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Targeting lymphatic function as a novel therapeutic intervention for rheumatoid arthritis

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Abstract

Although clinical outcomes for patients with rheumatoid arthritis (RA) have greatly improved with the use of biologic and conventional DMARDs, approximately 40% of patients do not achieve primary clinical outcomes in randomized trials, and only a small proportion achieve lasting remission. Over the past decade, studies in murine models point to the critical role of the lymphatic system in the pathogenesis and therapy of inflammatory-erosive arthritis, presumably by the removal of catabolic factors, cytokines and inflammatory cells from the inflamed synovium. Murine studies demonstrate that lymphatic drainage increases at the onset of inflammatory-erosive arthritis but, as inflammation progresses to a more chronic phase, lymphatic clearance declines

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Author contributions

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Competing interests

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and both structural and cellular changes are observed in the draining lymph node. Specifically, chronic damage to the lymphatic vessel from persistent inflammation results in loss of lymphatic vessel contraction followed by lymph node collapse, reduced lymphatic drainage, and ultimately severe synovitis and joint erosion. Notably, clinical pilot studies in patients with RA report lymph node changes following treatment, and thus draining lymphatic vessels and nodes could represent a potential biomarker of arthritis activity and response to therapy. Most importantly, targeting lymphatics represents an innovative strategy for therapeutic intervention for RA.

Introduction

Rheumatoid arthritis (RA) is an inflammatory joint disorder that affects 0.5-1% of the population worldwide.^{1,2} Despite major advances in our understanding of RA pathogenesis, major unmet clinical needs remain for a large proportion of patients, whose disease is refractory to current treatments.^{3,4} Although autoantibodies have long been implicated in RA, it is now broadly accepted that environmental and epigenetic factors are also critical in the development and pathogenesis of joint disease.⁵ Moreover, the aetiology of seronegative RA is not well understood.⁶ An additional relatively under-recognized mechanism, altered lymphatic function, has the potential to modulate disease activity and clinical course in RA via multiple pathways. Herein, we provide an overview of the synovial lymphatic system in the context of joint homeostasis and inflammatory arthritis, review the experimental and clinical evidence that support the central importance of the lymphatic system in the progression and resolution of inflammatory arthritis, and introduce the concept that the lymphatic vasculature is a potential clinical diagnostic biomarker and novel therapeutic target in RA.

The lymphatic system

Structure and function

In general, the peripheral lymphatic system has two primary functions: immune cell surveillance and interstitial fluid balance.⁷ The lymphatic system maintains interstitial fluid balance by shifting fluid from the periphery through vessels that connect to a series of draining lymph nodes, which empty into final ducts (for example, the right lymphatic duct and thoracic duct) where the lymph is returned to the vascular supply via the subclavian veins.⁸

Interstitial fluid becomes lymph when it enters the initial lymphatics or lymphatic capillaries that are present in most tissues, including the synovium (Figure 1A). These vessels are lined by a single layer of lymphatic endothelial cells (LECs) with specialized button-like junctions that are highly permeable to solutes and macromolecules, which physically attach to the extracellular matrix by anchoring filaments (Figure 1B).⁹⁻¹⁴ LECs overlap, creating primary valves that prevent the backflow of fluid into the tissue (Figure 1C).^{12,13} Entry of fluid into the lymphatic vessels takes place when external compressive forces are higher than the intraluminal fluid pressure, thereby pushing fluid, macromolecules and cells into the initial lymphatics.^{15,16} These external forces are generated by skeletal muscle contractions, ambulation or other joint movement.

The initial lymphatics converge to form larger collecting lymphatic vessels. These vessels are less permeable than the initial lymphatics owing to their tight zipper-like junctions between adjacent LECs.¹⁴ In addition, collecting lymphatics contain secondary bicuspid valves, which help prevent backflow, and one or more layers of lymphatic muscle cells (LMCs), which allow for vessel contraction to move lymph against the force of gravity (Figure 1D).^{9,10,17–19} LMCs have a myosin and actin cytoskeletal architecture with characteristics of both smooth muscle (SMA and SMB myosin heavy chain isoforms) and striated muscle (β -myosin heavy chain).²⁰ Thus, these cells possess characteristics of both vascular smooth muscle and cardiac striated muscle, facilitating the generation of tone as well as strong, phasic contractions.^{20–22} These contractile components respond to a tightly controlled cascade of signalling molecules that balances myosin light chain kinase, smooth muscle (MLCK) and myosin light chain phosphatase (a holoenzyme composed of 3 subunits, a catalytic subunit of type 1 phosphatase, PPlc; a targeting subunit, termed myosin phosphatase target subunit, MYPT; and a smaller subunit, M20, of unknown function²³) activity to produce the tone and contractile events (reviewed elsewhere¹⁰). Briefly, contractions can be generated by stretch-mediated, L-type or T-type calcium channels that generate an influx of Ca^{2+} ions, which bind to calmodulin.²⁴ Calmodulin, in turn, activates MLCK, phosphorylating myosin light chain and shortening its length. LMCs are relaxed by nitric oxide (NO), which is synthesized by LECs and derived from various sources, including inducible nitric oxide synthase (iNOS), produced either directly or indirectly from immune cells travelling along the lumen of the vessel, and endothelial nitric oxide synthase (eNOS) activated by shear stress.²⁵ NO translocates to the LMCs, where it activates soluble guanylyl cyclase and downstream cyclic GMP-dependent protein kinase (cGK-1, also known as protein kinase G), which blocks MLCK, enabling dephosphorylation of myosin light chain by its phosphatase and subsequent vessel relaxation (Figure 1E).²⁶

Fluid courses through the lymphatic vessels until it enters a series of lymph nodes, which are highly-organized organs containing immune cells, where adaptive immune responses are generated. Antigenic stimuli travel directly through the lymph, or are captured by antigen-presenting cells in the periphery and transported to the lymph node. Finally, lymph enters the sinuses via the afferent lymphatic vessel, exits in the efferent lymphatic vessel, and moves to the next lymph node, eventually returning to the circulation through the great veins. The pathways that lead to generation of an immune response are reviewed comprehensively elsewhere^{27–31} and are beyond the scope of this Review. Whereas much is known about antigen transport and immune interactions in the peripheral lymph nodes, understanding of the specific processes that lead to lymph generation and lymphatic contraction in human joints is extremely limited, and studies to elucidate the synovial lymphatic system are at the forefront of this field.

