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REVIEW

Engineering approaches to investigate the roles of lymphatics vessels in rheumatoid arthritis

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Abstract

Rheumatoid arthritis (RA) is one of the most common chronic inflammatory joint disorders. While our understanding of the autoimmune processes that lead to synovial degradation has improved, a majority of patients are still resistant to current treatments and require new therapeutics. An understudied and promising area for therapy involves the roles of lymphatic vessels (LVs) in RA progression, which has been observed to have a significant effect on mediating chronic inflammation. RA disease progression has been shown to correlate with dramatic changes in LV structure and interstitial fluid drainage, manifesting in the retention of distinct immune cell phenotypes within the synovium. Advances in dynamic imaging technologies have demonstrated that LVs in RA undergo an initial expansion phase of increased LVs and abnormal contractions followed by a collapsed phase of reduced lymphatic function and immune cell clearance in vivo. However, current animal models of RA fail to decouple biological and biophysical factors that might be responsible for this lymphatic dysfunction in RA, and a few attempted in vitro models of the synovium in RA have not yet included the contributions from the LVs. Various methods of replicating LVs in vitro have been developed to study lymphatic biology, but these have yet not been integrated into the RA context. This review discusses the roles of LVs in RA and the current engineering approaches to improve our understanding of lymphatic pathophysiology in RA.

KEYWORDS
in vitro models, lymphatic vessels, organ-on-chip, rheumatoid arthritis, synovium

Abbreviations: ALP, alkaline phosphatase; Bin cells, $IgM^+CD23^+CD21^{hi}CD1d^{hi}$ B cells; BMP-2, bone morphogenetic protein 2; BVs, blood vessels; CCR7, CC chemokine receptor 7; CE-MRI, contrast-enhanced magnetic resonance imaging; CIA, collagen-induced arthritis; CMFDA, 5-chloromethylfluorescein diacetate; CS, calcium silicate; CSF-1, colony-stimulating factor-1; CXCL13, CXC-chemokine ligand 13; CXCR5, CXC-chemokine receptor 5; DCs, dendritic cells; DMARDs, disease-modifying antirheumatic drugs; DTPA, diethylenetriamine penta-acetic acid; ECM, extracellular matrix; eNOS, endothelial nitric oxide synthase; FLSs, fibroblast-like synoviocytes; GM-CSF, granulocyte–macrophage colony-stimulating factor; GPI, glucose-6-phosphate isomerase; hBMSCs, human bone marrow stromal cells; ICG, indocyanine green; IFN-γ, interferon gamma; IL-1, IL-1β, IL-1Ra, IL-2, IL-6, IL-8, IL-15, IL-17, IL-18, Interleukin 1, 1 beta, 1 receptor antagonist, etc.; ILN, iliac lymph node; iNOS, inducible nitric oxide synthase; iPSC-CM, induced pluripotent cell-derived cardiomyocyte; iPSCs, induced pluripotent stem cells; IRF1, interferon regulatory factor 1; LECs, lymphatic endothelial cells; LMCs, lymphatic muscle cells; LNs, lymph nodes; LVs, lymphatic vessels; LYVE-1, lymphatic vessel endothelial hyaluronan receptor 1; MHC II, major histocompatibility complex class II; MLSs, macrophage-like synoviocytes; MMP13, matrix metalloproteinase-13; MSCs, mesenchymal stem cells; NIR-ICG, near-infrared indocyanine green; NO, nitric oxide; OA, osteoarthritis; PBMCs, peripheral blood mononuclear cells; PD-L1, programmed death-ligand-1; PDMS, polydimethylsiloxane; PLN, popliteal lymph node; PMA, phorbol-12-myristate-13-acetate; PROX-1, Prospero homeobox 1; PTEN, phosphatase and tensin homolog; RA, rheumatoid arthritis; TCR, T cell receptor; tDCs, thymic dendritic cells; TGF-β, transforming growth factor-β; TNF-Tg, TNF-transgenic; TNF-α, tumor-necrosis factor-alpha; VEGF-C, vascular endothelial growth factor C; VEGFR3, vascular endothelial growth factor receptor 3; β-TCP, β-tricalcium phosphate.

