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Lymphatic Vessels in Inflammation

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Abstract

Lymphatic vessels are important for tissue fluid homeostasis and for the uptake of dietary lipids in the intestine. Moreover, lymphatic vessels are intimately linked with induction of the immune response, as they transport antigen, inflammatory mediators, and leukocytes from peripheral tissues to draining lymph nodes (dLNs). Research of the last 10 years has revealed that lymphatic vessels form a highly plastic network, which rapidly adapts to inflammation in a stimulus- and tissue-specific manner. The inflammatory environment induces changes in gene expression in lymphatic endothelial cells (LECs) and leads to a profound proliferative expansion of the lymphatic network in both the inflamed tissue and the dLNs. Inflammatory changes in the lymphatic network have been shown to impact fluid drainage as well as leukocyte trafficking, suggesting that lymphatic vessels play an active role in the regulation of inflammatory processes. In fact, experimentally enhancing or blocking lymphangiogenesis was shown to modulate the course of inflammatory and immune responses in various disease models. Given these exciting pre-clinical findings, lymphatic vessels and inflammatory lymphangiogenesis are emerging as potential new therapeutic targets for the treatment of chronic inflammatory and autoimmune disorders, and for the prevention of graft rejection. In this review, we will summarize current knowledge about the inflammatory response of the lymphatic network and introduce the main molecular and cellular mediators of inflammatory lymphangiogenesis. Our review will particularly focus on how inflammation-induced changes in lymphatic vessels are thought to impact the course of inflammatory and immune responses and address the therapeutic implications of these findings.

Keywords: Inflammation; Lymphangiogenesis; Lymph node; Lymphatic drainage; Leukocyte migration; Lymphatic endothelial cells; Lymphatic muscle cells

Introduction

Lymphatic vessels are responsible for draining fluids and macromolecules from tissues and for taking up lipids in the intestine [1-3]. Moreover, lymphatic vessels transport antigen and leucocytes between peripheral tissues, lymph nodes (LNs), and blood and therefore are important for the induction and regulation of immune responses [2,4,5]. Although lymphatic vessels were already described almost 400 years ago [6], molecular and cellular research of the lymphatic vascular system is a fairly young discipline. In fact, this field has only started to advance in the last 18 years, propelled by the identification of lymphatic-specific markers like the vascular endothelial growth factor receptor-3 (VEGFR-3), podoplanin (gp38), the hyaluronan receptor LYVE-1, or the lymphatic-specific transcription factor Prox-1 [2,7]. These markers made it possible to unambiguously identify lymphatic vessels in tissues and to isolate lymphatic endothelial cells (LECs) for *in vitro* analyses. Ever since we have witnessed a true explosion of research investigating the role of lymphatic vessels in health and disease. It is nowadays well established that lymphatic vessels are highly dynamic structures, which undergo both morphological and functional changes under pathological conditions. The lymphatic network has been shown to play an important role in inflammatory and autoimmune diseases, cancer, lymphedema, graft rejection and wound healing [1,3,8]. One of the most striking changes of lymphatic vessels under inflammatory conditions is the strong proliferative expansion of the lymphatic network. Inflammatory lymphangiogenesis reportedly occurs in human psoriasis [9], rheumatoid arthritis [10], inflammatory bowel disease (IBD) [11,12], lymphedema [13], or transplant rejection [14,15]. Experimentally enhancing lymphangiogenesis was shown to ameliorate the disease course in animal models of skin inflammation [16,17], rheumatoid arthritis [18] and IBD [19]. On the other hand, blocking lymphangiogenesis exacerbated the disease state in rheumatoid arthritis [20], IBD [21] and in type I diabetes [22]. Furthermore, blocking lymphangiogenesis improved graft survival in models of corneal and pancreatic islet transplantation [23-25]. These exciting findings have suggested that lymphatic vessels might play a more important and active role in the initiation and resolution of inflammatory processes than previously assumed. Moreover, they have revealed that inflammatory lymphangiogenesis might be a promising new target for treating a variety of chronic inflammatory and immune-mediated disorders.

In this review we will provide a brief introduction of the lymphatic vascular system and then summarize current knowledge about inflammatory lymphangiogenesis. In particular, we will introduce its main molecular and cellular mediators and discuss the impact of inflammatory lymphangiogenesis on the morphology of the lymphatic network. Next we will address how inflammation-induced changes in the lymphatic network are thought to impact lymphatic function, namely leukocyte trafficking and fluid drainage. Finally, we will discuss the therapeutic implications of these findings.

Anatomy of the Lymphatic Vascular Network

Afferent lymphatic vessels begin in peripheral tissues as a branched network of blind-ended capillaries [2] (Figure 1). These capillaries converge into pre-collecting and collecting vessels, which are

connected to LNs. Collecting vessels that leave LNs, so-called efferent lymphatic vessels, may connect with further LNs [26] but eventually merge into the thoracic duct. The latter finally fuses with the subclavian vein, thereby connecting the lymphatic vascular system with the blood circulation [2]. Lymphatic endothelial cells (LECs) in capillaries differ from LECs in collectors in terms of gene expression and morphology. While LECs in capillaries express high levels of the lymphatic marker genes LYVE-1, Prox-1 andVEGFR-3, these genes are down-regulated in collector LECs [27,28]. Moreover, LECs in the capillaries are oak leaf-shaped cells that are connected by discontinuous button-like junctions [29] (Figure 1). These junctions give rise to overlapping flaps, also called primary valves, through which fluid and leukocytes enter lymphatic vessels [29,30]. Collector LECs, on the other hand, are elongated cells that are connected by continuous cell-cell junctions [29]. While lymphatic capillaries only have a thin, discontinuous basement membrane and are devoid of lymphatic muscle cell coverage, lymphatic collectors are surrounded by a continuous basement membrane and lymphatic muscle cells [27,31]. Moreover, collecting vessels contain intraluminal valves to prevent backflow of lymph. The valves divide the collecting vessels into functional units called lymphangions (Figure 1). Lymph is propelled from one lymphangion to the next through phasic contractions of the lymphatic muscle cells, as well as through contractions of the surrounding skeletal muscles and arteries [2,31].