Effects of inflammation on lymphatics

The lymphatic system and immune function are closely connected because antigen-presenting cells enter lymphatic vessels to travel to the lymph nodes where they initiate adaptive immune responses. Dendritic cells (DCs), neutrophils and macrophages travel through these vessels.^{32–36} The importance of this lymphatic network is highlighted in mice that lack dermal lymphatics and show decreased humoral immunity.³⁷ Interestingly, immune

cells travelling adjacent to and within lymphatics can substantially alter the vessels by releasing factors that induce lymphangiogenesis and/or alter lymphatic vessel functions (such as contraction or permeability), and can also damage the vessel wall.³⁸

Lymphangiogenesis, or the growth of lymphatic vessels, is a primary response during inflammation, and disruption of this process leads to a variety of diseases.³⁹ Vascular endothelial growth factor C (VEGF-C), and its cognate receptor VEGF receptor 3 (VEGFR-3), expressed on LECs, are the master regulators of lymphangiogenesis.³⁹ Macrophages are a well-known source of VEGF-C and are abundant in inflamed tissues.^{40,41} Importantly, activated macrophages and T cells cooperate to enhance expression of VEGF-C.⁴² During inflammation, VEGFR-3 also regulates LEC expression of CC-chemokine 21 (CCL21), a potent chemotactic signal for DCs.^{32,43} These complex interactions reveal the presence of a coordinated system controlling lymphangiogenesis and immune cell migration.

Immune cell regulation of lymphatic contraction is facilitated primarily through cytokine-mediated NO signalling, as demonstrated by the reduced lymphatic contraction in a model of sterile inflammation, which was not observed in *iNOS*^{-/-} mice.⁴⁴ The reduction in lymphatic contraction frequency and ejection fraction was shown to be dependent on iNOS-expressing CD11b⁺Gr1⁺ cells that surround collecting lymphatic vessels.⁴⁴ Additionally, direct application of TNF and IL-1 β *in vivo* can decrease lymphatic contraction frequency, which is mediated through iNOS.⁴⁵ This finding was confirmed in *ex vivo* studies in which lymphatic contraction was inhibited by direct application of TNF to mesenteric lymphatic vessels, and restored by antagonizing iNOS and guanylate cyclase.⁴⁶ The mechanism of this impairment is known to be partially dependent on arachidonic acid metabolites; the subject has been reviewed elsewhere.^{10,47–49}

From *ex vivo* cannulation experiments, it is known that lymphatics respond to a variety of mechanical cues, including pressure, flow, and stretch.^{50–53} The increased fluid load traversing lymphatics during inflammation can increase all three of these factors. Contraction frequency increases in response to elevated transmural pressure, partially owing to increased shear that results in more NO production.^{50,52,54,55} However, it should be noted that there seems to be a Gaussian relationship between pressure and flow, as at high pressures, frequency reduces.⁵² When fluid load is lowered, and thus transmural pressure decreases, homeostatic contraction frequency returns. Therefore, whether a reduction in lymphatic contraction is mechanically and/or cytokine-mediated is difficult to determine from *in vivo* experiments.

Both initial and collecting lymphatics have an inherent permeability that is integral to their function. However, during inflammation, barrier integrity can be breached, leading to leakage of lymph into the interstitium. In the initial lymphatics, unidirectional flow facilitates the entry of fluid, cells, and macromolecules into the lymphatic system.^{9–14} During acute inflammation, permeability of the initial lymphatics increases, and tracer molecules flow back into the interstitium.⁵⁶ True measurements of initial lymphatic permeability are rare, but many groups have reported increased leakiness of the initial lymphatics in the presence of inflammation, which is a prospective sign of increased

permeability.^{57–59} Similarly, in a murine model of psoriasis, permeability increased in the initial dermal lymphatics of psoriatic plaques, but not in adjacent uninvolved skin.⁶⁰ In the collecting lymphatics, their inherent permeability is beneficial as it enables antigen sampling by macrophages and DCs in the surrounding adipose tissue, which facilitates downstream T cell responses.⁶¹ DCs regulate collecting lymphatic vessel permeability via CC-chemokine receptor 7 (CCR7)-dependent mechanisms.⁶² Diabetic mice, and mice after bacterial infection, also have increased collecting lymphatic permeability.^{63,64} Mechanistically, *in vitro* studies showed that TNF and IL-1 β can both increase LEC monolayer permeability.^{65,66} Thus, in contrast to the blood vasculature, permeability of lymphatic vessels is a highly regulated and dynamic process that changes during normal physiology and pathologic conditions.