1 | INTRODUCTION

Rheumatoid arthritis (RA) is a chronic autoimmune inflammatory disorder that afflicts the synovial lining of joints and manifests in reduced range of motion, pain, swelling, and numerous other complications. No effective cure had been identified, and palliative biological drugs such as disease-modifying antirheumatic drugs (DMARDs) only alleviate inflammation and are becoming ineffective for a rising portion of the population. In recent years, attention has shifted to eradicating the source of synovial autoimmunity and inflammation, with a key area being the synovial-draining lymphatics. With imaging technologies such as contrast-enhanced magnetic resonance imaging (CE-MRI) and power Doppler ultrasonography, the dynamic changes in lymphatic structure and function before and during the onset of RA inflammation have been more thoroughly documented both in animal and human models. Therefore, the relationship between the synovial lymphatics and RA pathophysiology could provide key areas for therapeutic intervention, and thus will benefit from the development of physiologically relevant in vitro models to circumvent ethical concerns and costs. However, very few in vitro models to date have been made for the synovium microenvironment, and none yet include the synovial lymphatics. This review will provide an overview of the burgeoning evidence of the role of lymphatics in RA pathophysiology, as well as current attempts to replicate the synovium or lymphatics in vitro and how these elements may potentially be combined.

2 | ROLE OF LYMPHATICS IN RA

2.1 | Lymphatic structure and function

Descriptions of lymph glands and a system of lymphatic vessels running in parallel to the blood vasculature carrying colorless fluid can be traced back as far as the Hippocratic School (5th–4th century BC) and Aristotle (384–322 BC).[1] The lymphatic system performs two primary functions, namely interstitial fluid balance and immune cell transport.[2] Interstitial fluid is balanced by draining from the periphery to the initial lymphatic vessels that merge in collecting lymphatics and draining lymph nodes (LNs) and eventually empty into final ducts such as the thoracic duct, returning the fluid back to the blood vasculature via the subclavian veins.[3] The initial lymphatic vessels are composed of a single layer of lymphatic endothelial cells (LECs) with specialized "button-like" cell–cell junctions that are highly permeable to solutes and molecules but prevent backflow to the interstitium with their unique "primary" valve function.[4] External mechanical forces create a pressure imbalance between the interstitial spaces and intraluminal fluid pressure that facilitates interstitial fluid uptake.[5–7] The initial lymphatic vessels converge into the collecting vessels, which are less permeable due to the presence of "zipper-like" cell–cell junctions and mural cell coverage, which also prevent fluid backflow with intraluminal valves (or "secondary" valves) to prevent fluid backflow.[8,9] Moreover, the collecting vessels are lined with perivascular layers of lymphatic muscle cells (LMCs), which possess characteristics of both smooth muscle cells and cardiac striated muscle cells to endow them with the capability of modulating vascular tone and rhythmic contraction.[5,6,10–13]

Dysfunction of the lymphatics has attracted considerable attention as a lack of efficient interstitial fluid drainage and immune cell clearance contributes to numerous maladies such as poor immune function, impaired wound healing, and lymphedema.[14,15] The lymphatic system plays an integral role in the adaptive immune response, as antigen-presenting cells, such as dendritic cells (DCs), from the periphery travel through the afferent lymphatics to the lymph nodes, where T and B lymphocytes are activated by a specific foreign antigen and are transported via the efferent lymphatics to sites of inflammation to mount an immune response.[16–19] Furthermore, lymphatics serve as a route for T lymphocytes to egress from the infected lesion after the resolution of the infection to prevent overly prolonged chronic inflammation.[20,21] The notion that the lymphatic network is critical to the development of autoimmune and inflammatory diseases is supported by studies showing that the absence of dermal lymphatics in mice leads to impaired humoral immunity and the production of autoantibodies.[22] Additional signals from immune cells traversing the lymphatic network themselves also contribute to structural and functional changes in response to inflammation or disease. For instance, lymphangiogenesis is mediated by vascular endothelial growth factor C (VEGF-C) and its cognate receptor, vascular endothelial growth factor receptor 3 (VEGFR-3), and macrophages, as well as T cells, cooperate to increase the expression of VEGF-C in response to inflammation.[23–25] Immune cell signaling can even affect lymphatic contraction, with a prime example being cytokine-mediated nitric oxide (NO) or tumor necrosis factor (TNF) signaling reducing lymphatic contraction in an inflammatory environment.[26,27] Furthermore, LECs themselves perform essential immunomodulatory functions to mitigate the adaptive immune response and provide immune tolerance, including secretion of transforming growth factor-β (TGF-β) to suppress DC maturation,[28] production of IL-7 to increase IL-2 sensitivity in regulatory T cells,[29] and secretion of colony-stimulating factor-1 (CSF-1, also known as a macrophage-colony stimulating factor, M-CSF) that affects macrophage differentiation.[30] Further immune tolerance is provided by the lymph node microenvironment itself, as shown by studies from Turley and colleagues demonstrating that lymph node stromal cells assist in the deletion of self-reactive T cells in the intestinal lymph nodes, functionally similar to the central tolerance induction (negative selection) by medullary TECs and thymic DCs (tDCs) in preventing autoimmunity.[31] Thus, the functions of the lymphatic system in immune cell surveillance and interstitial fluid balance implicate involvement in autoimmune diseases.

