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Inflammation-induced lymphangiogenesis and lymphatic dysfunction

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Abstract

The lymphatic system is intimately linked to tissue fluid homeostasis and immune cell trafficking. These functions are paramount in the establishment and development of an inflammatory response. In the past decade, an increasing number of reports has revealed that marked changes, such as lymphangiogenesis and lymphatic contractile dysfunction occur in both vascular and nodal parts of the lymphatic system during inflammation, as well as other disease processes. This review provides a critical update on the role of the lymphatic system in disease process such as chronic inflammation and cancer and examines the changes in lymphatic functions the diseases cause and the influence these changes have on the progression of the diseases.

Introduction

Interstitial fluid containing various molecules and cells is collected by the lymphatic system at the level of the initial lymphatic vessels (lymphatics), actively transported via collecting lymphatics towards lymph nodes for immune surveillance, and drained back to the blood stream via postnodal, efferent lymphatics. Initial lymphatics are blind-ended tubes comprised of endothelial cells linked together by discontinuous junctions [1, 2], which allow interstitial fluid to enter the vessels (Figure 1). Compression of the initial lymphatic vessels closes the endothelial junctions and propels lymph forward into the collecting lymphatics where luminal unidirectional valves (also formed by endothelial cells) are present at close spacing. In addition to a luminal monolayer of endothelial cells, collecting lymphatics also possess smooth muscle in their outer walls (Figure 1) [3]. Transient, phasic contractions of the lymphatic muscle layer provide the primary mechanism through which lymph is propelled toward lymph nodes and back to the bloodstream.

Soluble antigens and antigen-activated dendritic cells (DCs) enter initial lymphatics at the periphery under the guidance of transmural flow. In addition, DCs find their way to the lumen thanks to a chemotactic gradient of CCL21 produced by the lymphatic endothelial cells (LECs) [4]. While soluble antigens freely and quickly travel through collecting lymphatic vessels to the draining lymph node, antigen-activated DCs travel at a much slower pace [5, 6]. They enter the lymph node from prenodal, afferent lymphatic vessels, surrounding the lymph node cortex and concentrate at the medullar area. Lymph nodes provide a highly organized microenvironment to support efficient antigen screening and

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trigger proper adaptive immune response. Once in the lymph node, small soluble antigens (molecular weight below 70kD) quickly move along the conduit network and are sampled by lymph node resident DCs [7-9]. The activated DCs migrate to the T cell zone directed by chemokines CCL19 and CCL21 produced there and efficiently interact with the T lymphocytes to establish an antigen specific T cell response. Large particles/antigens cannot enter lymph node parenchyma. Instead, they travel through lymph node sinuses and are screened by lymph node macrophages. Naïve lymphocytes leave the blood circulation and home to the lymph node from specialized blood vessel called high endothelial venules (HEVs).

The lymph node vasculature and stromal cells express chemokines, which direct the T and B cell compartmentalization. Specifically, fibroblastic reticular cells (FRCs) and HEVs express CCL19 and CCL21, which attract CCR7 expressing dendritic cells (DCs) and T cells to concentrate at the lymph node paracortical zone. CXCR5 expressing B cells are guided by CXCL13 expressed by follicular stroma cells, follicular DC (FDC) network and marginal reticular cell (MRC) stromal networks to locate at the lymph node cortex. After the immune surveillance and clonal expansion, lymphocytes exit the lymph node, a process facilitated by sphingosine-1-phosphate expressed by LECs of efferent lymphatic vessels [10-12] (Figure 2A). During steady state, lymph nodes function as critical location for generating peripheral tolerance. Tissue resident DCs constantly sample self-antigen and become semi-activated and traffic to the draining lymph node. These self-antigen bearing DCs cause tolerance to self-reactive T cells through clonal deletion, anergy and/or expanding regulatory T cells [13-19]. In addition, LECs and FRCs express peripheral tissue antigens and promote tolerance via clonal deletion of self-reactive CD8 T cell [20-23]. A functional lymphatic vessel network is thus necessary for efficiently delivering antigen and antigen presenting cells to the lymph node; the route taken by the antigen to be presented may determine the ultimate immune responses.

