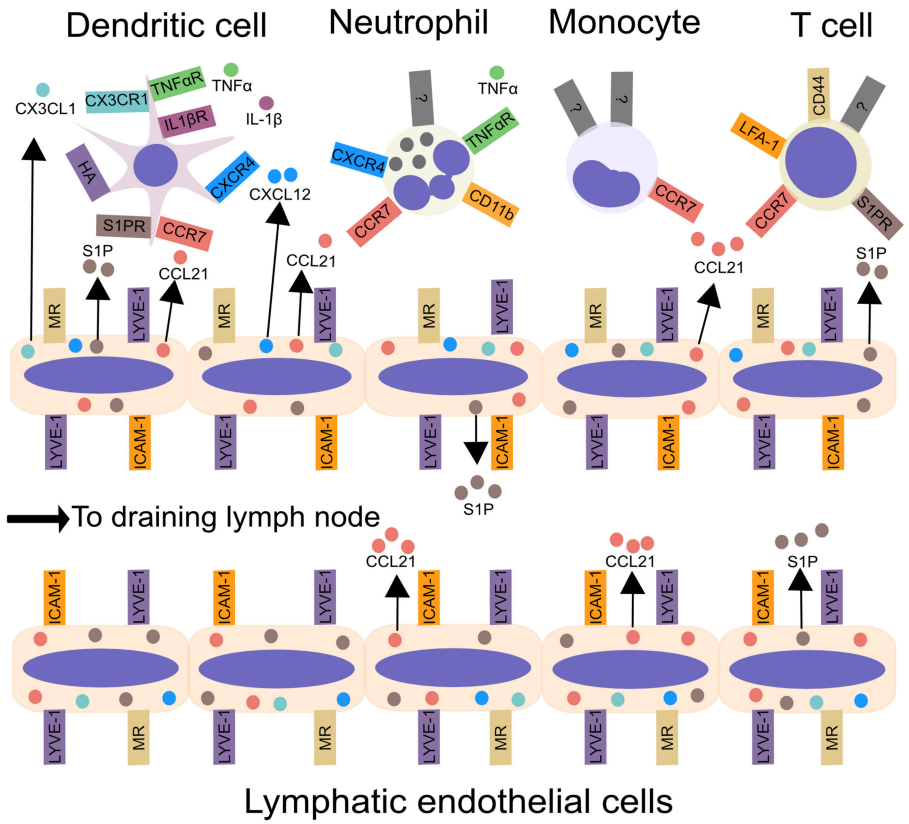

FIGURE 1 | Leukocyte migration from peripheral tissues to draining lymph nodes *via* afferent lymphatic vessels. Inflammatory cytokines, including IL-1β and TNF-α, produced by tissue-resident myeloid cells, enhance DC and neutrophil migration from tissues to lymphatic vessels. Chemokines, such as CCL21, CX3CL1, and CXCL12, synthesized by LECs in the skin control leukocyte migration to lymphatic vessels and aid transmigration into the vessel lumen. In addition to chemokines, lymphatic endothelial cells produce the lipid S1P, which acts upon S1P receptors, to promote the migration of DCs and T cells into lymphatic vessels and aid trafficking to the draining lymph node. Integrins, such as ICAM-1, CD11b, and LFA-1 may promote leukocyte entry into lymphatic vessels and subsequent migration within lymphatics. Interactions between CD44 and MR promote T cell entry into lymphatic vessels. Lastly, LYVE-1 can bind to hyaluronic acid on DCs, and promote DC entry into lymphatic vessels. ICAM-1, Intercellular Adhesion Molecule 1; LFA-1, lymphocyte function-associated antigen 1; LYVE-1, Lymphatic vessel endothelial hyaluronan receptor 1; MR, macrophage mannose receptor; S1P, sphingosine-1-phosphate.

CD37, CD38, and CD47 which enhance DC movement toward CCR7 ligands and migration to dLNs (15, 47–50). In contrast, immunosuppressive molecules including IL-10 (51), TGF-β (52, 53) and the anti-inflammatory lipid Resolvin E1 (54) can inhibit DC trafficking.

In addition to CCR7, a number of other chemokine receptor/ligand pairs have been implicated in lymphatic DC migration. CXCR4/CXCL12 and CX3CR1/CX3CL1 enhance DC trafficking from inflamed skin (55, 56). The role of CCR8 is less clear with CCR8-deficient mice displaying reduced lymphatic migration of DCs following an injection of latex beads (57), but enhanced migration of DCs following FITC painting (58), suggesting that CCR8 plays a limited, or stimulus-specific, role in this process.

Intravital imaging and FITC-painting experiments have demonstrated that integrins, and integrin signaling are required for inflammation-induced DC migration to dLNs (14, 59, 60). However, DCs from mice lacking all integrins were able to migrate to LNs when injected into resting skin (61), indicating that integrins are important for DC migration in response to inflammation but dispensable for steady state DC egress. Accordingly, inflammation increases the expression of integrin ligands on lymphatic endothelial cells (LECs) (14). DC-expressed L1 cell adhesion molecule guides transendothelial migration of DCs thereby promoting trafficking to dLNs (17). A recent study demonstrated that interactions between LEC-expressed LYVE-1 and hyaluronan on the DC plasma membrane mediated DC adhesion and transmigration across LECs and subsequent migration to dLNs (13).

Sphingosine-1-phosphate (S1P), a lipid mediator of leukocyte egress from lymphoid organs (62), has been implicated in DC trafficking from the skin and lung (33, 63–65). However, in mice that lack S1P in lymphatic fluid, but not blood, the migration of adoptively transferred DCs to dLNs was comparable to that seen in wild-type mice (66). These results, and the fact that there are five S1P receptors (63), suggest that further experiments are required to uncover the precise role of S1P signaling in DC migration.

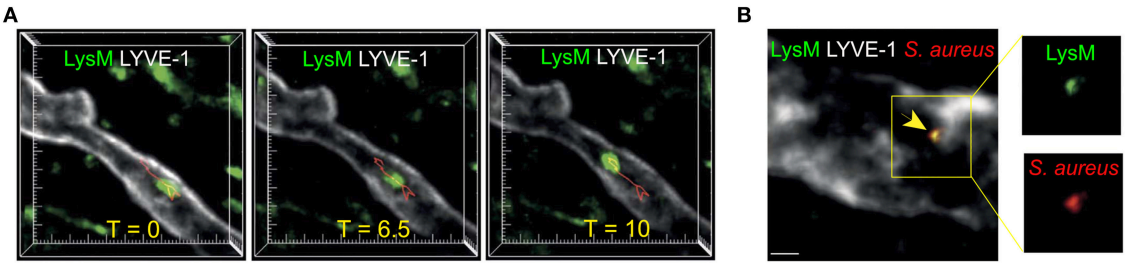

FIGURE 2 | Neutrophil migration in skin lymphatic vessels. **(A)** Two-photon microscopy was used to examine the lymphatic migration of neutrophils in response to *S. aureus*. Images are maximum intensity projections of three-dimensional volumes acquired *via* two-photon microscopy. Lysozyme M^+ GFP neutrophil (green) migrating inside a lymphatic vessel (LYVE-1, white) is show at three representative time points. Red track indicates neutrophil's path. Tick marks are 10 μm apart. **(B)** Two-photon image of a Lysozyme M reporter mouse skin with a Lysozyme M^+ (green) neutrophil containing *S. aureus* (red) inside the LYVE-1^+ lymphatic vessel (white). Scale bar is 10 μm. Figure was adapted from Hampton et al. (6).

In contrast to cDCs, the lymphatic migration of pDCs is poorly understood. While one study reported that adoptively transferred pDCs migrated to dLNs from ovine skin (67), another showed that pDCs were not detected in the lymph of rats (68). However, pDCs transported harmless inhaled antigen from murine lungs to the mediastinal LN where they suppressed T cell activation, suggesting that pDC migration may play a role in preventing inflammation (69).

Neutrophils

Neutrophils are the first immune cells recruited to sites of inflammation, where they kill pathogens and release mediators that recruit other leukocytes (70, 71). Until recently neutrophils were thought to die at inflammatory foci. However, several groups, including ours, have shown that neutrophils can enter tissue lymphatic vessels and migrate to dLNs from the site of inflammation (6, 72–74).

Intravital imaging of inflamed mouse skin has enabled direct visualization of neutrophil migration within lymphatic vasculature (**Figure 2A**) (6, 72). However, in comparison to DCs, the significance and extent of neutrophil lymphatic migration are incompletely understood. Cannulation experiments have demonstrated that inflammation leads to a dramatic increase in neutrophils in ovine afferent lymph (8, 75, 76). Furthermore, neutrophils can transport antigens and microorganisms (**Figure 2B**) from the site of infection to LNs (6, 8, 77). Accordingly, inhibiting neutrophil lymphatic migration reduced early lymphocyte proliferation (6). Notably, a recent study did not detect substantial lymphatic migration of neutrophils in response to *Staphylococcus aureus* (*S. aureus*) (78). This likely highlights the fact that most neutrophils arrive in dLNs from the circulation *via* HEVs in response to bacteria already in dLNs, while a smaller population of neutrophils migrates directly from the site of inflammation to dLNs *via* afferent lymphatics. However, since neutrophils are the first innate immune cell subset to arrive in the LN from inflamed tissues, and often carry microbes, neutrophil lymphatic migration can exert considerable influence on the subsequent adaptive immune response (6, 77, 79).

Lymphatic migration of neutrophils could potentially be exploited by pathogens to enhance dissemination, since some microorganisms including the bacterium *S. aureus* can survive inside neutrophils (80). Consistent with this, injection of *Leishmania major*-containing neutrophils was sufficient to establish infection in mice, while depleting neutrophils reduced *Leishmania* burden when the pathogen was injected into the skin (81). Neutrophils also transported live *Mycobacterium bovis bacille* Calmette-Guérin from the skin to dLNs (77). In *Toxoplasma gondii* infection neutrophils removed the macrophages that line the subscapular sinus of the LN (82), however, it is not clear whether this favors pathogen control or spread.

CCR7 appears to be less important for neutrophil lymphatic migration than for that of DCs. Although it was required for neutrophil entry into lymphatic vessels in response to TNF-α and Complete Freund's Adjuvant (CFA) (72), and for CFA-driven migration from skin to LNs (83), neutrophil migration from the skin to dLNs in response to *S. aureus* was CCR7-independent (6). This suggests that the requirements for neutrophil trafficking vary depending on the stimulus and additional molecules may play key roles in guiding this migration.

The chemokine receptor CXCR4 regulates neutrophil migration from the bone marrow into the circulation (84) and may also play a role in their lymphatic migration. Inhibiting CXCR4 decreased neutrophil trafficking in response to immune complexes and *S. aureus* (6, 73). However, CXCR4 was not required for neutrophil entry into lymphatic vessels in response to CFA, as revealed by confocal imaging (72), again highlighting differences in neutrophil trafficking in response to distinct stimuli.

The cell surface receptor CD11b, which is involved in neutrophil recruitment from the vasculature into tissues (85), is emerging as a major regulator of neutrophil migration *via* lymphatics since neutrophil migration from inflammatory foci to LNs is substantially reduced when CD11b is inhibited (6, 73, 74). Intravital imaging demonstrated that blocking CD11b or its ligand ICAM-1 impaired neutrophil intraluminal crawling within lymphatic vasculature following CFA injection by reducing neutrophil speed and directionality (72). Likewise,

inhibiting CD11b and ICAM-1 reduced neutrophil entry into lymphatic vessels and diminished egress from the skin in response to *Mycobacterium bovis* (74). Lymphocyte Function-associated Antigen (LFA-1), which binds to ICAMs and is involved in neutrophil entry into tissues from the circulation, was required for neutrophil migration *via* afferent lymphatics in response to immune complexes (73) but not *S. aureus* (6).

Inflammatory cytokines may enhance neutrophil entry into lymphatics. TNF-α promoted neutrophil entry and crawling within lymphatic vessels in mouse cremaster muscle (72). However, since inflammatory cytokines also control neutrophil recruitment to sites of inflammation and lifespan, identifying a distinct role for these molecules in neutrophil lymphatic migration requires further investigation.

Monocytes and Macrophages

Monocytes are circulating leukocytes that phagocytose and kill bacteria and fungi and regulate the activity of other immune cells *via* cytokine release (86–88). They can also differentiate into DC and macrophage subsets (89). Several studies have demonstrated that monocytes egress tissues *via* afferent lymphatic vessels and transport antigen to dLNs (7, 8, 90–92). Once there, monocytes may present and cross-present antigens since a subcutaneous injection of antigen-pulsed monocytes induced the proliferation of antigen-specific $CD4^+$ and $CD8^+$ T cells (91). The molecular mechanisms controlling monocyte migration *via* lymphatic vessels are yet to be identified. However, CCR7 may be important, since CCR7-deficient LPS-primed monocytes failed to migrate from the footpad to the popliteal LN (91). Accumulating evidence suggests that macrophages can also migrate from inflammatory lesions to dLNs (93–95) and that α1β1 integrin may limit macrophage egress *via* afferent lymphatics (93).

LYMPHATIC MIGRATION OF ADAPTIVE IMMUNE CELLS

T Cells

T cells possess a rearranged T cell receptor which includes either αβ or γδ polypeptides (96). While αβ T cells are more abundant, γδ T cells are enriched in epithelial and mucosal tissues where they act as the first line of defense against pathogens. One of the main functions of $CD8^+$ T cells is to kill infected cells (97), while $CD4^+$ helper T cells secrete cytokines and regulate the function of other immune subsets (98). Photoconversion experiments (22), along with lymphatic cannulation (99), have demonstrated that effector, rather than naïve, T cells comprise the majority of lymph migrating T cells under homeostatic (22, 99) and inflammatory conditions (22, 100). This migration plays an important role in immune surveillance and in resolution of inflammation (18, 101–103).

Like DCs, T cells use CCR7 to migrate to LNs under homeostatic and inflammatory conditions. Following antigen challenge, T cells overexpressing CCR7 were preferentially lost from the lung and accumulated in the mediastinal LNs (104), while CCR7-deficient T cells failed to migrate from the footpad to the popliteal LN (11). However, the need for CCR7 in T cell migration might be context dependent, as T cells used CCR7 for trafficking from acute, but not chronically, inflamed skin (100). Tumor-infiltrating T cells could also emigrate to dLNs independently of CCR7 (9).

The lipid S1P, which promotes αβ T cell exit from LNs (62), can also mediate their migration *via* afferent lymphatics. Consequently, antagonizing S1P receptors led to T cell accumulation near skin lymphatic vessels and reduced migration to dLNs (100, 105). LEC-expressed macrophage mannose receptor (MR) and the cell surface molecule CD44, which interacts with MR, promoted T cell lymphatic migration by increasing T cell adhesion to lymphatic vessels (106, 107). Another LEC-expressed protein, CLEVER-1, was also demonstrated to be important for T cell migration *via* afferent lymphatics (108). Additionally, intravital imaging has shown that LFA-1 and its ligand ICAM-1, increased T cell velocity within afferent lymphatic vessels thereby promoting T cell migration to dLNs (16).

T cell egress from tissues to dLNs can either promote inflammatory responses or suppress them. For example, lymphatic migration of Regulatory T (Treg) cells may suppress allograft rejection (103). Likewise, CCR7-deficient Treg cells failed to migrate to the draining LN and accumulated in the skin, reducing skin inflammation during a delayed-type hypersensitivity reaction (101). Conversely, overexpression of CCR7 on antigen-specific Th1-cells enhanced their egress to dLNs and led to faster resolution of the inflammatory response in the skin (102).

Like αβ T cells, γδ T cells can migrate from the skin (23, 95, 109) and tumors (9) *via* lymphatic vessels to dLNs and comprise a large proportion of the cells in bovine lymph (110). Photoconversion experiments have demonstrated that murine γδ T cells can migrate from the skin to dLNs in the absence of CCR7 (23). Similarly, bovine γδ T cells can egress tissues independently of this receptor (111). The consequences of γδ T cell lymphatic migration are poorly understood, though it may enhance $CD8^+$ T cell proliferation (23).

B CELLS

Cannulation experiments in sheep (112) and photoconversion experiments in mice (95), suggest that B cells use afferent lymphatic vessels to migrate from tissues to dLNs. The mechanisms of this migration are not yet known, however, at least in chronic inflammation, B cell egress may be independent of S1P and requires CCR7 (100). Similarly to T cells, blocking CLEVER-1 reduced B cell migration to dLNs (108).

LYMPHATIC MIGRATION OF IMMUNE CELLS IN DISEASE

The importance of immune cell migration *via* lymphatics in host defense is illustrated by the observations that mice lacking CCR7 are susceptible to microbial and viral infections (113–115). On the other hand, lymphatic migration of immune cells may also augment autoimmunity since preventing immune cell trafficking from the meninges to the cervical LNs reduced the

severity of EAE (116). Furthermore, higher densities of lymphatic vessels in transplanted corneas (117) and kidneys (118) were associated with rejection, while preventing DC migration to the dLN by blocking Vascular Endothelial Growth Factor C (VEGF-C) improved corneal transplantation outcomes (119). Interestingly, in mice, obesity was associated with decreased lymphatic function and reduced immune cell migration to dLNs (120), suggesting that obesity may be linked to decreased immunity. CCR7 as well as its two ligands, CCL19 and CCL21, have been identified in mouse and human atherosclerotic lesions (121), consistent with accumulating evidence of a role for immune cell lymphatic migration in heart disease (122–125).

Lymphatic vasculature also plays a crucial role in tumor immunity by enabling transport of antigens from tumors to dLNs and egress of immune cells (9, 126). Lymphatic vessels can also serve as conduits for tumor cell spread (1). Their dual role in cancer is highlighted by the findings that many tumors overexpress VEGF-C, which promotes the growth and survival of LECs (127, 128) leading to increased LN metastasis (128–130). Furthermore, a recent study demonstrated that IFNγ-induced PD-L1 expression by LECs may dampen anti-tumor immunity by limiting cytotoxic $CD8^+$ T cell accumulation in melanoma (131). On the other hand, overexpression of VEGF-C in a mouse melanoma model increased DC migration to dLNs (132). Consistent with an increase in lymphatic migration in cancer, enhanced trafficking of adoptively transferred B and T lymphocytes from footpads to dLNs was observed in melanoma-bearing mice (133).

Similarly to DC migration from the periphery, the DC-dependent transfer of antigen from B16F10 melanoma to the dLN required CCR7. This correlated with an increase in $CD8^+$ T cell priming and a reduction in tumor growth (126). Overexpression of TGF-β1 in a model of squamous cell carcinoma reduced DC trafficking to dLNs, which led to an increase in LN metastasis (53). However, since CCR7 and the TGF-β1 receptor are not restricted to DCs (134), other immune cells may also contribute to these effects on tumor growth and metastasis. In contrast to DCs, photoconversion experiments demonstrated that tumor-infiltrating T cells do not require CCR7 to migrate to dLNs *via* afferent lymphatics (9).

