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The Peripheral Lymphatics as an Active Player in the Immune Response

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Abstract

The lymphatics system has many diverse roles in the human body. These range from regulating fluid balance and fatty acid transport to serving as a conduit for leukocyte entry into lymphoid organs. While stromal cell populations are becoming increasingly recognised as important components of the immune response, the peripheral lymphatics are still commonly perceived as a passive conduit for immune cells. In contrast to this paradigm, the lymphatics are capable of responding to a wide range of pathogenic stimuli and secondary immune mediators, activating diverse, functional and stimulus dependant programs to these, with subsequent regulation of the effector function of multiple immune cell populations. In this review, we highlight papers that support the peripheral lymphatics as a crucial and active player in the immune response. We also interpret this evidence in the context of human disease pathogenesis and the potential for lymphatic-targeted therapeutic interventions.

Keywords: Lymphatics; Immune response; Stromal cell

Introduction

The lymphatics system runs parallel to the venous system [1]. Its primary immune-related functions are mediating the trafficking of immune cells between the periphery and secondary lymphoid tissues, and draining fluid and soluble immune mediators/antigens from affected sites. The afferent lymphatic vessels primarily transport dendritic cells into the secondary lymph nodes, whilst efferent vessels facilitate the efflux of lymphocytes and antibodies from the lymph nodes [2,3]. In addition to their direct roles in the immune system, the lymphatics are essential for the general maintenance of fluid balance and the transportation of fatty acids from the gut into the circulation [2,3].

While the lymphatics are often perceived as a passive conduit for fluid, fatty acids, and immune cells, evidence has emerged that suggests their role in orchestrating immune responses is active rather than passive. The lymphatics are influenced by changes in their immune microenvironment, while lymphatic dysfunction can incur additional functional impairments of the immune response [2,3].

This reciprocal relationship between the lymphatics and the rest of the immune system is evidenced in a number of disease states that show concurrent lymphatic and general immune abnormalities. For example, the chronic inflammation in chronic obstructive pulmonary disease patients is characterised by multiple immune defects including abnormalities in the phenotype and density of lymphatic vessels [4]. Similarly, adult-onset obesity is characterised by abnormal, 'leaky' lymphatic vessels as well as widespread immune dysfunction [5]. Moreover, lymphangiogenesis (the formation of new lymphatic vessels) is commonly observed in a number of inflammatory conditions such as chronic skin inflammation, chronic airway inflammation, and rheumatoid arthritis [6-9]. Interestingly, lymphangiogenesis has been linked to positive outcomes, through reductions in tissue damage and oedema, in diseases that involve joint and acute skin inflammation [10,11]. However, lymphangeogenesis can also be associated with negative immune outcomes during graft rejection and the pathogenesis of infective endocarditis [12-16]. Finally, lymphangiogenesis has a well-accepted role in mediating tumour metastases and the adverse outcomes associated with this [17].

While these observations strongly support an active role of the peripheral lymphatics in the immune response, diseases and experimental models of 'primary' lymphatic dysfunction best highlight the role of the lymphatics in the immune response. For example, primary lymphatic ablation in mouse models results in worsening inflammation, delayed wound healing, and oedema [18-20]. In humans, primary (and secondary) lymphoedema patients are also characterised by global immune deficits including impaired wound healing, decreased immune defences and an increased susceptibility to infections [21]. This same relationship between lymphatic and immune dysfunction is also observed systemic sclerosis and lymphatic filariasis patients [22,23].

Taken together, peripheral lymphatic abnormalities appear to be strongly linked to general immune function and are present in the majority of immunological-based human diseases. However, despite these observations, the lymphatics remain understudied, with the majority of research focusing on other immune players, and not the critical role of the lymphatics themselves.

In this review, we highlight papers that demonstrate an active role for the peripheral lymphatics both in responding to, and regulating the immune response.

Integration into the Immune System

Pathogen recognition

The capability of an immune cell to integrate signals from both primary pathogen associated molecular patterns and secondary immune molecules is essential to its functional capabilities in the

immune response. While the lymphatics are often viewed as immunologically inert conduits for fluid and cell transport, evidence increasingly suggests that the lymphatics can sense and respond to immunological signals within their microenvironment. The primary outcome of this recognition is the regulation of molecules involved in mediating cell trafficking or lymphangeogenesis.