The involvement of the lymphatic system in tissue fluid homeostasis and immune response/surveillance, make it a critical player in the establishment of an inflammatory response. Importantly, marked changes in the functions of both vascular and nodal parts of the lymphatic system have been reported during inflammation processes.

Lymphangiogenesis in inflammation

Inflammation has been shown to cause a remodeling of the lymphatic network characterized by the growth of new initial lymphatics, a process known as lymphangiogenesis. Inflammation-induced lymphangiogenesis occurs in both the draining lymph node and the inflamed tissue (Figure 2B) and is mediated by vascular endothelial growth factor (VEGF). A signaling through VEGF receptor 2 (VEGFR2) and VEGFC/D signaling through VEGFR3 [24]. In acute inflammation, B cells express VEGFA, which activates VEGFR2 and lymph node lymphangiogenesis [25]. Overexpression of VEGFA specifically in B cells is sufficient to induce lymph node lymphangiogenesis [26]. However, B cells are not absolutely required for lymph node lymphangiogenesis, as it was shown that lymphangiogenesis could be induced at a later time point in mice deficient in B cells (μ MT mice) in acute inflammation [27]. Interestingly, T cells play an opposite role in regulating lymphangiogenesis in inflammation. Activated T cells express interferon gamma ($\text{IFN}\gamma$), which suppress lymph node lymphangiogenesis [28]. Macrophages moving from the inflamed tissue to the draining lymph node also express VEGFA and activate lymph node lymphangiogenesis [29, 30]. During inflammation, lymph node lymphangiogenesis also enhances VEGFA production by non-hematopoietic cell populations, such as FRCs [31]. The expression of VEGFA in FRCs is stimulated by activation of the lymphotoxin beta receptor ($\text{LT}\beta\text{R}$), a receptor expressed in lymph node vasculature and stromal cells [27], and crucial for lymphoid organ development [11]. Blocking $\text{LT}\beta\text{R}$ decreases lymph node

lymphangiogenesis. [32] B cells, T cells and DCs express LT β R ligands (LT $\alpha_1\beta_2$ complex and LIGHT) and can potentially induce lymphangiogenesis via this pathway.

Unlike in lymph node, tissue lymphangiogenesis is independent of B cells [33]. In the inflamed tissue, lymphangiogenesis is induced by infiltrating macrophages via expression of VEGFA and VEGFC [30]. VEGFA and VEGFC produced in the tissue can be delivered to the draining lymph node and initiate lymph node lymphangiogenesis. Transforming growth factor beta (TGF- β) induces VEGFC in macrophages and promotes lymphangiogenesis in rat unilateral ureteral obstruction [34]. Surprisingly, blocking TGF- β enhances lymphangiogenesis in wound healing model [35, 36]. Whether other immune cell types play a role in lymphangiogenesis in inflamed tissue is not clear.

Several pathological conditions, such as autoimmunity, microbial infection and chronic allograft rejection, lead to chronic inflammation. Importantly, increase lymphatic vessel density is closely associated with kidney allograft rejection in human [37]. Lymphangiogenesis has also been noted in rejection of corneal grafts [38], where VEGFA and VEGFC, overexpressed by the infiltrated macrophages, are deemed responsible [38, 39]. Lymphangiogenesis is also preeminent in chronic inflammations of the gastrointestinal tract, such as Inflammatory Bowel Diseases (IBD), Crohn's disease (CD) and ulcerative colitis (UC). During disease flares, intestinal lymphatics proliferate from their normal distribution in the submucosa to every layer of the inflamed small and large bowel [40-44]. These observations suggest that lymphangiogenesis may be triggered by inflammation and, as intestinal lymphatics were reported to be extremely dilated in specimens from CD patients [43], lymphangiogenesis may represent an attempt to compensate for impaired lymphatic drainage in order to maintain clearance of interstitial fluid and inflammatory infiltrate and restore gut homeostasis.