CONCLUDING REMARKS

Lymphatic migration of immune cells presents opportunities for control of immune responses in infection and homeostasis. However, with the exception of DCs and T cells, the mechanisms controlling lymphatic migration of immune cells remain poorly understood. New tools, such as photoconversion and intravital imaging are poised to provide novel insight into the migration of previously overlooked immune subsets. A better understanding of the distinct mechanisms guiding lymphatic migration of specific immune subsets may suggest new approaches for treatment of cancer, autoimmunity and excessive inflammation.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REFERENCES

1. Alitalo K. The lymphatic vasculature in disease. *Nat Med*. (2011) 17:1371–80. doi: 10.1038/nm.2545
2. Cueni LN, Detmar M. New insights into the molecular control of the lymphatic vascular system and its role in disease. *J Invest Dermatol*. (2006) 126:2167–77. doi: 10.1038/sj.jid.5700464
3. Kastenmüller W, Torabi-Parizi P, Subramanian N, Lämmermann T, Germain RN. A spatially-organized multicellular innate immune response in lymph nodes limits systemic pathogen spread. *Cell*. (2012) 150:1235–48. doi: 10.1016/j.cell.2012.07.021
4. Von Andrian UH, Mempel TR. Homing and cellular traffic in lymph nodes. *Nat Rev Immunol*. (2003) 3:867–78. doi: 10.1038/nri1222
5. Miyasaka M, Tanaka T. Lymphocyte trafficking across high endothelial venules: dogmas and enigmas. *Nat Rev Immunol*. (2004) 4:360–70. doi: 10.1038/nri1354
6. Hampton HR, Bailey J, Tomura M, Brink R, Chtanova T. Microbe-dependent lymphatic migration of neutrophils modulates lymphocyte proliferation in lymph nodes. *Nat Commun*. (2015) 6:7139. doi: 10.1038/ncomms8139
7. Bonneau M, Epardaud M, Payot F, Niborski V, Thoulouze M-I, Bernex F, et al. Migratory monocytes and granulocytes are major lymphatic carriers of *Salmonella* from tissue to draining lymph node. *J Leukoc Biol*. (2006) 79:268–76. doi: 10.1189/jlb.0605288
8. Neeland MR, Elhay MJ, Nathanielsz J, Meeusen ENT, de Veer MJ. Incorporation of CpG into a liposomal vaccine formulation increases the maturation of antigen-loaded dendritic cells and monocytes to improve local and systemic immunity. *J Immunol*. (2014) 192:3666–75. doi: 10.4049/jimmunol.1303014
9. Torcellan T, Hampton HR, Bailey J, Tomura M, Brink R, Chtanova T. *In vivo* photolabeling of tumor-infiltrating cells reveals highly regulated egress of T-cell subsets from tumors. *Proc Natl Acad Sci USA*. (2017) 114:5677–82. doi: 10.1073/pnas.1618446114
10. Förster R, Schubel A, Breitfeld D, Kremmer E, Renner-Müller I, Wolf E, et al. CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs. *Cell*. (1999) 99:23–33. doi: 10.1016/S0092-8674(00)80059-8
11. Debes GF, Arnold CN, Young AJ, Krautwald S, Lipp M, Hay JB, et al. Chemokine receptor CCR7 required for T lymphocyte exit from peripheral tissues. *Nat Immunol*. (2005) 6:889–94. doi: 10.1038/ni1238
12. Haig DM, Hopkins J, Miller HR. Local immune responses in afferent and efferent lymph. *Immunology*. (1999) 96:155–63. doi: 10.1046/j.1365-2567.1999.00681.x
13. Johnson LA, Banerji S, Lawrance W, Gileadi U, Prota G, Holder KA, et al. Dendritic cells enter lymph vessels by hyaluronan-mediated docking to the endothelial receptor LYVE-1. *Nat Immunol*. (2017) 18:762–70. doi: 10.1038/ni.3750

14. Johnson LA, Clasper S, Holt AP, Lalor PF, Baban D, Jackson DG. An inflammation-induced mechanism for leukocyte transmigration across lymphatic vessel endothelium. *J Exp Med*. (2006) 203:2763–77. doi: 10.1084/jem.20051759
15. Van VQ, Lesage S, Bouguermouh S, Gautier P, Rubio M, Levesque M, et al. Expression of the self-marker CD47 on dendritic cells governs their trafficking to secondary lymphoid organs. *EMBO J*. (2006) 25:5560–8. doi: 10.1038/sj.emboj.7601415
16. Teijeira A, Hunter MC, Russo E, Proulx ST, Frei T, Debes GF, et al. T cell migration from inflamed skin to draining lymph nodes requires intralymphatic crawling supported by ICAM-1/LFA-1 interactions. *Cell Rep*. (2017) 18:857–65. doi: 10.1016/j.celrep.2016.12.078
17. Maddaluno L, Verbrugge SE, Martinoli C, Matteoli G, Chiavelli A, Zeng Y, et al. The adhesion molecule L1 regulates transendothelial migration and trafficking of dendritic cells. *J Exp Med*. (2009) 206:623–35. doi: 10.1084/jem.20081211
18. Hunter MC, Teijeira A, Halin C. T cell trafficking through lymphatic vessels. *Front Immunol*. (2016) 7:613. doi: 10.3389/fimmu.2016.00613
19. Bromley SK, Yan S, Tomura M, Kanagawa O, Luster AD. Recirculating memory T cells are a unique subset of CD4+ T cells with a distinct phenotype and migratory pattern. *J Immunol*. (2013) 190:970–6. doi: 10.4049/jimmunol.1202805
20. Tomura M, Yoshida N, Tanaka J, Karasawa S, Miwa Y, Miyawaki A, et al. Monitoring cellular movement *in vivo* with photoconvertible fluorescence protein "Kaede" transgenic mice. *Proc Natl Acad Sci USA*. (2008) 105:10871–6. doi: 10.1073/pnas.0802278105
21. Tomura M, Hata A, Matsuoka S, Shand FHW, Nakanishi Y, Ikebuchi R, et al. Tracking and quantification of dendritic cell migration and antigen trafficking between the skin and lymph nodes. *Sci Rep*. (2015) 4:6030. doi: 10.1038/srep06030
22. Tomura M, Honda T, Tanizaki H, Otsuka A, Egawa G, Tokura Y, et al. Activated regulatory T cells are the major T cell type emigrating from the skin during a cutaneous immune response in mice. *J Clin Invest*. (2010) 120:883–93. doi: 10.1172/JCI40926
23. Nakamizo S, Egawa G, Tomura M, Sakai S, Tsuchiya S, Kitoh A, et al. Dermal Vγ4 + γδ T cells possess a migratory potency to the draining lymph nodes and modulate CD8 + T-cell activity through TNF-α production. *J Invest Dermatol*. (2015) 135:1007–15. doi: 10.1038/jid.2014.516
24. Roediger B, Ng LG, Smith AL, St. Groth BF, Weninger W. Visualizing dendritic cell migration within the skin. *Histochem Cell Biol*. (2008) 130:1131–46. doi: 10.1007/s00418-008-0531-7
25. Tal O, Lim HY, Gurevich I, Milo I, Shipony Z, Ng LG, et al. DC mobilization from the skin requires docking to immobilized CCL21 on lymphatic endothelium and intralymphatic crawling. *J Exp Med*. (2011) 208:2141–53. doi: 10.1084/jem.20102392
26. Alvarez D, Vollmann EH, von Andrian UH. Mechanisms and consequences of dendritic cell migration. *Immunity*. (2008) 29:325–42. doi: 10.1016/j.immuni.2008.08.006
27. Worbs T, Hammerschmidt SI, Förster R. Dendritic cell migration in health and disease. *Nat Rev Immunol*. (2017) 17:30–48. doi: 10.1038/nri.2016.116
28. Ho AWS, Prabhu N, Betts RJ, Ge MQ, Dai X, Hutchinson PE, et al. Lung CD103+ dendritic cells efficiently transport influenza virus to the lymph node and load viral antigen onto MHC class I for presentation to CD8 T cells. *J Immunol*. (2011) 187:6011–21. doi: 10.4049/jimmunol.1100987
29. Lukens MV, Kruijsen D, Coenjaerts FEJ, Kimpen JLL, Bleek GM van. Respiratory syncytial virus-induced activation and migration of respiratory dendritic cells and subsequent antigen presentation in the lung-draining lymph node. *J Virol*. (2009) 83:7235–43. doi: 10.1128/JVI.00452-09
30. Cleret A, Quesnel-Hellmann A, Vallon-Eberhard A, Verrier B, Jung S, Vidal D, et al. Lung dendritic cells rapidly mediate anthrax spore entry through the pulmonary route. *J Immunol*. (2007) 178:7994–8001. doi: 10.4049/jimmunol.178.12.7994
31. Bravo-Blas A, Utriainen L, Clay SL, Kästele V, Cerovic V, Cunningham AF, et al. *Salmonella enterica* serovar typhimurium travels to mesenteric lymph nodes both with host cells and autonomously. *J Immunol*. (2018) 202:260–7. doi: 10.4049/jimmunol.1701254
32. Voedisch S, Koenecke C, David S, Herbrand H, Förster R, Rhen M, et al. Mesenteric lymph nodes confine dendritic cell-mediated dissemination of *Salmonella enterica* serovar typhimurium and limit systemic disease in mice. *Infect Immun*. (2009) 77:3170–80. doi: 10.1128/IAI.00272-09
33. St. John AL, Ang WXG, Huang M-N, Kunder CA, Chan EW, Gunn MD, et al. S1P-Dependent Trafficking of Intracellular Yersinia pestis through Lymph Nodes Establishes Buboes and Systemic Infection. *Immunity*. (2014) 41:440–50. doi: 10.1016/j.immuni.2014.07.013
34. Hintzen G, Ohl L, del Rio M-L, Rodriguez-Barbosa J-I, Pabst O, Kocks JR, et al. Induction of tolerance to innocuous inhaled antigen relies on a CCR7-dependent dendritic cell-mediated antigen transport to the bronchial lymph node. *J Immunol*. (2006) 177:7346–54. doi: 10.4049/jimmunol.177.10.7346
35. Permanyer M, Bošnjak B, Förster R. Dendritic cells, T cells and lymphatics: dialogues in migration and beyond. *Curr Opin Immunol*. (2018) 53:173–9. doi: 10.1016/j.coi.2018.05.004
36. Clatworthy MR, Aronin CEP, Mathews RJ, Morgan NY, Smith KGC, Germain RN. Immune complexes stimulate CCR7-dependent dendritic cell migration to lymph nodes. *Nat Med*. (2014) 20:1458–63. doi: 10.1038/nm.3709
37. Jang MH, Sougawa N, Tanaka T, Hirata T, Hiroi T, Tohya K, et al. CCR7 is critically important for migration of dendritic cells in intestinal lamina propria to mesenteric lymph nodes. *J Immunol*. (2006) 176:803–10. doi: 10.4049/jimmunol.176.2.803
38. Ivanov S, Scallan JP, Kim K-W, Werth K, Johnson MW, Saunders BT, et al. CCR7 and IRF4-dependent dendritic cells regulate lymphatic collecting vessel permeability. *J Clin Invest*. (2016) 126:1581–91. doi: 10.1172/JCI84518
39. Russo E, Nitschké M, Halin C. Dendritic cell interactions with lymphatic endothelium. *Lymphat Res Biol*. (2013) 11:172–82. doi: 10.1089/lrb.2013.0008
40. Russo E, Teijeira A, Vaahtomeri K, Willrodt A-H, Bloch JS, Nitschké M, et al. Intralymphatic CCL21 promotes tissue egress of dendritic cells through afferent lymphatic vessels. *Cell Rep*. (2016) 14:1723–34. doi: 10.1016/j.celrep.2016.01.048
41. Weber M, Hauschild R, Schwarz J, Moussion C, de Vries I, Legler DF, et al. Interstitial dendritic cell guidance by haptotactic chemokine gradients. *Science*. (2013) 339:328–32. doi: 10.1126/science.1228456
42. Britschgi MR, Favre S, Luther SA. CCL21 is sufficient to mediate DC migration, maturation and function in the absence of CCL19. *Eur J Immunol*. (2010) 40:1266–71. doi: 10.1002/eji.200939921
43. Antonopoulos C, Cumberbatch M, Dearman RJ, Daniel RJ, Kimber I, Groves RW. Functional caspase-1 is required for Langerhans cell migration and optimal contact sensitization in mice. *J Immunol*. (2001) 166:3672–7. doi: 10.4049/jimmunol.166.6.3672
44. Pang IK, Ichinohe T, Iwasaki A. IL-1R signaling in dendritic cells replaces pattern-recognition receptors in promoting CD8+ T cell responses to influenza A virus. *Nat Immunol*. (2013) 14:246–53. doi: 10.1038/ni.2514
45. Wang B, Fujisawa H, Zhuang L, Kondo S, Shivji GM, Kim CS, et al. Depressed Langerhans cell migration and reduced contact hypersensitivity response in mice lacking TNF receptor p75. *J Immunol*. (1997) 159:6148–55.
46. Scandella E, Men Y, Gillessen S, Förster R, Groettrup M. Prostaglandin E2 is a key factor for CCR7 surface expression and migration of monocyte-derived dendritic cells. *Blood*. (2002) 100:1354–61. doi: 10.1182/blood-2001-11-0017
47. Partida-Sánchez S, Goodrich S, Kusser K, Oppenheimer N, Randall TD, Lund FE. Regulation of dendritic cell trafficking by the ADP-ribosyl cyclase CD38: impact on the development of humoral immunity. *Immunity*. (2004) 20:279–91. doi: 10.1016/S1074-7613(04)00048-2
48. Jones EL, Wee JL, Demaria MC, Blakeley J, Ho PK, Vega-Ramos J, et al. Dendritic cell migration and antigen presentation are coordinated by the opposing functions of the tetraspanins CD82 and CD37. *J Immunol*. (2016) 196:978–87. doi: 10.4049/jimmunol.1500357
49. Gartlan KH, Wee JL, Demaria MC, Nastovska R, Chang TM, Jones EL, et al. Tetraspanin CD37 contributes to the initiation of cellular immunity by promoting dendritic cell migration. *Eur J Immunol*. (2013) 43:1208–19. doi: 10.1002/eji.201242730
50. Hagnerud S, Manna PP, Cella M, Stenberg A, Frazier WA, Colonna M, et al. Deficit of CD47 results in a defect of marginal zone dendritic cells, blunted immune response to particulate antigen and impairment of skin dendritic cell migration. *J Immunol*. (2006) 176:5772–8. doi: 10.4049/jimmunol.176.10.5772