Lymphatic phenotypes in RA

Evidence from mouse models

Although several animal models of inflammatory erosive arthritis exist, none precisely recapitulates the pathophysiology of RA.⁶⁷ As such, they are broadly divided into models of autoimmunity, which are characterized by sudden onset of disease, and models of general joint inflammation that display a more stochastic progression. K/B $\times$ N⁶⁸ and TNF-transgenic (TNF-Tg)⁶⁹ mice are representative examples of these different RA models, respectively, and have distinct aetiologies of inflammatory erosive arthritis. Because joint pathogenesis is a dynamic process, an approach to study arthritic changes has focused on longitudinal imaging studies using contrast enhanced MRI (CE-MRI), power Doppler ultrasonography (PDUS), near-infrared indocyanine green (NIR-ICG) imaging, and *in vivo* micro-CT (μ CT).^{59,70–73} With the use of these longitudinal imaging modalities, we found that K/B $\times$ N mice demonstrated a marked increase in lymphatic vessel contraction frequency during the acute phase of synovitis, which in the chronic phase of arthritis returns to levels similar to those seen in wild-type mice.⁵⁹ By contrast, the slow onset of inflammatory arthritis in TNF-Tg mice enabled us to identify that ankle and knee arthritis progresses by two distinct mechanisms, as illustrated in Figure 2. During the initial stages of arthritis and inflammation in the ankle joints of TNF-Tg mice, increased lymphangiogenesis maintains lymphatic drainage from the joint without a detectable increase in lymphatic vessel contractions compared with wild-type littermates (Supplemental Video 2).⁷⁴ This phase of arthritic progression is termed the ‘expansion’ phase because the popliteal lymph node enlarges in size owing to increased lymphangiogenesis, increased pressure from afferent vessels, and infiltration of IgM⁺CD23⁺CD21^{hi}CD1d^{hi} B cells.^{59,75–81} Interestingly, this subset of B cells is distinct from marginal zone and follicular B cells in the lymph node, suggesting they are a unique population of B cells in inflamed lymph nodes, which we therefore named ‘Bin’ cells.^{79,82} The increase in lymphatic vessel contraction frequency in K/B $\times$ N mice, and lymphangiogenesis in both K/B $\times$ N and TNF-Tg mice, represent a compensatory response that enhances the removal of inflammatory cells and catabolic factors from the synovium. Functionally, this process limits joint inflammation and focal erosion in the expanding phase. In these models, however, this compensatory mechanism is incomplete because synovial inflammatory cells and catabolic factors directly damage LECs and LMCs when they pass through afferent lymphatic vessels and draining lymph nodes.⁸³

Following the expansion phase in the TNF-Tg model, the popliteal lymph node reduces in volume and power Doppler signal decreases, indicating reduced blood flow, marking what has been termed the ‘collapsed’ phase of arthritic progression.^{70,72} This collapse coincides with several pathologic processes, including increased synovial hyperplasia, poor lymphatic clearance, lymphatic vessel damage with subsequent increased leakiness and loss of contractions (Supplemental Video 3), leukocyte stasis within lymphatic vessels, translocation of Bin cells from the lymph node follicles to the lymph node sinuses, and reduction in draining lymph node volume with increased fluid pressure.^{70,75,79,81,84,85} Interestingly, the lymph node collapse is asymmetrical, and occurs along the ipsilateral axis of the lower limb.⁸⁵ Although a direct cause and effect relationship between the altered lymphatic dynamics outlined above and arthritic processes is not firmly established, the sequence of events fits with a unifying model to explain the collapsed phase (Figure 2). In this model, increased inflammatory cells, cytokines/chemokines and catabolic factors generated during persistent inflammation pass through the afferent lymphatic vessels, and damage LECs and underlying LMCs. Concomitantly, these catabolic factors trigger LECs to express high levels of iNOS, resulting in ‘NO squelching’ of eNOS-mediated contractions. Thus, lymphatic vessel structural damage and NO production by LECs results in loss of lymphatic vessel contractions owing to constitutive activation of soluble guanylyl cyclase and downstream cGK-1-mediated inhibition of calcium signalling in LMCs (Figure 1E). At the same time, activated macrophages become static in the vessel lumen because of the loss of contractions. These cells also express iNOS, which furthers inhibition of lymphatic vessel contraction due to NO squelching. Additionally, activated macrophages in the draining lymph nodes express the potent B cell chemotactic factor CXC-chemokine 13 (CXCL13)⁸⁶. A decrease or absence of lymphatic flow from afferent lymphatic vessels enables a CXCL13 gradient to form, increasing CXCL13 levels in the lymph node sinuses, which drives the migration of Bin cells from the follicles into the sinuses. As a result, these Bin cells accumulate and clog the lymphatic vessel. Both active and passive lymphatic transport from the inflamed joint is consequently markedly reduced or abolished, and is followed by progressive joint inflammation and destruction.

Lymph node collapse in RA

Although lymph node involvement and lymphadenopathy in patients with RA was first described in 1896 by Chauffard and Ramond,^{87,88} follow-up studies have been hampered by the absence of reliable clinical outcome measures and biomarkers to assess lymphatic function, and to monitor disease progression and response to therapy. Knowledge of human lymphatics has advanced considerably in the last 10 years, based on recent pioneering studies using cadaveric dissection, which mapped the lymphatic anatomy of the upper limb in detail.^{89,90} Moreover, clinical imaging strategies (CE-MRI, PDUS and NIR-ICG)^{91–98} have provided the first evidence that the lymphatics draining inflamed joints can serve as biomarkers of arthritis and response to therapy.⁹⁵ Additionally, *ex vivo* histopathology and immunofluorescence analyses of popliteal lymph node tissues harvested from patients undergoing total knee replacement surgery or lower limb amputation have confirmed the presence of B follicular hyperplasia and histological alterations in lymph nodes, and the existence of Bin-like B cells, in tissue from patients with RA, findings not observed in tissues from patients with other (non-RA) severe lower limb diseases.⁸⁰