Indeed, impaired lymph drainage and persistent lymph stasis could contribute to chronic inflammation by causing activated DCs and antigens to accumulate in lymphoid follicles rather than to be drained to the lymph nodes [45]. The idea of intestinal lymphangiogenesis being a consequence of impaired lymph drainage is further supported by observations that poor rates of lymphatic clearance leads, in the Chy3 mouse model of congenital lymphedema [46], to elevated basal levels of pro-inflammatory cytokines (IL-6), but decreased levels of IL-4 [47], which may regulate proliferation and adhesion molecule expression in lymphatic endothelial cells [48]. While it is tempting to suggest a role for lymphangiogenesis in the modulation/worsening of inflammation in IBD, whether lymphangiogenesis is pathological or protective still remains an open question. Indeed, the role played by lymphangiogenesis in chronic inflammation is not clear. In chronic organ transplantation rejection, blocking lymphangiogenesis favors organ transplantation survival [37, 38, 49], but inhibiting lymphangiogenesis and blocking lymph flow increases the severity of inflammation in a mouse model of arthritis [50].

Inflammation-induced contractile dysfunction

Collecting lymphatic vessels transport the collected lymph towards draining lymph node and eventually return it to the blood. As mentioned earlier and in addition to passive force driven by tissue movement, this function is promoted by lymphatic muscle, which rhythmically contract to drive lymphatic transport against gravity (Figure 1). Instead of increasing vessel density, collecting lymphatic vessels usually change their contractile behavior upon inflammatory stimulation. These inflammation-induced alterations are characterized first by dilation of the vessel and decrease in contraction frequency. In particular, impaired lymphatic contractile activity and vessel dilation has been reported in a model of peritonitis [51] and in a model of experimental ileitis [52]. In oxazolone skin painting induced acute inflammation, lymphatic contractions were also suppressed, an action involving infiltrated

iNOS expressing CD11b⁺Gr-1⁺ cells [53]. The dilated vessel and reduced lymphatic contraction strength appear to decrease the lymphatic transport capacity. However, dilated, slow pumping vessel may also in some circumstances favor faster material transport. Experiments on isolated vessels from control/healthy animals showed that increased intraluminal pressure dilate them and reduce the frequency of contractions to allow material to quickly travel through the vessel network [54]. However, the valves appear to be less efficient to prevent backflow when lymphatic vessel is largely dilated, particularly in situation mimicking lymphedema [55]. However, the inflammatory environment may cause different outcomes. Increased pumping function has been observed during acute intestinal challenge with the inflammatory substance fMLP [56], and during edemagenic stress [57], and has been suggested to lead to increase in lymph flow (however this parameter was not measured directly). Moreover, simultaneous measurement of lymph flow and lymphatic contractility during acute challenge with LPS showed lymph flow rapidly augmenting due to an increase in pumping activity during the first two hours of the challenge, and remaining elevated in later phase despite pumping inhibition [58, 59]. Many studies performed with the aim to dissect out the role of individual components of the inflammatory soup in lymphatic pumping modulation and classical inflammatory mediators, such as prostanooids, histamine or nitric oxide have been shown to modulate lymphatic pumping and lymph drainage (see reviews [60, 61]). In addition, neuromediators important in immune and inflammatory responses, such as substance P, calcitonin gene related peptide (CGRP), neuropeptide Y or vasoactive intestinal polypeptide (VIP), have also been reported to strongly modulate lymphatic vessel contractility [62-65].

In a physiological and homeostatic context, it has been widely demonstrated that endothelium-derived mediators such as nitric oxide (NO), prostacyclin or prostaglandin E₂ inhibit lymphatic contractility [66-75], while on the other hand, prostaglandin H₂/Thromboxane A₂, increase it [70, 73-76]. These molecules being strongly associated with and upregulated during the inflammatory process, they are very credible candidates to influence lymphatic pumping during inflammation. Indeed, studies demonstrated that the inhibition of mesenteric lymphatic pumping during TNBS-induced ileitis was due to an increase in production of both NO and prostaglandins [52, 77].