51. Cumberbatch M, Clelland K, Dearman RJ, Kimber I. Impact of cutaneous IL-10 on resident epidermal Langerhans' cells and the development of polarized immune responses. *J Immunol*. (2005) 175:43–50. doi: 10.4049/jimmunol.175.1.43
52. Kel JM, Girard-Madoux MJH, Reizis B, Clausen BE. TGF-β is required to maintain the pool of immature Langerhans cells in the epidermis. *J Immunol*. (2010) 185:3248–55. doi: 10.4049/jimmunol.1000981
53. Imai K, Minamiya Y, Koyota S, Ito M, Saito H, Sato Y, et al. Inhibition of dendritic cell migration by transforming growth factor-β1 increases tumor-draining lymph node metastasis. *J Exp Clin Cancer Res*. (2012) 31:3. doi: 10.1186/1756-9966-31-3
54. Sawada Y, Honda T, Hanakawa S, Nakamizo S, Murata T, Ueharaguchi-Tanada Y, et al. Resolvin E1 inhibits dendritic cell migration in the skin and attenuates contact hypersensitivity responses. *J Exp Med*. (2015) 212:1921–30. doi: 10.1084/jem.20150381
55. Kabashima K, Shiraishi N, Sugita K, Mori T, Onoue A, Kobayashi M, et al. CXCL12-CXCR4 engagement is required for migration of cutaneous dendritic cells. *Am J Pathol*. (2007) 171:1249–57. doi: 10.2353/ajpath.2007.070225
56. Johnson LA, Jackson DG. The chemokine CX3CL1 promotes trafficking of dendritic cells through inflamed lymphatics. *J Cell Sci*. (2013) 126:5259–70. doi: 10.1242/jcs.135343
57. Qu C, Edwards EW, Tacke F, Angeli V, Llodrá J, Sanchez-Schmitz G, et al. Role of CCR8 and other chemokine pathways in the migration of monocyte-derived dendritic cells to lymph nodes. *J Exp Med*. (2004) 200:1231–41. doi: 10.1084/jem.20032152
58. Yabe R, Shimizu K, Shimizu S, Azechi S, Choi B-I, Sudo K, et al. CCR8 regulates contact hypersensitivity by restricting cutaneous dendritic cell migration to the draining lymph nodes. *Int Immunol*. (2015) 27:169–81. doi: 10.1093/intimm/dxu098
59. Nitschke M, Aebischer D, Abadier M, Haener S, Lucic M, Vigl B, et al. Differential requirement for ROCK in dendritic cell migration within lymphatic capillaries in steady-state and inflammation. *Blood*. (2012) 120:2249–58. doi: 10.1182/blood-2012-03-417923
60. Xu H, Guan H, Zu G, Bullard D, Hanson J, Slater M, et al. The role of ICAM-1 molecule in the migration of Langerhans cells in the skin and regional lymph node. *Eur J Immunol*. (2001) 31:3085–93. doi: 10.1002/1521-4141(2001010)31:10<3085::AID-IMMU3085>3.0.CO;2-B
61. Lämmermann T, Bader BL, Monkley SJ, Worbs T, Wedlich-Söldner R, Hirsch K, et al. Rapid leukocyte migration by integrin-independent flowing and squeezing. *Nature*. (2008) 453:51–5. doi: 10.1038/nature06887
62. Cyster JG, Schwab SR. Sphingosine-1-phosphate and lymphocyte egress from lymphoid organs. *Annu Rev Immunol*. (2012) 30:69–94. doi: 10.1146/annurev-immunol-020711-075011
63. Czeloth N, Bernhardt G, Hofmann F, Genth H, Förster R. Sphingosine-1-phosphate mediates migration of mature dendritic cells. *J Immunol*. (2005) 175:2960–7. doi: 10.4049/jimmunol.175.5.2960
64. Idzko M, Hammad H, van Nimwegen M, Kool M, Müller T, Soullié T, et al. Local application of FTY720 to the lung abrogates experimental asthma by altering dendritic cell function. *J Clin Invest*. (2006) 116:2935–44. doi: 10.1172/JCI28295
65. Rathinasamy A, Czeloth N, Pabst O, Forster R, Bernhardt G. The origin and maturity of dendritic cells determine the pattern of sphingosine 1-phosphate receptors expressed and required for efficient migration. *J Immunol*. (2010) 185:4072–81. doi: 10.4049/jimmunol.1000568
66. Pham THM, Baluk P, Xu Y, Grigorova I, Bankovich AJ, Pappu R, et al. Lymphatic endothelial cell sphingosine kinase activity is required for lymphocyte egress and lymphatic patterning. *J Exp Med*. (2010) 207:17–27. doi: 10.1084/jem.20091619
67. Pascale F, Pascale F, Contreras V, Bonneau M, Courbet A, Chilmonczyk S, et al. Plasmacytoid dendritic cells migrate in afferent skin lymph. *J Immunol*. (2008) 180:5963–72. doi: 10.4049/jimmunol.180.9.5963
68. Yrlid U, Cerovic V, Milling S, Jenkins CD, Zhang J, Crocker PR, et al. Plasmacytoid dendritic cells do not migrate in intestinal or hepatic lymph. *J Immunol*. (2006) 177:6115–21. doi: 10.4049/jimmunol.177.9.6115
69. de Heer HJ, Hammad H, Soullié T, Hijdra D, Vos N, Willart MAM, et al. Essential role of lung plasmacytoid dendritic cells in preventing asthmatic reactions to harmless inhaled antigen. *J Exp Med*. (2004) 200:89–98. doi: 10.1084/jem.20040035
70. Amulic B, Cazalet C, Hayes GL, Metzler KD, Zychlinsky A. Neutrophil function: from mechanisms to disease. *Annu Rev Immunol*. (2012) 30:459–89. doi: 10.1146/annurev-immunol-020711-074942
71. Tecchio C, Micheletti A, Cassatella MA. Neutrophil-derived cytokines: facts beyond expression. *Front Immunol*. (2014) 5:508. doi: 10.3389/fimmu.2014.00508
72. Arokiasamy S, Zakian C, Dilliway J, Wang W, Nourshargh S, Voisin M-B. Endogenous TNFα orchestrates the trafficking of neutrophils into and within lymphatic vessels during acute inflammation. *Sci Rep*. (2017) 7:44189. doi: 10.1038/srep44189
73. Gorlino CV, Ranocchia RP, Harman MF, García IA, Crespo MI, Morón G, et al. Neutrophils exhibit differential requirements for homing molecules in their lymphatic and blood trafficking into draining lymph nodes. *J Immunol*. (2014) 193:1966–74. doi: 10.4049/jimmunol.1301791
74. Rigby DA, Ferguson DJP, Johnson LA, Jackson DG. Neutrophils rapidly transit inflamed lymphatic vessel endothelium via integrin-dependent proteolysis and lipoxin-induced junctional retraction. *J Leukoc Biol*. (2015) 98:897–912. doi: 10.1189/jlb.1HI0415-149R
75. Cahill RN, Frost H, Trnka Z. The effects of antigen on the migration of recirculating lymphocytes through single lymph nodes. *J Exp Med*. (1976) 143:870–88. doi: 10.1084/jem.143.4.870
76. Neeland MR, Shi W, Collignon C, Taubenheim N, Meeusen ENT, Didierlaurent AM, et al. The lymphatic immune response induced by the adjuvant AS01: a comparison of intramuscular and subcutaneous immunization routes. *J Immunol*. (2016) 197:2704–14. doi: 10.4049/jimmunol.1600817
77. Abadie V, Badell E, Douillard P, Ensergueix D, Leenen PJM, Tanguy M, et al. Neutrophils rapidly migrate via lymphatics after *Mycobacterium bovis* BCG intradermal vaccination and shuttle live bacilli to the draining lymph nodes. *Blood*. (2005) 106:1843–50. doi: 10.1182/blood-2005-03-1281
78. Bogoslowski A, Butcher EC, Kubes P. Neutrophils recruited through high endothelial venules of the lymph nodes via PNAd intercept disseminating *Staphylococcus aureus*. *Proc Natl Acad Sci USA*. (2018) 115:2449–54. doi: 10.1073/pnas.1715756115
79. Yang C-W, Strong BSI, Miller MJ, Unanue ER. Neutrophils influence the level of antigen presentation during the immune response to protein antigens in adjuvants. *J Immunol*. (2010) 185:2927–34. doi: 10.4049/jimmunol.1001289
80. Gresham HD, Lowrance JH, Caver TE, Wilson BS, Cheung AL, Lindberg FP. Survival of *Staphylococcus aureus* inside neutrophils contributes to infection. *J Immunol*. (2000) 164:3713–22. doi: 10.4049/jimmunol.164.7.3713
81. Peters NC, Egen JG, Secundino N, Debrabant A, Kimblin N, Kamhawi S, et al. *In vivo* imaging reveals an essential role for neutrophils in leishmaniasis transmitted by sand flies. *Science*. (2008) 321:970–4. doi: 10.1126/science.1159194
82. Chtanova T, Schaeffer M, Han S-J, van Dooren GG, Nollmann M, Herzmark P, et al. Dynamics of neutrophil migration in lymph nodes during infection. *Immunity*. (2008) 29:487–96. doi: 10.1016/j.immuni.2008.07.012
83. Beauvillain C, Cunin P, Doni A, Scotet M, Jaillon S, Loiry M-L, et al. CCR7 is involved in the migration of neutrophils to lymph nodes. *Blood*. (2011) 117:1196–204. doi: 10.1182/blood-2009-11-254490
84. Eash KJ, Greenbaum AM, Gopalan PK, Link DC. CXCR2 and CXCR4 antagonistically regulate neutrophil trafficking from murine bone marrow. *J Clin Invest*. (2010) 120:2423–31. doi: 10.1172/JCI41649
85. Dinauer MC. Primary immune deficiencies with defects in neutrophil function. *Hematology*. (2016) 2016:43–50. doi: 10.1182/asheducation-2016.1.43
86. Van de Weert–van Leeuwen PB, Van Meegen MA, Speirs JJ, Pals DJ, Rooijakkers SHM, Van der Ent CK, et al. Optimal complement-mediated phagocytosis of *Pseudomonas aeruginosa* by monocytes is cystic fibrosis transmembrane conductance regulator–dependent. *Am J Respir Cell Mol Biol*. (2013) 49:463–70. doi: 10.1165/rcmb.2012-0502OC
87. Smeekens SP, van de Veerdonk FL, Joosten LAB, Jacobs L, Jansen T, Williams DL, et al. The classical $CD14^{++}$ $CD16^{-}$ monocytes, but not the patrolling

$CD14^+$ $CD16^+$ monocytes, promote Th17 responses to Candida albicans. *Eur J Immunol*. (2011) 41:2915–24. doi: 10.1002/eji.201141418

88. Mohanty S, Joshi SR, Ueda I, Wilson J, Blevins TP, Siconolfi B, et al. Prolonged proinflammatory cytokine production in monocytes modulated by interleukin 10 after influenza vaccination in older adults. *J Infect Dis*. (2015) 211:1174–84. doi: 10.1093/infdis/jiu573
89. Ginhoux F, Jung S. Monocytes and macrophages: developmental pathways and tissue homeostasis. *Nat Rev Immunol*. (2014) 14:392–404. doi: 10.1038/nri3671
90. Calabro S, Tortoli M, Baudner BC, Pacitto A, Cortese M, O'Hagan DT, et al. Vaccine adjuvants alum and MF59 induce rapid recruitment of neutrophils and monocytes that participate in antigen transport to draining lymph nodes. *Vaccine*. (2011) 29:1812–23. doi: 10.1016/j.vaccine.2010.12.090
91. Leirião P, del Fresno C, Ardavín C. Monocytes as effector cells: activated Ly-6C(high) mouse monocytes migrate to the lymph nodes through the lymph and cross-present antigens to CD8+ T cells. *Eur J Immunol*. (2012) 42:2042–51. doi: 10.1002/eji.201142166
92. Jakubzick C, Gautier EL, Gibbings SL, Sojka DK, Schlitzer A, Johnson TE, et al. Minimal differentiation of classical monocytes as they survey steady-state tissues and transport antigen to lymph nodes. *Immunity*. (2013) 39:599–610. doi: 10.1016/j.immuni.2013.08.007
93. Becker HM, Rullo J, Chen M, Ghazarian M, Bak S, Xiao H, et al. α1β1 integrin-mediated adhesion inhibits macrophage exit from a peripheral inflammatory lesion. *J Immunol*. (2013) 190:4305–14. doi: 10.4049/jimmunol.1202097
94. Bellingan GJ, Caldwell H, Howie SE, Dransfield I, Haslett C. *In vivo* fate of the inflammatory macrophage during the resolution of inflammation: inflammatory macrophages do not die locally, but emigrate to the draining lymph nodes. *J Immunol*. (1996) 157:2577–85.
95. Hayes AJ, Rane S, Scales HE, Meehan GR, Benson RA, Maroof A, et al. Spatiotemporal modeling of the key migratory events during the initiation of adaptive immunity. *Front Immunol*. (2019) 10:598. doi: 10.3389/fimmu.2019.00598
96. Gascoigne NRJ, Rybakin V, Acuto O, Brzostek J. TCR signal strength and T cell development. *Annu Rev Cell Dev Biol*. (2016) 32:327–48. doi: 10.1146/annurev-cellbio-111315-125324
97. Wong P, Pamer EG. CD8 T cell responses to infectious pathogens. *Annu Rev Immunol*. (2003) 21:29–70. doi: 10.1146/annurev.immunol.21.120601.141114
98. Zhu J, Yamane H, Paul WE. Differentiation of effector CD4 T cell populations. *Annu Rev Immunol*. (2010) 28:445–89. doi: 10.1146/annurev-immunol-030409-101212
99. Mackay CR, Marston WL, Dudler L. Naive and memory T cells show distinct pathways of lymphocyte recirculation. *J Exp Med*. (1990) 171:801–17. doi: 10.1084/jem.171.3.801
100. Brown MN, Fintushel SR, Lee MH, Jennrich S, Geherin SA, Hay JB, et al. Chemoattractant receptors and lymphocyte egress from extralymphoid tissue: changing requirements during the course of inflammation. *J Immunol*. (2010) 185:4873–82. doi: 10.4049/jimmunol.1000676
101. Menning A, Höpken UE, Siegmund K, Lipp M, Hamann A, Huehn J. Distinctive role of CCR7 in migration and functional activity of naive- and effector/memory-like Treg subsets. *Eur J Immunol*. (2007) 37:1575–83. doi: 10.1002/eji.200737201
102. Gómez D, Diehl MC, Crosby EJ, Weinkopff T, Debes GF. Effector T cell egress via afferent lymph modulates local tissue inflammation. *J Immunol*. (2015) 195:3531–6. doi: 10.4049/jimmunol.1500626
103. Zhang N, Schröppel B, Lal G, Jakubzick C, Mao X, Chen D, et al. Regulatory T cells sequentially migrate from inflamed tissues to draining lymph nodes to suppress the alloimmune response. *Immunity*. (2009) 30:458–69. doi: 10.1016/j.immuni.2008.12.022
104. Bromley SK, Thomas SY, Luster AD. Chemokine receptor CCR7 guides T cell exit from peripheral tissues and entry into afferent lymphatics. *Nat Immunol*. (2005) 6:895–901. doi: 10.1038/ni1240
105. Ledgerwood LG, Lal G, Zhang N, Garin A, Esses SJ, Ginhoux F, et al. The sphingosine 1-phosphate receptor 1 causes tissue retention by inhibiting the entry of peripheral tissue T lymphocytes into afferent lymphatics. *Nat Immunol*. (2008) 9:42–53. doi: 10.1038/ni1534
106. Marttila-Ichihara F, Turja R, Miiluniemi M, Karikoski M, Maksimow M, Niemela J, et al. Macrophage mannose receptor on lymphatics controls cell trafficking. *Blood*. (2008) 112:64–72. doi: 10.1182/blood-2007-10-118984
107. Salmi M, Karikoski M, Elima K, Rantakari P, Jalkanen S. CD44 binds to macrophage mannose receptor on lymphatic endothelium and supports lymphocyte migration via afferent lymphatics. *Circ Res*. (2013) 112:1577–82. doi: 10.1161/CIRCRESAHA.111.300476
108. Karikoski M, Irjala H, Maksimow M, Miiluniemi M, Granfors K, Hernesniemi S, et al. Clever-1/Stabilin-1 regulates lymphocyte migration within lymphatics and leukocyte entrance to sites of inflammation. *Eur J Immunol*. (2009) 39:3477–87. doi: 10.1002/eji.200939896
109. Gray EE, Ramírez-Valle F, Xu Y, Wu S, Wu Z, Karjalainen KE, et al. Deficiency in IL-17-committed Vγ4+ γδ T cells in a spontaneous Sox13-mutant CD45.1+ congenic mouse substrain provides protection from dermatitis. *Nat Immunol*. (2013) 14:584–92. doi: 10.1038/ni.2585
110. Van Rhijn I, Rutten VPMG, Charleston B, Smits M, van Eden W, Koets AP. Massive, sustained γδ T cell migration from the bovine skin *in vivo*. *J Leukoc Biol*. (2007) 81:968–73. doi: 10.1189/jlb.0506331
111. Vrieling M, Santema W, Van Rhijn I, Rutten V, Koets A. γδ T cell homing to skin and migration to skin-draining lymph nodes is CCR7 independent. *J Immunol*. (2012) 188:578–84. doi: 10.4049/jimmunol.1101972
112. Mackay CR, Kimpton WG, Brandon MR, Cahill RN. Lymphocyte subsets show marked differences in their distribution between blood and the afferent and efferent lymph of peripheral lymph nodes. *J Exp Med*. (1988) 167:1755–65. doi: 10.1084/jem.167.6.1755
113. Noor S, Habashy AS, Nance JP, Clark RT, Nemati K, Carson MJ, et al. CCR7-dependent immunity during acute *Toxoplasma gondii* infection. *Infect Immun*. (2010) 78:2257–63. doi: 10.1128/IAI.01314-09
114. Bardina SV, Brown JA, Michlmayr D, Hoffman KW, Sum J, Pletnev AG, et al. Chemokine receptor CCR7 restricts fatal west nile virus encephalitis. *J Virol*. (2017) 91:e02409–16 doi: 10.1128/JVI.02409-16
115. Olmos S, Stukes S, Ernst JD. Ectopic activation of *Mycobacterium tuberculosis*-specific $CD4^+$ T cells in lungs of $CCR7^{-/-}$ mice. *J Immunol*. (2010) 184:895–901. doi: 10.4049/jimmunol.0901230
116. Louveau A, Herz J, Alme MN, Salvador AF, Dong MQ, Viar KE, et al. CNS lymphatic drainage and neuroinflammation are regulated by meningeal lymphatic vasculature. *Nat Neurosci*. (2018) 21:1380–91. doi: 10.1038/s41593-018-0227-9
117. Dietrich T, Bock F, Yuen D, Hos D, Bachmann BO, Zahn G, et al. Cutting edge: lymphatic vessels, not blood vessels, primarily mediate immune rejections after transplantation. *J Immunol*. (2010) 184:535–9. doi: 10.4049/jimmunol.0903180
118. Kerjaschki D, Regele HM, Moosberger I, Nagy-Bojarski K, Watschinger B, Soleiman A, et al. Lymphatic neoangiogenesis in human kidney transplants is associated with immunologically active lymphocytic infiltrates. *J Am Soc Nephrol*. (2004) 15:603–12. doi: 10.1097/01.ASN.0000113316.52371.2E
119. Chen L, Hamrah P, Cursiefen C, Zhang Q, Pytowski B, Streilein JW, et al. Vascular endothelial growth factor receptor-3 mediates induction of corneal alloimmunity. *Nat Med*. (2004) 10:813–5. doi: 10.1038/nm1078
120. Weitman ES, Aschen SZ, Farias-Eisner G, Albano N, Cuzzone DA, Ghanta S, et al. Obesity impairs lymphatic fluid transport and dendritic cell migration to lymph nodes. *PLoS ONE*. (2013) 8:e70703. doi: 10.1371/journal.pone.0070703
121. Damas JK, Smith C, Øie E, Fevang B, Halvorsen B, Wæhre T, et al. Enhanced expression of the homeostatic chemokines CCL19 and CCL21 in clinical and experimental atherosclerosis. *Arterioscler Thromb Vasc Biol*. (2007) 27:614–20. doi: 10.1161/01.ATV.0000255581.38523.7c
122. Luchtefeld M, Grothusen C, Gagalick A, Jagavelu K, Schuett H, Tietge UJF, et al. Chemokine receptor 7 knockout attenuates atherosclerotic plaque development. *Circulation*. (2010) 122:1621–8. doi: 10.1161/CIRCULATIONAHA.110.956730
123. Wan W, Lionakis MS, Liu Q, Roffê E, Murphy PM. Genetic deletion of chemokine receptor Ccr7 exacerbates atherogenesis in ApoE-deficient mice. *Cardiovasc Res*. (2013) 97:580–8. doi: 10.1093/cvr/cvs349
124. Feig JE, Shang Y, Rotllan N, Vengrenyuk Y, Wu C, Shamir R, et al. Statins promote the regression of atherosclerosis via activation of the CCR7-dependent emigration pathway in macrophages. *PLoS ONE*. (2011) 6:e28534. doi: 10.1371/journal.pone.0028534