2.2 | RA pathophysiology

Rheumatoid arthritis is one of the most common chronic inflammatory joint disorders, affecting 0.5%–1% of the nearly 8 billion population worldwide with autoimmune cartilage degradation and synovial inflammation.[32] It primarily affects the small diarthrodial joints of the

hands and feet and manifests in hyperplasia of the intimal synovial lining from the overgrowth of macrophage-like synoviocytes (MLSs, or type A synoviocytes) and fibroblast-like synoviocytes (FLSs, or type B synoviocytes). Characteristically, autoreactive T cells, autoantibodies, and inflammatory macrophages infiltrate the synovium leading to an influx of inflammatory cytokines that attract degradative enzymes that destroy the extracellular matrix (ECM) and articular cartilage. Synovial antigens that have been explored as targets for antibody and T cell autoreactivity include type II collagen, proteoglycans, aggrecan, cartilage link protein, and heat shock proteins.[33–36] The inflammatory environment in the synovium is primarily the result of macrophage and fibroblast-derived cytokines such as IL-1, IL-6, IL-15, IL-18, granulocyte–macrophage colony-stimulating factor (GM-CSF, also known as colony-stimulating factor-2, CSF-2), and most importantly TNF-α, a vital bodily mediator of inflammation. Thus, biological agents such as inhibitors of TNF-α have shown success in mitigating collagen-induced arthritis in mouse models, and overexpression of TNF-α alone has been sufficient to induce RA in mice models.[37,38] Within the synovium microenvironment, the synoviocytes can proliferate without anchorage dependence and have defective contact inhibition.[39] Evidence also suggests that the unique RA microenvironment induces local antigen-driven B cell activation, as most B cells isolated from germinal centers in the RA synovium have unmutated V_H genes.[40] The most common treatments for RA clinically include DMARDs, with methotrexate as a prime example, and biological agents, with TNF inhibitors such as etanercept as a key example.[41] Nevertheless, an enlarging number of patients are becoming refractory to these current treatments or suffering from side effects. For instance, since the popular etanercept TNF inhibitor non-selectively suppresses all TNF-α induced inflammation throughout the body, it runs the significant risk of disrupting essential innate immunity, leading to increased danger of infection including from tuberculosis or upper respiratory pathogens. Thus, new areas for therapeutic targeting of RA are being explored, one of which includes the role of synovial lymphatics.

2.3 | Lymphatic changes in RA pathophysiology

Lymph node enlargement (also known as lymphadenopathy) in RA was first described in 1896,[42] but it was not until more definitive markers of LECs such as lymphatic vessel endothelial hyaluronan receptor 1 (LYVE-1) and prospero homeobox 1 (Prox-1) were discovered that thorough investigation of the synovial lymphatics could be conducted.[43,44] Most recently, the group of Edward Schwarz and colleagues have performed seminal work in identifying the role of lymphatic dysfunction in RA and were the first to demonstrate altered lymphatic function with near-infrared lymphangiography in human patients with RA.[45,46]

Currently, it is theorized that synovial lymphatics undergo two major phases in the progression of RA (Figure 1). In response to initial pre-arthritic inflammation, the lymph nodes first experience an "expansion phase" marked by increased lymphatic contractions and rapid lymphatic drainage to remove inflammatory cells and cellular debris[47,48] (Figure 1A–B). This process is accompanied by rapid migration of immune cells into the lymphatics, including direct entry of DCs into lymphatic collecting vessels via CC chemokine receptor 7 (CCR7) and integrin-binding mechanisms for more rapid transport to draining lymph nodes.[49] DC-LEC interactions have been shown to limit DC maturation for effector T cell activation through a Mac–/ICAM-1-dependent mechanism, and only in conditions of inflamed LECs in the absence of pathogen-derived signals, supposedly to prevent undesired immune reactions under inflammatory conditions. Therefore, rapid migration of DCs into the lymphatics and the resultant decrease in DC-LEC interactions could further exacerbate T cell activation and thus inflammation.[50] The draining lymph nodes themselves during this expansion phase physically increase in size due to increased fluid pressure and the influx of a unique subtype of $IgM^+CD23^+CD21^{hi}CD1d^{hi}$ B cells, called Bin cells.[51–57] Without the critical increased lymphatic clearance and lymphangiogenesis characteristic of the expansion phase, inflammation can increase into a synovitis pathway in mouse models.[58] However, as the increased lymphatic drainage of the expansion phase resolves the acute synovial inflammation, the removed inflammatory cells and catabolic factors instead damage LECs and LMCs of the afferent lymphatics and draining lymph nodes, leading to the collapsed phase.[59]