In collecting lymphatics, the lymphatic endothelium is thought as playing a role as barrier between the lymph and the interstitium. Although this role is not well defined, lymphatic endothelial barrier function is very likely modulated during inflammation, as demonstrated in a study using cultured lymphatic endothelial cell monolayer, where endothelial permeability was increased following treatment with pro-inflammatory cytokines (TNF- α , IL-6, IL-1 β , IFN- γ) and with LPS [78]. The authors further showed that this effect was dependent on the production of NO. Whether the increased permeability or the cytokines themselves also influence lymphatic pumping was not assessed in this study and indeed to our knowledge, investigations to evaluate a direct effect of cytokines on lymphatic pumping are sparse. A study by Hanley *et al.* [79] reported that in isolated pressurized bovine mesenteric lymphatics IL-1 α and β cause a significant inhibition of the pressure-dependent increase in lymphatic pumping. Interestingly, this effect occurred within minutes of IL-1 application, precluding a transcription-mediated action. In our own hands, we were not able to observe such rapid effect with isolated guinea pig mesenteric vessels (unpublished observation). A more recent study using noninvasive near-infrared fluorescence imaging described cessation of murine lymphatic propulsion as early as 4 h following intradermal administration of LPS, or either IL-1 β , TNF- α or IL-6, which were found to be elevated in the serum of mice treated with LPS [80]. Importantly, these effects were noted to be systemic and driven by NO production. NO, released by the lymphatic endothelium, was also thought to be involved in the action of VEGF to cause a dilation of pre-contracted isolated dog thoracic ducts [81]. In contrast, pumping activity of rat mesenteric collecting

lymphatics was shown by intravital microscopy to be increased by VEGF-C via activation of VEGFR-3 in ways that do not require changes in microvascular hemodynamics [82].

Collectively, the above reports highlight the pivotal contribution of the collecting lymphatic vessels in the development of inflammatory and immune responses. Further investigations are critically required to better understand how inflammation regulated lymphatic function and subsequently immune function.

Lymphatic drainage and immune function in diseases

To activate an immune response, antigen/DCs need to access lymphatic vessel at the initial lymphatic vessel site, be transported through collecting lymphatics and eventually home to specific area in the draining lymph node. Different stimuli inducing different gene expression patterns in LECs may have a variable impact on the antigen/DC trafficking [83]. The outcome of an immune response may depend on temporal and spatial changes occurring in the three areas where the lymphatic system plays a role: 1) in the tissue where lymphangiogenesis occurs, 2) at the level of the collecting lymphatics, where contractility/drainage is compromised and 3) in the lymph node, where the architecture supporting antigen/DC locomotion is modified (Figure 2).

Although the functional consequences of lymphangiogenesis occurring in pathological condition are not clear, the assumption is that an expanded lymphatic network would allow better lymphatic transport, presumably improving the removal of the excessive extracellular fluid brought by the inflammation-induced blood vessel permeability (Figure 2). By promoting lymph drainage, lymphatic vessels may increase the delivery of antigen and antigen bearing DCs to the draining lymph node and enhance immune responses. However, the ultimate immune response mounted during inflammation appears to be more complicated.

Tissue lymphangiogenesis may affect the ability of antigen/DC to enter initial lymphatic vessels. As described in the introduction section, initial lymphatics exhibit discontinuous cell junctions with limited basement membrane and no pericyte coverage (Button pattern, Figure 1 and 2A). This structure renders them highly permeable to the surrounding environment and allows efficient fluid and DC access to the lymphatic vessels. Even small number of functional initial lymphatic vessel is sufficient to allow DC migration to draining lymph node [84]. The collecting lymphatics however, possess an endothelium with continuous cell junctions, surrounding pericyte coverage and basement membrane (Zipper pattern) [2]. The Zipper pattern favors lymphatic flow, but it is not clear if it allows materials to access lymphatic vessel lumen. During the embryologic development, LECs first acquire a Zipper pattern and later differentiate into Button pattern. This embryologic program seem to be recapitulated in the proliferating LECs which show a Zipper pattern [2]. The proliferating LECs may thus have reduced permeability and thereby fluid access to the lumen of the lymphatic may be reduced. On the other hand, while proliferating, LECs increase the surface area, thus potentially favoring extracellular material entry into lymphatic vessels. In oxazolone skin painting caused inflammation, a temporary reduction of antigen and DC accumulation in the inflamed lymph node was associated with lymphatic function insufficiency in the first 2-5 days. This initial decline was followed at later time point (7-10 days) by the recovery/enhance T cell response [27]. Daily injection of lipopolysaccharide (LPS) in the mouse peritoneal cavity also showed a reduced accumulation of FITC in the draining lymph node [29, 30]. Thus, the ability of proliferating LECs to enhance or decrease lymph collection may be temporally and spatially dependent on the progress of lymphangiogenesis.