125. Trogan E, Feig JE, Dogan S, Rothblat GH, Angeli V, Tacke F, et al. Gene expression changes in foam cells and the role of chemokine receptor CCR7 during atherosclerosis regression in ApoE-deficient mice. *Proc Natl Acad Sci USA*. (2006) 103:3781–6. doi: 10.1073/pnas.0511043103
126. Roberts EW, Broz ML, Binnewies M, Headley MB, Nelson AE, Wolf DM, et al. Critical role for $CD103^{+}/CD141^{+}$ dendritic cells bearing CCR7 for tumor antigen trafficking and priming of T cell immunity in melanoma. *Cancer Cell*. (2016) 30:324–36. doi: 10.1016/j.ccell.2016.06.003
127. Su J-L, Yen C-J, Chen P-S, Chuang S-E, Hong C-C, Kuo I-H, et al. The role of the VEGF-C/VEGFR-3 axis in cancer progression. *Br J Cancer*. (2007) 96:541–5. doi: 10.1038/sj.bjc.6603487
128. Tammela T, Alitalo K. Lymphangiogenesis: molecular mechanisms and future promise. *Cell*. (2010) 140:460–76. doi: 10.1016/j.cell.2010.01.045
129. Skobe M, Hawighorst T, Jackson DG, Prevo R, Janes L, Velasco P, et al. Induction of tumor lymphangiogenesis by VEGF-C promotes breast cancer metastasis. *Nat Med*. (2001) 7:192–8. doi: 10.1038/84643
130. Yanai Y, Furuhata T, Kimura Y, Yamaguchi K, Yasoshima T, Mitaka T, et al. Vascular endothelial growth factor C promotes human gastric carcinoma lymph node metastasis in mice. *J Exp Clin Cancer Res*. (2001) 20:419–28.
131. Lane RS, Femel J, Breazeale AP, Loo CP, Thibault G, Kaempf A, et al. IFNγ-activated dermal lymphatic vessels inhibit cytotoxic T cells in melanoma and inflamed skin. *J Exp Med*. (2018) 215:3057–74. doi: 10.1084/jem.20180654
132. Lund AW, Duraes FV, Hirosue S, Raghavan VR, Nembrini C, Thomas SN, et al. VEGF-C promotes immune tolerance in B16 melanomas and cross-presentation of tumor antigen by lymph node lymphatics. *Cell Rep*. (2012) 1:191–9. doi: 10.1016/j.celrep.2012.01.005
133. Habenicht LM, Albershardt TC, Iritani BM, Ruddell A. Distinct mechanisms of B and T lymphocyte accumulation generate tumor-draining lymph node hypertrophy. *Oncoimmunology*. (2016) 5:e1204505. doi: 10.1080/2162402X.2016.1204505
134. Worthington JJ, Fenton TM, Czajkowska BI, Klementowicz JE, Travis MA. Regulation of TGFβ in the immune system: an emerging role for integrins and dendritic cells. *Immunobiology*. (2012) 217:1259–65. doi: 10.1016/j.imbio.2012.06.009

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PD-1 Blockade During Post-partum Involution Reactivates the Anti-tumor Response and Reduces Lymphatic Vessel Density

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Post-partum breast cancer patients, or breast cancer patients diagnosed within 10 years of last childbirth, are ∼3–5 times more likely to develop metastasis in comparison to non-post-partum, or nulliparous, patients. Additionally, post-partum patients have increased tumor-associated lymphatic vessels and LN involvement, including when controlled for size of the primary tumor. In pre-clinical, *immune-competent*, mouse mammary tumor models of post-partum breast cancer (PPBC), tumor growth and lymphogenous tumor cell spread occur more rapidly in post-partum hosts. Here we report on PD-L1 expression by lymphatic endothelial cells and CD11b+ cells in the microenvironment of post-partum tumors, which is accompanied by an increase in PD-1 expression by T cells. Additionally, we observed increases in PD-L1 and PD-1 in whole mammary tissues during post-partum mammary gland involution; a known driver of post-partum tumor growth, invasion, and metastasis in pre-clinical models. Importantly, implantation of murine mammary tumor cells during post-partum mammary gland involution elicits a CD8+ T cell population that expresses both the co-inhibitory receptors PD-1 and Lag-3. However, upon anti-PD-1 treatment, during post-partum mammary gland involution, the involution-initiated promotional effects on tumor growth are reversed and the PD-1, Lag-3 double positive population disappears. Consequently, we observed an expansion of poly-functional CD8+ T cells that produced both IFNγ and TNFα. Finally, lymphatic vessel frequency decreased significantly following anti-PD-1 suggesting that anti-PD-1/PD-L1 targeted therapies may have efficacy in reducing tumor growth and dissemination in post-partum breast cancer patients.

Keywords: lymphatic endothelial cells, post-partum breast cancer, metastasis, immunotherapy, PD-L1, PD-1, T cells

INTRODUCTION

Post-partum breast cancer patients in our cohort, or breast cancer patients diagnosed within 10 years of last childbirth, are ∼3–5 times more likely to develop metastasis in comparison to non-post-partum, or nulliparous, patients (1, 2). Additionally, post-partum patients have increased LN involvement and peri-tumor lymphatic vessel density (LVD) (2–4). In 2010, Asztalos et al. identified alterations to gene expression patterns in normal mammary tissues from post-pregnant women that persist up to 10 years post-partum in comparison to nulliparous women (5). Specifically, they observed changes to genes involved in inflammation, angiogenesis, extracellular matrix (ECM), and breast cancer; suggesting that the mammary microenvironment after pregnancy may be conducive to malignancy. Consistent with this hypothesis, recent pregnancy can increase a woman's risk for developing breast cancer for more than 20 years following childbirth (6–8). Normal mammary gland development associated with pregnancy consists of a period of expansion of the mammary epithelium, to prepare the gland for lactation, followed by full differentiation of the mammary epithelium into milk secreting cells. Following lactation, or pregnancy in the absence of lactation, post-partum mammary gland involution occurs to return the mammary epithelium to the pre-pregnant state. Previous studies of normal post-partum mammary gland involution in rodents and women have revealed that attributes of this normal developmental process are similar to those observed in breast tumors (9–12). These attributes include establishment of a tissue microenvironment that is characterized by ECM remodeling, increased LVD, immune infiltration and evidence of immune suppression (3, 13–15). Additionally, non-metastatic tumor cells implanted into this tissue microenvironment in pre-clinical models grow and invade more rapidly, seed micro-metastases, and are durably altered to a more invasive and metastatic state; suggesting that post-partum involution can drive intrinsic, pro-metastatic changes, in tumor cells (3, 16). These findings predict that the process of normal involution may drive post-partum breast cancer (PPBC) metastasis.

Post-partum mammary gland involution, induced by weaning, has been extensively studied in rodents where it is characterized by two phases of tissue remodeling. The first, known as the reversible phase, is triggered by milk stasis and results in death of the secretory mammary epithelium (17, 18). The second phase, known as the irreversible phase, consists of stromal remodeling and repopulation of the gland with adipocytes. Insight into molecular programs that govern this developmental process in mice has been gained through gene expression profiling studies on whole mammary tissues where roles for death receptors and immune mediators were revealed (10, 11, 19). Additionally, influx of immunosuppressive Foxp3+ regulatory T cells and IL-10+ macrophages occurs during involution resulting in effector T cell suppression (13). Furthermore, M2-like or tissue repair type macrophages and macrophages with pro-lymphatic phenotypes are evident (3, 20, 21). As epithelial cell apoptosis during involution likely results in the increased presentation of self-antigens (22) it is not surprising that numerous cell types initiate an immune-tolerant microenvironment and, consequently, an environment that could be primed for post-partum tumor growth. Importantly, LVD also increases during mammary gland involution, presumably to promote the clearance of increased fluid—generated by milk stasis, apoptotic cell debris, and immune cell infiltrates. However, we have recently shown that the mammary lymphatics that arise during post-partum involution are also capable of transporting tumor cells to distant lymph nodes during the active phase of tissue remodeling and that post-partum patients are enriched for lymph node involvement (2, 21). These studies suggest that metastatic seeding via lymphatics may be an early event in post-partum patients, which may account for the increased metastasis observed (21).

Tumor-associated lymphatic vessels not only promote dissemination (23–27), but have recently been shown to reduce anti-tumor immune responses in tumor models (28, 29). Lymphatic endothelial cells (LECs) normally promote peripheral immune tolerance in the lymph node during homeostasis. Specifically, lymph node LECs express PD-L1 to inhibit auto-reactive T cells via engagement of the inhibitory receptor Programmed Death-1 (PD-1) (30–34). In addition, inhibitory receptor expression of PD-1 is also an important marker of T cell effector function. However, upregulation of multiple inhibitory receptors, such as PD-1, Lag-3, and TIGIT can occur as a result of chronic antigen stimulation and lead to non-responsive T cells that fail to successfully clear the pathogen (35–40). In the cancer setting, a similar phenomenon occurs as tumor-infiltrating T cells upregulate multiple co-inhibitory receptors and are limited in their ability to produce multiple effector cytokines (such as IFNγ and TNFα) (41), making them less polyfunctional and unable to clear the tumor (42). Recently, several studies have pointed to a role for tumor-associated lymphatic and/or macrophage expression of PD-L1 in contributing to T cell inhibition (28, 29, 43, 44). In addition, we and others have published that PD-L1 expression by LECs promotes their survival during an immune response and a role for PD-L1 expression in promoting tumor cell survival has been demonstrated (45–48). Thus, PD-L1 clearly plays a role in promoting cell survival and immunosuppression in multiple cell types present in the tumor microenvironment (TME).

Since T-cell infiltration, immune suppression, macrophage infiltration, and lymphangiogenesis have all been described during post-partum involution (3, 13, 21), we sought to determine whether T cell expression of co-inhibitory receptors allows for immune evasion by tumor cells during post-partum involution and whether this mechanism could be reversed with anti-PD-1 treatment. In this manuscript, we investigate the immune regulatory state of the mammary tissue during post-partum involution and in tumors implanted during involution (post-partum tumors). We demonstrate that PD-L1 and PD-1

Abbreviations: LEC, Lymphatic endothelial cell; PDPN, Podoplanin; PD-1, Programmed Death-1; PD-L1, Programmed Death-Ligand 1; Lag-3, Lymphocyte-activation gene-3; LN, Lymph node; LVD, Lymphatic Vessel Density; TME, Tumor Micro Environment.

are a part of the involution program and show increased expression of PD-L1 on lymphatic endothelial cells, and cells of myeloid lineage, as well as PD-1 on T cells during mammary gland involution and in post-partum tumors. Importantly, we demonstrate that this mechanism is an integral part of the tumor promotional program effects of involution. Administration of an anti-PD-1 antibody to mice during involution, when the tumors are established, reduced growth of the post-partum tumors to levels observed in nulliparous hosts. Upon evaluation of the tumor infiltrating CD8+ T cells we discovered co-expression of two inhibitory receptors, PD-1 and Lag-3, that were specific to tumors established during involution. Following treatment with anti-PD-1, the PD-1+Lag-3+ inhibitory CD8+ T cell population disappeared and the frequency of total CD8+ T cells and polyfunctional CD8+ T cells increased significantly suggesting a reversal of at least some of the immunosuppressive effects of involution. Surprisingly, anti-PD-1 treatment also reduced tumor/involution associated LVD suggesting this treatment may have implications for stopping lymphatic-mediated metastasis. Our results lay the ground work for additional studies aimed at uncovering the potential role of the mammary LECs during involution, and in the tumor microenvironment, in promoting immunosuppression and suggest a potential treatment option for PPBC patients.

MATERIALS AND METHODS

Animal Studies

All animal procedures were approved by the University of Colorado Anschutz Medical Campus Institutional Animal Care and Use Committee. BALB/c and C57Bl/6 (age 6–8 weeks) were obtained from Charles River Laboratories. Mice were crossbred for involution studies; C57Bl/6 female mice were bred in triad with BALB/c males and BALB/c females were bred with C57Bl/6 males. Age-matched nulliparous females were used as controls. At 10–14 days post-parturition, post-partum mammary gland involution was instigated in the bred females by force-weaning the pups. For normal involution studies, mammary glands, and draining lymph nodes (inguinal lymph nodes) were harvested from nulliparous and involution day 6 mice. Tumor studies for the 66cl4 and E0771 mouse mammary carcinoma models were performed as previously described (21) with the tumors, mammary glands, and lymph nodes being taken for flow cytometry, immunohistochemistry, and downstream biochemical analyses. For the E0771 PD-1 intervention studies, 250,000 tumor cells were injected into the number 4 mammary glands of either nulliparous or involution day 1 C57Bl/6 dams. Tumor sites were palpated daily (E0771) or twice weekly (66cl4) for tumors. Calipers were used to take measurements and the tumor volumes were calculated using length x width x width $\times$ 0.5. Additionally, 66cl4 tumor cells were luciferase and GFP tagged allowing for tumors to be detected using the Xenogen 200. Once tumors became measurable, mice were randomized into control or treatment groups and injected with 250 μg of either isotype control (anti-IgG2a [clone 2A3; Bio X Cell cat. #BP0089]) or anti-PD-1 (clone RMP1-14; Bio X Cell cat. #BP0146) antibody, respectively. Injections were administered intra-peritoneally every third day. Tumor studies were ended based on primary tumor cell growth or ulceration at 3–4 weeks post injection (66cl4) or 1–2 weeks post injection (E0771). *In vivo* studies were performed in triplicate with pooled or representative data shown.

Mammary Gland Processing and Staining (IHC)

Mammary glands were harvested and placed into 10% neutral buffered formalin for 48 h. After 48 h, tissues were moved to 70% EtOH, processed, and stained for LYVE-1 as previously described (3, 14, 16, 21, 49).

Lymphatic Vessel Density Quantification

Lymphatic vessel density (LVD) was performed as previously described (3, 21). Briefly, slides stained for PDPN (D2-40) or LYVE-1 were scanned into the Aperio ImageScope software. Lymphatic vessels were counted in the tumor-adjacent tissue (peri-tumor region) and LVD was quantified as the number of lymphatic vessels per area of tissue.

Human Tissue Acquisition

Research using de-identified human breast tissue (**Supplemental Table 1**) was conducted under a protocol deemed exempt from subject consent as approved by the Colorado Multiple Institution Review Board (COMIRB) and tissues were acquired by Virginia Borges as previously reported (1). Dr. Borges obtained written informed consent from the patients, the studies were conducted in accordance with recognized ethical guidelines (e.g., Declaration of Helsinki, CIOMS, Belmont Report, U.S. Common Rule), and the studies were approved by an institutional review board.

Staining of Human Tissue Using Vectra

Four-micron thick sections were taken from Formalin Fixed Paraffin Embedded tissue, dewaxed in xylenes and rehydrated. Slides were placed in 10% NBF for 20 min for extra fixation, rinsed with DI water, then submerged in Target Retrieval Solution pH6 (Dako cat# S1699) and placed in a pressure cooker for 20 min. Slides were rinsed with Dako wash buffer (Dako cat# K8000), blocked for 10 min with Perkin Elmer Diluent/Block (Perkin Elmer cat# ARD1001EA), then sequentially stained for the following markers: PD-L1 (clone E1L3N), PD-1 (clone NAT105;), PDPN (clone D2-40), and CD68 (clone KP1). Incubation time for all primary antibodies was 1 h at room temperature. Slides were rinsed and stripped in Target Retrieval Solution in between every primary. Slides were then incubated in Perkin Elmer Opal Polymer HRP Mouse+Rabbit secondary (cat# ARH1001EA) for 30 min at room temperature, followed by a 10 min incubation in Opal Fluorophore reagents (Perkin Elmer). After the final stain, Spectral DAPI (Perkin Elmer cat# FP1490) was applied to slides for 5 min, then slides were rinsed and cover-slipped with ProLong Diamond Antifade Mountant (Thermo cat# P36970). Multispectral imaging was then performed using the Vectra 3 Automated Quantitative Pathology Imaging System (Perkin Elmer). Whole slide scans were collected using the

10x objective and 5–10 regions were selected for multispectral imaging with the 20x objective. The multispectral images were analyzed with inForm software (Perkin Elmer) to unmix adjacent fluorochromes, subtract autofluorescence, segment the tissue into lymphatic vessels and non lymphatic vessels, segment the cells into nuclear, and membrane compartments, and to phenotype the cells according to morphology and cell marker expression. Cells with a PD-L1 threshold <0.95 were classified as PD-L1 negative while cells with a value >0.95 threshold were classified as PD-L1 positive using inForm software. To quantitate PD-L1 in lymphatics a blinded observer imaged 5–10 representative fields from the peritumor region that were positive for PDPN vessels by only the PDPN channel. For PDPN we also counted PD-L1+ lymphatic vessels by adding the PD-L1 channel and counted PDPN+PD-L1+ vessels as well as PDPN+PD-L1- vessels and calculated the percent positive per case, which was then normalized to area. PD-1+ cells were also counted in the same manner as PDPN+ vessels.

Flow Cytometry

Tumors were separated from the mammary gland. Both tumors and mammary glands were placed in six-well plates with 2 mL of Click's media without mercaptoethanol or L-glutamine (Irvine Scientific, Santa Ana, CA), where they were minced with scalpels, digested with 500 units/ml collagenase type II and IV and 20 μg/ml DNase (Worthington Biochemical Corporation, Lakewood, NJ) and incubated for 1 h at 37°C. The tissue suspension was then filtered through a 100 μm strainer and washed with Click's. The filtered cells were centrifuged at 1,400 RPM for 5 min, the supernatant was removed, and the pellet was resuspended in 1 mL FACS buffer (500 mL 1x HBSS pH 7.4, 0.1% BSA, 0.02% sodium azide, up to 1L ddH_2O). The tumor cells were stained with BD viability 510 dye prior to staining with CD45 (clone30-F11), CD8a APC/Cy7 (clone 53-6.7) (1:400), CD4 APC or PerCp-Cy5.5 (clone RM4-5) (1:300), PD-1 FITC or BV421 (clone 29F.1A12) (1:100), Lag-3 PerCP/Cy5.5 (clone C9B7W) (1:100), and/or CD11a FITC (clone M17/4) (1:200). The mammary glands were stained with BD viability dye 510 followed by CD45 APC-Cy7 or Pacific Blue (clone 30-F11) (1:300), CD31 Pacific Blue or PerCp-Cy5.5 (clone 390) (1:200), PDPN APC or PE-Cy7 (clone 8.1.1) (1:200), CD11b Pacific Blue or PerCp or FITC (clone M1/70)(1:400), F4/80 APC, APC-Cy7, PerCP-Cy5.5 or FITC (clone BM8) (1:100), PD-L1 PE, FITC, or BV421 (clone RMP1-30 or 29F.1A12) (1:200), and EpCAM PE-Cy7 or APC-Cy7 (clone G8.8) (1:100). Flow cytometry antibodies were purchased from Biolegend (San Diego, CA). CD8 T cells were identified from live, CD3+/CD8+, where they were further characterized by their expression of PD-1 and Lag-3. Lymphatic endothelial cells were identified from live, CD45-/EpCAM-, and CD31+PDPN+. Cells were run on the DakoCytomation CyAn ADP flow cytometer (Fort Collins, CO) or FACs Canto II, acquired using Summit software or Diva Software, and analyzed with FlowJo software (Tree Star, Ashland, OR). Geometric mean fluorescence intensity (gMFI) was calculated with FlowJo software.

