Review

Regulation and implications of inflammatory lymphangiogenesis

Honsoul Kim[1,2*], Raghu P. Kataru[1,2] and Gou Young Koh[1,2,3]

[1] National Research Laboratory of Vascular Biology and Stem Cells, Korea Advanced Institute of Science and Technology, Daejeon, Korea
[2] Graduate School of Medical Science and Engineering, Korea Advanced Institute of Science and Technology, Daejeon, Korea
[3] Graduate School of Nanoscience and Technology (WCU), Korea Advanced Institute of Science and Technology, Daejeon, Korea

Lymphatic vessels (LVs) are highly dynamic structures that intimately interact with their surrounding microenvironment. They have a profound influence on the immune system and therefore can manipulate inflammatory processes. Inflammation is a major cause of adulthood lymphangiogenesis and LV remodeling. In turn, LVs can reciprocally manipulate inflammatory processes. For instance, LV growth and/or activation regulate antigen presentation and inflammatory cell recruitment to lymph nodes (LNs), and therefore critically affect adaptive immunity. The vascular endothelial growth factor (VEGF)-C–VEGF receptor-3 and VEGF-A–VEGF receptor-2 signaling pathways are particularly important in inflammatory lymphangiogenesis. LVs contribute to the pathophysiology of various inflammatory conditions. Knowledge of lymphatic biology can be applied to manipulate inflammatory disorders and divert immune responses. This review summarizes basic concepts of inflammation-relevant lymphatic biology, and describes recent progress and practical implications.

Reciprocal control of inflammation and lymphangiogenesis

Accumulating evidence suggests that LVs are versatile structures that actively respond to the tissue microenvironment. Recently, significant progress in our knowledge has been achieved on how various internal and external stimuli influence and regulate the growth, structure and function of LVs. In turn, LVs have profound influence on the immune system and therefore can significantly regulate certain biological processes, such as inflammation. LVs are tubular structures composed of monolayered lymphatic endothelial cells (LECs) and some surrounding mural cells. They comprise a one-way network that drains and transports extracellular fluid, macromolecules, and cellular components from the peripheral tissue into the systemic circulation. Under physiological conditions, the lymphatic system regulates tissue fluid homeostasis, immune surveillance, and absorption of dietary fats. Additionally, growth of new LVs (lymphangiogenesis) is involved in various pathological conditions, including inflammation, wound healing, and tumor metastasis [1–3].

Inflammation is a complex reaction against injurious agents such as microbes and damaged cells. Typically, vascular responses, migration and activation of leukocytes, and systemic responses occur during inflammation. These reactions serve to destroy, dilute, or wall off the injurious agent, and simultaneously activate a cascade of events that try to heal and reconstitute the damaged tissue. Therefore, the inflammatory response is basically a protective reaction that is deeply associated with tissue repair. Nevertheless, inflammation can be potentially harmful. As reflected by its cardinal signs (namely redness, pain, heat, swelling, and loss of function), inflammation is frequently an extremely dynamic and violent process [4,5]. Lymphangiogenesis is often observed amid various types of inflammation, and accumulating evidence implies that LVs are directly and/or indirectly associated with inflammatory and/or repair processes [1,6,7].

LECs have specialized architecture that is highly efficient to transport fluid and cellular components. They form a monolayer superficial to an incomplete basement membrane [3], and therefore LECs are exposed to the intraluminal environment as well as the extracellular matrix. As terminally differentiated cells that are distinct from blood endothelial cells, LECs strictly require a proper microenvironment to maintain their original features and capacities [8]. In this regard, LECs are significantly influenced by physiological and/or pathological alterations of the microenvironment. Therefore, inflammatory disorders can affect LVs, but *vice versa* is also possible and the regulation between LVs and inflammation should be considered as a reciprocal process. In the present review, we focus on the characteristics, regulation and response of LVs with regard to inflammatory processes that have been described in the recent literature.