Although contractile function of lymphatic vessels is reduced and lymphatic vessels are dilated during inflammation [51-53, 58, 59]. The consequence of these changes with respect to lymph transport is not clear. Direct measurements of lymph flow, cell movement and volume of lymph transported are required.

Several experimental approaches have been considered to assess lymph flow in physiological conditions. They have provided quantitative measures of bulk flow rates, but also have their caveats. Lymph flow was measured during lymphatic cannulation, where lymph was collected from the main mesenteric lymphatics draining the gut with a small glass micropipette [56, 59, 85]. While accurate, this method does not provide information on lymph flow in prenodal, afferent lymphatics as it has so far only been obtained from postnodal larger vessels. Fluorescent [86-91] or radionuclide labeling [92, 93] techniques have been applied to lymph flow measurement. Two methods were developed to measure fluid velocities in individual mouse tail lymphatic capillaries using fluorescent recovery after photobleaching (FRAP) [86] and residence time distribution theory (RTD) [89]. Again, whether these methods are applicable to measures of lymph flow in collecting afferent vessels has not been tested. In the context of immune cell trafficking to the draining lymph node to induce an immune response, whether lymph flow measurement accurately reflects cells movement and cells always move at lymph velocity can be questioned. It might be therefore important to consider directly tracking cell movement and velocity along lymphatic vessels. Such measurements have been successfully performed during intravital video microscopy of rat mesenteric lymphatics using a high-speed video camera system [94, 95]. This technique had the advantage to provide temporal and spatial resolution of cell flux throughout cycle of the lymphatic phasic contraction. It should also allow measurement of cell velocity in dilated and quiescent lymphatics, as it occurs in inflammation. However to date, this has not been performed in these conditions.

Once lymph and cells enter the draining lymph node from afferent lymphatic vessels, they traffic through the node sinus or the conduit system (Figure 2). Fluid and small molecular weight antigen travel deeply into lymph node quickly through lymph node conduit. It is not clear how inflammation affect these types of materials traveling through lymph node. The lymph node structure undergoes dramatic changes following antigen activation, including angiogenesis and lymphangiogenesis [27, 33], decreased expression of genes contributing to peripheral node addressin (PNAd), a complex mixture of glycoproteins, which attracts L-selectin expressing lymphocyte homing [27, 96-98] and decreased chemokine production of CCL21 and CXCL13 [99]. These changes in lymph node architecture appear to hinder naïve cell and DC access and interaction, but enhance the ability of effector cells to leave the lymph node and prevent pathological immune-mediated damage. Subcutaneous injection of complete Freund's adjuvant in the mouse footpad enhanced DCs migration to the draining lymph node and increased T cell response, while expanding lymph node lymphangiogenesis [25]. However, CD19 B cells driven over expression of VEGFA in lymph node reduced the T cell response to ovalbumin or LPS despite the artificially increased lymphatic vessel density in lymph node.[26]. How the changes of lymphatic system at the tissue initial lymphatic absorbance, collecting lymphatic transport, or lymph node microenvironment affect the subsequent immune function remain to be clarified.

In chronic inflammation, formation of tertiary lymphoid organs (TLOs) is frequently induced in the inflamed tissue. Typical TLOs exhibit features of lymph node, such as T and B cell compartmentalization, lymphatic vessels and high endothelial cells. As the essential signal directs lymphoid organogenesis, $LT\beta R$ activation is required for initiation of TLOs [11]. $LT\alpha_1\beta_2$ complex expression in lymphoid organ inducer cells (LTIC) activates $LT\beta R$ expressed on lymphoid organ organizer cell and initiates a cascade of signals leading to lymphoid organogenesis. TLOs develop in tissues affected by autoimmune diseases such as

the synovium in rheumatoid arthritis and the salivary gland in Sjorgren's syndrome [100]. The lymphatic drainage and immune function in tertiary lymphoid organ is not clear.