Intracellular Cytokine Staining

Cells were isolated from the tissue and treated with or without (unstimulated controls) phorbol 12-myristate 13-acetate (PMA) (20 ng/ml) (Sigma, St. Louis, MO) plus ionomycin (1 ug/ml) (Sigma, St. Louis, MO) for 4–6 h at 37 degrees in the presence of 2 ug/ml of brefeldin A (Adipogen, San Diego, CO) in RPMI+2.5% FBS. Cells were then stained with CD8, CD45, CD4, CD44, PD-1, and Lag-3 (as above) and incubated at 37°C for 30 min. Following surface marker staining cells were fixed with 1% paraformaldehyde and 4% sucrose for 10 min in the dark at room temperature. Following fixation, cells were permeabilized with BD Perm Wash (BD Biosciences, San Jose CA) and stained for cytokines IFNγ (1:200) (APC; Biolegend clone XMG1.2) and TNFα (1:200) (FITC; Biolegend clone MP6-XT22). After washing cells were resuspended in FACs buffer (0.5% Bovine Serum Albumin and 0.1% Sodium Azide in PBS) and were run on an ADP Cyan. Gating was determined based on unstimulated controls. All antibodies were purchased from Biolegend (San Diego, CA).

TCGA RNASeq Analysis

Analysis was performed on cbioportal.org TCGA Breast provisional dataset using the co-expression tool and the RNA-Seq data.

Statistics

One-way ANOVA, unpaired *t*-test, and linear regression were run in the GraphPad Prism software, assuming normal distributions among independent samples. For **Figure 6**, Pearson and Spearman analysis was performed on cbioportal.org. *P*-values of <0.05 were deemed significant.

RESULTS

PD-L1 Expression and PD-1 T Cells Are Observed in Patients With PPBC and in Pre-clinical Models

To determine if the increased lymphatics (PDPN) that we observe in our patients with PPBC exhibit upregulation of PD-L1 and/or whether PD-1+ T cell infiltration occurs in the tumor microenvironment (TME) of PPBCs, we utilized multispectral imaging of the peri-tumor region in tissues from three different patients with PPBC, who were within 1-year (PPBC1), 3 years (PPBC2), and 4 years post-partum (PPBC3) (**Supplemental Table 1**). In comparison to three non-PPBC patients, who were all nulliparous, we observed increased PDPN+ LVD in the peri-tumor region from our PPBCs. We also observed frequent lymphatic vessel expression of PD-L1 (arrow) and that lymphatic vessels were frequently infiltrated with PD-L1 expressing tumor cells (asterisk), identified by their altered nuclear morphology. Furthermore, we also observed the presence of CD68+ macrophages that appear to express PD-L1 (+ symbol) as well as cells expressing PD-1 (arrowhead) in the surrounding areas (**Figure 1A** and **Supplemental Figure 1**). We quantitated PD-L1+ cells in the peritumor region and observed that total numbers of PD-L1+ cells per area did not differ between groups (**Figure 1B**), but that total PDPN+ vessel

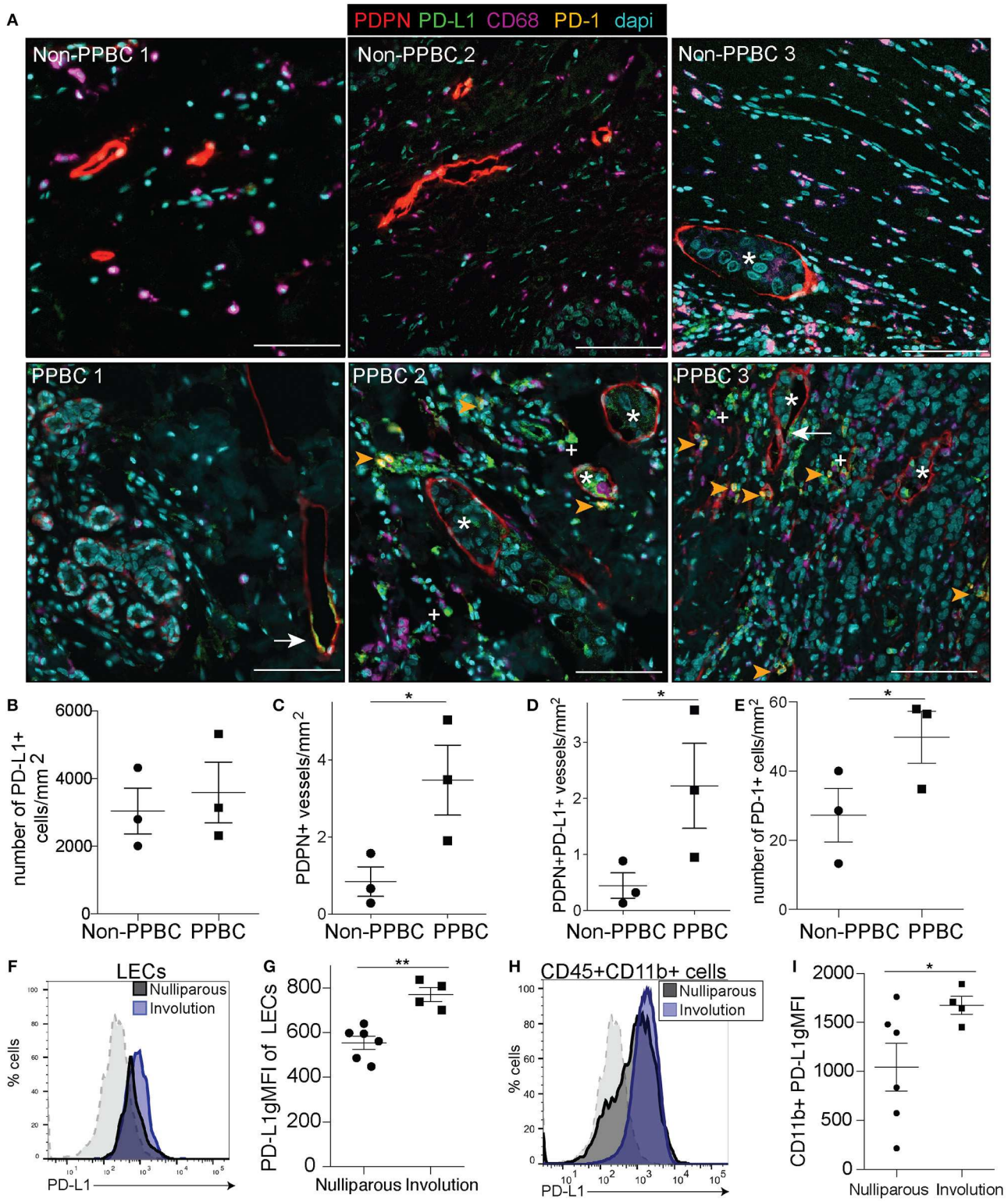

FIGURE 1 | PDPN/PD-L1/PD-1 in post-partum patients and mouse mammary tumor models. **(A)** Tumor adjacent mammary tissues from three nulliparous non-post-partum breast cancer patients (Non-PPBBC) and three patients with post-partum breast cancer (PPBC) stained for PDPN (red), PD-L1 (green), CD68 (magenta), PD-1 (orange), and dapi (cyan). Asterisks indicate tumor cells found within the lymphatic vessel, white arrows indicate PD-L1+ lymphatic vessels, plus signs indicate PD-L1+ macrophages (CD68) and orange arrows indicate PD-1+ cells. **(B)** Number of PD-L1+ cells in the peritumoral region of the patients

(Continued)

FIGURE 1 | from A. **(C)** Number of PDPN+ vessels in the peritumoral region of the patients from A. **(D)** Number of PDPN+ vessels that are also PD-L1+ in the peritumoral region of the patients from A. **(E)** Number of PD-1+ cells in the peritumoral region of the patients from A. **(F)** Histogram of PD-L1 expression by LECs (CD45-EpCAM-CD31+PDPN+PD-L1+) from 66CL4 tumors implanted during mammary gland involution. Gray dotted line indicates a PD-L1 negative population (CD45+CD11b-F4/80-) that does not change. **(G)** gMFI of PD-L1 of LECs in tumor. **(H)** Histogram of PD-L1 expression by CD45+ CD11b+ cells from tumors implanted during mammary gland involution. Gray dotted line indicates a PD-L1 negative population (CD45+CD11b-F4/80-) that does not change. **(I)** gMFI calculated for PD-L1 from D. Data shown from animal experiments are from 1 representative experiment of 2 replicates with at least 4 tumors per group. Unpaired *t*-test: $*p < 0.05$; $**p < 0.01$. Scale bars are 100 microns in length.

density, as well as PDPN+PD-L1+ vessel density, were increased in our patients with PPBC compared to nulliparous controls (**Figures 1C,D**) suggesting that PD-L1 lymphatics are a part of the TME in patients with PPBC. Finally, we observe a significant increase in PD-1+ cells in the TME of our PPBC patients (**Figure 1E**).

Since we observed PD-L1 expression in cells of the TME from patients with PPBC, we next asked if PD-L1 expression was a characteristic of the TME of post-partum tumors in our murine model of PPBC. Using the 66cl4 isograft model where we have shown increased lymph vessel density (LVD) and lymph node metastasis in post-partum hosts (3), we examined post-partum tumor-associated LEC expression of PD-L1 in Balb/c mice. As previously observed, tumor cells implanted on day 1 of involution (involution group tumors) exhibited decreased latency and increased growth compared to nulliparous (**Supplemental Figure 2A**) (3, 13, 16). At study endpoint, 4 weeks post-injection, tumors were harvested, and populations were analyzed by flow cytometry (**Supplemental Figures 2B,C**). We observed an increase in the fluorescence intensity of PD-L1 on CD45-Epcam-CD31+PDPN + tumor associated LECs (**Figures 1F,G**), which we also observed with implantation of E0771 tumors into BL6 mice (**Supplemental Figure 3A**). We found that while there were similar frequencies of the monocyte (CD11b+) populations in both tumor models (**Supplemental Figure 3B** and not shown), CD11b+ cells in both 66cl4 and E0771 tumors implanted during mammary gland involution had higher average levels of PD-L1 (**Figures 1H,I** and **Supplemental Figure 3C**). The number of PD-L1+ cells was also increased in the involution group, but this increase was lost when normalized to tumor size (**Supplemental Table 2**). We also analyzed additional cell populations for PD-L1 including monocytes, fibroblasts, blood endothelial cells (BECs), and EpCAM+ tumor cells based on described markers. We did not see significant staining differences or staining above background in the fibroblasts, BECs or EpCAM+ cells (**Supplemental Figure 3D**). These results complement our recently published data showing that CD11b+ monocytes in the TME of involution group tumors contribute to lymphangiogenesis and extend our observations to describe their expression of PD-L1 (21).

As PD-L1 is the inhibitory ligand, we next examined expression of the inhibitory receptor, PD-1, by CD4+ and CD8+ tumor-associated T cells (gating-**Supplemental Figure 2D**) all of which are also positive for CD11a, a molecule that has been shown to render them unable to control tumor growth (50). We found an increase in the expression of PD-1 on CD4+ T cells (**Figure 2A**) and after quantification we found that an average of 30% of CD4+ T cells in the involution group tumors expressed PD-1 compared to 10% in the nulliparous controls (**Figure 2B**). As cytotoxic CD8 T cells are typically thought to be important in controlling tumors we next asked about expression of PD-1 on CD8+ T cells. We observed a striking difference in the expression profile of CD8+ T cells in tumors implanted during mammary gland involution compared to tumors implanted in nulliparous hosts (**Figure 2C**). When we quantified this difference, we found a >4-fold increase in expression of PD-1 by CD8+ T-cells from involution group tumors (**Figure 2D**). Additionally, we observed similar phenotypes in the mammary draining lymph nodes of tumor bearing animals (**Figure 2E**) suggesting this mechanism of tumor-associated immune suppression may extend beyond the local tumor microenvironment to drive the increased LN metastasis that we observe in post-partum patients. Finally, we observed similar phenotypes in the E0771 tumors, but not lymph nodes (**Supplemental Figures 4A,B**).

PD-L1 and PD-1 Expression Are Observed in Mouse Mammary Tissues During Normal Post-Partum Mammary Gland Involution

We next asked if the LECs of C57/BL6 (B6) or Balb/c female mice exhibit expression of PD-L1 during normal post-partum mammary gland involution, similar to what is observed in LN LECs to induce peripheral tolerance during tissue homeostasis (31). We evaluated mouse mammary LECs at involution day 6, the peak of the remodeling phase of involution, for expression of PD-L1. PD-L1+ LECs were evaluated based on the markers described above and validated by staining with isotype and fluorescence minus one (FMO) controls (**Supplemental Figure 2B**). We observed that expression of PD-L1 was increased in mammary LECs isolated from involution group mice compared to nulliparous (**Figure 3A**). We also found that the percentage and number of LECs expressing PD-L1, as well as the geometric mean fluorescence intensity (gMFI) of PD-L1, was increased on LECs during involution (**Figures 3B–D**), which is similar to that observed in our pre-clinical model of PPBC (**Figures 1F,G**). We found these increases in both the B6 mice as well as the Balb/C mice (**Supplemental Figures 5A–C**). Since we observed increased PD-L1 expression in the CD11b+ population in our model of PPBC, we first confirmed that the CD11b+ population increased (gating-**Supplemental Figure 2C**) during involution in both B6 (**Figure 3E**) and Balb/C (**Supplemental Figure 5D**) mouse mammary glands. Then, we also observed an increase in expression of PD-L1 by this

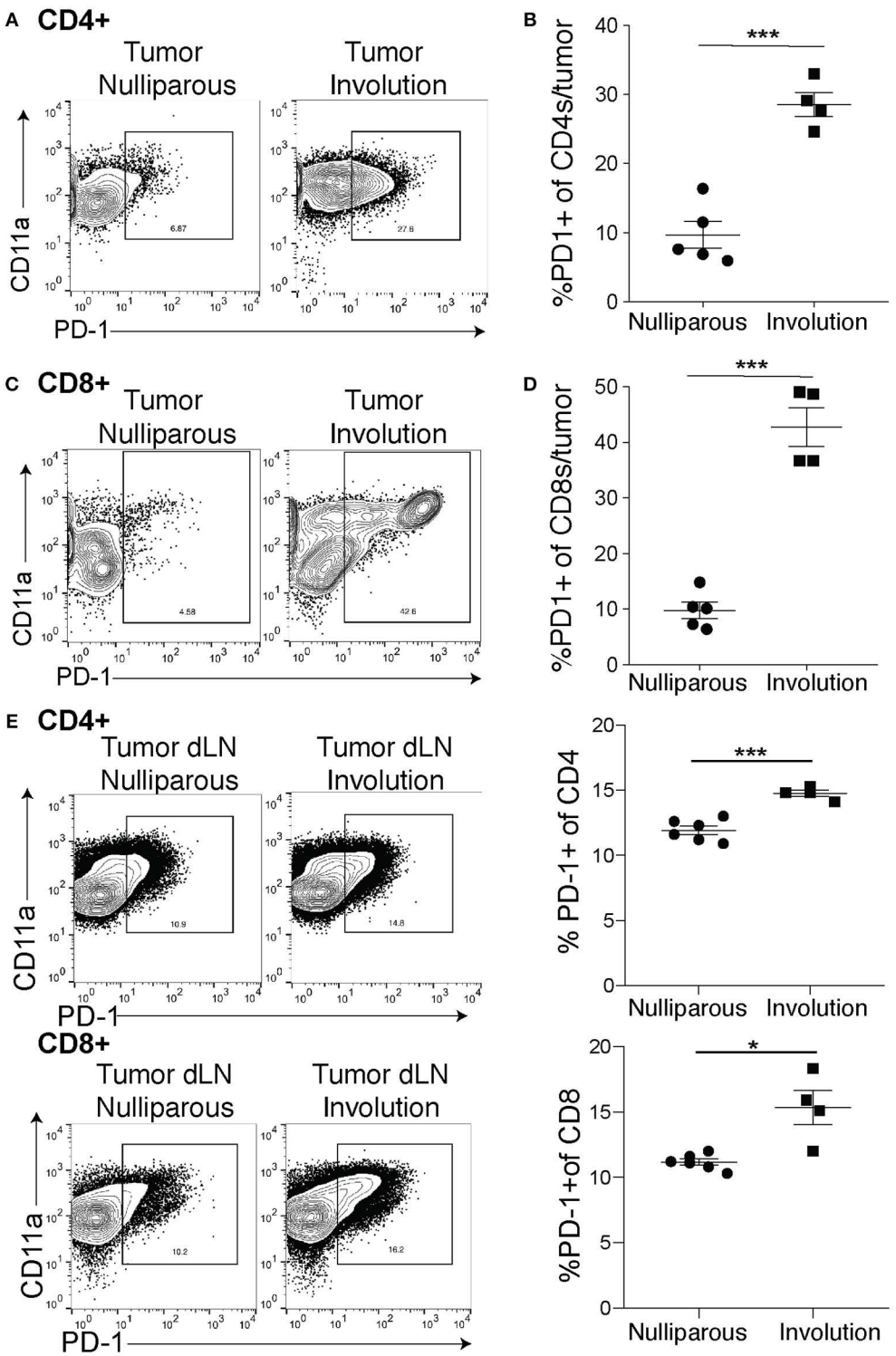

FIGURE 2 | Tumor-associated PD-1$^+$ T cells are increased in tumors implanted during involution. **(A)** Representative flow plots for cells from nulliparous or involution group tumors of CD11a and PD-1 expression by CD4$^+$ T cells. **(B)** Quantification of CD4$^+$ T cells that express PD-1 from A. **(C)** Representative flow plots of CD8$^+$ T cell expression of PD-1 and CD11a from nulliparous and involution tumors and **(D)** quantification of PD-1$^+$ CD8$^+$ T cells. **(E)** Representative flow plots of CD11a and PD-1$^+$ CD4$^+$ T cells or CD8$^+$ T cells in the tumor draining lymph nodes and quantification of PD-1+ frequency of each cell type. Data shown are from 1 representative experiment of 2 replicates with at least 4 tumors per group. Unpaired *t*-test: $^*p < 0.05$; $^{***}p < 0.001$.