The consequent LEC/LMC damage leads to the "collapsed phase" of synovial lymph nodes, in which the draining lymphatic system effectively collapses leading to impaired lymphatic clearance, increased vessel leakiness and decreased contractions, and stasis of inflammatory fluid in the joint and afferent lymphatics[47,51,54,60–62] (Figure 1C). The proposed mechanism for this vessel collapse is that inflammatory cytokines that damage the LECs and LMCs also trigger LECs to express higher levels of inducible nitric oxide synthase (iNOS) and impair constitutive endothelial nitric oxide synthase (eNOS) activity in "NO squelching." iNOS may produce NO continuously, in contrast to the regulated production of NO by eNOS. Static macrophages within the lymphatic vessels also express iNOS and further impair lymphatic contraction.[27,63] These macrophages begin to express B cell chemotactic factor, CXC-chemokine ligand 13 (CXCL13), which drives migration of CXC-chemokine receptor 5 (CXCR5)-positive Bin cells from the lymph node follicles to the sinuses and clogs the lymph vessels.[64] The result is synovial hyperplasia and joint degradation, which is only partially resolved by conventional TNF-α inhibition therapies that additionally pose the risk of increased susceptibility to foreign infections. The first clinical trial involving near-infrared indocyanine green (NIR-ICG) imaging of lymphatics in RA patients is currently ongoing and reflects the importance of finding therapeutic targets for the lymphatics in RA.[65]

3 | CURRENT EXPERIMENTAL MODELS OF RA

A lack of experimental models of normal and impaired synovium and synovial immunity has been a major obstacle to better understanding and treatment of RA. Though animal models of RA have contributed to the field, they are often difficult to use to identify the