Lymphatic endothelial cells (LECs) in both mice and humans express functional toll-like receptors (TLRs) 1–6 and 9 and respond to pathogenic stimuli through these receptors [17,24-27]. For example, TLR-mediated recognition of lipopolysaccharide (LPS) induced the expression of pro-inflammatory mediators, including interleukin (IL)-6, IL-8, vascular cell adhesion molecule (VCAM)-1, and intercellular adhesion molecule (ICAM)-1, which are important for the lymphatic-dependent regulation of cell migration and activation [24]. Interestingly, stimulation via TLR4 can also induce chemokine expression, chemokine-dependent macrophage recruitment, and macrophage-dependent lymphatic re-organisation, highlighting the complex interplay between the lymphatics and other immune cell populations [28]. Importantly, stimulation with the TLR2 agonist, lipoteichoic acid and the TLR4 agonist, lipopolysaccharide induced overlapping, yet distinct, gene expression profiles in cultured human LECs [24-26]. While this is entirely consistent with known differences in signalling outcomes mediated through different TLRs, it emphasises the specificity of pathogen recognition in LECs and suggests that the lymphatics contribute to directing pathogen-specific immune responses.

In addition to the modulation of effector genes in LECs, TLR-dependent pathogen recognition is also important for proper lymphatic organisation. TLR-deficient mouse models show decreased lymphatic transport function, abnormal lymphatic architecture, and fewer capillary lymphatics, all of which can be linked to the immune deficits in these models [27]. While the abnormal lymphatics might be at least partially attributed to abnormal macrophage function in this model (discussed further below), these results still suggest a major role of pathogen recognition in all aspects of lymphatic function [27].

In addition to *in vitro* stimulation assays, pathogen recognition by LECs also appears to play an important role in other physiological *in vivo* models. For example, LECs isolated from a mouse model of contact hypersensitivity differentially expressed more than 1000 genes when compared to the control [29]. Given that only a relatively small number of chemokines, integrins, and cytokines have been linked to lymphatic-dependent regulation of immune cell migration and function, the identification of a large number of novel genes modulated by LECs in response to inflammation, suggests an underappreciated role for the lymphatics in other aspects of the immune response [29].

Similar to what was observed with the TLR2 and TLR4 agonists, the inflammation-mediated gene expression in LECs appears to be highly stimulus dependent. When comparing LECs isolated from oxazolone-induced contact hypersensitivity and Complete Freund's Adjuvant-induced inflammation, large differences in the transcriptional expression of key chemokines and integrins were observed [29]. Once again this suggests distinct, stimulus-dependent activation of the lymphatics in these two models. Interestingly, despite the large differences in inflammation-induced gene expression, both mouse models showed similar levels of oedema, which further highlights a role for the lymphatics beyond fluid transport [29].

The lymphatics also responded to the filarial parasite *Brugia malayi* and differentially regulated the expression of a number of key immunological molecules related to lymphangeogenesis and immune cell migration and activation [23]. Interestingly, clinical lymphatic filariasis, induced by *Brugia malayi* and related parasites, is characterised by both immune depression and lymphatic dysfunction [23]. Thus, there appears to be a clear link between pathogen recognition, lymphatic gene expression, and global immune function in a clinical context. Interestingly, similar differences in key immunological molecules related to immune cell migration and activation were observed in LECs isolated from primary lymphoedema patients, which share the immune depression and lymphatic dysfunction of lymphatic filariasis patients [30]. Therefore, lymphatic-intrinsic expression of key immune mediators appears essentially linked to overall immune function in human disease.