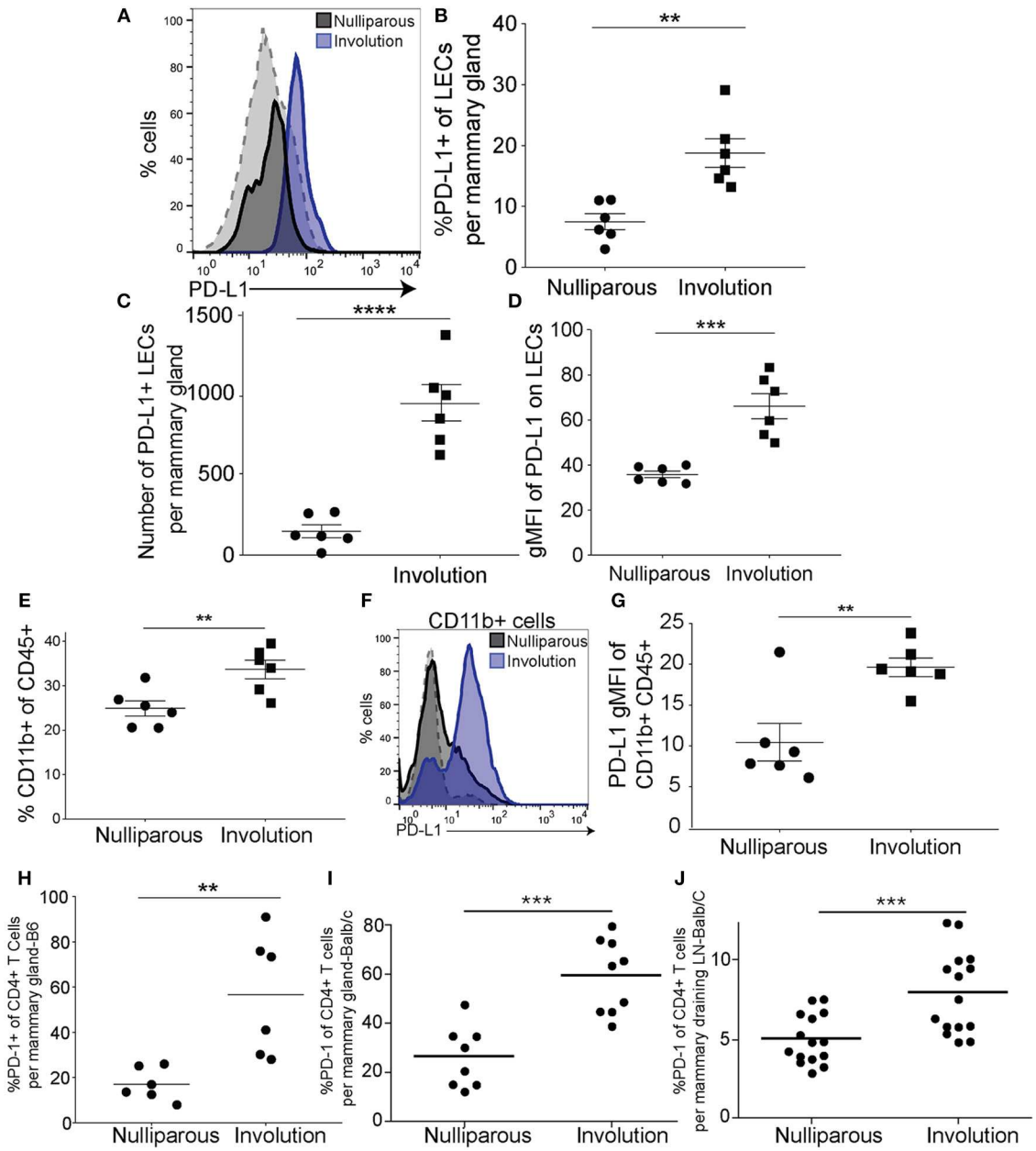

FIGURE 3 | PD-L1$^+$ lymphatic endothelial cells and PD-L1$^+$ myeloid cells increase in the mammary gland during post-partum involution. **(A)** Representative histogram of PD-L1 by lymphatic endothelial cells (CD45$^-$EpCAM$^-$CD31$^+$PDPN$^+$PD-L1$^+$) acquired by flow cytometry from B6 mammary glands of nulliparous (n = 6) or recently weaned, involution day 6 (n = 6), mice. Gray dotted line indicates a PD-L1 negative population (CD45+CD11b-F4/80-) that does not change. **(B)** Quantification of the frequency of PD-L1+ LECs of total LECs per mammary gland. **(C)** Number of PD-L1$^+$ LECs per mammary gland. **(D)** Quantification of gMFI of PD-L1 expression by LECs from A. **(E)** Frequency of CD11b$^+$ cells of the CD45$^+$ cells found in the mammary gland in nulliparous or involution group mice. **(F)** Histogram of PD-L1 expression by CD11b$^+$ cells in mammary gland of nulliparous or involution group mice. Gray dotted line indicates a PD-L1 negative population (CD45$^+$CD11b$^-$F4/80$^-$) that does not change. **(G)** Quantification of PD-L1 expression by gMFI on CD11b+ cells as shown in F. **(H)** Percent of CD4$^+$ T cells that are PD-1$^+$ from the mammary glands of B6 mice and **(I)** as in H except from Balb/c mice. **(J)** Percent of PD-1$^+$ CD4$^+$ T cells in the axillary lymph nodes from nulliparous and involution group tissues. Data shown are from 1 representative experiment of 2 replicates with at least 6 mammary glands per group. Unpaired t-test: $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$.

population (**Figure 3F**) that was significant (**Figure 3G**) in both models (**Supplemental Figure 5E**).

To better understand if PD-1 expression on T cells is similarly increased during post-partum mammary gland involution, we evaluated the T-cell compartment at involution day 6 by assessing the frequency of PD-1 expression by CD4+ and CD8+ T-cells in B6 and Balb/c mice compared to isotype controls (gating-**Supplemental Figure 2D**). Similar to previous results in Balb/c

mice (51), we observed a significant increase in the percent of PD-1+ CD4+ T cells in mammary glands from B6 (**Figure 3H**) and confirmed this in our Balb/c mice (**Figure 3I**). This increase in PD-1+ CD4+ T cells also extended to the LN (**Figure 3J**). Further, we found an increase in CD8+ T cells expressing PD-1 in the B6 mice (**Supplemental Figure 5F**), which was not significant in the Balb/c mice (**Supplementary Figures 5G,H**). These results suggest that a mechanism of LEC and/or monocyte/macrophage mediated T cell inhibition could be driving the decreased latency and increased growth rate that we observe in our pre-clinical models when tumor cells are implanted during involution (16, 21, 52).

PD-1 Targeted Therapy Reduces Tumor Growth in Post-partum Hosts by Reactivating T-Cells

A prediction of our results is that inhibition of PD-L1/PD-1 signaling during involution would dampen the increased tumor growth observed when tumors are implanted during involution by reversing this involution-driven mechanism of immune suppression. To test our hypothesis, we utilized the E0771 mouse mammary tumor model since tumors implanted in this model become palpable during active involution; when the immune suppressive mechanism is most activated. Thus, we orthotopically implanted E0771 tumor cells into the intact mammary glands of C57BL/6 mice at involution day 1 or into nulliparous hosts. Then, we blocked PD-L1-mediated inhibition of T cells by administering an anti-PD-1 monoclonal antibody every third day after tumors were palpable and size-matched in both nulliparous and involution groups. Following two and three treatments, in involution and nulliparous mice, respectively, mice were euthanized and flow cytometry performed on tumors (**Figure 4A**). Similar to previous results, involution group tumors exhibited decreased latency and increased growth compared to tumors in nulliparous hosts (**Figure 4B**) (3, 13, 16). Importantly, the anti-PD-1 treatment significantly reduced the growth rate in involution group tumors, to levels more similar to those observed in the nulliparous hosts, and did not significantly affect tumor growth in the nulliparous group (**Figure 4B**). We then evaluated whether the anti-PD-1 treatment affected immune cell infiltration into the tumors in the involution group. Immunohistochemistry (IHC) on a single tumor from each involution group revealed increased intratumoral staining for CD45, which was validated by our flow cytometry where we observed a >2-fold increase in the number of CD45+ cells in the tumor (**Figures 4C,D**). We also observed a significant increase in the number of intra-tumoral CD8+ T cells following treatment with anti-PD-1 when the tumors were implanted into involution hosts (**Figure 4E**). Additionally, we found a significant decrease in LEC frequency in the adjacent mammary tissues of involution group mice treated with anti-PD-1 by both flow cytometry (**Figure 4F**) and by tissue staining with PDPN to assess LVD (**Figure 4G**). Conversely, we found no significant difference in PD-L1 expression by LECs after anti-PD-1 treatment (**Supplemental Figure 6A**). These findings suggest that blockade of PD-1 could have potential for blocking both tumor growth and lymphogenous tumor cell spread during involution.

To evaluate whether anti-PD-1 treatment was affecting the phenotype and functionality of the tumor-associated T cells, we used flow cytometry to assess the co-expression of co-inhibitory markers, PD-1 and Lag-3, by the CD8+ T cells (**Figure 5A**) as well as PD-1 single positive expression by the CD8+ and CD4+ T cells (**Supplemental Figures 6B,C**). With treatment, we observed a decrease in the frequency of PD-1 and Lag-3 double positive cells in the involution group tumors, but not in the nulliparous tumors (**Figure 5B**). Importantly, the Lag-3 gMFI was also significantly decreased following anti-PD-1 treatment in the involution group, but not the nulliparous, (**Figure 5C**) and this effect was not due to the treatment antibody (clone RMP1-14) blocking the staining antibodies (clone RMP1-30 or 29F1A12) (**Supplemental Figure 6D**). We also observed that the frequency of the CD45+ cells that were also positive for CD8 was increased in involution group tumors, and the nulliparous, that were treated with anti-PD-1 (**Figure 5D**). However, the number of CD8+ cells was not increased (see **Figure 4E**) nor was tumor growth significantly affected by anti-PD-1 treatment in the nulliparous group (**Figure 5D**). While PD-1 and Lag-3 are markers of T cells exhaustion, these markers do not evaluate the functionality of the T cells. Therefore, we next measured the production of the effector cytokines IFNγ and TNFα by tumor-associated CD8+ and CD4+ T cells in our involution group tumors. We evaluated CD8+PD-1+ T cells *ex vivo* in the presence or absence of stimulation with PMA/Ionomycin (**Figure 5E**). After anti-PD-1 treatment we found significant increases in the production of both IFNγ and TNFα by CD8+PD1+ T cells, from the involution group treated with anti-PD-1, suggesting that they are poly-functional (**Figure 5F**). The frequency of CD4+ T cells and the production of IFNγ by CD4+ T cells after anti-PD-1 treatment was not significantly different with treatment (**Supplemental Figures 6E,F**).

Co-expression of Immune Inhibitory Programs and PD-L1, PDPN, and CD68 Is Observed in Patients With Breast Cancer

Having shown that PD-L1+ LEC and monocytic cell populations likely contribute to the immune inhibitory microenvironment in the mammary gland during involution and in breast cancer, we examined whether breast cancer patient samples frequently exhibit co-expression of immune-inhibitory programs and of the LEC marker PDPN and the macrophage marker CD68. To accomplish this, we examined co-expression by RNASeq in breast cancers using The Cancer Genome Atlas cBioPortal for Cancer Genomics. As expected we observed co-expression of PD-L1 (gene name *CD274*) with CD8 (gene name *CD8A*), PD-1 (gene name *PDCD1*), and *LAG3* (**Figures 6A–C**). We also observed significant co-expression of *CD274* with *PDPN* and *CD68* as well as between *PDPN* and *CD68* (**Figures 6D–F**). Correlation coefficients and *p*-values for each relationship are reported in **Table 1**. Furthermore, consistent with our results during in mouse mammary tissue during involution we did not observe significant co-expression of CD274 with *PDGFA*, an established fibroblast marker, or with *PDGFB* the heterodimeric

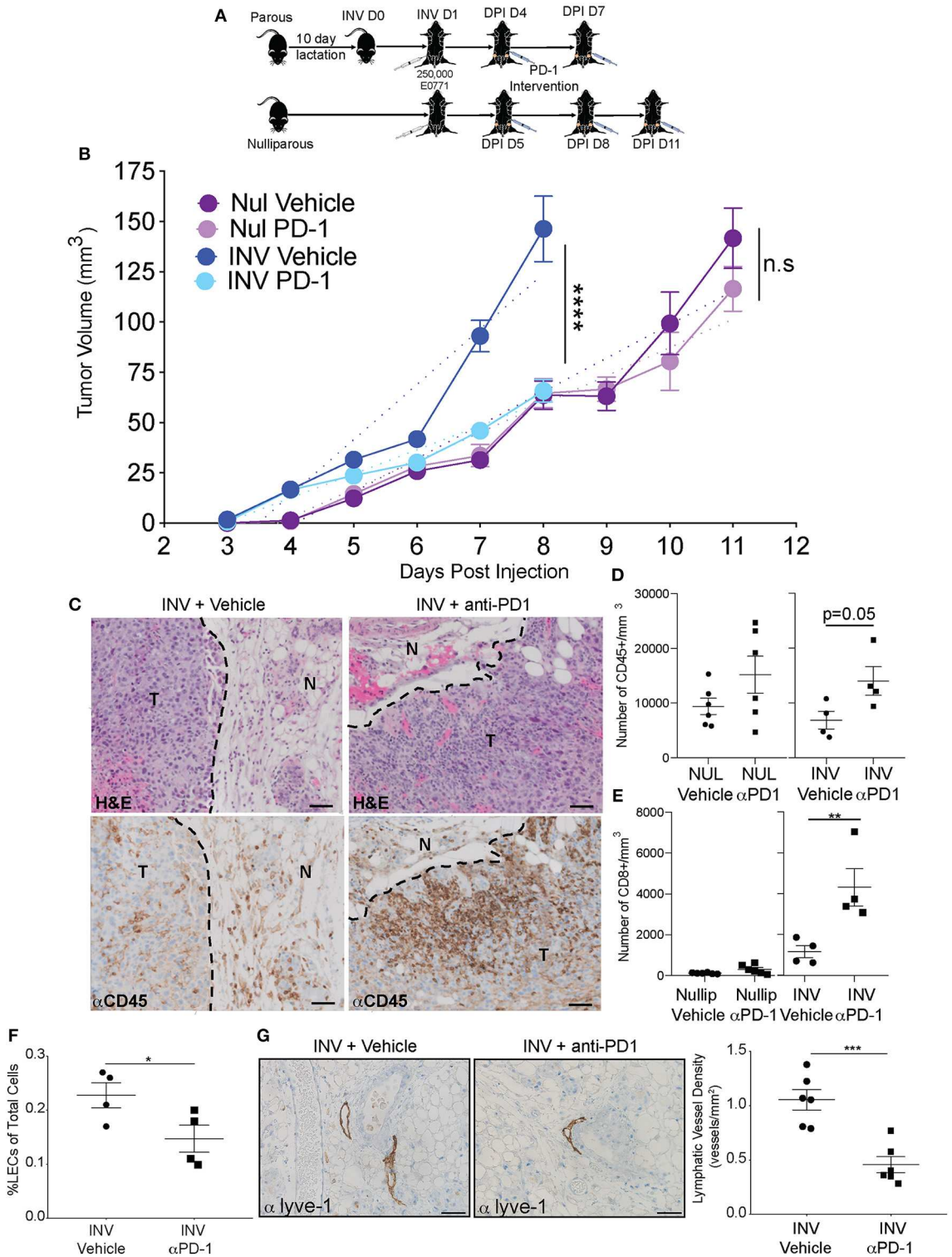

FIGURE 4 | Anti-PD-1 treatment reduces tumor growth, enhances immune infiltration, and reduces lymphatic vessels in a model of post-partum breast cancer **(A)** C57/Bl6 mice were bred and allowed to lactate for 10 days before pups were removed to initiate mammary gland involution (parous/involution, $n = 6$); age-matched nulliparous controls were used ($n = 6$). Parous animals were injected with 250,000 E0771 tumor cells at Inv D1 (involution group) or into nulliparous animals (nulliparous group). Once tumors became measurable (involution = 4 days post injection (DPI); nulliparous=DPI D5), anti-PD-1 intervention was administered and continued every third day. E0771 mammary tumor growth curves from nulliparous and involution group C57Bl/6 mice treated with vehicle or anti-PD-1 are shown in

(Continued)

FIGURE 4 | (B). Results are representative from two independent studies. Dotted lines represent the slope of the tumor growth. **(C)** Representative images of H&E analysis and immunohistochemistry for CD45 (brown) in fixed tumor tissue to identify tumor infiltrating lymphocytes after anti-PD-1 treatment compared to vehicle controls, T = tumor and N = normal. $CD45^+$ area was 9.99% of tumor area for involution with vehicle and 22.62% of tumor area for involution with anti-PD-1 treatment. Scale bars are 50 microns. Number of **(D)** $CD45^+$ and **(E)** $CD8^+$ cells per area in tumors from B quantified by flow cytometric analysis. **(F)** % LECs of total in tumors from B quantified by flow cytometry. **(G)** Representative images of Lyve-1 stained fixed tumor adjacent tissues and quantitation of Lyve-1^+ vessels per area in involution group tumors +/- PD-1 treatment. Scale bars are 100 microns. $^*p < 0.05$; $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