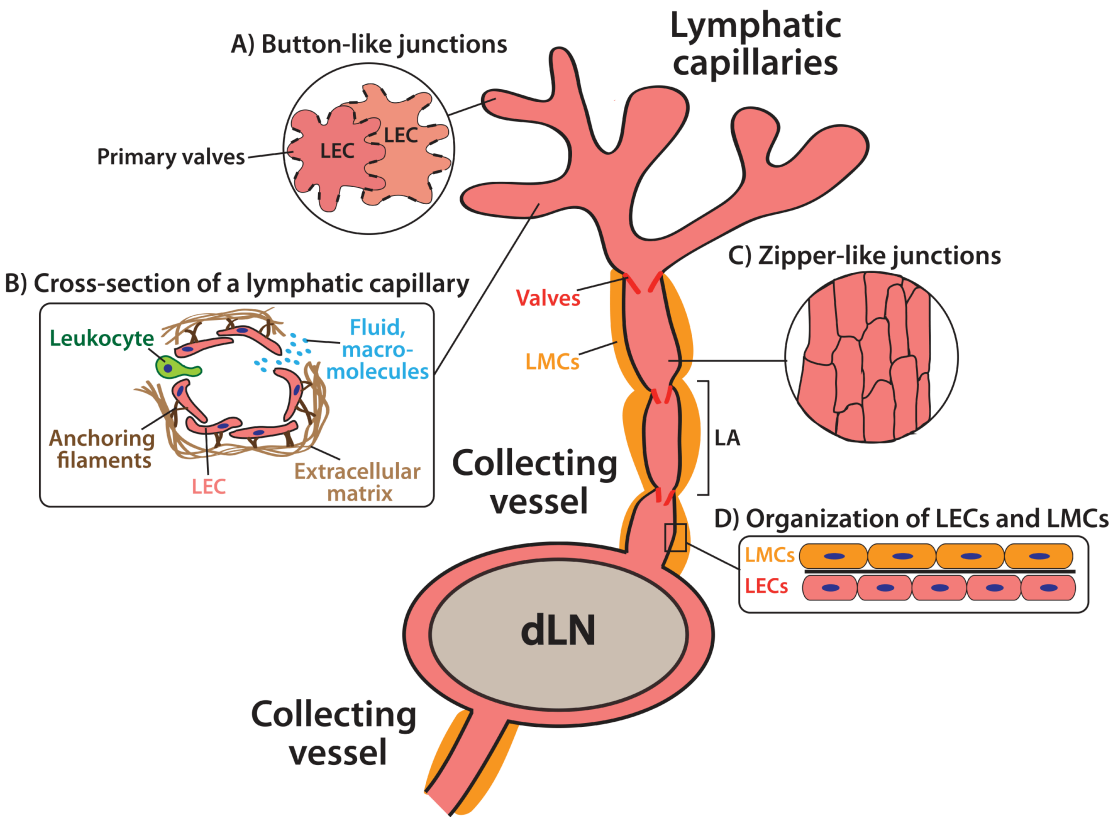

Figure 1: Organization of the lymphatic network. (a) Afferent lymphatic vessels begin as blind-ended capillaries in peripheral tissues. These then merge into collecting vessels, which feed into a dLN. Collecting vessels contain valves and are surrounded by smooth muscle cells (SMCs). The vessel segment between two valves is called lymphangion (LA). Lymph fluid and leukocytes leave the LNs through the efferent collecting vessel. At the level of the thoracic duct the lymphcontent is ejected into the blood vascular circulation. Inserts: (A) LECs in lymphatic capillaries are oak-leaf shaped. Neighboring cells partially overlap and are connected by discontinuous, "button-like" cell-cell junctions (thick black lines). This setup generates open flaps (primary valves) **(B)** The flaps account for the entry of fluids, macromolecules and leukocytes into lymphatic capillaries. Capillary LECs are connected with the extracellular matrix through filaments, which account for the rapid dilation of lymphatic vessels during tissue edema. **(C)** LECs in lymphatic collectors have an elongated shape. Neighboring LECs are connected by continuous, "zipper-like" cell-cell junctions. This organization makes the collecting vessels less permeable as compared to initial capillaries and well suited for conducting fluids. **(D)** LECs in collecting vessels are in intimate association with lymphatic muscle cells (LMCs) lymphatic muscle cell, which mediate lymphatic contractility.

Inflammatory Lymphangiogenesis

Numerous studies have shown that the lymphatic network in peripheral tissues and draining LNs (dLNs) undergoes dramatic expansion and morphologic changes in the context of inflammation. As in other biologic processes, the rate and nature of inflammatory lymphangiogenesis appear to be highly stimulus- and tissue-specific. Depending on the inflammatory stimulus, different molecular and cellular mediators of inflammatory lymphangiogenesis are involved. Consequently, the extent and morphologic characteristics of lymphatic vessel remodeling and its impact on lymphatic function may vary. In the following sections selected aspects of inflammatory lymphangiogenesis will be discussed in greater detail.