Normal lymphangiogenesis during development and adulthood

During the embryonic period, the formation of LVs is initiated with the expression of homeobox transcriptional factor Prox-1 in a subset of venous endothelial cells of the cardinal vein. The Prox-1-expressing LECs migrate and establish the primary lymph sac that is later remodeled into a delicate LV network. Mature LECs express markers such as Prox-1, VEGF receptor-3, lymphatic vessel endothelial hyaluronan receptor (LYVE)-1 and podoplanin, which are frequently used to identify LVs [3,4,9]. VEGF-C and its corresponding

Corresponding author: Koh, G.Y. (gykoh@kaist.ac.kr)
* Current affiliation: Department of Radiology, Research Institute of Radiological Science, Yonsei University Health System, Seoul 120-752, Korea.

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VEGF receptor-3 axis is the most validated key signaling pathway for lymphangiogenesis [3,10]. VEGF-D is another ligand that can activate VEGF receptor-3, but at least during development is rather functionally redundant with VEGF-C [11]. The VEGF-A–VEGF receptor-2 axis is also important for normal lymphangiogenesis, however, it is not clear whether its effect is direct or secondary [9]. Fibroblast growth factor 2, platelet-derived growth factor B, and hepatocyte growth factor are also known to stimulate lymphatic growth [9,12].

In adulthood, lymphangiogenesis is known to occur only during certain pathological circumstances such as tissue repair, inflammation and tumor-related conditions [7]. Lymphangiogenesis was previously thought to only proceed via sprouting of pre-existing LVs. However, it has recently been debated whether lymphangiogenesis exclusively depends on local proliferation of pre-existing LECs (lymphangiogenesis) or if circulating endothelial progenitors can directly contribute (so-called, lymphovasculogenesis) [3,13,14]. Transdifferentiation of macrophages into LECs has been described in human transplanted kidney [15] and in a mouse corneal injury model [16]. Recently, a bone marrow-derived podoplanin$^+$ subpopulation was described as participating in postnatal lymphatic neovascularization [17]. Notably, all three studies describing lymphovasculogenesis via bone marrow-derived cells [15–17] were performed under inflammation-relevant conditions. Nevertheless, the notion of lymphovasculogenesis is not yet widely accepted, and further research is necessary to establish it as a real phenomenon.

During inflammation, lymphangiogenesis can be observed in the inflamed peripheral tissue (extranodal LVs; ENLVs) and its draining LNs (intranodal LVs; INLVs). Many characteristics of ENLVs and INLVs overlap; however, with regard to inflammation, the responses of these LVs can be considered in separate categories. Based on their anatomical distribution, ENLVs function as relatively simple conduits, whereas, INLVs act as bottleneck filters that converge afferent flows from multiple directions. Moreover, INLVs are specialized tissues where vigorous immune reactions take place. They are heavily populated with various immune cells and, consequently, become subjected to a much tenser microenvironment that markedly differs from that of the extranodal lymphatic system (Figure 1).

ENLVs

LVs are particularly abundant in the skin and mucous membranes, which are the primary tissues exposed to