Taken together, the specific recognition of distinct pathogens by LECs results in distinct functional programs that would be predicted to allow the lymphatics to actively respond and regulate pathogen-specific immune responses (Figure 1).
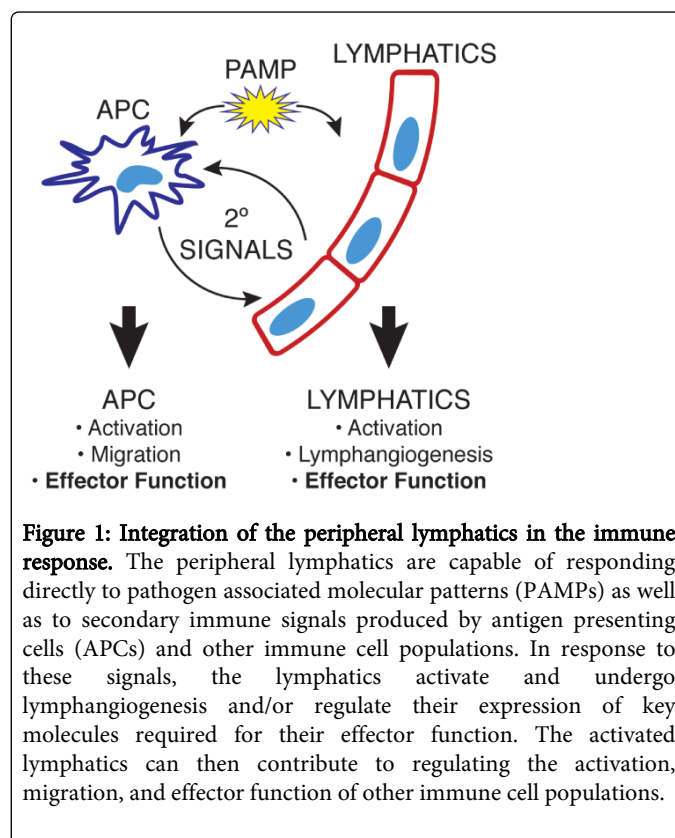
Figure 1: Integration of the peripheral lymphatics in the immune response. The peripheral lymphatics are capable of responding directly to pathogen associated molecular patterns (PAMPs) as well as to secondary immune signals produced by antigen presenting cells (APCs) and other immune cell populations. In response to these signals, the lymphatics activate and undergo lymphangiogenesis and/or regulate their expression of key molecules required for their effector function. The activated lymphatics can then contribute to regulating the activation, migration, and effector function of other immune cell populations.

Response to immune mediators

While external pathogenic signals may be the initial driving force behind an immune response, the resulting response is additionally influenced by cytokines and chemokines induced in the immune milieu. Moreover, it is the integration of direct pathogenic signals and these secondary immune signals that determines the outcome of an immune response. Consistent with their ability to sense pathogenic signals and modulate their function, LECs are responsive to a range of key immune mediators including adrenomedullin, chemokine (C-X-C Motif) ligand 12 (CXCL12), high-mobility group box 1 (HMGB1),

histamine, interferon alpha (IFNα), IFNβ, IFNγ, IL-1β, IL-6, IL-8, IL-20, IL-27, oncostatin M, retinoic acids, thrombin, transforming growth factor beta (TFGβ), and tumour necrosis factor alpha (TNFα) [31-44]. Importantly, these cytokines modulate the function of the lymphatics by inducing stimulus-dependent patterns of inflammatory gene expression and by mediating lymphangiogenesis in a stimulus-dependent manner.

As with the recognition of pathogenic stimuli, this recognition of secondary immune mediators appears to be equally important in a clinical context and has an impact on global immune functioning. In the context of models of abnormal wound healing, increased TGFβ expression inhibited LEC proliferation/function promoting lymphatic fibrosis and delaying lymphatic regeneration. Interestingly, lymphatic dysfunction has been shown to negatively affect the wound healing process and so pathogenic TGFβ signalling to the lymphatics appears to be driving the altered wound healing [20,36]. Similarly, IL-27 has been shown to act as an anti-lymphangiogenic modulator and has been implicated as a key contributor in numerous inflammatory disorders such as psoriasis, periapical lesions and osteoclastogenesis [45-47]. Interestingly, many of these same inflammatory disorders are also characterised by altered lymphatic growth/function suggesting a potential direct link between abnormal IL-27 signalling to the lymphatics and the altered lymphatic/general immune function in these inflammatory diseases [6-9].

Thus, the lymphatics appear to be capable of driving pathogen-specific responses by regulating their expression of key immunologic mediators. This specificity appears to be mediated via both the direct recognition of pathogens via specific pathogen recognition receptors and through the recognition of secondary immune signals produced in a pathogen-specific manner by other immune populations (Figure 1).