Cellular and molecular mediators of inflammatory lymphangiogenesis

Mediators of inflammatory lymphangiogenesis are either produced by leukocytes or by stromal cells. The best-characterized mediators of this process are vascular endothelial growth factor (VEGF)-A and VEGF-C / VEGF-D, which signal through VEGFR-2 and/or VEGFR-3 [32]. Particularly macrophages are considered an important source of VEGF-A and VEGF-C. Macrophages have been shown to drive lymphangiogenesis in the inflamed tissues [33-36] as well as in dLNs [34]. For example, in a mouse model of LPS-induced skin inflammation depletion of macrophages or blockade of VEGF-C/-D or VEGF-A greatly inhibited inflammation-induced lymphangiogenesis [34]. During pulmonary inflammation induce in mice by infection with *Mycoplasma pulmonis*, dendritic cells (DCs), macrophages and neutrophils were all shown to produce VEGF-C and VEGF-D [33]. More recently, neutrophils have also been implicated in inflammatory lymphangiogenesis occurring in mice upon induction of a contact hypersensitivity (CHS) response or upon immunization with Complete Freund's Adjuvant (CFA) and protein antigen. Under these conditions neutrophils were found to contribute to lymphangiogenesis by producing VEGF-D and by regulating the bioactivity of VEGF-A in tissues [37]. Besides leukocytes also non-immune cells, such as epithelial cells [33,38], keratinocytes [39], and fibroblastic reticular cells [37] have been identified as major sources of VEGF-A or VEGF-C.

In addition to VEGFR-2 and VEGFR-3 ligands, many other inflammatory mediators, such as LPS [40], IL-17 [41] orIL-8 [42] were shown to induce lymphangiogenesis *in vitro* and *in vivo* in animal models. Moreover, lymphotoxin (LTα and LTβ) have been implicated in inflammatory lymphangiogenesis, particularly in the context of tertiary lymphoid organ (TLO) formation [43,44]. Given the pleiotropic effects of most of these mediators, it is difficult to dissect whether their *in vivo* lymphangiogenic activity is mediated by direct action on LECs or indirectly through factors released by other cell types. Interestingly, some inflammatory cytokines have been shown to inhibit lymphangiogenesis. IFN-γ, which is mainly produced by T helper1 (Th1) and natural killer (NK) cells, was shown to inhibit lymphangiogenesis *in vitro* and *in vivo* [45,46]. Furthermore, inhibition of TGF-β was shown to induce lymphangiogenesis in a mouse model of peritonitis [47] and to improve lymphatic drainage in a murine lymphedema model [48]. Moreover, in a mouse model of lymphedema depletion of $CD4^+$T cells promoted lymphangiogenesis and resolution of edema, indicating that T-cell-derived products negatively impact lymphatic network formation and function [49]. Overall, inflammatory lymphangiogenesis appears to be regulated by the expression of both pro-and anti-lymphangiogenic factors, which are expressed from various cellular sources, depending on the nature of the inflammatory stimulus.

Lymphangiogenesis in peripheral tissues

To date three different mechanisms have been described by which the lymphatic network in peripheral tissues expands in the context of inflammation: This may be through dilation and proliferative expansion of existing vessels [50,51] or through sprouting of new vessels [34,36,52]. Moreover, it has been suggested that the lymphatic network may expand by incorporation and trans-differentiation of bone marrow (BM)-derived cells into the existing lymphatic network [14,35,53]. Interestingly, the lymphangiogenic mediators VEGF-A and VEGF-C/-D seem to induce different patterns of vascular remodeling: Adenoviral delivery of mouse VEGF-A_{164} or human VEGF-A_{165} or to the murine skin induced a strong dilation and proliferative expansion of existing vessels but little or no sprouting, respectively [50,51]. Conversely, VEGF-C and VEGF-D were shown to preferentially induce sprouting lymphangiogenesis [50]. On the other hand, constitutive or conditional overexpression of VEGF-C in the skin [54,55] or in lungs [56] was shown to induce giant lymphatic vessels. The latter findings indicates that – likely depending on the dose or duration and time-window of exposure - VEGF-C may additionally induce lymphatic dilation in addition to sprouting. Recently, some studies have suggested that inflammatory lymphangiogenesis may also involve the incorporation and trans-differentiation of BM-derived cells into LECs [14,35,53]. Macrophages have been reported to trans-differentiate into LECs in LPS-induced peritonitis [53] and corneal inflammation [35], and trans-differentiation of BM-derived cells into LECs has also been observed in human kidney transplants [14]. However, the occurrence, exact mechanism and general importance of trans-differentiation presently remain controversial.

Interestingly, inflammation-induced or VEGF-C-induced expansion of the lymphatic network in peripheral tissues like the lung [33,56-58] or skin [51,55,59] of mice appears to give rise to long-lived lymphatic vessels, which remain after inflammation has resolved or expression of pro-lymphangiogenic factors has subsided. By contrast, in the cornea lymphatic vessels were observed to partially regress after a first inflammatory episode but to regrow in an accelerated manner during recurrent inflammation [60]. The latter differences might be linked with the fact that the cornea – in contrast to skin or lung – under steady-state conditions is an avascular tissue [8]. Overall, these findings raise the intriguing possibility that the lymphatic vessel network in the peripheral tissues might develop some form of "lymphatic memory" to accelerate tissue drainage and immune induction in the case of recurrent inflammatory episodes.

In addition to the macroscopic expansion of the lymphatic network, lymphatic vessels also change at the cellular level in the context of inflammation: Yao et al. [58] reported that the characteristic button-like junctions in capillaries transform into zipper-like junctions in a mouse model of chronic airway inflammation. Zipper-like junctions are also seen in lymphatic sprouts of capillaries during embryonic development [29,58], indicating that the button-like arrangement represents a mature and differentiated state. Moreover, besides these junctional changes, down-regulation of LYVE-1, VEGFR-3 and Prox-1 has been reported in several studies of inflammation [33,61-63]. Thus, during certain inflammatory conditions, the lymphatic vessels appear to acquire a more collector-like phenotype. The functional implications of these changes are unknown, but they could influence fluid uptake and migration of leucocytes.