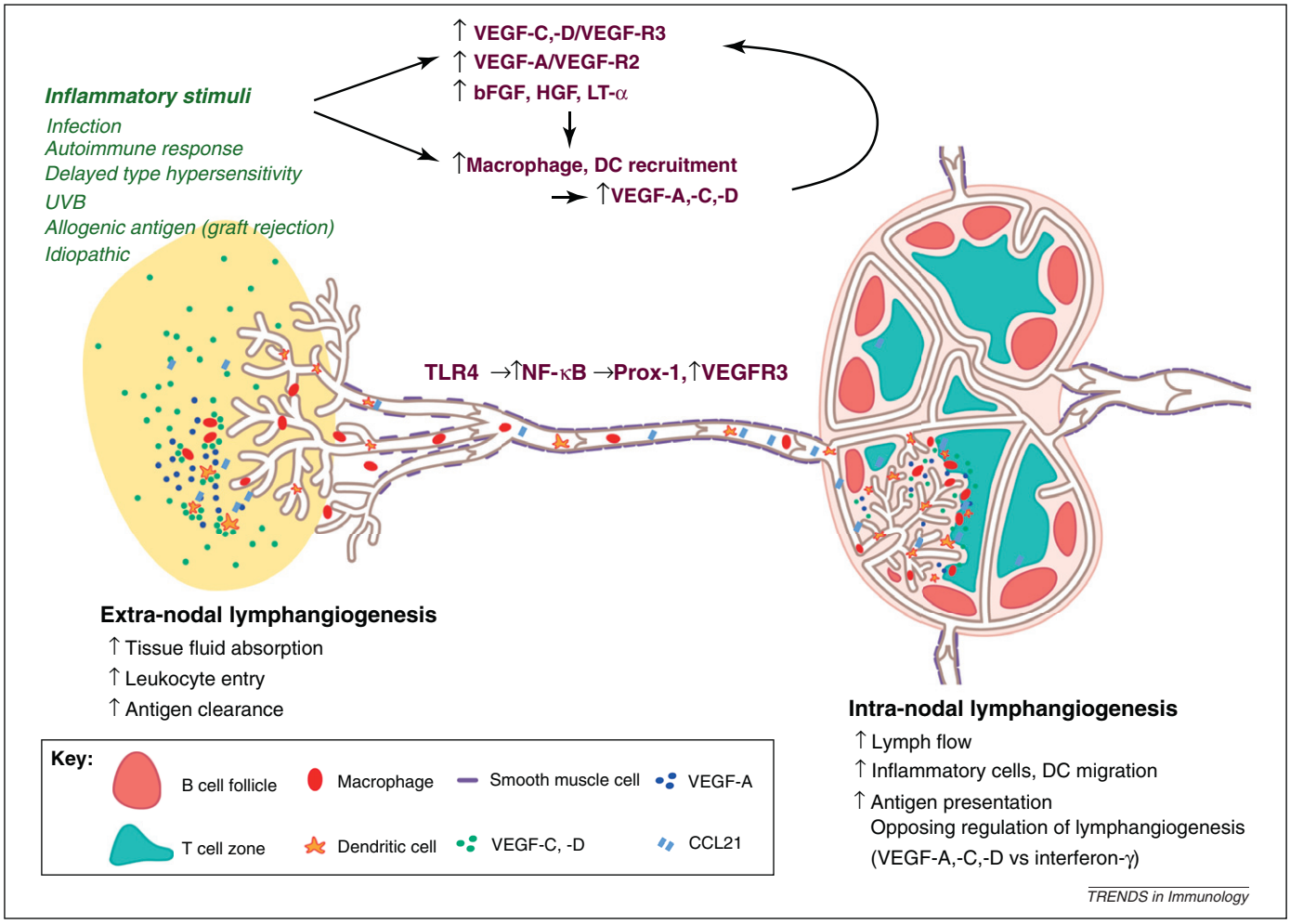

Figure 1. Inflammatory stimuli stimulate extranodal and intranodal lymphangiogenesis. Various inflammatory stimuli in the peripheral tissue trigger cascade events that mainly involve recruitment of leukocytes, secretion of prolymphangiogenic growth factors (VEGF-C, -D and -A), activation of VEGF receptor-3 and VEGF receptor-2 signaling pathways in lymphatic endothelial cells (LECs). CCL21 gradient serves to recruit dendritic cells (DCs) into draining lymph nodes (LNs). This results in lymphangiogenesis of the peripheral tissue (extranodal lymphangiogenesis) and LN (intranodal lymphangiogenesis), which mediate tissue fluid absorption, lymph flow, leukocyte migration, antigen clearance and antigen presentation.

foreign antigens. At stable state, the structure of the ENLV is microscopically optimized to regulate the passage of immune cells along with tissue fluid. The lymphatics are covered by a discontinuous basement membrane, especially in the initial lymphatics that serve as entry sites for inflammatory cells. Penetration of the LEC layer occurs through flap valves that lack continuous intercellular junctions, allowing communication between the interstitium and lymphatic lumen with relatively little resistance [18]. Overlapping flaps at borders of oak leaf-shaped LECs of initial lymphatics lack junctions at the tip but are anchored on the sides by discontinuous button-like junctions (buttons) that differ from the conventional, continuous, zipper-like junctions (zippers) found in collecting lymphatics and blood vessels [19]. After the lymph is absorbed, it moves from lymphatic capillaries into the precollecting vessels, which have sparse mural components like smooth muscle cells. The precollecting vessels next drain into collecting LVs that have a periendothelial smooth muscle layer, basement membrane, continuous zipper-like interendothelial junctions and valves [1,20,21]. Interestingly, *Mycoplasma pulmonis* infection induces lymphangiogenesis in the airway, and the growing tips of lymphatic sprouts exhibit zippers instead of buttons at 14 days after infection. Later, as inflammation resolves, this pattern changes; sprouts become less numerous and most sprouting lymphatics develop buttons and resemble stable initial lymphatics. This suggests that buttons are specialized junctions rather than immature forms [19].