Cellular regulation of lymphatic function

While the responsiveness of the lymphatics to soluble immune mediators implies cellular interactions, a number of immune cell populations have been specifically implicated in the regulation of lymphatic function. One of the primary outcomes of the cellular regulation of lymphatic function appears to be the regulation of lymphangeogenesis. In the context of inflammation, lymphangeogenesis increases the density and size of lymphatic vessels in order to increase fluid drainage, cell migration, and antigen clearance from the affected site and is intrinsically linked to the resolution or progression of the inflammation [10,48]. Macrophages, T cells, mast cells, dendritic cells (DCs), and other stromal cell populations, can express lymphangeogenesis-promoting molecules, including the canonical vascular endothelial growth factors (VEGFs)-A, -C, and -D, in response to activation [48-54]. In fact, the induction of VEGF molecules appears to be a general characteristic of immune responses, such as those related to human inflammatory disease [6-9]. In addition to VEGF molecules, a large number of classical immune mediators, including IFNγ, IL-8, IL-17, and TNFα, have been shown to play additional roles in regulating lymphangiogenesis [35,49,55]. The consistent regulation of molecules involved in lymphangiogenesis in all immune responses by nearly all immune cell populations, suggests that the lymphatics are integrally linked with immunity.

However, despite these descriptions, the complex interplay between the lymphatics and other immune cell populations remains poorly understood. It thus remains of high priority to understand how specific immune cells direct lymphatic function and lymphangiogenesis; especially the spatiotemporal regulation of these intereactions and the role direct cell-to-cell contact might play.

Regulating the Immune Response

Regulation of cell migration and activation

The role of lymphatics as a passage for inflammatory cells to and from the periphery is well established. Nevertheless, this role is frequently perceived to be that of a relatively inert conduit. However, evidence suggests that peripheral lymphatic endothelial cells are more intimately involved in this aspect of the immune response. Given the essential nature of cell migration for initiating the majority of peripheral immune responses, a complete understanding of these processes is crucial.

The mechanisms of DC migration are different in the steady state compared to inflammatory conditions [43,56-59]. While this has been primarily associated with DC-dependant up-regulation of chemokine receptors and integrins [43,56-59], the concurrent regulation of the chemokine and integrin partners on the lymphatics in response to stimuli (see above) suggests that it is a combined DC-lymphatic activation sequence, which mediates DC migration. DC migration also appears to be different depending on the type of inflammation induced. For example, DCs show varied migratory behaviours in oxazolone-induced contact hypersensitivity and CFA-induced inflammation mice models [29]. This difference appears to be mediated by differences in both DC and lymphatic activation and function, especially given the large numbers of immune migration-related molecules differentially expressed in the LECs from either model [29]. Moreover, DC migration is not only regulated by lymphatic-expression of chemokines and integrins, but by the functional organisation of the lymphatics structure. For example, in response to inflammation and TNFα, LECs form ICAM-1 enriched microvilli structures and local CCL21 (CCR7 agonist) depots, which facilitate the migration of DCs [59,60]. Thus, while DC maturation is a well-accepted first step in the process of inflammatory DC migration into the lymph node, it is becoming increasingly clear that the activation of the lymphatic endothelium is an additional, crucial step in this process.

In addition to regulating migration into the lymph node, there is indirect data to suggest that the lymphatics may also contribute to migratory DC function and local immune homeostasis. Signalling through chemokine receptors and integrins, including CCR7 [61], CCR5 [62], CLEC2 [56], and ICAM-1 [63], additionally influence DC maturation. For example, CL19 and CCL21 signalling through CCR7 module DC proliferation, differentiation, survival and effector function [61,64]. In addition to chemokine receptor signalling, other related lymphatic-based signals may also have a crucial functional role in regulating the class of the immune response generated. For example, steady-state DCs receiving signals through lymphatic expressed ICAM-1 show reduced CD86 expression and maturation and thus a reduced ability to activate T cells, while TLR-stimulated DCs were immune to this LEC suppression [63]. Thus, LECs appear to be contributing to T cell tolerance in the steady state by regulating the functional status of DCs through direct cell-to-cell interactions. Finally, the lymphatics also express a number of poly-functional immune mediators in response to stimulation, such as IL-6 and IL-8, which are known to regulate the function of DCs and other immune cell populations [24]. Thus the peripheral lymphatics may play a more general role in regulating immune homeostasis though their stimulus-

specific expression of general immune mediators, although this has not been directly tested experimentally. Indeed, investigating these other immune modulatory roles represents a key area for future lymphatics research.