LN lymphangiogenesis

An abundant literature demonstrates that inflammatory lymphangiogenesis is not limited to the inflamed peripheral tissues but also occurs in dLNs [34,39,45,59,64-67] (Figure 2A and 2B). Interestingly, the proliferative expansion and structural changes induced in LNs appear to be more profound compared to lymphatic remodeling occurring in the inflamed tissue [34,39,59]. The magnitude of this response may be linked with the fact that LNs themselves are highly dynamic structures that rapidly change in size and cellular content. Although most studies to date have uniformly identified VEGF-A as the main driver of LN lymphangiogenesis, the cellular sources of this mediator may vary, depending on the inflammatory model and the time point analyzed. Particularly B cells have been identified as inducers of inflammatory LN lymphangiogenesis [37,64,68]. In a model of skin inflammation induced by immunization with CFA and protein antigen, VEGF-A-producing B cells were responsible for LN lymphangiogenesis occurring in the early phase of the immune response (i.e. day 1-4) [64]. Interestingly, a follow-up study using the same mouse model revealed that in absence of B cells neutrophils accounted for the induction of LN lymphangiogenesis at later stages of the inflammatory response [37]. Similarly, experiments in a mouse model of immunization with oxazolone showed that the lymphangiogenic response was strikingly delayed but still present in mice lacking B cells [68]. On the other hand, work from our group revealed that during oxazolone-induced CHSLN lymphangiogenesis was induced by VEGF-A that was drained to the LN via the lymphatic network from its site of production, i.e.by epidermal keratinocytes [39]. More recently also fibroblastic reticular cells in LNs have been shown to contribute to CFA-induced LN lymphangiogenesis by producing VEGF-A [66,67]. Besides pro-lymphangiogenic VEGF-A produced by various LN-resident cell types, also anti-lymphangiogenic IFN-γ derived from T cells was shown to regulate LN lymphangiogenesis [45,46]. In fact, the data suggest that the extent of T cell activation occurring in the context of inflammation may impact the LN lymphangiogenic response [45]: Specifically, inflammation induced by dermal injection of the T cell activating mitogen concanavalin A was accompanied by only weak LN lymphangiogenesis, whereas inflammation induced by dermal LPS injection resulted in profound LN lymphangiogenesis in mice [45].

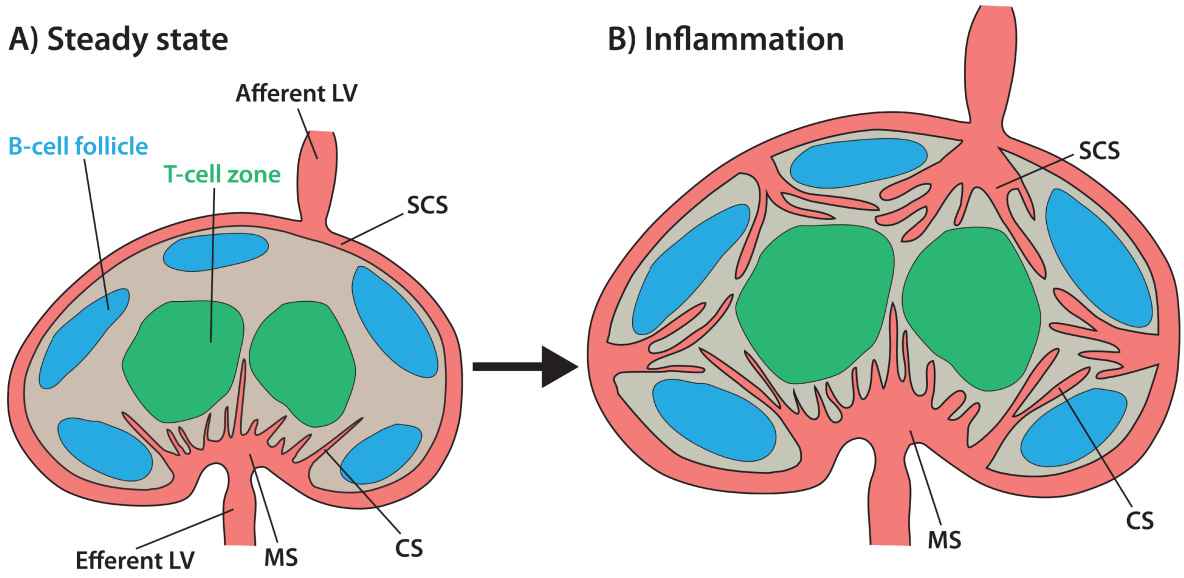

Figure 2: Lymphatic network in LNs at steady-state and in inflammation. (A) Steady-state condition: The lymphatic network in LNs is organized into the subcapsular sinus (SCS), the medullary sinus (MS) and the cortical sinuses (CS). B cell areas (blue) and T cell zones (green) are shown. **(B)** Inflammation leads to a massive expansion of the SCS, MS and CS. Pro-lymphangiogenic (+) and anti-lymphangiogenic (-) factors produced by B and T cells respectively, impact the strength of the LN lymphangiogenic response.

Interestingly, Tan et al. found that the pattern of lymphangiogenesis in CFA-induced inflammation displayed remarkable spatial and temporal differences [66]. While the lymphatics of the subcapsular sinus (SCS) already expanded during the early phase of the inflammatory response, the cortical and medullary sinuses only expanded at later time points of inflammation. Functional data indicate that the temporal and spatial differences in LN lymphangiogenesis might serve to sequentially modulate DC and T cell migration to and from LNs, respectively. Notably, DCs arriving in the LN through afferent lymphatic vessels typically enter the node by traversing the SCS [26] (Figure 2A). By contrast, T cells egress from LNs by migrating across the cortical and medullary sinuses [26]. Blockade of VEGFR-2/VEGFR-3-mediated lymphangiogenesis during early time points of inflammation was shown to reduce both lymphangiogenesis in the SCS and DC migration to dLNs in mice [64]. By contrast, at later time points, blockade of VEGFR-2/VEGFR-3

inhibited lymphangiogenesis in the cortical and medullary sinuses and reduced T cell egress from LNs [66]. These findings suggest that LN lymphangiogenesis may serve to fine-tune leukocyte migration and the induction of immune responses in LNs draining sites of inflammation. In line with such a regulatory function, it was found that LN lymphangiogenesis is a transient phenomenon: As the inflammation and the induction of adaptive immunity progresses and eventually resolves, lymphatic vessels in LNs regress back to normal [34,35,45].