Most studies of the signaling pathway of inflammatory lymphangiogenesis actually refer to extranodal lymphangiogenesis. Most researchers have reached a consensus that the VEGF-C–VEGF receptor-3 axis in LECs is the most important signaling axis for inflammatory lymphangiogenesis, as is the case for normal developmental lymphangiogenesis. Transgenic overexpression of VEGF-C results in an increased number and enlarged size of subcutaneous LVs, which significantly limits acute skin inflammation and relieves edema; VEGF-D overexpression also achieves a similar but less potent effect than that of VEGF-C [22]. In reverse, inhibition of the VEGF-C (or VEGF-D)–VEGF receptor-3 signaling pathway reduces lymphangiogenesis and increases edema and inflammation [23–25]. Recently, a study using a peritonitis mouse model further described inflammatory lymphangiogenesis as being accomplished through activation of nuclear factor (NF)-κB, and subsequent upregulation of Prox-1 and VEGF receptor-3 [26].

The VEGF-A and VEGF receptor-2–VEGF receptor-1 axis is also known to have substantial influence on inflammatory lymphangiogenesis, but its action appears to be somewhat context-dependent. Enlarged LVs are observed in transgenic mice that overexpress VEGF-A, along with prolonged inflammatory response when delayed type hypersensitivity is induced [27]. In this model, LEC proliferation and LV hyperplasia that occur during chronic skin inflammation persist for significantly longer periods in VEGF-A-overexpressing mice compared with wild-type mice. Double-blocking of VEGF receptor-1 and VEGF receptor-2 effectively suppresses inflammation [27], but it should be noted that the interpretation of the underlying mechanism of this outcome is not a simple matter, because inflammatory angiogenesis and plasma leakage are inhibited as well. In another study using a tumor necrosis factor (TNF)-α-overexpressing rheumatoid arthritis model, VEGF receptor-2 neutralization reduces the amplitude of lymphangiogenesis [24]. However, in a chronic airway inflammation model produced by *M. pulmonis* infection, neither VEGF receptor-1 nor VEGF receptor-2 inhibition has a significant effect on lymphangiogenesis [23].

Whether lymphangiogenesis achieved under conditions of VEGF receptor-2 activation is a primary event or merely the outcome of indirect activation of VEGF receptor-3 is a tenacious question. To clarify whether VEGF receptor-2 and/or VEGF receptor-1 signaling transduction can trigger lymphangiogenesis on their own, placenta growth factor (PlGF) and VEGF-E – selective agonists for VEGF receptor-1 and VEGF receptor-2 have been used [28], respectively. In postnatal mice, VEGF-E successfully induces lymphatic hyperplasia, but very few additional sprouts are produced. Interestingly, this effect is not suppressed by inhibition of VEGF-C and VEGF-D [28]. By contrast, single activation of VEGF receptor-1 by PlGF overexpression does not induce a meaningful phenotype on LVs. Therefore, it seems that VEGF-A has some direct effect on LVs, which is mediated mainly through VEGF receptor-2 in a VEGF-receptor-3-independent manner; however, the direct effect of VEGF receptor-2 signaling does not seem to involve significant lymphangiogenesis, because sprouting is not induced. Instead, VEGF receptor-2 signaling may participate to achieve LV hypertrophy/enlargement [28]. Nevertheless, further studies are necessary to clarify precisely the biological meaning of VEGF receptor-3-independent lymphatic effects under various inflammatory conditions. Chronic inflammation may induce a state of accumulation of lymphoid cell clusters that are structurally similar to LNs, which are better known as tertiary lymphoid organs. Lymphangiogenesis is known to occur in tertiary lymphoid organs that have developed in thyroiditis, sialitis, rheumatoid arthritis and chronic renal graft rejection. Lymphotoxin-α is an inflammatory mediator reported to have significant roles in such inflammation-related lymphangiogenesis and LV function [29].