The next critical question is whether the phases of lymph node changes identified in murine studies are relevant to lymph node changes in RA. To address this question, Manzo and colleagues designed a proof-of-concept pilot study to formally examine whether RA is associated with alterations in the joint-draining lymph nodes.⁹⁸ Clinical and PDUS examination revealed hypertrophy of the lymph node cortex in the absence of clinical lymphadenopathy in 7 out of 10 patients with active disease, but there were no lymph node aberrations in the 5 patients in clinical remission or 5 healthy controls. Moreover, PDUS signal was amplified in the cortical and hilar regions of lymph nodes at baseline but returned to normal levels after 3 months of anti-TNF and/or methotrexate therapy⁹⁸. In a follow-up study of 40 consecutive patients with RA refractory to treatment with DMARDs and 20 healthy individuals,⁹⁷ PDUS demonstrated considerable alterations of RA lymph node morphology and volume following anti-TNF treatment, and a degree of concordance of these alterations with synovitis activity in peripheral joints. Interestingly, low PDUS signal in the lymph nodes (which has also been observed in collapsed murine lymph nodes)^{70,72} at baseline was strongly associated with a poor clinical response to anti-TNF therapy. These studies provide initial evidence that lymph node dynamics, including changes in volume and PDUS signal, a marker of blood flow, are associated with joint pathology and responsiveness to treatment, and thus are a biomarker of RA disease activity and response to therapy.

Advances in RA flare assessment

The murine models outlined above demonstrate a strong temporal association between altered lymphatic flow and progressive joint inflammation and damage. A major question that emerges from these studies is whether the lymphatic dynamics contribute to joint inflammation in RA. Joint flare, a prevalent but poorly understood process in inflammatory arthritis, provides an opportunity to examine a potential relationship between the lymphatics and arthritis exacerbation.

In spite of more aggressive treatment approaches based on combination therapies of conventional and biological DMARDs, one of the perplexing problems in RA care is the loss of response to therapies over time, and the well-recognized phenomenon of periods of episodic worsening of disease activity, often referred to as disease flare. Ongoing research has advanced our understanding of the descriptive characterization of flares from the perspectives of patients and health care providers, yet we lack insight into the central pathophysiological correlates. Moreover, we lack the biomarkers of impending flare that are needed to facilitate early recognition and targeted intervention. As outlined in this Review, the discovery that altered lymph node dynamics and lymphatic flow precedes exacerbated arthritis in murine models provides critical clues to the existence of alternative pathways responsible for acute and chronic synovial inflammation, which might have relevance in patients with RA.

Inflammatory flares of RA are a well-recognized feature of the underlying disease. Some flares are self-limited, mild in nature, and resolve without intervention, whereas others persist and worsen, and ultimately represent failure of a previously effective therapy and necessitate a change in treatment. Work ongoing over a number of years by multiple groups has sought to better characterize flares in RA. To facilitate research in the area, the Outcome

Measures in Rheumatology Clinical Trials (OMERACT) group proposed to define the concept of RA flare as “worsening of signs and symptoms of sufficient intensity and duration to lead to change in therapy”,^{99,100} and further identified events that characterized flares along their continuum of severity.¹⁰¹ This framework led to the development of a set of core domains to assess flare in RA, which included standard assessments of RA disease activity (swollen and tender joints, laboratory markers), patient and physician global assessments of disease activity, patient pain ratings, and assessment of physical function.¹⁰² Additional assessments including fatigue, stiffness, and participation in life activities were subsequently added and validated.^{103–105}

Demonstrating that patient reports and clinical assessments actually reflect events in the synovial microenvironment are important for linking clinical phenomenology with pathobiology. Ultrasonography and MRI have been used for such evaluations.⁹⁵ Ultrasonography is a powerful tool that provides point-of-care testing and objective evidence of active inflammation including the presence of joint effusions, synovial hypertrophy, and bone erosions.^{107,108} However, the utility of ultrasonography is limited by operator proficiency and the inability to visualize deeper anatomical structures.^{109,110} Compared with ultrasonography, MRI can image deep structures with high resolution, and therefore provide greater detail regarding disease activity, although its use is limited by cost, feasibility, and availability.^{110,111} Therefore, both imaging modalities are currently applied in a complementary fashion to evaluate arthritic disease.

Although limited in number, studies investigating the relationship between imaging outcomes and instruments assessing the OMERACT core domains have helped refine our understanding of the progression of RA flare. Applying the OMERACT definition of flare, Saleem and colleagues showed that some patients in clinical remission demonstrated power Doppler signals in the upper extremities (wrist and hand) that were predictive of subsequent flare.¹¹² Furthermore, in a cohort of patients with RA in clinical remission ($n=427$), imaging of the hand, wrists and associated tendons by PDUS^{113–115} detected tenosynovitis in 22.7% (95% CI 0.19–0.27), and these patients had an increased likelihood of flare as determined by the self-reported Flare Assessment in Rheumatoid Arthritis (FLARE) questionnaire (OR 1.95; 95% CI 1.17–3.26). These studies suggest that in clinically asymptomatic patients, ultrasonography (PDUS in particular) can reveal unstable or fluctuating inflammation that develops into flares.

MRI has also been used to evaluate synovial inflammation and its ability to assess or predict flare.^{116–119} Gaffney *et al.* used DCE-MRI, a method to assess ‘wash in’ and ‘wash out’ of contrast dye, to compare changes in knee synovial contrast enhancement to histological synovial changes via synovial biopsies in 20 patients with RA.¹¹⁷ They found good correlation ($r=0.63$, $P<0.01$) between histology and imaging. However, they were unable to correlate MRI synovial enhancement with flare as determined by clinical findings and use of the Health Assessment Questionnaire (HAQ, global assessment), or by the modified Ritchie Articular Index (local assessment). In a study performed in a cohort of patients with juvenile idiopathic arthritis, those who subsequently experienced a disease flare (12 of 32 patients), as defined by no longer meeting Wallace criteria for remission, had significantly different maximal enhancement of synovium on DCE-MRI at baseline compared with patients in

remission over the course of the study ($P=0.05$); a higher maximal enhancement was suggestive of flare potential.¹²⁰ The applicability of these findings to adults with RA require confirmation, but this study suggests the potential usefulness of DCE-MRI for flare prediction.