Changes in LEC Gene Expression: Impact on Leukocyte Trafficking

From blood vessels it is well known that inflammation-induced changes in gene expression, in particular up-regulation of chemokines and adhesion molecules, regulate the recruitment of leukocytes to sites of inflammation. Similarly, inflammatory stimuli have been shown to profoundly alter the expression of cell adhesion molecules and chemokines in LECs. ICAM-1, VCAM-1, and L1CAM are LEC-expressed adhesion molecules that are upregulated under inflammatory conditions and mediate DC migration into lymphatic vessels [61,69,70]. ICAM-1 and VCAM-1 have also been implicated in DC crawling within lymphatic capillaries [71]. During steady state, however, expression of ICAM-1 and VCAM-1 on LECs is very low and DCs migrate independently of these molecules [72]. In addition to their role in leukocyte trafficking, some inflammation-induced adhesion molecules, such as the integrins $\alpha_5\beta_1$ [73] and $\alpha_1\beta_1$ [74] or ALCAM [75], were shown to support lymphangiogenesis.

Besides adhesion molecules also chemokines are important for leukocyte trafficking through lymphatic vessels. The best-characterized chemokine involved in this process is CCL21 [5,76]. CCL21 is constitutively expressed by lymphatic vessels and mediates the migration of CC-chemokine receptor 7 (CCR7) expressing DCs, T cells, and neutrophils towards and into lymphatic vessels [5,76,77]. A considerable fraction of CCL21 is stored in intracellular vesicles in LECs [61,78], but CCL21 may be rapidly released *in vitro* upon treatment with TNF-α [78]. CCL21 expression is reportedly upregulated *in vivo* in the context of inflammation [61,79] and under conditions of increased lymph flow [80]. Although DC migration to dLNs is highly CCR7-dependent in both steady-state and in inflammation [61], other inflammation-induced chemokines in LECs were shown to contribute to this migratory step. Specifically, CXCL12 and CX3CL1 are upregulated in lymphatic vessels during inflammation and support DC migration to dLNs [81,82].

Besides CCL21, CXCL12 and CX3CL1 many other inflammatory chemokines are strikingly upregulated in LECs during ongoing inflammatory responses [61,69,83,84]. Performing a gene expression analysis of LECs isolated from inflamed and resting murine skin we observed that the response of LECs to inflammation is highly stimulus-specific [61]. For example, chemokines like CCL8, CXCL9 or CXCL10 and ICAM-1 were strongly upregulated in inflammation induced by a CHS response to oxazolone but only weakly upregulated during inflammation induced by CFA injection into the skin [61]. A similar stimulus-specific chemokine expression pattern was also observed in *in vitro* studies when incubating LECs with different TLR ligands [83,84]. It is tempting to speculate that such stimulus specific responses might serve to fine-tune tissue exit and migration of specific leukocyte subsets into lymphatic vessels. However, besides the involvement of CXCL12 and CX3CL1 in DC migration [81,82], only little evidence exists so far for the role of other inflammation-induced chemokines in leukocyte trafficking through lymphatic vessels. During LPS-induced peritonitis LEC-derived chemokines like CCL2, CCL5 or CX3CL1 supposedly induced the association of VEGF-C-producing peritoneal macrophages with lymphatic vessels, thereby accounting for enhanced lymphangiogenesis and macrophage trafficking to dLNs [40]. Moreover, it was recently reported that lymphocyte egress from chronically inflamed murine skin was only partially dependent on CCR7 and completely independent of CXCR4 expression but sensitive to treatment with pertussis toxin [85]. These data indicate that, in addition to the CCL21/CCR7 signaling, other unknown chemotactic stimuli participate in lymphocyte egress from chronically inflamed tissues.

In addition to secreting chemokines, lymphatic vessels also actively contribute to removing chemokines from inflamed tissues. In response to inflammation LECs have been shown to upregulate the chemokine scavenging receptor D6, which binds, internalizes and degrades most CC-chemokine ligands [86]. Analysis of D6-deficient mice has revealed a crucial role for this receptor in the resolution of tissue inflammation [86-88]. In fact, D6 appears to function by constantly removing inflammatory chemokines and allowing for a selective presentation of CCL21 on lymphatic vessels. In absence of D6, a strong accumulation of myelomonocytic cells around lymphatic vessels was observed, what impeded cell trafficking and fluid flow to dLNs [87]. Interestingly, also the expression of other chemokine scavenging receptors on LECs has recently been identified [89,90], but the role of these receptors in the inflammatory response of lymphatic vessels has not been elucidated to date.