Once inflammatory lymphangiogenesis has been accomplished, the bulk of the newly formed extranodal LVs persist for at least 12 weeks, despite treatment and resolution of the inflammation [23]. This observation is in harmony with reports that VEGF receptor-3 activation is crucial for growth of new LVs, but is not necessary for maintaining already established LVs [13,30]. The biological significance of persisting lymphatics is unclear; it may reflect long-term microenvironment remodeling to prepare more efficiently the immune system for potentially repeatable inflammatory episodes.

LN (intranodal) lymphangiogenesis

LNs play a pivotal role during inflammation and immune responses. They are structures that are interposed along the course of relatively larger LVs and serve as immunological guard posts. Structurally, INLVs are continuous, with peripheral afferent lymphatics that extend to form the subcapsular sinus and surround the B cell follicles, and stretch into the medullar region of LNs that eventually form an exit

as efferent LVs [31,32]. INLVs traverse through densely packed aggregations of immune cells, predominantly lymphocytes. Such architecture allows intimate interaction between INLVs and immune cells, which obviously influences the inflammatory response. For instance, the physical proximity between INLVs and the T cell zone enables rapidly moving T lymphocytes to be exposed to their specific antigen, thus initiating adaptive immune responses [33,34]. During the acute phase of inflammation, robust LN lymphangiogenesis occurs and subcapsular INLVs proliferate and penetrate deep into the cortex [31].

Like the signaling pathways involved in the extranodal lymphangiogenesis, the VEGF-A–VEGF receptor-2 and VEGF-C/VEGF-D–VEGF receptor-3 pathways have been implicated as the principal pathways of inflammatory LN lymphangiogenesis [35]. Although the majority of molecular mechanisms of LN lymphangiogenesis essentially overlap with those of extranodal lymphangiogenesis, studies on intranodal lymphangiogenesis have mainly focused on the VEGF-A–VEGF receptor-2 signaling pathway instead of the VEGF-C–VEGF receptor-3 axis. During inflammation, follicular B cells [36,37], CD11b macrophages (migrated from the peripheral tissue) [38,39], and fibroblast-type reticular stromal cells [40] are reportedly the major sources of intranodal VEGF-A, a potent prolymphangiogenic regulator that is responsible for INLV growth. Extranodal VEGFs can also significantly stimulate lymphangiogenesis in draining LNs. One group has induced chronic inflammation by oxazolone in VEGF-A-overexpressing transgenic mice. Under their experimental design, the VEGF-A produced at the peripheral inflamed tissue, but not inside the LNs, was responsible for growth and regression of LN lymphatics; intranodal mRNA levels of VEGF-A, VEGF-C and VEGF-D were not significantly altered [41,42]. This implies that LN lymphangiogenesis can be regulated by not only the local intranodal signals but also remote inflammatory products that drain into the LNs. However, a situation of intranodal components having little contribution on lymphangiogenesis is probably infrequent, because most reports in the literature emphasize the significance of intranodal prolymphangiogenic components [31,36–40]. The biological meaning and influence of inflammation on lymphangiogenesis in relation to the specific stimuli and the duration of inflammation remain to be clarified.

Interestingly, not many studies have specifically described the role of VEGF-C–VEGF receptor-3 signaling on LN lymphangiogenesis. Data from *K14-VEGF-C* transgenic mice have shown that increased VEGF-C levels increase INLVs [38]. Additionally, studies using rheumatoid arthritis mouse [24] and lipopolysaccharide (LPS)-induced acute inflammation models [38] have shown that VEGF receptor-3 inhibition lowers the amplitude of LN lymphangiogenesis. Considering the magnitude of the impact of the VEFG-C–VEGF receptor-3 pathway on extranodal lymphangiogenesis, it seems likely that the VEGF-C and/or VEGF-D–VEGF receptor-3 axis has paramount significance in intranodal lymphangiogenesis as well. Regarding the cellular mechanisms, several key TNF family members, lymphotoxin and LIGHT (homologous to lymphotoxins, inducible expression, competes with herpes simplex virus glycoprotein D for herpesvirus entry mediator, a receptor expressed on T lymphocytes) have also been shown to play crucial roles in LN hypertrophy and homeostasis of stromal, vascular components during inflammation [43–46].