Taken together, the efforts to define flare by use of questionnaires or imaging have provided valuable tools for diagnosis and possible intervention, yet the mechanisms that underlie this sudden alteration in joint inflammation remain enigmatic.

Lymphatic alterations in flare

The association between arthritic flare and lymph node dynamics is poorly understood. To explore this association, we performed a pilot clinical study to investigate the relationship between arthritis flare and popliteal lymph node volume by use of CE-MRI in 10 patients with RA, before and after anti-TNF therapy.⁹² Knee flare in patients with chronic arthritis (disease duration >20 years) demonstrated advanced synovitis adjacent to focal bone erosions and small popliteal lymph nodes. In keeping with the findings in our preclinical studies,⁷⁵ all patients with detectable lymph nodes on CE-MRI showed decreased lymph node volume after 16 weeks of anti-TNF therapy (mean decrease 37%; $P=0.002$).⁹² Also, a significant improvement in knee pain was observed, as determined by the Rheumatoid and Arthritis Outcome Score,¹²¹ which inversely correlated with a decreased in popliteal lymph node volume ($R^2=0.94$; $P<0.05$). In aggregate, these results suggest that patients with RA in whom draining lymph node volume is maintained during anti-TNF therapy experience the greatest amelioration of pain in flaring joints.

To formally establish quantitative biomarkers for draining lymphatics in RA, several human NIR-ICG imaging studies are ongoing. An example of this pioneering work comes from Sevick-Muraca and colleagues, who evaluated lymphatic vessels in patients with lymphoedema.^{122–124} This group was the first to demonstrate lymphatic vessel contraction rates in healthy people.¹²⁵ An example of this experimental NIR-ICG imaging technology and its potential as a clinical diagnostic tool is presented in Box 1 (See also Supplemental Video 4, 5, and 6). Although no NIR-ICG imaging studies have been reported in patients with RA to date, open clinical protocols are actively enrolling participants¹²⁶, and it is likely that data will be forthcoming in the next few years.

Lymphatic-modulating treatments for RA

The findings in murine models of arthritis coupled with imaging findings in patients with RA strongly implicate the synovial lymphatic system in the events that promote exacerbation of arthritis. The next important challenge is to address therapeutic strategies to restore lymphatic flow (Table 1). Therapeutic options — both FDA-approved RA therapies and novel lymphatic-modulating interventions — should be considered. It is fascinating to note that despite the efficacy of our current targeted biologics, the mechanisms that promote resolution of synovitis remain enigmatic. Studies have focused on unique pathways that are interrupted by anti-TNF agents^{127–129} and antibody-independent effects of anti-CD20 B cell depletion therapy.^{130,131} In particular, the marked reduction in synovial macrophage numbers following anti-TNF therapy does not result from apoptosis,^{132,133} or altered

monocyte influx into the synovium.¹³⁴ Rather, anti-TNF therapy restores lymphatic vessel contractions, attributed to lymphatic vessel repair and the restoration of contractions promotes leukocyte migration and egress from inflamed joints, as determined by transmission electron microscopy.⁸³

The pathways leading to joint inflammation and damage in the TNF-Tg murine arthritis model are lymphocyte-independent,¹³⁵ providing an ideal setting to assess the antibody-independent efficacy of B cell depletion.¹²⁴ With this in mind, the results of a study in which B cells were depleted in TNF-Tg mice before and after the onset of arthritis demonstrated that anti-CD20 prophylactic treatment prevented knee flare,⁷⁹ and therapeutic treatment of flaring mice with collapsed popliteal lymph nodes ameliorated inflammatory-erosive arthritis.⁷³ Moreover, longitudinal NIR-ICG imaging and immunofluorescent intravital microscopy before and after anti-CD20 treatment demonstrated that B cell depletion restores passive lymphatic flow and leukocyte migration in lymphatic vessels without restoring contractions.⁷³ Given that anti-CD20 treatment removes B cells from the lymphatic sinuses in draining lymph nodes,⁷³ we conclude that B cell depletion maintains lymph node expansion by preventing B cell influx, and ameliorates arthritic flare by unclogging draining lymphatic vessels and restoring passive lymphatic flow. However, it should be noted that, although these B cells are polyclonal and do not seem to have the ability to express pro-inflammatory cytokines,⁷⁹ they can reduce inflammation or induce lymphangiogenesis by alternative mechanisms, as has been described elsewhere.¹³⁶

Promoting lymphangiogenesis and drainage

Lymphangiogenesis is a compensatory response to joint inflammation, and inhibition of lymphatic vessel formation during arthritic progression considerably exacerbates disease.⁷⁷ Therefore, an intervention for RA that enhances lymphatic drainage from affected joints has therapeutic potential. An initial proof-of-concept study examining this potential found that injection of VEGF-C adeno-associated virus into inflamed murine joints reduced synovitis, bone erosion, cartilage loss and the number of infiltrating macrophages.¹³⁷ Interestingly, a 2014 study demonstrated that VEGF-C–VEGFR-3 signalling also inhibits macrophage activation via regulation of the Toll-like receptor 4–nuclear factor- κ B pathway.¹³⁸ Thus, VEGF-C might serve a dual therapeutic function in RA by improving lymphatic vessel function and inhibiting inflammation. Although gene therapies for arthritis have proven to have major clinical challenges,¹³⁹ it is of interest that a clinical trial of VEGF-C gene therapy for lymphoedema following mastectomy are currently ongoing.¹⁴⁰