Functional Implications of Inflammatory Lymphangiogenesis

It is still unclear whether inflammatory lymphangiogenesis is entirely a protective response that contributes to the resolution of inflammation, or whether it forms part of its pathology. For example, in human IBD the density of lymphatic vessels was shown to correlate with disease severity [11]. At the same time, histologic and imaging studies in human patients have described lymphatic dysfunction and lymphatic obstruction as a characteristic feature of IBD [91-93]. Moreover, VEGFR-3-mediated blockade of lymphangiogenesis was recently shown to exacerbate inflammation in a mouse model of IBD [21], whereas disease amelioration was observed upon VEGF-C-dependent stimulation of lymphatic function [19]. Together, these findings indicate that lymphangiogenesis may have a protective effect in IBD, and that lymphatic vessel dysfunction might even contribute to disease development. On the other hand, highly increased lymphatic vessel densities have been recognized as a hallmark of rejected human kidney transplants [14,15]. Moreover, animal studies have shown that blockade of lymphangiogenesis is a potent strategy for securing allograft survival [14,15,22-25,94]. These findings argue that lymphangiogenesis occurring in transplanted tissues does not reduce but rather contributes to inflammation and the rejection process. Overall, the two examples indicate that the functional significance of lymphangiogenesis might be different, depending on the organ analyzed and the type of inflammation and immune response induced. From an immunologic point of view it seems relevant to distinguish between two different scenarios during which inflammatory lymphangiogenesis typically occurs: This can be (i) in tissues with pre-existing lymphatic immune connectivity, i.e. with a functioning lymphatic network, such as the skin, the intestine or the lung. Alternatively, inflammatory lymphangiogenesis may occur in (ii) tissues with non-existing lymphatic immune connectivity, namely in

transplanted organs or in immune-privileged tissues with no pre-existing connectivity with dLNs.

(i) Tissues with pre-existing lymphatic immune connectivity

Inflamed tissues generally become edematous due to increased fluid influx from blood, which exceeds the drainage capacity of the lymphatic vessel network. It is likely that inflammatory lymphangiogenesis serves to adapt the lymphatic network to meet the enhanced demand for removing fluids, inflammatory mediators, and leukocytes from the inflamed tissue. Removal of antigen-presenting cells (APCs) from the inflamed site is thought to contribute to the resolution of inflammation [34,95,96]. Moreover, lymphatic drainage contributes to dampening tissue inflammation. This is best exemplified by lymphedema, a condition in which lymphatic drainage is defective or malfunctioning [1]: Lymphedema not only results in the accumulation of fluids but also of leukocytes and inflammatory mediators in the tissue, leading to tissue inflammation, as was shown in both animal and human studies [13,97]. By now several studies have demonstrated that blockade of inflammatory lymphangiogenesis, e.g. by targeting the VEGF-C/VEGFR-3 axis, aggravated tissue inflammation in various models of immune-mediated, chronic inflammatory disorders. For example, in mouse models of skin inflammation blockade of VEGFR-3 reduced lymphatic drainage and delayed resolution of inflammation [16,34,63]. Similarly, anti-VEGFR-3 treatment worsened edema formation and tissue inflammation in mouse models of IBD[21], rheumatoid arthritis [20], or infection-induced airway inflammation [33]. On the other hand, stimulation of lymphangiogenesis through activation of the VEGFR-3 pathway was shown to accelerate the resolution of chronic and acute skin inflammation [16,17,98], to attenuate joint damage in a mouse model of arthritis [18], or to ameliorate IBD symptoms [19]. Interestingly, in contrast to the documented anti-inflammatory effects of VEGF-C, VEGF-A appears to exacerbate inflammation [16,63,99]. This may be due to different effects of VEGF-A and VEGF-C on the remodeling of the lymphatic network [16,50,51], as well as the well-known pro-inflammatory and permeability-enhancing activity of VEGF-A on blood vessels [1,16,32,63,100].

(ii) Tissues with non-existing lymphatic immune connectivity

In tissues with pre-established lymphatic immune connectivity (e.g. skin, lung, intestine), the lymphatic network in steady-state is thought to play a major role in maintaining immune tolerance, by constantly transporting self-proteins to dLNs [4,101]. Moreover, several studies have shown that LECs in LNs fulfill antigen-presenting functions and thereby directly contribute to tolerance induction [4]. This may explain why in the context of tissues with pre-existing lymphatic immune connectivity lymphangiogenesis seems to generally exert a beneficial, anti-inflammatory effect, due to its drainage-enhancing capabilities. The situation appears to be different in tissues with non-existing lymphatic immune connectivity, such as in transplanted organs, where connectivity with dLNs is only established by inflammatory lymphangiogenesis. Under these conditions, inflammatory lymphangiogenesis accelerates the arrival of alloantigen and alloantigen-presenting leukocytes in dLNs, leading to the induction of adaptive immunity and eventually to graft rejection. Several human and animal studies have investigated the involvement of inflammatory lymphangiogenesis in graft rejection [14,15,22-25,94,102]. Particularly in mouse studies in the cornea it was shown that blockade of inflammatory lymphangiogenesis was an effective strategy for reducing APC migration to dLNs and retarding the graft rejection process [8,23,24]. However, the cornea might be a special tissue, since it lacks blood vessels and lymphatic vessels in the uninflamed state and therefore can be considered an immune-privileged tissue [8]. Similar findings are now also emerging for pancreatic islets, which normally also lack lymphatic vessel but display a strong lymphangiogenic response during transplantation [25,102] or during autoimmune attack [22]. Blockade of lymphangiogenesis was shown to enhance the survival of pancreatic islets allografts transplanted under the kidney capsule in mice [25]. Moreover, blockade of lymphangiogenesis was shown to delay the onset of diabetes in a mouse model of experimentally induced autoimmune diabetes [22]. Similarly, adenoviral treatment with a VEGFR-3-Fc fusion protein was shown to reduce heart allograft rejection [94].