Although many features of INLVs and ENLVs overlap, unshared features also exist, such as the negative regulatory mechanism of T lymphocytes on LN lymphangiogenesis. T cells that constitutionally reside in large quantities inside LNs suppress INLVs mainly through interferon (IFN)-γ, which modulates the Janus kinase–signal transducer and activator of transcription (JAK–STAT) pathway [31]. Ultimately, the final state of INLVs is determined by the balance of prolymphatic drives [VEGF-A, VEGF-C, and VEGF-D secreted by intranodal B cells, macrophages, dendritic cells (DCs), and molecules that are directly drained from the peripheral tissue], and the antilymphatic drive (IFN-γ secreted by T cells) [31,47]. This notion explains why LPS injection promotes LN lymphangiogenesis, whereas concanavalin-A (T cell mitogen) does not increase INLVs, although both agents induce potent inflammatory reactions in the skin [31]. A recent study has suggested that this concept of coordination between prolymphatic and antilymphatic drives [interferon-γ, transforming growth factor (TGF)-β1 [48–50] and endostatin [51]] regulating lymphangiogenesis could also be valid in extranodal lymphangiogenesis [47]. Such bidirectional regulation would establish a very unique and flexible system, allowing diverse lymphangiogenic responses to occur according to the inflammatory stimuli.

ENLVs and INLVs also notably differ in their plasticity. Compared to ENLVs, INLVs react much more dynamically during inflammation. Once lymphangiogenesis occurs and inflammation resolves, newly formed INLVs readily regress [31,38,42], whereas new ENLVs seem to be persistent even after inflammation has resolved [23,52]. The mechanisms of such differences are unclear, but these findings imply significant differences in the microenvironment and possibly in the involved signaling pathways. One potential explanation could be that the density of T cells is much higher in LNs, resulting in a more potent T-cell-mediated antilymphatic effect and consequent INLV regression [31].

LVs, immune cell migration and adaptive immunity

Paracrine secretion of prolymphangiogenic molecules by immune cells is a major task. Leukocytes – such as $CD11b^+$ macrophages, follicular B cells, and DCs – that infiltrate and/or reside in the inflamed tissue and regional LNs can markedly potentiate the prolymphatic drive by secreting VEGF-C and VEGF-D. As previously described, this observation (at least partially) accounts for how the VEGF-A–VEGF receptor-2 axis may activate lymphangiogenesis [23,36,53]. Migration of antigen-presenting DCs to the draining LNs is essential for initiation of adaptive immunity. Appropriate inflammatory stimuli induce activation and/or expansion of LVs, subsequently increasing the migration/activation of DCs [31,36,54]. Extranodal lymphangiogenesis aids the entry and migration of DCs to draining LNs. Downstream intranodal lymphangiogenesis can facilitate fruitful interactions between DCs and immune cells, provoking immune reactions. During this process, VEGF receptor-2 and VEGF receptor-3 signaling is suggested to be important [36]. In this sense, LVs may be

considered to be a vague link between innate immunity and adaptive immunity.

During acute inflammation induced by LPS, LECs express high levels of Toll-like receptor (TLR)4, which is involved in NF-κB activation. The final outcome is the production of various chemokines which achieve TLR4-dependent chemotactic recruitment of macrophages that serve as a major source of VEGF-C and VEGF-D [55]. LECs produce chemokines that are sensed by hematopoietic cells and act as major guiding cues for cell migration [33,56]. LVs express chemokine CC ligand (CCL)21, a chemokine that recruits chemokine CC receptor (CCR)7$^+$ DCs [1,36,57]. Inflammatory cytokines (TNF-α, lymphotoxin-α and interleukin-1α) secreted from recruited leukocytes trigger LECs to express leukocyte adhesion receptors intercellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1, and E-selectin, which mediate LN trafficking of DCs via afferent lymphatics [58]. Interestingly, DC entry into afferent LVs could occur in an integrin-independent manner [59]. Inflammatory mediators can trigger DC maturation and migratory capacity, for example, by inducing a rapid decrease of CCR1 expression, whereas CCR7 abruptly increases [54]. The physical environment under inflammatory conditions is also influential, because the transmural flow affects lymphatic function, including CCL21 expression [60]. Recruited DCs can easily enter initial LVs through the discontinuous LEC junctions [19] or through the preformed portals on LVs [18], after which they crawl in contact with LECs in initial LVs and then passively flow by lymphatic flow in the collecting LVs toward the draining LNs [61].