Comparing migratory DCs in the LNs to their tissue-resident counterparts, suggests that even in the steady state, DCs undergo a major phenotypic and functional maturation following migration into the lymph node [65]. Interestingly, the phenotype and numbers of these migratory DCs appears to be relatively constant in germ-free mice, where the contribution of pathogen-dependent signals on the DC maturation should be minimal. [66,67] given the ability of lymphatic-expressed signals to modulate DC maturation and migration, the lymphatics may be the key player in steady-state migration.

The interaction between DCs and LECs is the most readily studied in the literature. However, it is logical to suggest that the LECs would analogously influence the functions of T cells which also traffic through the lymphatics at various stages of their lifecycle [68]. Indeed, the complex role lymph node-resident lymphatics cells play in T cell immunity suggest a certain capacity for peripheral lymphatic cells to modulate T cell function, even without MHC expression [69]. Moreover, various T cell subsets also express CCR7 and would be predicted to show the same multifunctional activation to stimulation that is observed in DCs to lymphatic released CCR7-ligands [68]. Moreover, a specialised subset of LECs in lymphatic precollectors may be specifically involved in recruiting CCR10+ T cells during skin inflammation via CCL27, highlighting the diversity of LECs and suggesting an active regulatory role for the lymphatics [70]. Finally, LECs have been shown to express a wide range of poly-functional cytokines and chemokines, including IL-7, which may imply a role for the lymphatics in regulating T cell homeostasis, distinct from the well-appreciated role in T cell migration [71,72]. However, the interaction between LECs and T cells in the peripheral lymphatics remains poorly studied.

Collectively, these observations suggest that the regulation of cell migration and activation are intrinsically linked and are both crucially mediated by the lymphatics (Figure 1).

Clinical Perspectives

Given our burgeoning awareness of the role of lymphatic dysfunction in various pathological conditions, lymphatic-targeted therapeutic approaches are extremely promising for clinical practice [3,73]. Controlling the lymphatic vasculature could improve outcomes in a wide range of conditions from inflammatory disorders to cancer by increasing or decreasing lymphatic vessel numbers and/or regulating the function of the lymphatics. Indeed, given the significant active role the lymphatics play in regulating the immune response (as discussed in this review), complete, successful therapies for all immune conditions may require assessment and regulation of lymphatic function.

While lymphatic-based intervention strategies are relatively limited to date, a number of different approaches show promise. Promoting lymphatic vessel formation in conditions of lymphatic hypoplasia may be accomplished with extracorporeal shock wave therapy [74], treatment with IL-8 [35] and VEGF-C [75], or via induction of circulating endothelial progenitors [3,76]. In contrast, blocking lymphangiogenesis through VEGF inhibition with antibodies, peptides, pre-existing anti-cancer drugs (e.g. Sunitinib and

Rapamycin), or RNA interference may be beneficial in preventing tumour metastasis and improving outcomes [77-83]. Additionally, lymphatic microsurgery is often effective in the early-treatment/ management of lymphoedema and could be extended to additional conditions characterised by lymphatic dysfunction [84].

While the majority of these intervention strategies have yet to reach the clinic, they remain an area of increasing promise for targeting the lymphatics as a crucial component of the immune response. As our understanding of lymphatics-based regulation of the immune response increases, the number of therapeutic targets and therapeutic options should also increase.

Conclusions/Future Perspectives

We have highlighted a number of papers that describe both the regulation of the lymphatics by other immune populations and the reciprocal, lymphatic-mediated regulation of immune responses. There is strong evidence that the lymphatics play an active role in regulating the migration of immune cells thus contributing to the modulation of immune responses. In addition, there is evidence that the lymphatics can express a large number of poly-functional immune mediators not directly related to cell migration, which implies a more diverse role than previously appreciated. Taken together, these observations suggest that the lymphatics play a highly integrated role in regulating immunity (Figure 1), and as such it is crucial that the lymphatics are considered when assessing immunological studies. Whenever possible, future studies should be designed and interpreted so that all factors in the immune milieu are considered, including the role of the peripheral lymphatics.

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