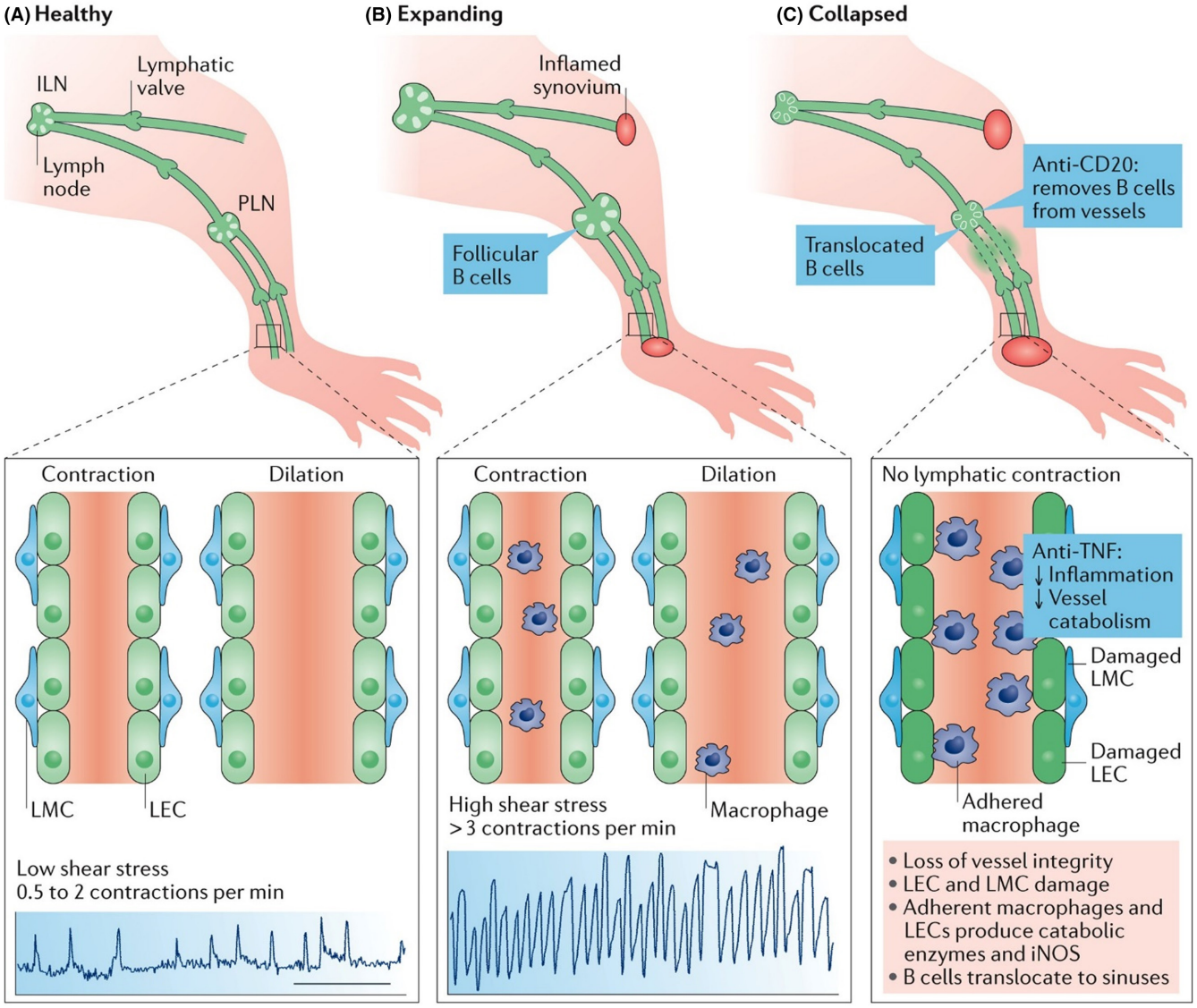

FIGURE 1 Overview of lymphatic phenotype in healthy, expanded, and collapsed lymphatics in mouse RA models. (A) Synovial lymphatics at homeostasis, where distal lymphatic vessels drain the footpad to the popliteal lymph node (PLN), and the proximal lymphatic vessels drain lymph from the PLN and knee synovium to the iliac lymph node (ILN). Lymphatic vessels contract 0.5–2 times per minute with tight junctions between lymphatic endothelial cells (LECs) and lymphatic muscle cells (LMCs). (B) The expansion phase after the onset of inflammatory arthritis, characterized by inflammation-induced lymphangiogenesis and rapid clearance of inflammatory cells (~5 contractions per minute). CD11b+/LYVE1+ macrophages are present in contracted and dilated lymph vessels and travel at great speed (~0.2 mm/s). (C) The collapsed phase, characterized by absent or rare lymphatic contraction. PLNs and ILNs decrease in size owing to fluid loss, and B cells translocating from B cell follicles into the lymphatic sinuses effectively "clog" the lymphatic vessels, and thus anti-CD20 therapy removes B cells and restores lymph flow. Figures (A–C) were adapted with permission from Bouta et al.[45]

pathophysiologic mechanisms underlying this multifactorial disease, because it is difficult to isolate the relative contributions of biological and biophysical factors, such as lymphatic drainage. To examine the role of lymphatics in RA pathogenesis effectively and thoroughly, a controlled system of synovial draining lymphatics and the inflamed joint is necessary to observe the autoimmune response and test therapeutic targets. Though each of them presents intriguing advantages, currently available in vitro models fail to include contributions from lymphatic vasculature and are overly simplified in culture conditions, mostly on two-dimensional dishes. Two-dimensional (2D) cell culture models are highly controllable but do not recapitulate the three-dimensional (3D) organization and function of the synovium in vivo. We review currently available in vivo models of RA and in vitro or ex vivo models of synovium and RA, including 2D co-culture in vitro, 3D multi-component models in vitro, tissue explants ex vivo, and microfluidic organ-on-chip models in vitro.

3.1 | Current standards of in vivo models

Non-human animals may not naturally develop autoimmune disorders within a short timeline for experimental approaches, thus most in vivo models induce arthritis in animals through injection of soluble agents or genetic manipulation, and even so can only be used to

study select pathophysiological aspects such as articular cartilage erosion.[66–68]

One of the most common soluble agent-induced arthritis models is collagen-induced arthritis (CIA) mouse model, in which type II collagen emulsion with complete Freund's adjuvant is inoculated into mice, most often C57BL/6 mice, to stimulate the production of anti-collagen II antibodies mimicking the joint swelling and stiffness of RA.[69–71] Monoclonal anti-type II collagen antibodies can alternatively be injected, though the resultant immune response will not be T and B cell-mediated and does not involve the presence of MHC II haplotype as in native human RA.[72,73] These induced models recapitulate most features of RA, such as infiltration of inflammatory cells, synovial hyperplasia and pannus, and cartilage and bone destruction. However, these methods are only efficacious in certain strains of rodents or present inter-group variability of disease severity. Furthermore, CIA often results in acute and self-limiting polyarthritis that ignores systemic components of RA on other organ systems.

Another option is the use of genetic modification to induce RA, with an example being the K/BxN mouse model generated by crossing mice expressing the MHC class II molecule A^{g7} with the T cell receptor (TCR) transgenic KRN line expressing a TCR specific for a G6PI-peptide.[74–76] Therefore, one advantage of this method is the ability to create autoantibodies, similar to in vivo autoimmune diseases, to glucose-6-phosphate isomerase (GPI) within the serum. Alternatively, a popular method is to add the transgene for human TNF-α to mice, which as described before, is sufficient to induce polyarthritis within mice models and affirms the role of TNF-α at the apex of the pro-inflammatory cascade in RA.[38,77] Regardless, both K/BxN and TNF transgenic models do not produce rheumatoid factors characteristic of RA patient serum or recapitulate the entire pathophysiology of the disease.[68] One of the most recently developed murine models from Kataru et al. is especially beneficial for lymphatics research, as the mice possess an increased number of functional lymphatics due to deletion of phosphatase and tensin homolog (PTEN), a negative regulator of VEGFR3 signaling in LECs.[78] PTEN inhibits the downstream effects of the activation of VEGFR3 by VEGF-C, and thus its deletion led to the development of mature, intact lymphatic vessels compared with lymphangiogenesis induced by VEGF-C injection.