Traditional Chinese medicines appeal to many patients, on the basis of their perceived safety and efficacy, coupled with the high costs and attendant risks associated with newer therapies. Indeed, traditional Chinese medicine is a mainstay therapy for arthritis worldwide.¹⁴¹ To test if traditional Chinese medicines have direct effects on lymphatic dysfunction, TNF-Tg mice with established arthritis were treated with Du-Huo-Ji-Sheng-Tang (DHJST)¹⁴² and its purified active compound ferulic acid²⁵ for 3 months. The treated mice showed reduced joint inflammation and bone and cartilage erosion.²⁵ Of particular relevance, DHJST and ferulic acid promoted lymphatic drainage from affected joints to draining lymph nodes while restoring lymphatic vessel contraction.^{25,142} Mechanistically, ferulic acid increased

expression levels of contraction-related genes in LMCs, and inhibited TNF-induced NO production by LECs.²⁵ DHJST and ferulic acid also increased the lymphatic thoracic duct formation in zebrafish.¹⁴² Another traditional Chinese medicine, panaxnotoginseng, promoted LEC migration, tube formation, and VEGF-C expression.¹⁴³ Collectively, these findings suggest that traditional Chinese medicines regulate lymphatic vessel draining function by affecting LECs and LMCs, and might have DMARD activity.

Targeting lymphatic function

As described above, NO is a central regulator of lymphatic vessel contractions, and excessive iNOS-derived NO inhibits lymphatic vessel contractions in inflamed tissues (Figure 1E). Thus, there is a strong rationale for selective iNOS inhibition as a novel RA therapy. Indeed, a selective iNOS inhibitor (GW274150) was evaluated in a phase IIA clinical trial for early RA, and showed a trend towards reduction in synovial thickness (33%; $P=0.072$) and synovial vascularity (42%; $P=0.075$) compared with placebo at 28 days as shown by PDUS.¹⁴⁴ However, our preclinical data suggest that expanding lymphatics would predominate in early RA (Figure 2), and therefore that selective iNOS inhibition would have greater effects on advanced RA (collapsed phase), although this remains speculative. To test this hypothesis in TNF-Tg mice, we evaluated the effects of L-N6-(1-iminoethyl)lysine 5-tetrazole-amide (L-NIL), a moderately selective inhibitor of iNOS that does not affect eNOS activity¹⁴⁵ and reduces adjuvant-induced arthritis in rats.¹⁴⁶ Remarkably, we found that a single injection of L-NIL into the footpad of TNF-Tg mice with collapsed lymph nodes significantly increased dye uptake and restored lymphatic contractions within 300 s of administration, whereas injection of saline had no effect.²⁵ In another cohort of TNF-Tg mice, treatment with L-NIL, but not with saline or with the non-specific NOS inhibitor N ω -nitro-L-arginine methyl ester (L-NAME), significantly increased lymphatic contraction in TNF-Tg mice, demonstrating that the recovery of lymphatic function was attributable to iNOS inhibition. Furthermore, no changes in lymphatic contractions were observed in wild-type mice treated with L-NIL, L-NAME or saline.²⁵ These proof-of-concept studies indicate that agents that selectively inhibit iNOS or iNOS-mediated downstream signals could represent new therapies for RA by regulating lymphatic vessel function.

Drug screens in wild-type mice for lymphatic-modulating drugs via NIR-ICG imaging revealed that a short-acting phosphodiesterase 5 (PDE5) inhibitor (sildenafil) induces lymphatic rerouting (see Supplementary video 7) versus placebo control (see Supplementary Video 1). This finding suggests a novel alternative intervention for collapsed lymphatics, in which dysfunctional lymphatic vessels can be circumvented by dilation of collateral lymphatic vessels; this approach warrants investigation in animal models of inflammatory arthritis. PDE5 in vascular smooth muscle cells hydrolyzes cGMP and inhibits vessel relaxation. Therefore, we hypothesize that PDE5 inhibitors, such as sildenafil and tadalafil, promote LMC relaxation and could therefore reduce lymphatic contraction (Figure 1E). Future studies are necessary to validate these preliminary findings and confirm the mechanism of action of PDE5 in lymphatics.

Lymphatic drug targets in other disease states

The lymphatic system is now a major research focus owing to the central importance of lymphatic transit in homeostasis and evidence for its involvement in many disease processes, including Alzheimer disease, glaucoma, hypertension, myocardial infarction, lymphoedema, chronic bronchitis, and cancer.^{147–152} Although this research is still in its early stages, it has catalyzed the need to understand basic lymphatic physiology (Figure 1) and potential treatments targeting lymphatics.

Secondary lymphoedema is a prevalent conventional lymphatic disease, but effective therapies are not available. It often occurs when lymph nodes are removed during cancer therapy and can lead to impaired lymphatic transport. Therefore, treatments that increase lymphangiogenesis show promise. As mentioned above, VEGF-C gene therapy is being studied in a phase I clinical trial¹⁴⁰ in patients with lymph node transplant and breast cancer-associated lymphoedema.^{153–156} In a study published in 2017, bestatin, a leukotriene A4 hydrolase antagonist, alleviated experimental lymphoedema by improving lymphatic anatomy and promoting lymphangiogenesis;¹⁵⁷ this drug is currently under evaluation in a phase II clinical trial in patients with secondary lymphoedema of the lower limb¹⁵⁸. Similarly, investigators have set out to improve lymphangiogenesis in other models with lymphatic insufficiencies. Treatment with VEGF-C reduced intraocular pressure and restored heart function after myocardial infarction in mouse models.^{150,159}

Lymphangiogenesis can also be pathological. The cornea is avascular, but during cornea transplants there is often both angiogenesis and lymphangiogenesis, which is considered a poor prognostic factor.¹⁶⁰ In preclinical models, blocking lymphangiogenesis in a cornea graft improved the survival of the transplant.^{161,162} In this case, the regenerating lymphatics in cornea grafts deliver immune infiltrates that orchestrate graft rejection.¹⁶³