A growing body of evidence indicates the importance of VEGF receptor-3 signaling in promoting adaptive immunity by mobilizing DC trafficking to the draining LNs [62,63]. Interfering with VEGF receptor-3 signaling by soluble extracellular domain of VEGF receptor-3 Ig decreases CCL21 production in the LECs of transplanted allograft heart, and consequently suppresses adaptive immunity [62]. Similarly, local inhibition of VEGF receptor-3 after corneal transplantation reduces the graft-derived DC migration to the host's draining LNs; donor-specific delayed-type hypersensitivity declines, and survival of the transplanted cornea is improved [63]. DCs are also crucially involved in lymphangiogenesis of tertiary lymphoid structures in a lymphotoxin-ligand-dependent manner [64].

LECs may also negatively regulate inflammatory reactions in several ways. CCR7 ligands, CCL19 and CCL21, are secreted by LECs and can induce an inhibitory program in T cells [65,66]. Furthermore, LN-resident LECs can mediate peripheral immune tolerance by expressing multiple peripheral tissue antigens and directly presenting these epitopes to CD8$^+$ T cells, leading to their deletion [66,67]. However, further research is needed to establish the role of lymphatics in regulating adaptive immunity.

Therapeutic implications

Clinical and preclinical studies have indicated a relation between lymphangiogenesis and inflammatory disorders, including but not limited to airway inflammation [23,68], rheumatoid arthritis [24], inflammatory bowel disease [69,70], inflammatory disorders of the skin [22,25,71],