Impact of Inflammation on Lymphatic Drainage Function

Inflammation leads to a rapid influx of fluid into the interstitial space and results in tissue swelling. Since capillary LECs are connected by filaments with the extracellular matrix [103] (Figure 1), tissue swelling leads to the opening of the primary valves, resulting in interstitial fluid influx and dilation of lymphatic vessels [104]. Intuitively, one would assume that inflammation increases the drainage rate of lymphatic vessels. However, both enhanced and reduced drainage rates have been reported in different animal models of acute and chronic inflammation. For example, lymphatic drainage was reduced during acute and persistent CHS-induced skin inflammation [63,105,106]. Moreover, impaired lymphatic drainage and increased leakiness of lymphatic vessels was also observed in UVB-induced skin inflammation [99]. On the other hand, increased lymphatic flow was observed during intestinal inflammation in rats upon treatment with N-formyl-methionyl-leucyl-phenylalanine (fMLP) [107] or in guinea pigs treated with LPS [108]. Moreover, lymphatic drainage was enhanced in a mouse model of atopic dermatitis caused by dermal overexpression of IL-4 [109]. Thus, inflammation appears to alter lymphatic drainage function in a stimulus-dependent manner. In many cases the inflammation-induced changes in lymphatic drainage have been observed to occur very rapidly i.e. in the course of several minutes to hours after applying an inflammatory stimulus [63,99,105-108]. Such rapid responses are less likely explained by profound morphological changes induced by inflammatory lymphangiogenesis. More likely, these changes in lymphatic drainage are caused by immediate effects of the inflammatory stimuli on the vasculature; namely, on the vessel-forming LECs or on lymphatic muscle cells, which control lymphatic pumping (Figure 1).

Effect of inflammation on LEC barrier function

LECs in lymphatic capillaries and collectors express the same junctional proteins, but the organization of cell-cell junctions is different (Figure 1). In both capillaries and collectors VE-cadherin, which is crucial for maintaining endothelial cell barrier function [110], co-localizes with tight junction proteins like ZO-1, claudin-5, JAM-A or ESAM [29]. While these molecules form continuous, zipper-like junctions in collectors, VE-cadherin and tight junction proteins only display a punctuate expression pattern in oak leaf-shaped capillary LECs, thereby giving rise to the characteristic open flaps [29] (Figure 1). To date only few studies have investigated how inflammatory

mediators affect lymphatic vessel permeability *in vivo* [51,99,111,112]. Far more studies have addressed this in *in vitro* experiments performed with LEC monolayers. LECs cultured in monolayers form continuous cell-cell junctions [111], which resemble the setup observed in collecting lymphatic vessels. This indicates that *in vitro* experiments rather model the barrier function of collecting vessels than fluid transport across lymphatic capillaries. Many inflammatory and lymphangiogenic mediators modulate LEC barrier function *in vitro* [99,113-116]. Besides thrombin and histamine [113] alsoVEGF-A [111], VEGF-C [116], inflammatory cytokines like TNF-α, IL-1β, IL-6, IFN-γ, or LPS [114,115] increase permeability of LEC monolayers. A recent study revealed that the permeability-inducing effect of most inflammatory cytokines was largely nitric oxide (NO)-dependent and accompanied by down-regulation of VE-cadherin protein levels in LECs [115]. Moreover, inflammatory cytokines increased the amount of phosphorylated myosin light chain 20 in LECs [115], which is indicative of enhanced cellular actin-myosin contractility and enhanced permeability [110,115]. Interestingly, also transmural flow was shown to increase the *in vitro* permeability of LEC monolayers, by inducing the down-regulation and internalization of VE-Cadherin and PECAM-1 [80]. On the other hand, adrenomedullin, a peptide with anti-inflammatory and lymphangiogenic activity, was shown to prevent the VEGFA-mediated increase in LEC permeability by stabilizing VE-cadherin and ZO-1 at cell–cell junctions of LECs [111].

Effect of inflammation on lymphatic muscle cells

Lymphatic muscle cells surround the collecting lymphatic vessels, and their phasic contractions result in the propulsion of lymph from one lymphangion to the next (Figure 1). The lymph volume pumped through the lymphatic vessels depends on the frequency and strength of the contraction [31]. Inflammatory cells as well as inflammatory mediators have been shown to affect the contractility of lymphatic muscle cells [31,107,112,117-121]. For example, prostaglandin and prostacyclin were shown to decrease the pumping rate of lymphatic collectors [117]. Similarly, systemic or intradermal injection of IL-6, IL-1β, and TNF-α resulted in decreased lymph pumping frequency and flow velocity, as assessed in *in vivo* near-infrared-imaging studies in mice [118]. By contrast, substance P [119], a neuropeptide secreted by inflammatory cells, as well as the pro-lymphangiogenic factor VEGF-C [112] and fMLP [107] were shown to increase lymphatic pumping of rat mesenteric vessels. Moreover, NO produced by inflammatory cells was shown to profoundly affect lymphatic pumping: In a mouse model of oxazolone-induced skin inflammation Liao et al. observed that NO production by CD11b+Gr-1+ inflammatory cells, which accumulated on collecting lymphatic vessels, accounted for reduced lymphatic pumping activity [65]. On the other hand, also histamine [120] or NO [121] released from activated mast cells have been identified as modulators of lymphatic contractility.

In conclusion, inflammatory and lymphangiogenic mediators may not only affect lymphatic drainage by inducing morphologic changes in the lymphatic network, but may also acutely impact drainage by modulating LEC barrier function or lymphatic pumping. Intriguingly, the above-mentioned examples reveal that many mediators (e.g. VEGF-C [112], histamine [113,120], NO [65,115,121] or inflammatory cytokines [115,118]) simultaneously act on both LECs and lymphatic muscle cells.

Inflammatory Lymphangiogenesis: Therapeutic Implications

There is growing appreciation that strategies attempting to enhance rather than to block lymphangiogenesis and lymphatic drainage function may represent an effective therapeutic approach for inflammatory disorders like rheumatoid arthritis [20,122], Morbus Crohn [19,21] or psoriasis [16,63], i.e. inflammatory disorders occurring in tissues with pre-established lymphatic immune connectivity. Particularly delivery of VEGF-C into inflamed skin was shown to enhance drainage and to reduce inflammatory symptoms in a murine model of chronic skin inflammation [16,17]. In addition, systemic adenoviral VEGF-C delivery enhanced drainage and reduced joint damage in a mouse model of arthritis [18] and ameliorated disease conditions in a mouse model of IBD [19]. The anti-inflammatory effects of VEGF-C have so far mainly been attributed to direct stimulation of lymphangiogenesis and lymphatic function in LECs. However, also certain leukocytes, such as certain DCs, tissue macrophages or monocytic cells in blood, have been shown to express VEGFR-3 [19,123-125]. In fact, recent experimental findings indicate that VEGF-C may also exert anti-inflammatory activity by directly acting on these cell types [19,125]. In a mouse model of sepsis VEGF-C acting on VEGFR-3-expressing activated macrophages was shown to potently suppress the inflammatory response induced by intraperitoneally administered LPS [125]. Similarly, in mouse models of IBD, VEGF-C was shown to activate anti-inflammatory responses in intestinal macrophages [19]. Thus, it is important to bear in mind that the anti-inflammatory effects of VEGF-C may not be exclusively mediated by its action on lymphatic vessels.