The aforementioned in vivo models are useful for mimicking select aspects of RA, whether it be an autoimmune activity, joint degradation, or cytokine inflammation, but are not truly reflective of the full disease pathophysiology. This is an important drawback as complications such as osteoporosis and cardiovascular disease are implicated in RA progression, necessitating a closer study of systemic effects.[79,80] Yet this also raises another issue as physiologically complex in vivo models render it difficult to decouple certain causes and biological factors contributing to the disease. Rather, developing in vitro models for isolating select aspects of the synovium in RA, such as the lymphatic system, may be more beneficial to first discover the contributions from individual systems. Eventually, such systems can be utilized for drug screening with patient-specific cells to develop more personalized therapies considering the complexity and variability of this autoimmune disease. Thus, a reliable in vitro model for recapitulating RA pathogenesis and high throughput drug screening is desirable to balance accuracy in the physical manifestations of the disease with experimental and fiscal feasibility. The current attempts at modeling RA inflammation and the synovium in vitro can be broadly divided into four categories: two-dimensional (2D) co-culture models, three-dimensional (3D) multi-component models, tissue explant models, and microfluidic organ-on-chip models.

3.2 | 2D co-culture models in vitro

While 3D tissue models can more adequately capture the biological complexity of organ systems and disease pathophysiology, 2D co-culture models are in part preferred and perhaps even outperform 3D tissue models in terms of high-throughput-ness and experimental feasibility, including aspects of nutrient perfusion and studying singular components or environmental factors of a disease. For instance, 2D co-culture models are particularly useful for determining the effect of inflammatory cytokines on synoviocyte and chondrocyte monolayer cultures. Such systems have been used to test optimal concentrations of therapeutics, analyze RA-associated gene expression profiles, study the effect of chondrocyte and cytokine interactions on synoviocyte phenotype and behavior, and determine antigen and aggregate uptake in RA.[81–84] However, 2D culturing systems run the risk of altering the phenotype of cells from their native state, as is the case with chondrocytes that downregulate type II collagen in favor of type I collagen in 2D culture.[85,86] When stimulated with inflammatory cytokines, such as IL-1β, TNF-α, or IFN-γ, chondrocytes in these 2D models have been shown to decrease the expression of type II collagen and aggrecan and increase the expression of matrix metalloproteinase-13 (MMP13), driving chondrocyte apoptosis as is observed in vivo.[87–92] Further advancements in this 2D system have also shown that RA synovial fibroblast-conditioned media alone can suppress TNF-α-induced IFN-γ expression in macrophages.[93] Most recently, a tri-culture model was developed to study interactions among osteoblasts, osteoclasts, and endothelial cells in a bone erosion model of RA.[94]

3.3 | 3D multi-component models in vitro

Given the aforementioned limitations of 2D models, 3D tissue-engineered models have been developed (Figure 2), which include scaffold-free models,[95] self-organizing scaffold models,[96] natural scaffold models,[97] and synthetic scaffold models.[98] To fully construct a 3D model of the RA inflamed joint, most previous work has focused on individual aspects of the joint system: the synovium, cartilage, and bone.

The normal synovium consists of a continuous surface layer of cells (intima) and the underlying tissue (subintima). The intima is approximately 1–2 cells thick and is composed of two distinct subtypes