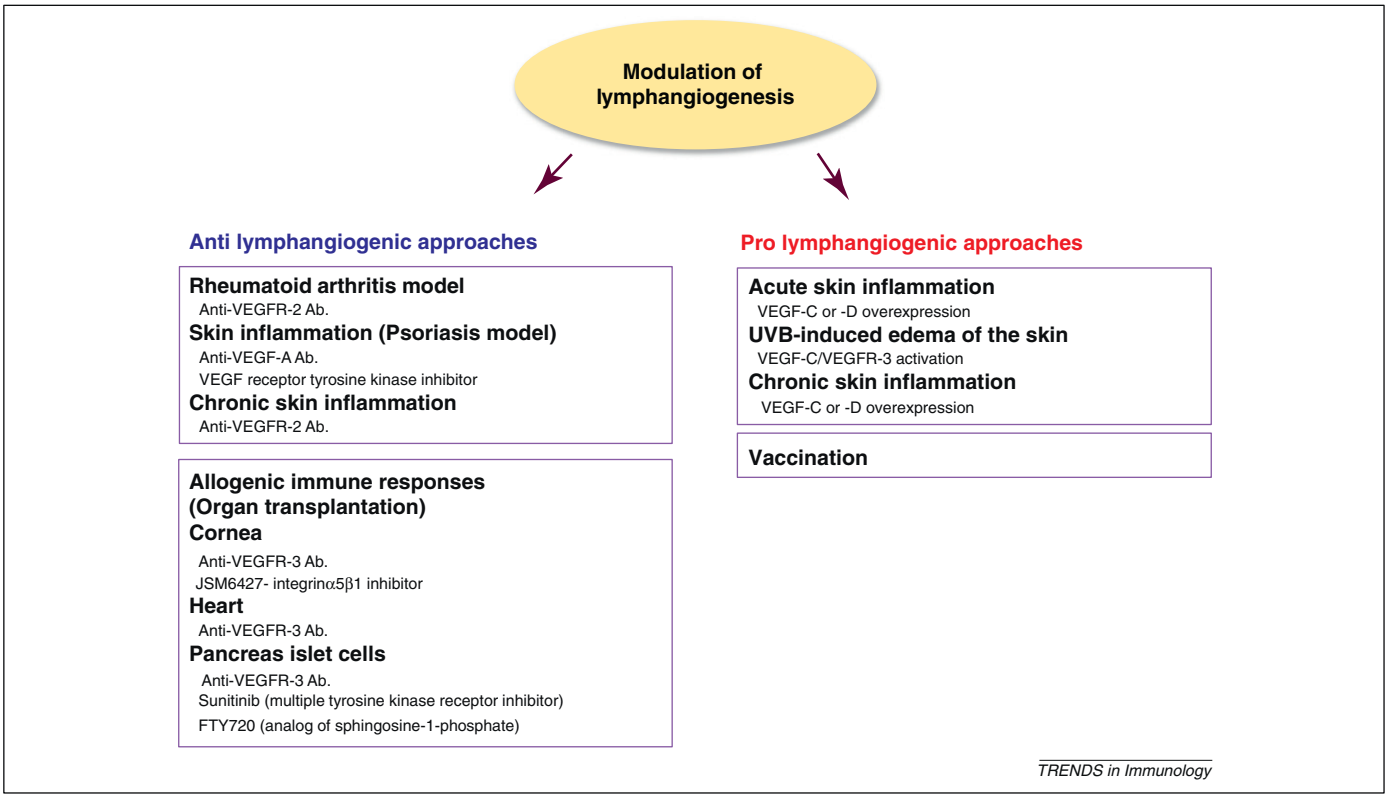

Figure 2. Making transition from the bench to bedside in the field of inflammatory lymphangiogenesis. Summary of lymphatic vessel (LV)-modulating agents that have been tested and found effective in preclinical studies for selected disease models. Antilymphangiogenic approaches in models of rheumatoid arthritis [24], psoriasis [71,78], and chronic skin inflammation [25] have been studied. Another major application of antilymphangiogenic treatments has been attempted to control allogenic immune response in organ transplantation models of cornea [76], heart [62] and pancreas islet cells [75]. Prolymphangiogenic approaches can be potentially utilized in the treatment of acute skin inflammation [22], UVB-induced edema of skin [22,79], and chronic skin inflammation [25], and to improve the efficacy of vaccination [82].

Kawasaki disease [72,73], viral infection [74] and organ transplantation [62,75–77]. Blocking lymphangiogenesis impairs the lymphatic function, which results in aggravation of inflammation at the primary site [23,24]. Anti-VEGF treatments have produced promising results in preclinical studies, with both anti-VEGF antibodies [25,78] and VEGF receptor tyrosine kinase inhibitors [71] used against chronic skin inflammation. By contrast, increased levels of VEGF-C or VEGF-D relieve the severity of acute skin inflammation and reduce dermal edema, probably by improving lymph flow and decreasing edema [22,25,79]. However, regardless of symptomatic improvement, we should cautiously investigate whether prolymphangiogenic approaches are truly beneficial in infectious/inflammatory disorders, because the promoted lymphatic drainage could theoretically increase the systemic exposure to unfiltered pathogens and/or inflammatory mediators. Any therapeutic modulation of inflammatory lymphangiogenesis should be designed and refined according to the context of inflammation and purpose of intervention.

Preventing graft rejection after transplantation is another emerging field pertaining to inflammatory lymphangiogenesis. Increased lymphangiogenesis after organ transplantation enhances the delivery of antigen-presenting cells to the draining LNs and provokes unwanted immune responses [75,76]. Lymphangiogenesis suppression improves the survival of experimentally transplanted pancreatic islet cells [75] and cornea [80]. Recently, angiopoietin-2 suppression has been suggested as a candidate method to prevent rejection [81]. Temporary selective inhibition of lymphangiogenesis before transplantation might help prevent early postoperative access of inflammatory cells to the lymphatic system and improve overall graft survival [76].

The lymphatic vessels also serve as essential route for vaccine delivery as free-form or mediated through DCs and might be exploited to improve vaccination [82]. However, the therapeutic potential of lymphatic vessels for quick and efficient vaccine delivery is still largely untapped and requires further research (Figure 2).

Concluding remarks

Owing to their specific functions and plasticity, LVs can be markedly remodeled by inflammatory insults; they are also capable of altering inflammatory responses. Such reciprocal modifications allow substantial complexity and flexibility in the signaling pathway and final outcome of inflammatory lymphangiogenesis. In spite of recent progress that has markedly expanded our knowledge of lymphatic biology, the field remains young and a lot of research is still required. Clinically relevant questions about INLVs must be answered in order to make a successful transition from the bench to the bedside.

Acknowledgments

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