Thus far, neither VEGF-C nor any other pro-lymphangiogenic mediator has entered clinical development. In the context of infection-induced inflammatory disorders, a potential concern of pro-lymphangiogenic treatments might be that enhanced lymphatic drainage and lymphangiogenesis might over-run the filtering and immune surveillance function of dLNs and facilitate systemic spread of pathogens. Clearly, more preclinical experiments are required to demonstrate the therapeutic potential and safety of pro-lymphangiogenic approaches for the treatment of chronic inflammatory and autoimmune diseases. Besides the VEGF-C/VEGFR-3 axis also other recently identified pro-lymphangiogenic signaling pathways may be interesting to investigate as potential therapeutic targets [111,126,127].

In the context of transplant rejection several preclinical studies have revealed a clear benefit of anti-lymphangiogenic treatments for preventing graft rejection, particularly in mouse models of corneal transplantation [8,23,24] or in pancreatic islet transplantation [25]. Furthermore, blockade or VEGFR-3 was able to prolong cardiac allograft survival in mice by reducing immune cell trafficking due to decreased CCL21 production [94]. However, the promising results observed might not be generalizable for all types of transplantation. Several studies have shown that allo-responses to transplanted organs are not exclusively induced in dLNs [128]. In some cases, priming occurs in distant secondary lymphoid organs like the spleen, through APCs that have exited the graft by entry into blood vessels [128]. Thus, blocking lymphangiogenesis will likely only proof to be a useful therapeutic approach in transplant situations, in which DC migration through lymphatic vessels represents the major route for the induction of allo-responses.

Conclusion and Outlook

Over the past 10 years major progress has been made in our understanding of inflammatory lymphangiogenesis and of the general role of lymphatic vessels in the development and resolution of inflammatory responses. Research has revealed that lymphatic vessels form a highly dynamic network, which rapidly responds to tissue inflammation in a stimulus and tissue-specific manner. Moreover, the functional significance of inflammatory lymphangiogenesis appears to be highly context specific, as evidenced by the either pro- or anti-inflammatory effects observed, depending on the tissue analyzed. Several studies performed in animals have identified inflammatory lymphangiogenesis as a potential therapeutic target for modulating inflammatory and immune-mediated responses. Although promising results have been achieved in these preclinical disease models, a lot remains to be investigated to better understand the exact mechanism(s) through which lymphatic vessels and inflammatory lymphangiogenesis participate in the regulation of tissue inflammation and immune induction in LNs.

The emerging picture suggests that the role of the lymphatic network in the regulation of immune responses goes beyond transporting antigen, inflammatory mediators, and leukocytes between peripheral tissues and LNs [4].In fact, several studies are now documenting the direct involvement of LN LECs in dampening the immune response and in tolerance induction [4]. Amongst the inflammatory changes occurring in the lymphatic network, LN lymphangiogenesis probably is least well characterized and functionally understood. In the future, it will be important to further understand how inflammation-induced LN lymphangiogenesis and inflammation-induced gene expression changes in LN LECs impact antigen-presentation and tolerance induction.

Although the lymphatic network typically undergoes a proliferative expansion in both peripheral tissues and dLNs in the context of inflammation, this does not always seem to produce functional lymphatic vessels that enhance tissue drainage. Interestingly, even VEGF-C which is generally considered a potential therapeutic target for stimulating productive lymphangiogenesis and lymphatic function, may give rise to a dysfunctional lymphatic network: Overexpression of VEGF-C in the lungs of neonatal mice -but not in adult mice - induced a disease condition reminiscent of human pulmonary lymphangiectasia [56]. Not so surprisingly, this indicates that the dose, the tissue environment and the time-point at which exposure to VEGF-C occurs may influence the extent of productive as compared to pathologic lymphangiogenesis. Recent studies have also revealed that the anti-inflammatory effects of VEGF-C may not only be mediated by direct effects on lymphatic vessels, but also involve activation of VEGFR-3-expressing leukocytes [19,125]. In light of these findings it will be interesting to further dissect the exact mechanism(s) by which VEGF-C and pro-lymphangiogenic treatment reduce inflammation. It is likely that the relative contribution of leukocytes may vary, depending on the type of inflammation induced and the tissue studied.

With regards to potential therapeutic applications, it will also be important to identify more lymphangiogenic factors, which preferentially stimulate lymphatic vessels yet do not induce angiogenesis in blood vessels. In fact, to date only few lymphatic-specific growth factors have been identified. Moreover, it may be desirable to identify molecules that do not lead to a vast proliferative expansion of the lymphatic network, but rather to an enhancement of lymphatic vessel integrity and drainage function. This could be accomplished by molecules acting on LECs or also on the LEC-surrounding lymphatic muscle cells. Clearly, a lot remains to be investigated to better understand how lymphatic drainage function is regulated in the context of inflammation. In this regard, the advent of new live imaging methodologies, which allow to study inflammatory lymphangiogenesis and drainage in a longitudinal manner [100,118,129], are expected to contribute to a better understanding of the complex relationship between inflammation and lymphatic function.

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