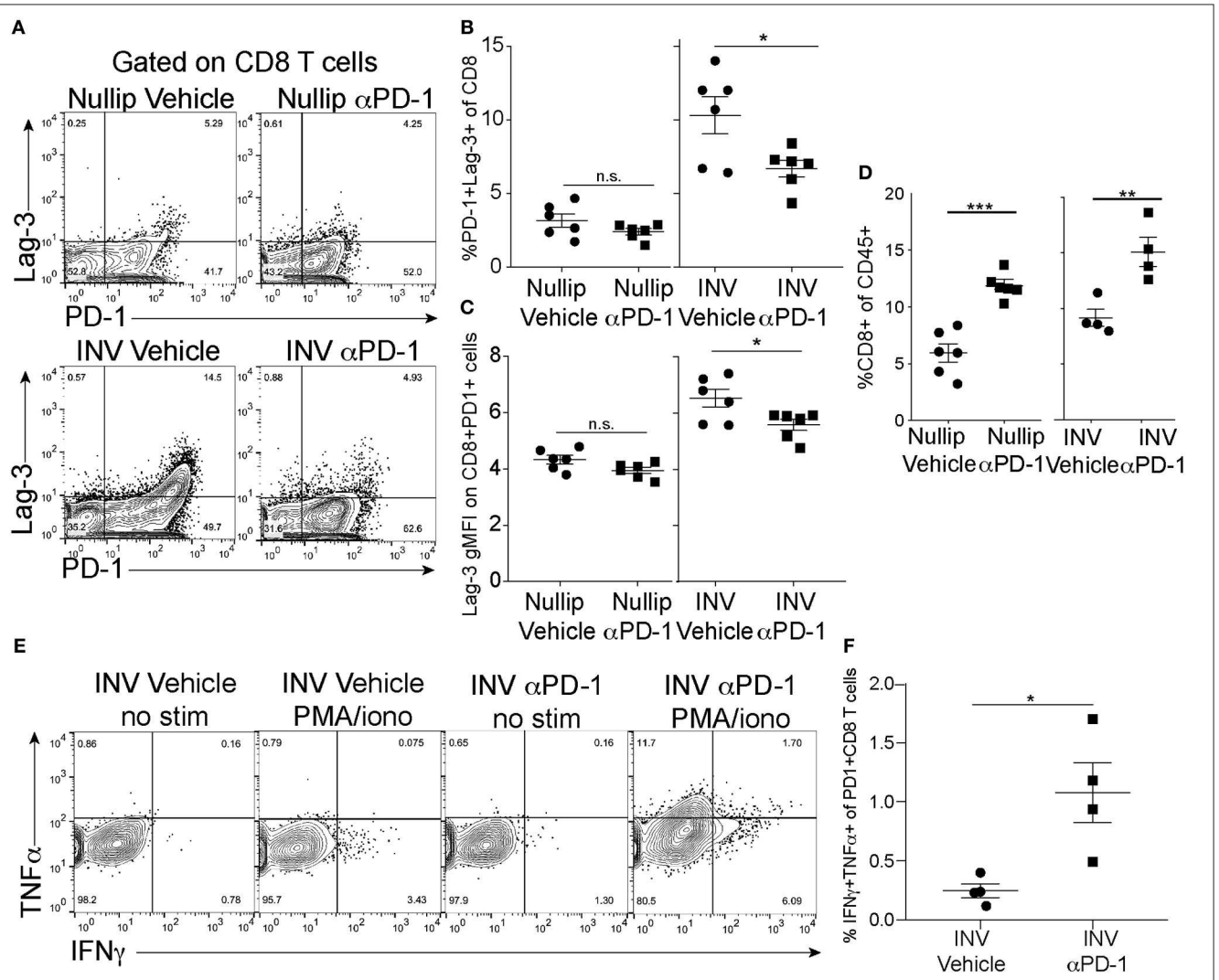

FIGURE 5 | Tumor Infiltrating T cells after PD-1 treatment. **(A)** Example flow cytometry plots of $CD45^+CD3^+B220^-CD8^+$ T cells from each treatment group. Shown is expression of Lag-3 and PD-1. **(B)** Frequency of $CD8^+$ T cells that express both PD-1 and Lag-3 as shown in A-upper right quadrant. **(C)** gMFI of Lag-3 on PD-1^+ $CD8^+$ T cells (upper right quadrants in A). **(D)** Frequency of CD8+ of CD45+ T cells in tumors treated with anti-PD-1 as measured by flow cytometry. **(E)** Flow cytometry plots of $CD45^+CD3^+CD8^+$PD-1^+ cells at end of experiment (cells from right quadrants from C) after PMA/ionomycin treatment and intracellular cytokine staining with IFNγ and TNFα. **(F)** Quantification of double positive IFNγ and TNFα cells from upper right quadrant in G in involution group tumors with or without treatment. Data shown are from 1 representative experiment of 2 replicates with at least 4 tumors per group. Two-way ANOVA (A). Unpaired *t*-test **(C–G)**: $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

partner of *PDGFA*. Therefore, we predict that this mechanism of immune suppression is mediated, in part, by cells of the TME may be at play in breast cancer patients and that analysis of LEC and monocyte/macrophage content could be utilized to predict whether a breast cancer patient is likely to respond to PD-1-targeted therapy.

DISCUSSION

Our results suggest that an immune inhibitory microenvironment involving LEC and monocyte/macrophage expression of PD-L1 and T-cell expression of PD-1 is induced during involution and may be co-opted by post-partum tumors.

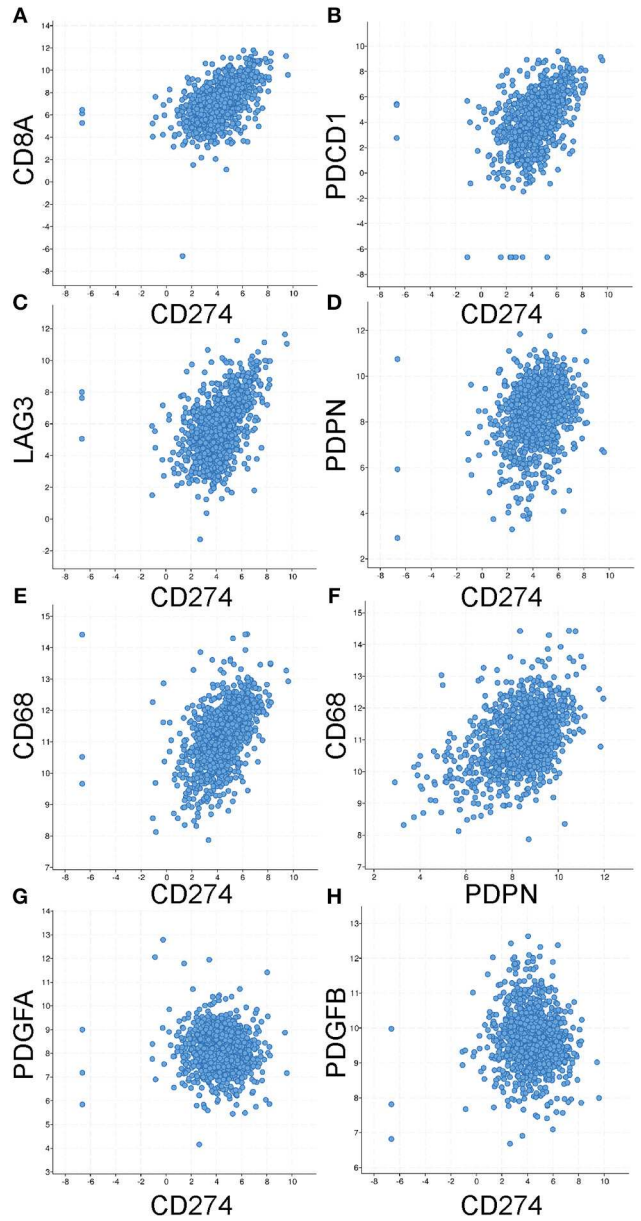

FIGURE 6 | Co-expression of identified markers of immune suppression in primary breast cancers. Using the co-expression tool in cBioPortal to analyze mRNA (by RNASeq) levels of multiple markers reveals significant positive correlations between CD274 (the gene encoding for PD-L1) and **(A)** CD8A **(B)** PDCD1 (the gene encoding for PD-1) **(C)** LAG3 and **(D)** PDPN and **(E)** CD68. As well as between PDPN and CD68 **(F)**. No correlation was observed with **(G)** PDGFA, a marker of fibroblasts, or its heterodimeric partner PDGFB **(H)**. Statistical analyses are provided in **Table 1**.

TABLE 1 | Statistics for mRNASeq correlation analyses.

Genes	Spearman	*p-value*	Pearson	*p-value*
CD274 CD8A	0.55	1.33e−88	0.51	3.06e−75
CD274 PDCD1	0.51	4.39e−73	0.45	1.00e−56
CD274 LAG3	0.49	6.50e−68	0.45	3.64e−57
CD274 PDPN	0.26	5.92e−19	0.27	1.7e−19
CD274 CD68	0.52	1.68e−77	0.47	1.86e−62
PDPN CD68	0.42	4.98E−48	0.45	2.13E−55
CD274 PDGFA	−0.05	0.100	−0.05	0.0849
CD274 PDGFB	−0.08	5.746Ee−3	−0.04	0.216

These inhibitory receptor-ligand interactions appear to promote increased tumor growth and immune evasion by increasing PD-1 and Lag-3 expression by tumor infiltrating T cells. In 2017, Dieterich et al. published that LECs in the tumor microenvironment could promote T cell exhaustion through expression of PD-L1 (28) and more recently Lane et al. identified a mechanism by which T cell infiltration can manipulate PD-L1 expression via IFNg (29). These results are similar to normal mechanisms of T-cell inactivation by LECs in the lymph node where LEC expression of peripheral tissue antigens and PD-L1 act to prevent autoimmunity and promote peripheral tolerance (30, 31, 33). Here, we suggest that a similar mechanism is induced during post-partum mammary gland involution by showing that PD-L1+ LECs and monocyte/macrophage populations and PD-1+ T-cells are abundant. Additionally, we believe that both increased lymphatics and their expression of PD-L1, along with increased PD-1 and Lag-3 expression by T cells in the tumor microenvironment could lead to increased tumor metastasis in tumors established during involution. We also show that specific targeting of the PD-L1/PD-1 interaction in our model of PPBC decreases mammary tumor growth during involution. Identification of the mechanisms that underlie the increased expression of co-inhibitory ligands and receptors during involution, and in post-partum tumors, is important for proposing rational clinical trials for post-partum patients.

We predict that the immune suppressive environment, which occurs as a consequence of mammary gland involution and is mediated, in part, by lymphatics and monocyte/macrophage populations, aids in prevention of autoimmunity in a tissue healing environment (3, 10, 13, 20, 53, 54). While our studies do not identify the specific pathways activated in the mammary gland during involution that control PD-L1 expression by LECs or monocytes/macrophages, and other cells, several possibilities are evident from the existing literature. First, pro-inflammatory enzyme cyclooxygenase-2 (COX-2) is expressed and active during involution, and correlations between COX-2 expression and PD-L1 expression have been reported in lung cancer, melanoma, a mouse model of mammary cancer, and in tumor-associated macrophages and myeloid derived suppressor cells (55–59). Additionally, blocking COX-2 during involution also decreases some of the tumor-promotional effects of involution

including increased growth (16). Second, STAT-3 is activated during involution and is a known activator of PD-L1 expression (60–65). Third, high expression of PD-L1 in the subcapsular sinus LECs in the lymph node (30, 45) occurs through lymphotoxin beta receptor (LTBR or TNFRSF3) signaling and is mediated by the presence of B cells (30). Interestingly, LTBR signaling is also a known regulator of epithelial cell apoptosis during involution (19) and B cells are increased in the mammary gland during involution (13) suggesting an additional mechanism that may drive LECs to upregulate PD-L1 expression. We propose that a number of mechanisms could be at play to result in the upregulation of PD-L1 on lymphatic endothelial cells, as well as other cells, in the mammary gland during mammary gland involution and in post-partum tumors. As we have previously published that macrophages contribute to lymphatic remodeling, and lymphatic mimicry, during involution it is notable that the monocyte/macrophage population expressing PD-L1 is also higher during involution and in tumors implanted during involution. We suggest that this increase in myeloid cells could either be to remodel the lymphatic vasculature or to drive expression of PD-L1 through as of yet undefined mechanisms. It is also possible that the increased PD-L1 myeloid population is contributing to the expression of PD-L1 by incorporating into the vasculature and expressing lymphatic markers. Dissecting these molecular mechanisms that drive PD-L1 expression is under active investigation.

In addition to increased PD-L1 expression, we show that tumor infiltrating T cells express more co-inhibitory receptors in mammary tumors implanted during post-partum involution. We also found that treatment with anti-PD-1 in these tumors resulted in decreased frequencies of PD-1 and Lag-3 double-positive CD8+ T cells and decreased Lag-3 expression. The frequency and expression of Lag-3+ cells in anti-PD-1-treated involution group tumors was more similar to nulliparous group mice, suggesting that anti-PD-1 treatment could reverse the immune suppression observed in involution group tumors by decreasing or inhibiting the expansion of Lag-3 and PD-1 double positive T cells. An alternative explanation is that the anti-PD-1 depletes PD-1+ T cells and this possibility is being explored through complementary anti-PD-L1 studies by our group. Further, while IFNγ expression is relatively low in bulk tissue during involution, we do see IFNγ expression by T cells in mammary tumors implanted into both nulliparous and involution group hosts demonstrating that IFNγ may at least partially contribute to PD-L1 expression by the cells in the tumor (29). These findings indicate that while the production of IFNγ within the tumor is not increased, that instead the frequency and poly-functionality (IFNγ and TNFα double positive cells) of CD8+ T cells is increased following treatment with anti-PD-1. These findings predict increased tumor killing consistent with what is seen in patients (41) and is consistent with the decreased tumor volume we observed. Our findings that LVD is also decreased after anti-PD-1 treatment could either be a cause or consequence of tumor regression, but is likely due to the treatment induced pro-inflammatory environment in contrast to the anti-inflammatory environment observed in post-partum tumors. Loss of LVD could significantly impact the ability of tumor cells to migrate to the tumor draining lymph node, which could also reduce metastasis. A limitation of our current studies is the lack of metastatic data in our preclinical models as well as the lack of analysis of PD-L1 expression on post-partum tumors; these important studies are being actively pursued using additional models.

Our findings that an involution-targeted therapy, anti-PD-1, can mitigate the decrease in tumor growth and LVD that we observe after implantation during involution are consistent with our previous results showing that inhibition of COX-2 can decrease growth, invasion, LVD, and metastasis (3, 16). Whether anti-PD-1 therapy could also mitigate the increased metastasis observed in our models is unanswered by our current data. However, if such mechanisms are maintained long term, as is suggested by our 66cl4 model where tumors are isolated long after completion of involution, post-partum patients with metastasis may also benefit from anti-PD-1 therapy, which has shown efficacy in the metastatic setting (see below). Our findings may also lend insight into the increased aggressiveness and metastatic potential of breast cancer diagnosed within 5–10 years of recent childbirth. We propose that a plausible mechanism is immune suppression in both the mammary tissue and the draining lymph node, which could account for the increased lymph node positivity observed in post-partum patients (2). This, along with the increased lymphangiogenesis and lymphatic vessel invasion observed in the adjacent mammary tissue of post-partum patients, could provide a favorable route for metastatic spread. While further studies addressing mechanisms of lymphatic expression of PD-L1 and lymphatic growth in tumors must be performed, we believe these studies provide substantial evidence that current immunotherapies could benefit patients with PPBC. Immunotherapy, specifically anti-PD-1/PD-L1 based, has been investigated in breast cancer with positive results. First, pembrolizumab—the highly selective monoclonal-antibody-based therapy against PD-1—was the first shown to be successful as a monotherapy for metastatic triple-negative cases of breast cancer (TNBC), with some long-lasting responses reported, and has also shown benefit for advanced ER+/Her2- in the KEYNOTE trials (66–70). It is also currently in clinical trial with several chemotherapy partners, including in the neoadjuvant setting and as a single drug in the post-neoadjuvant setting for patients with residual cancer (71, 72). Anti-PD-L1 based therapy, specifically atezolizumab, is now a standard of care option in combination with the chemotherapy drug nab-paclitaxel for TNBC that have at least 1% PD-L1 expressing tumor infiltrating immune cells, based on the results of the Impassion 130 clinical trial, which demonstrated a 10-month improvement in overall survival for the combination (73). While the early immunotherapy trials with check-point block inhibitors have met with success, many patients do not benefit or do not achieve long-term benefit and death due to progressive cancer remains the norm. This highlights that additional markers, such as evaluation of LVD, may be better or synergistic with existing markers for predicting patient response (74). Importantly, several trials are ongoing to investigate the use of anti-PD-1 and anti-PD-L1 in the (neo) adjuvant setting and the treatment has proven safe and well-tolerated with preliminary results demonstrating

promising efficacy (71, 72, 74). Here we have identified a specific population of patients who may benefit from (neo) adjuvant PD-1/PD-L1 blockade—women diagnosed within 10 years post-partum who are at high risk for metastasis. Ongoing research is investigating this possibility as well as whether a combined approach with an anti-lymphangiogenesis-based therapy could improve survival for PPBC patients.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations ethical guidelines (e.g., Declaration of Helsinki, CIOMS, Belmont Report, U.S. Common Rule) of the Colorado Multiple Institution Review Board (COMIRB) committee with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the COMIRB. This study was carried out in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals, Animal Welfare Act and PHS Policy by the University of Colorado Anschutz Medical Campus Institutional Animal Care and Use Committee. The protocol was approved the institutional animal care and use committee.

AUTHOR CONTRIBUTIONS

BT and TL designed and executed experiments, analyzed results, and drafted the manuscript. AE, JF, and AW performed experiments, analyzed data, and critically reviewed the manuscript. VW performed imaging experiments. VB provided samples and critically reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.01313/full#supplementary-material

REFERENCES

1. Callihan EB, Gao D, Jindal S, Lyons TR, Manthey E, Edgerton S, et al. Postpartum diagnosis demonstrates a high risk for metastasis and merits an expanded definition of pregnancy-associated breast cancer. *Breast Cancer Res Treat*. (2013) 138:549–59. doi: 10.1007/s10549-013-2437-x
2. Goddard ET, Bassale S, Schedin T, Jindal S, Johnston J, Cabral E, et al. Association between postpartum breast cancer diagnosis and metastasis and the clinical features underlying risk. *JAMA Netw Open*. (2019) 2:e186997. doi: 10.1001/jamanetworkopen.2018.6997
3. Lyons TR, Borges VF, Betts CB, Guo Q, Kapoor P, Martinson HA, et al. Cyclooxygenase-2-dependent lymphangiogenesis promotes nodal metastasis of postpartum breast cancer. *J Clin Invest*. (2014) 124:3901–12. doi: 10.1172/JCI73777
4. Black SA, Nelson AC, Gurule NJ, Futscher, B. WLyons TR. Semaphorin 7a exerts pleiotropic effects to promote breast tumor progression. *Oncogene*. (2016) 35:5170–8. doi: 10.1038/onc.2016.49
5. Asztalos S, Gann PH, Hayes MK, Nonn L, Beam CA, Dai Y, et al. Gene expression patterns in the human breast after pregnancy. *Cancer Prev Res*. (2010) 3:301–11. doi: 10.1158/1940-6207.CAPR-09-0069
6. Nichols HB, Schoemaker MJ, Cai J, Xu J, Wright LB, Brook MN, et al. Breast cancer risk after recent childbirth: a pooled analysis of 15 prospective studies. *Ann Intern Med*. (2019) 170:22–30. doi: 10.7326/M18-1323
7. Albrektsen G, Heuch I, Hansen, SKvale G, Breast cancer risk by age at birth, time since birth and time intervals between births: exploring interaction effects. *Br J Cancer*. (2005) 92:167–75. doi: 10.1038/sj.bjc.6602302
8. Schedin P. Pregnancy-associated breast cancer and metastasis. *Nat Rev Cancer*. (2006) 6:281–91. doi: 10.1038/nrc1839
9. Schedin P, O'Brien J, Rudolph M, Stein, TB, Orges V. Microenvironment of the involuting mammary gland mediates mammary cancer progression. *J Mamm Gland Biol Neoplasia*. (2007) 12:71–82. doi: 10.1007/s10911-007-9039-3
10. Stein T, Morris JS, Davies CR, Weber-Hall SJ, Duffy MA, Heath VJ, et al. Involution of the mouse mammary gland is associated with an immune cascade and an acute-phase response, involving LBP, CD14 and STAT3. *Breast Cancer Res*. (2004) 6: R75–91. doi: 10.1186/bcr753
11. Stein T, Salomonis N, Gusterson BA. Mammary gland involution as a multi-step process. *J Mamm Gland Biol Neoplasia*. (2007) 12:25–35. doi: 10.1007/s10911-007-9035-7
12. Stein T, Salomonis N, Nuyten DS, van de Vijver MJ, Gusterson BA. A mouse mammary gland involution mRNA signature identifies biological pathways potentially associated with breast cancer metastasis. *J Mammary Gland Biol Neoplasia*. (2009) 14:99–116. doi: 10.1007/s10911-009-9120-1
13. Martinson HA, Jindal S, Durand-Rougely C, Borges V, Schedin P. Wound healing-like immune program facilitates postpartum mammary gland involution and tumor progression. *Int J Cancer*. (2015) 136:1803–13. doi: 10.1002/ijc.29181
14. O'Brien J, Lyons T, Monks J, Lucia MS, Wilson RS, Hines L, et al. Alternatively activated macrophages and collagen remodeling characterize the postpartum involuting mammary gland across species. *Am J Pathol*. (2010) 176:1241–55. doi: 10.2353/ajpath.2010.090735
15. Jindal S, Gao D, Bell P, Albrektsen G, Edgerton SM, Ambrosone CB, et al. Postpartum breast involution reveals regression of secretory lobules mediated by tissue-remodeling. *Br Cancer Res*. (2014) 16:R31. doi: 10.1186/bcr3633
16. Lyons TR, O'Brien J, Borges VF, Conklin MW, Keely PJ, Eliceiri KW, et al. Postpartum mammary gland involution drives progression of ductal carcinoma *in situ* through collagen and COX-2. *Nat Med*. (2011) 17:1109–15. doi: 10.1038/nm.2416