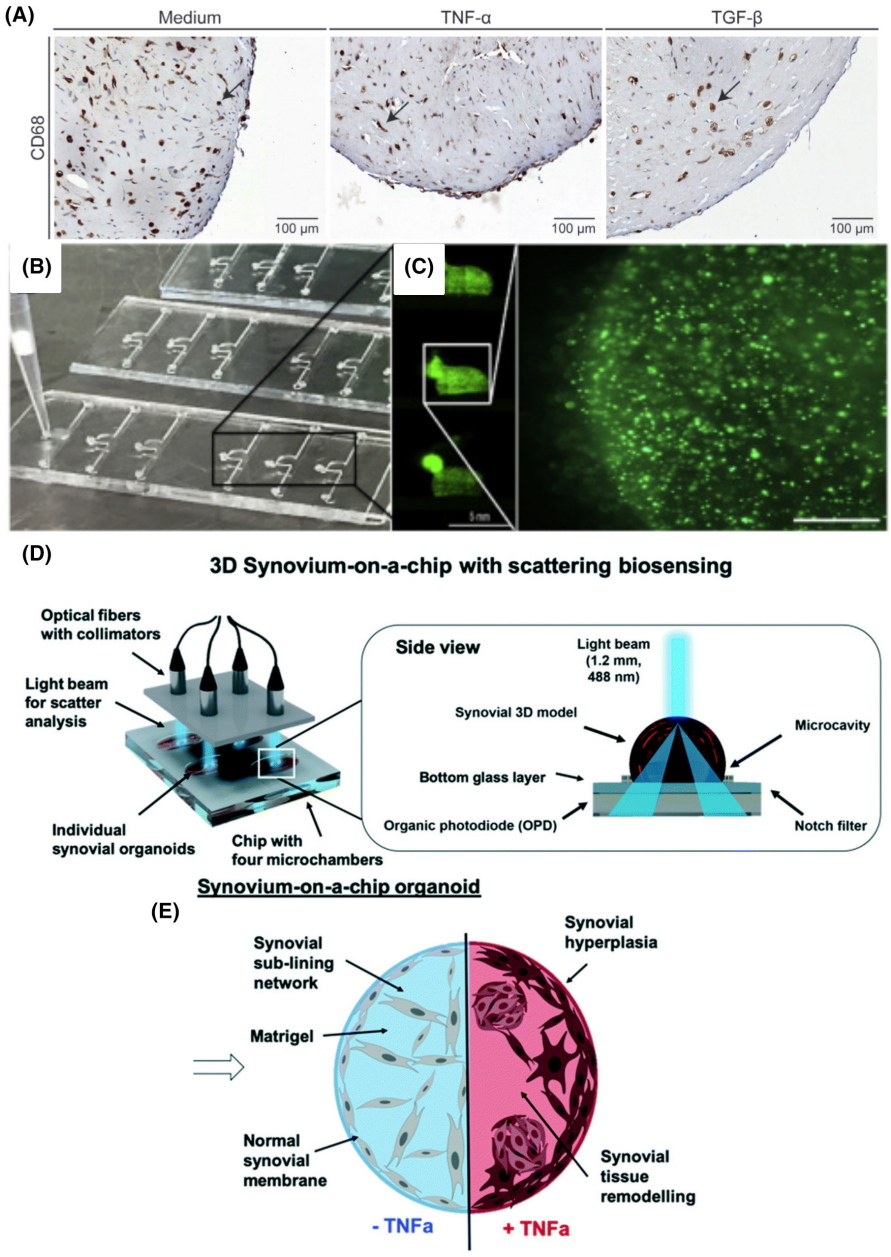

FIGURE 2 Representative examples of 3D in vitro models of synovium and cartilage. (A) Synovial micromasses were generated from primary RA FLS and CD14+ PBMCs and stimulated for 3 weeks with 10 ng/ml recombinant human TNF-α or TGF-β. Micromasses were stained for CD68, showing infiltration of inflammatory macrophages.[103] (B) Photograph of the cartilage-on-a-chip device with glass slide as the top layer and PDMS slab with microstructures as the bottom layer showing loading of cell-laden hydrogel in the top chamber using a pipette tip.[160] (C) Overview fluorescent image of cartilage-on-a-chip showing CMFDA-stained primary equine chondrocytes.[160] Scale bars 5 mm and 500 μm. CMFDA, 5-chloromethylfluorescein diacetate. (D) Schematic overview of the synovium-on-a-chip system comprising four individual microchambers harnessing three-dimensional human synovium organoids with light scattering biosensing.[161] (E) Illustration of tissue remodeling process for tumor necrosis factor-alpha (TNF-α) cytokine-induced three-dimensional synovial remodeling on chip.[161] Figures were adapted with permission from each indicated reference as follows: (A) Broeren et al.,[103] (B, C) Rosser et al.,[160] and (D, E) Rothbauer et al.[161]

of synoviocytes: type A synoviocytes, which are of MLS, and type B synoviocytes, which are of fibroblast lineage (FLS). In contrast, the subintima is relatively acellular with scattered blood and lymphatic vessels, fat cells, and fibroblasts.[99] FLSs are primarily involved in the hyperplasia and pannus formation of the synovium during RA, and are thus the most extensively studied and utilized for 3D gel-suspended micromass models of the synovium.[100] Karonitsch and colleagues used this model to study the effects of inflammatory cytokines on synovial tissue remodeling, finding that IFN-γ promotes FLS invasion while TNF-α promotes FLS aggregation.[101] Similarly, Bonelli and colleagues found with an analogous model that TNF-α regulates the expression of the transcription factor interferon regulatory factor 1 (IRF1), a key regulator of the IFN-mediated inflammatory cascade, and confirmed this with TNF-α transgenic mouse arthritis model.[102] One of the most complex models of the synovium was recently created by Broeren et al. combining RA-patient derived FLS with peripheral CD14+ monocytes or a complete human RA synovial cell suspension, recapitulating the intimal layer with fibroblasts and macrophages. As is seen in vivo, long-term exposure to TNF-α led to intimal hyperplasia, altered macrophage phenotype, and an increase in IL-6, IL-8, and IL-1β, corroborating previous findings[100,103] (Figure 2A). All the models discussed rely on diseased FLS, which are limited in availability and vary in disease severity depending on the source.[104] Additionally, in the human pathogenesis of RA, the intimal layer thickens with MLSs, accounting for up to 80% of the intima in the diseased state, that begin to infiltrate into the subintima.[99] As of yet, most models have utilized solely FLS to model the synovium, so a key limitation is the lack of physiologically relevant macrophage-like synoviocytes to mediate the immune infiltration. Therefore, mesenchymal stem cells are being examined as a