It is now well-established that lymphatic dysfunction is an important factor in many disease states, and advantages in imaging have allowed for the functional assessment of lymphatics, both preclinically and clinically. Preclinically, lymphatic contraction is inhibited in obesity¹⁶⁴ and inflammation.⁴⁴ Clinically, Dercum disease (adiposis dolorosa)¹⁶⁵, chronic venous insufficiency¹⁶⁶, and lymphoedema¹⁶⁷ demonstrate lymphatic contraction deficiencies. Moreover, in intestinal inflammation, a model of inflammatory bowel disease, lymphatic contraction can be restored with glibenclamide, which targets potassium channels located on LMCs.¹⁶⁸

The preponderance of research is centered on understanding how lymphatics respond to a disease state or condition such as inflammation, whereas other investigations focus on how lymphatic dysfunction can drive disease. The most notable example of lymphatic dysfunction driving pathology and dysfunction is primary lymphoedema, which arises from a genetic mutation associated with loss of function in some component of the lymphatic vessel (such as the valves¹⁶⁹ or VEGFR-3¹⁷⁰). However, preclinical work has shown that, at least in murine models, lymphatic malformations can drive consequences other than lymphoedema. For instance, mice with haploinsufficiency in a gene essential for induction of the LEC phenotype (*Prox1*) were found to be a model for both lymphatic dysfunction and

adult-onset obesity. *Prox1*^{+/-} mice have tortuous and leaky lymphatic vessels and manifest increased fat stores in lymphatic-rich regions; furthermore, lymph collected from *Prox1*^{+/-} mice promoted adipogenesis *in vitro*.¹⁷¹ Similarly, Chy mice that are *Vegfc* haploinsufficient have lymphatic insufficiency, increased cutaneous fat deposition, chylous ascities and lymphoedema.¹⁷²

Conclusions

Although lymphatics have long been recognized as important in the pathogenesis of inflammatory arthritis, this area of research has only in the last 10 years been enabled by longitudinal imaging as described above. A high priority is investigation of the synovial lymphatic system, which originated from research aimed at elucidating the nature of the waxing and waning joint pathology characteristic of relapsing flare in RA.^{130,131} Mounting evidence demonstrates that lymphatic dysfunction exacerbates arthritic flare and disease progression in RA. Murine models have demonstrated that dramatic changes in lymphatic vessel function occur during the pathogenesis of inflammatory arthritis; these changes include an initial compensatory expanding phase in which the lymph node expands in size and lymphatic contraction and clearance are proficient, followed by a collapsed phase in which lymph nodes decrease in volume and there is a loss of the intrinsic lymphatic contraction. This dichotomy is mirrored by joint inflammation, where knee inflammation is not apparent until the collapsed phase, suggesting that targeting lymphatic function could be a potential treatment option and a promising area for future research.

Initial studies in patients demonstrate that alterations in the lymph nodes and lymphatic vessel function occur in RA. Moreover, near-infrared imaging can quantify lymphatic vessel function in patients, and permits identification of those who are likely to respond to specific treatments. Thus, we anticipate that therapies targeting lymphatic function will prove efficacious in RA.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Key points

- Dramatic changes in lymphatic vessel contraction and in the lymph nodes that drain inflamed joints are associated with disease progression and response to therapy in murine models of rheumatoid arthritis (RA)
- During mild to moderate experimental arthritis, lymphatic vessels and nodes that drain the joint undergo an initial ‘expansion’ phase that facilitates efficient lymphatic clearance and lymphatic vessel contractions
- In preclinical models, the expansion phase is followed by a ‘collapsed’ phase, in which B cells in the draining lymph node translocate from the follicles to lymphatic sinuses and the lymph node collapses
- The collapsed phase is characterized by lymphatic vessel structural damage, loss of contraction, and reduction in lymphatic clearance
- Pilot clinical studies indicate that alterations in lymph node volume and/or lymphatic flow could serve as biomarkers of treatment response with the potential to predict RA flare
- Several lymphatic system-modulating therapies show promise in preclinical models of inflammatory arthritis and RA

Box 1.**Near-infrared imaging of lymphatic vessels in humans.**

Visualization and quantification of lymphatic function with near-infrared (NIR) imaging provides a unique opportunity to examine the role of lymphatic contraction and flow in human disease.^{122,123} Following intradermal injections of a NIR dye, usually indocyanine green (ICG), images are captured by cameras sensitive to the NIR spectrum where ICG emits (peak of ~825 nm) to reveal lymphatic vessel anatomy and function. From cadaveric studies, we know that all areas of the dorsal hand drain through the major collecting vessels that initiate at the mid-dorsal aspect and cross the wrist, primarily running adjacent to the basilic or cephalic veins.^{89,90,173} It is therefore likely that the metacarpal joints are also drained by these collecting vessels. Experimental NIR-ICG imaging (as described by Rahimi *et al.*⁹⁵) has been used for the analysis of human lymphatic flow in a study in which ICG was injected into the four web spaces of the hands of a healthy volunteer following informed consent¹²⁶. In the NIR-ICG image on the left-hand side (a still image from Supplementary video 4), the major veins of the hand appear as dark vessels and lymphatic vessels (which fill with ICG) appear white. ICG signal is also apparent at the injection sites (*). The collecting lymphatic network is adjacent to the dorsal venous arch (#), and further joins together forming larger vessels

adjacent to the basilic vein (red arrow), and cephalic vein (yellow arrow). Also shown is a schematic representation of these veins and lymphatic vessels. The graph (a still image from Supplementary video 5) quantifies lymphatic vessel contractions in the hand during a 10-minute imaging session, by plotting the signal intensity in a region of interest over time; spikes in signal intensity (white arrows) are scored as lymphatic contractions. Contraction frequency changes over time during inflammatory arthritis in mice,^{25,38,59,73} and could prove to be an important biomarker or outcome measure in human disease.^{44,45,83} In the NIR-ICG image of the forearm from the same subject (obtained in the same session as the hand image), the lymphatic vessels that drain the web spaces flank the cephalic (yellow arrow) and basilic (red arrow) veins across the mid-forearm and the antecubital fossa. The graph (a still image from Supplementary video 6) presents quantification of the cephalic lymphatic vessel contraction frequency at the antecubital fossa, achieved through region of interest analysis.