17. Green, K. AStreuli CH, Apoptosis regulation in the mammary gland. *Cell Molecul Life Sci.* (2004) 61:1867–83. doi: 10.1007/s00018-004-3366-y
18. Lund LR, Romer J, Thomasset N, Solberg H, Pyke C, Bissell MJ, et al. Two distinct phases of apoptosis in mammary gland involution: proteinase-independent and -dependent pathways. *Development*. (1996) 122:181–93.
19. Clarkson RW, Wayland MT, Lee J, Freeman T, Watson CJ. Gene expression profiling of mammary gland development reveals putative roles for death receptors and immune mediators in post-lactational regression. *Breast Cancer Res*. (2004) 6:R92–109. doi: 10.1186/bcr754
20. O'Brien J, Lyons T, Monks J, Lucia MS, Wilson RS, Hines L, et al. Alternatively activated macrophages and collagen remodeling characterize the postpartum involuting mammary gland across species. *Am J Pathol*. (2010) 176:1241–55.
21. Elder AM, Tamburini AJ, Crump LS, Black SA, Wessells VM, Schedin PJ, et al. Semaphorin 7A promotes macrophage-mediated lymphatic remodeling during postpartum mammary gland involution and in breast cancer. *Cancer Res*. (2018) 78:6473–85. doi: 10.1158/0008-5472.CAN-18-1642
22. de Almeida C, Linden R. Phagocytosis of apoptotic cells: a matter of balance. *Cell Molecul Life Sci.* (2005) 62:1532–46. doi: 10.1007/s00018-005-4511-y
23. KaramanS, Detmar M. Mechanisms of lymphatic metastasis. *J Clin Invest*. (2014) 124:922–8. doi: 10.1172/JCI71606
24. Wei JC, Yang J, Liu D, Wu MF, Qiao L, Wang JN, et al. Tumor-associated lymphatic endothelial cells promote lymphatic metastasis by highly expressing and secreting SEMA4C. *Clin Cancer Res*. (2017) 23:214–24. doi: 10.1158/1078-0432.CCR-16-0741
25. Shayan R, Achen MG, Stacker SA. Lymphatic vessels in cancer metastasis: bridging the gaps. *Carcinogenesis*. (2006) 27:1729–38. doi: 10.1093/carcin/bgl031
26. Cai L, Yang S, Ding H, Cai J, Wang Z. Tumor-associated lymphatic endothelial cell promotes invasion of cervical cancer cells. *APMIS*. (2013) 121:1162–8. doi: 10.1111/apm.12068
27. Sleeman JP, Thiele W. Tumor metastasis and the lymphatic vasculature. *Int J Cancer*. (2009) 125:2747–56. doi: 10.1002/ijc.24702
28. Dieterich LC, Ikenberg K, Cetintas T, Kapaklikaya K, Hutmacher C, Detmar M. Tumor-associated lymphatic vessels upregulate PDL1 to inhibit T-cell activation. *Front Immunol*. (2017) 8:66. doi: 10.3389/fimmu.2017.00066
29. Lane RS, Femel J, Breazeale AP, Loo CP, Thibault G, Kaempf A, et al. IFNgamma-activated dermal lymphatic vessels inhibit cytotoxic T cells in melanoma and inflamed skin. *J Exp Med*. (2018) 215:3057–74. doi: 10.1084/jem.20180654
30. Cohen JN, Tewalt EF, Rouhani SJ, Buonomo EL, Bruce AN, Xu X, et al. Tolerogenic properties of lymphatic endothelial cells are controlled by the lymph node microenvironment. *PLoS ONE*. (2014) 9:e87740. doi: 10.1371/journal.pone.0087740
31. Tewalt EF, Cohen JN, Rouhani SJ, Guidi CJ, Qiao H, Fahl SP, et al. Lymphatic endothelial cells induce tolerance via PD-L1 and lack of costimulation leading to high-level PD-1 expression on CD8 T cells. *Blood*. (2012) 120:4772–82. doi: 10.1182/blood-2012-04-427013
32. Humbert M, Hugues S, Dubrot J. Shaping of peripheral T cell responses by lymphatic endothelial cells. *Front Immunol*. (2016) 7:684. doi: 10.3389/fimmu.2016.00684
33. Cohen JN, Guidi CJ, Tewalt EF, Qiao H, Rouhani SJ, Ruddell A, et al. Lymph node-resident lymphatic endothelial cells mediate peripheral tolerance via Aire-independent direct antigen presentation. *J Exp Med*. (2010) 207:681–8. doi: 10.1084/jem.20092465
34. Dubrot J, Duraes FV, Potin L, Capotosti F, Brighouse D, Suter T,et al. Lymph node stromal cells acquire peptide-MHCII complexes from dendritic cells and induce antigen-specific CD4(+) T cell tolerance. *J Exp Med*. (2014) 211:1153–66. doi: 10.1084/jem.20132000
35. Kao C, Oestreich KJ, Paley MA, Crawford A, Angelosanto JM, Ali MA, et al. Transcription factor T-bet represses expression of the inhibitory receptor PD-1 and sustains virus-specific CD8+ T cell responses during chronic infection. *Nat Immunol*. (2011) 12:663–71. doi: 10.1038/ni.2046
36. Pardoll DM. The blockade of immune checkpoints in cancer immunotherapy. *Nat* Rev *Cancer*. (2012) 12:252–64. doi: 10.1038/nrc3239
37. Odorizzi PM, Pauken KE, Paley MA, Sharpe, AWherry EJ. Genetic absence of PD-1 promotes accumulation of terminally differentiated exhausted CD8+ T cells. *J Exp Med*. (2015) 212:1125–37. doi: 10.1084/jem.20142237
38. Bengsch B, Johnson AL, Kurachi M, Odorizzi PM, Pauken KE, Attanasio J, et al. Bioenergetic insufficiencies due to metabolic alterations regulated by the inhibitory receptor PD-1 are an early driver of CD8(+) T cell exhaustion. *Immunity*. (2016) 45:358–73. doi: 10.1016/j.immuni.2016.07.008
39. Blackburn SD, Crawford A, Shin H, Polley A, Freeman GJ, Wherry EJ. Tissue-specific differences in PD-1 and PD-L1 expression during chronic viral infection: implications for CD8 T-cell exhaustion. *J Virol*. (2010) 84:2078–89. doi: 10.1128/JVI.01579-09
40. Ahn E, Araki K, Hashimoto M, Li W, Riley JL, Cheung J, et al. Role of PD-1 during effector CD8 T cell differentiation. *Proc Natl Acad Sci USA*. (2018) 115:4749–54. doi: 10.1073/pnas.1718217115
41. Egelston CA, Avalos C, Tu TY, Simons DL, Jimenez G, Jung JY, et al. Human breast tumor-infiltrating CD8(+) T cells retain polyfunctionality despite PD-1 expression. *Nat Commun*. (2018) 9:4297. doi: 10.1038/s41467-018-06653-9
42. Johnston RJ, Comps-Agrar L, Hackney J, Yu X, Huseni M, Yang Y, et al. The immunoreceptor TIGIT regulates antitumor and antiviral CD8(+) T cell effector function. *Cancer Cell*. (2014) 26:923–37. doi: 10.1016/j.ccell.2014.10.018
43. Roux C, Jafari SM, Shinde R, Duncan G, Cescon DW, Silvester J, et al. Reactive oxygen species modulate macrophage immunosuppressive phenotype through the up-regulation of PD-L1. *Proc Natl Acad Sci USA*. (2019) 116:4326–35. doi: 10.1073/pnas.1819473116
44. Hartley GP, Chow L, Ammons DT, Wheat WH, Dow SW. Programmed cell death ligand 1 (PD-L1) signaling regulates macrophage proliferation and activation. *Cancer Immunol Res*. (2018) 6:1260–73. doi: 10.1158/2326-6066.CIR-17-0537
45. Lucas ED, Finlon JM, Burchill MA, McCarthy MK, Morrison TE, Colpitts, T. MB, et al. Type 1 IFN and PD-L1 coordinate lymphatic endothelial cell expansion and contraction during an inflammatory immune response. *J Immunol*. (2018) 201:1735–47. doi: 10.4049/jimmunol.1800271
46. Azuma T, Yao S, Zhu G, Flies AS, Flies SJ, Chen L. B7-H1 is a ubiquitous antiapoptotic receptor on cancer cells. *Blood*. (2008) 111:3635–43. doi: 10.1182/blood-2007-11-123141
47. Jin Y, Chauhan SK, El Annan J, Sage PT, SharpeAH, Dana R. A novel function for programmed death ligand-1 regulation of angiogenesis. *Am J Pathol*. (2011) 178:1922–9. doi: 10.1016/j.ajpath.2010.12.027
48. Gato-Canas M, Zuazo M, Arasanz H, Ibanez-Vea M, Lorenzo L, Fernandez-Hinojal G, et al. PDL1 signals through conserved sequence motifs to overcome interferon-mediated cytotoxicity. *Cell Rep*. (2017) 20:1818–29. doi: 10.1016/j.celrep.2017.07.075
49. Black SA, Nelson AC, Gurule NJ, Futscher, B. WLyons TR. Semaphorin 7a exerts pleiotropic effects to promote breast tumor progression. *Oncogene*. (2016). doi: 10.1158/1557-3125.ADVBC15-B15
50. Liu X, Gibbons RM, Harrington SM, Krco CJ, Markovic SN, Kwon, E, et al. Endogenous tumor-reactive CD8+ T cells are differentiated effector cells expressing high levels of CD11a and PD-1 but are unable to control tumor growth. *Oncoimmunology*. (2013) 2:e23972. doi: 10.4161/onci.23972
51. Betts CB, Pennock ND, Caruso BP, Ruffell B, Borges V, Schedin P. Mucosal immunity in the female murine mammary gland. *J Immunol*. (2018) 201:734–46. doi: 10.4049/jimmunol.1800023
52. Lyons TR, Borges VF, Betts CB, Guo Q, Kapoor P, Martinson HA, et al. Cyclooxygenase-2-dependent lymphangiogenesis promotes nodal metastasis of postpartum breast cancer. *J Clin Invest*. (2014) 124:3901–12.
53. O'Brien J, Martinson H, Durand-Rougely C, Schedin P. Macrophages are crucial for epithelial cell death and adipocyte repopulation during mammary gland involution. *Development*. (2012) 139:269–75. doi: 10.1242/dev.071696
54. Fornetti J, Martinson HA, Betts CB, Lyons TR, Jindal S, Guo Q, et al. Mammary gland involution as an immunotherapeutic target for postpartum breast cancer. *J Mammary Gland Biol Neoplasia*. (2014) 19:213–28. doi: 10.1007/s10911-014-9322-z
55. Shimizu K, Okita R, Saisho S, Maeda AI, Nojima Y, Nakata M. Impact of COX2 inhibitor for regulation of PD-L1 expression in non-small cell lung cancer. *Anticancer Res*. (2018) 38:4637–44. doi: 10.21873/anticanres.12768
56. Shimizu K, Okita R, Saisho S, Maeda A, Nojima Y, Nakata M. Prognostic value of Cox-2 and PD-L1 expression and its relationship with tumor-infiltrating lymphocytes in resected lung adenocarcinoma. *Cancer Manag Res*. (2017) 9:741–50. doi: 10.2147/CMAR.S146897

57. Prima V, Kaliberova LN, Kaliberov S, Curiel DT, Kusmartsev S. COX2/mPGES1/PGE2 pathway regulates PD-L1 expression in tumor-associated macrophages and myeloid-derived suppressor cells. *Proc Natl Acad Sci USA*. (2017) 114:1117–22. doi: 10.1073/pnas.1612920114
58. Markosyan N, Chen EP, Evans RA, Ndong V, Vonderheide RH, Smyth EM. Mammary carcinoma cell derived cyclooxygenase 2 suppresses tumor immune surveillance by enhancing intratumoral immune checkpoint activity. *Breast Cancer Res.* (2013) 15:R75. doi: 10.1186/bcr3469
59. Botti G, Fratangelo F, Cerrone M, Liguori G, Cantile M, Anniciello AM, et al. COX-2 expression positively correlates with PD-L1 expression in human melanoma cells. *J Transl Med*. (2017) 15:46. doi: 10.1186/s12967-017-1150-7
60. Wang ZQ, Milne K, Derocher H, Webb JR, Nelson BH, Watson PH. PD-L1 and intratumoral immune response in breast cancer. *Oncotarget*. (2017) 8:51641–51. doi: 10.18632/oncotarget.18305
61. Li M, Liu X, Robinson G, Bar-Peled U, Wagner KU, Young WS, et al. Mammary-derived signals activate programmed cell death during the first stage of mammary gland involution. *Proc Natl Acad Sci USA*. (1997) 94:3425–30. doi: 10.1073/pnas.94.7.3425
62. Kritikou EA, Sharkey A, Abell K, Came PJ, Anderson E, Clarkson RW, et al. A dual, non-redundant, role for LIF as a regulator of development and STAT3-mediated cell death in mammary gland. *Development*. (2003) 130:3459–68. doi: 10.1242/dev.00578
63. Hughes K, Watson CJ. The role of Stat3 in mammary gland involution: cell death regulator and modulator of inflammation. *Horm Mol Biol Clin Investig*. (2012) 10:211–5. doi: 10.1515/hmbci-2012-0008
64. Chapman RS, Lourenco PC, Tonner E, Flint DJ, Selbert S, Takeda K, et al. Suppression of epithelial apoptosis and delayed mammary gland involution in mice with a conditional knockout of Stat3. *Genes Dev.* (1999) 13:2604–16. doi: 10.1101/gad.13.19.2604
65. Hughes K, Watson CJ. The multifaceted role of STAT3 in mammary gland involution and breast cancer. *Int J. Molecul Sci.* (2018) 19:E1695. doi: 10.3390/ijms19061695
66. Nanda R, Chow LQ, Dees EC, Berger R, Gupta S, Geva R, et al. Pembrolizumab in patients with advanced triple-negative breast cancer: Phase Ib KEYNOTE-012 study. *J Clin Oncol.* (2016) 34:2460–7. doi: 10.1200/JCO.2015.64.8931
67. Adams S, Loi S, Toppmeyer D, Cescon DW, De Laurentiis M, Nanda R, et al. Title: pembrolizumab monotherapy for previously untreated, PD-L1-positive, metastatic triple-negative breast cancer: cohort B of the phase 2 KEYNOTE-086 study. *Ann Oncol*. (2018). doi: 10.1093/annonc/mdy518
68. Adams S, Schmid P, Rugo HS, Winer EP, Loirat D, Awada A, Cescon DW, et al. Pembrolizumab monotherapy for previously treated metastatic triple-negative breast cancer: cohort A of the Phase 2 KEYNOTE-086 study. *Ann Oncol*. (2018) 30:405–11. doi: 10.1093/annonc/mdy517
69. Rugo HS, Delord P. *Preliminary Efficacy And Safety Of Pembrolizumab (MK-3475) in Patients with PD-L1-Positive, Estrogen Receptor-Positive (ER+)/HER2 Negative Advanced Breast Cancer Enrolled In KEYNOTE-028.* 2015 San Antonio Breast Cancer Symposium Abstract S5-(2015). doi: 10.1158/1538-7445.SABCS15-S5-07
70. Nanda R, Specht J, Dees E, Berger R, Gupta S, Geva R, et al. Pembrolizumab for metastatic triple-negative breast cancer (mTNBC): long-lasting responses in the phase Ib KEYNOTE-012 study. *Eur J Cancer.* (2017) 72:S38. doi: 10.1016/S0959-8049(17)30206-X
71. Nanda R, Liu MC, Yau C, Asare S, Hylton N, Van't Veer L, et al. Pembrolizumab plus standard neoadjuvant therapy for high-risk breast cancer (BC): results from I-SPY 2. *J Clin Oncol.* (2017) 35:506. doi: 10.1200/JCO.2017.35.15_suppl.506
72. Schmid P, Park YH, Muñoz-Couselo E, Kim SB, Sohn J, Im SA, et al. Pembrolizumab (pembro) + chemotherapy (chemo) as neoadjuvant treatment for triple negative breast cancer (TNBC): preliminary results from KEYNOTE-173. *J Clin Oncol.* (2017) 35:556. doi: 10.1200/JCO.2017.35.15_suppl.556
73. Schmid P, Adams S, Rugo HS, Schneeweiss A, Barrios CH, Iwata H, et al. Investigators MT, atezolizumab and nab-paclitaxel in advanced triple-negative breast cancer. *N Engl J Med*. (2018) 379:2108–21. doi: 10.1056/NEJMoa1809615
74. Solinas C, Gombos A, Latifyan S, Piccart-Gebhart M, Kok M, Buisseret L. Targeting immune checkpoints in breast cancer: an update of early results. *ESMO Open*. (2017) 2:e000255. doi: 10.1136/esmoopen-2017-000255

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