Similar alterations of lymphatic structure/function have also been described in cancer, where tumor induces peritumor lymphangiogenesis, lymphatic vessel dilation and lymph node lymphangiogenesis to provide favorable microenvironment for tumor cells to invade and metastasize to the sentinel lymph node [101]. Lymphangiogenesis occurs in the tumor draining lymph node even before tumor cell colonization suggesting secretion of growth factors by the tumor, thus preparing lymph node for metastasis [102]. Blocking lymphangiogenesis through anti-VEGFC or VEGFD treatment reduces lymph node metastasis, however, once the tumor cells have formed colonies in the lymph node, anti-VEGFC/D treatment does not affect tumor growth in the seeded lymph node [103]. The growth of metastasizing tumors in lymph node is a poor prognosis for patients and profoundly impacts treatment decisions [104, 105]. The lymph node metastatic tumor cells have a high prevalence to metastasize to second organ. In addition, the immune tolerance environment caused by the primary tumor in draining lymph node may have profound impact on the systemic immune tolerance to the tumor. Growth factors secreted by the tumor may induce lymphangiogenesis and the apoptotic tumor cells may send tumor antigen to induce peripheral tolerance. Induction of lymphoid stroma structures within tumor seems to inhibit tumor growth or tumor rejection. Ectopic growth of HEVs in breast cancer is a favorable prognosis for tumor development [106]. The effect of tumor immune therapy with depleting regulatory T cells relies on induction of HEV in the tumor to facilitate CD8⁺ T cell trafficking [107]. Induction of lymphoid stroma structure with lymphotoxin- and LIGHT (both members of the tumor necrosis factor (TNF) superfamily), that binds to LTβR to trigger lymphoid organ neogenesis, help tumor rejection through the recruitment of naïve T cells and the local priming of tumor specific CD8 T cells [108, 109]. However, when CCL21 is overexpressed in melanoma, it favors an immune tolerance microenvironment by enhancing the recruitment of Tregs and skewed the tolerogenic cytokine profile [110]. In addition, tumor invading the lymphatic system may express tumor antigen and induce immune tolerance [111]. The complication of lymphoid function in tumor immune environment brings more difficulties when designing tumor immune therapy.

Conclusion

The lymphatic system maintains fluid balance and plays critical roles in immune surveillance. Upon stimulation, lymphatics quickly collect and deliver antigen and antigen presenting cells to the draining lymph node to initiate protective immunity. During inflammation and cancer, the transport function of lymphatic vessels is modified and lymphangiogenesis occurs. Lymphangiogenesis is modulated by factors secreted by immune cells, such as T cells, B cells and macrophages. It provides an expanded lymphatic vessel network, which presumably allows greater fluid and materials access and transport. However, how lymphangiogenesis and changes in lymphatic draining function are involved in disease progression is not clear and needs to be further investigated.

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Figure 1. Initial lymphatic vessels and collecting lymphatic vessels

A. Ear whole-mount immunofluorescent staining shows initial lymphatic and collecting lymphatic vessels. Green, Lyve-1; Red, α SMA; Blue, CD31. Initial lymphatics expressing Lyve-1 has no pericyte coverage. When initial lymphatics merge into pre-collecting lymphatics, they start losing Lyve-1 staining and gain smooth muscle coverage, as demonstrated by α SMA staining. Collecting lymphatics have organized smooth muscle cell coverage. **B.** Schematic figure of initial lymphatic vessel (left) and collecting lymphatic vessel. The initial lymphatic vessels have oak-leaves shaped cell junctions (Button pattern). Organized smooth muscle coverage in collecting lymphatics allows phasic lymphatic contractions to propel lymph towards blood recirculation.

Figure 2. Schematic illustration of the changes occurring in the lymphatic system during inflammation

A. Normal lymphatic system, including initial lymphatic vessels, collecting lymphatic vessel and the draining lymph node. **B.** Lymphatic system during inflammation, displaying tissue lymphangiogenesis, collecting lymphatic vessel dilation and impaired lymphatic contractile function, and lymph node remodeling. All of these changes may affect immune function through regulating lymph and cell transport and trafficking within the lymph node in diseases.