Figure 1. Structure and function of the synovial lymphatic system.

(A) The synovial lymphatic system of arthrodial joints includes initial lymphatics interspersed throughout the synovium, which join to form collecting lymphatics that lead to joint-draining lymph nodes and, eventually, to the thoracic or right lymphatic duct, which joins the venous supply. (B) Initial lymphatics are composed of a network of lymphatic endothelial cells (LECs) with anchoring filaments to the basement membrane. Overlapping portions of LEC membranes create button-like junctions that act as primary valves to enable macromolecules, cells and fluid to enter the initially lymphatic system. (C) Lymphocytes

enter the initial lymphatics from the interstitial space. **(D)** Collecting lymphatic vessels comprise a single layer of LECs joined together by impermeable zipper-like junctions and one or more layers of continuous lymphatic muscle cells (LMCs). **(E)** Contractions in LMCs are triggered by calcium signalling, which is inhibited by nitric oxide (NO) signalling through soluble guanylyl cyclase (sGC). Under homeostatic conditions, NO produced by endothelial nitric oxide synthase (eNOS) in adjacent LECs inhibits LMC contractions to the lymphatic vessels to fill. In the setting of inflammation, the cycle of lymphatic vessel contractions can be disrupted by ‘squelching’ NO produced from inducible nitric oxide synthase (iNOS) expressed in adjacent LECs and/or immune cells, such as activated-adherent macrophages. CaM, calmodulin.

Figure 2. Overview of lymphatic phenotypes in normal (-healthy), expanding and collapsed lymph nodes in mice.

(A) The distal lymphatic vessels drain the footpad to the popliteal lymph node (PLN), and the proximal lymphatic vessels drain lymph from the PLN and knee synovium to the iliac lymph node (ILN). Inset: In contracted and dilated lymphatic vessels, tight connections exist between lymphatic endothelial cells (LECs) and lymphatic muscle cells (LMCs) during transport of lymph. At homeostasis, these lymphatic vessels contract 0.5–2 times per minute. Near infrared-indocyanine green (NIR-ICG) image shows the lower limb of a wild-type

mouse (Supplemental Video 1). **(B)** The initiation of inflammatory arthritis is followed by an 'expansion' phase, in which lymphangiogenesis in the synovium facilitates removal of inflammatory cells and lymph to the draining lymph nodes, which expand up to 10-fold in volume owing to lymphangiogenesis, excess lymphatic flow, and accumulation of B cells. Inset: CD11b⁺ macrophages are present in contracted and dilated lymph vessels and travel at great speed (~0.2 mm/s). In models of autoimmune arthritis, lymphatic vessel contractions increase to ~5 contractions/min at initiation of autoimmunity before stabilizing at ~1 contractions per min in the chronic phase of inflammatory arthritis **(C)** During chronic synovitis, ipsilateral PLNs and ILNs simultaneously collapse via poorly understood mechanisms. Lymphatic contractions are absent or rare in this 'collapsed' phase (Supplemental Video 3). PLNs and ILNs decrease in size owing to fluid loss, and B cells translocate from B cell follicles into the lymphatic sinuses effectively 'clog' the lymphatic vessels. A lack of lymphatic transport results in a sudden, severe exacerbation of synovitis. Anti-CD20 therapy removes B cells and restores passive lymphatic flow from the joint. Inset: Although the initiating event(s) of the collapsed phase are not known, several pathologic features of the degenerated lymphatic vessel suggest the involvement of intersecting mechanism.

Table 1.

Potential lymphatic-modulating therapies and their mechanisms of action

Therapy	Target	Mechanism of action	Potential adverse effects	Ref
VEGF-C gene therapy	VEGFR-3	Increases lymphangiogenesis	Unknown	137,138
TNF antagonists	TNFR	Reduces inflammation to restore lymphatic vessel contraction	Skin reaction at injection site; risk of infection (including tuberculosis) and cancer (including skin and lymphoma)	83
B cell depletion therapy	CD20	Unclogs lymphatic sinuses	Mucocutaneous reactions (Stevens–Johnson syndrome, infections (rarely, progressive multifocal leukoencephalopathy))	79,84
L-NIL	iNOS	Reduces nitric oxide that keeps vessels dilated	Unknown, possible hypertension	25,144,146
PDE5 inhibitors	PDE5	Dilates lymphoid vessels and sinuses to encourage lymphatic rerouting and B cell removal, respectively	Flushing, headache, visual disturbance	Unpublished data (Bouta et al, unpublished)
Ferulic acid	VEGFR-3	Increases VEGF-C production Reduces inflammation and restore lymphatic contraction Reduces nitric oxide production by LECs	Unknown	25,141,142

Abbreviations: iNOS, inducible nitric oxide synthase; LEC, lymphatic endothelial cell; L-NIL, L-N6-(1-iminoethyl)lysine 5-tetrazole-amide; PDE5, phosphodiesterase 5; TNFR, TNF receptor; VEGF-C, vascular endothelial growth factor C; VEGFR, vascular endothelial growth factor receptor.