substitute for FLS in synovium models, as they share surface markers, differentiation capacity, and the ability to produce hyaluronic acid with FLS.[105]

In terms of the chondrocyte component of the joint, articular cartilage is a notoriously difficult tissue to fabricate with tissue engineering, as it is mostly avascular and acellular with limited natural regeneration capacity. The basic structure of articular cartilage consists of several layers (superficially tangential, transitional, and radial) to absorb mechanical loads and protect the subchondral bone.[106] In RA, proinflammatory signals such as TNF-α and IFN-γ activate catabolic processes in chondrocytes that lead to cell death and matrix degradation.[88,107] Within the joint microenvironment, the chondrocytes produce and are heavily influenced by the mechanical cues within the ECM, which consists of type II, type IX, and type XI collagens as well as proteoglycans such as aggrecan. Considering that the chondrocytes are particularly sensitive to the ECM environment, most cartilage tissue engineering efforts are based on the consensus that chondrocytes require a scaffold to mimic the in vivo 3D structure.[92] Porous scaffolds have ranged in materials from type II collagen,[108] gelatin microspheres,[109] alginate breads,[110] hyaluronic acid, and chitosan.[111] Peck and colleagues, for instance, utilized to gelatin microsphere method to model 3D articular cartilage, along with a synovial cell line and lipopolysaccharide-activated monocytic THP-1 cells and found the inflammatory environment encouraged chondrocyte apoptosis, downregulation of matrix components, and upregulation of matrix degradative enzymes similar to in vivo.[109] A similar alginate 3D cartilage model showed that even supernatant from RA synovial fibroblasts was sufficient to induce chondrocyte degradation.[112] The field of cartilage tissue engineering has also shown promise for high-throughput drug screening, as Ibold et al. developed a 3D cartilage model co-culturing scaffold-free porcine cartilage with RA-derived FLS.[113] Recent efforts have even varied cell amounts, mechanical loading, and other factors to mimic more thoroughly the many layers of articular cartilage.[114] The most advanced models have attempted to remove the restraints of the scaffold altogether and rely on spontaneous self-assembly or mechanical induced self-organization[115–118] and have been utilized for preclinical in vitro screening.[119,120] To improve the efficacy and efficiency of these 3D cartilage models, mesenchymal stem cells (MSCs) have arisen, as with synoviocytes, as a viable cell source since they differentiate into a chondrocyte lineage, can be derived from multiple sources, and are easy to handle.[121,122] The group of Bonassar and colleagues, who have been instrumental in the development of 3D bioprinting techniques for articular cartilage constructs, have also introduced human induced pluripotent stem cells (iPSCs) as viable cell source for cartilage tissue-engineered constructs.[123]

Due to the inherent articular cartilage and bone erosion pathophysiology of RA, a full 3D in vitro model of the inflammatory joint includes the subchondral bone.[124] Progress in this field has been slow, however, since bone is highly vascularized, complex in terms of cell and matrix composition, and undergoes a constant change in response to mechanical load. In healthy bone tissue, osteoblasts and osteoclasts control bone growth and resorption, respectively, while osteocytes control bone homeostasis through mechanotransduction.[125] Traditionally, bone tissue engineering has focused on more orthopedic therapy applications, such as implants for bone regeneration.[126,127] In recent years, however, attention has turned to utilize bone tissue engineering to model orthopedic diseases in vitro, such as osteoporosis and RA. As with cartilage tissue engineering, most approaches rely on an ECM-mimicking scaffold, whether synthetic, natural, biodegradable, or non-biodegradable, that possess osteoconductive properties or modulus similar to human bone.[128,129] Other techniques such as scaffold-free organoids or spheroids and 3D printing, hydrogels, or beads have shown success as well.[97,130–133] Some techniques have rendered the scaffolds more bioactive with the inclusion of bone morphogenetic protein 2 (BMP-2) or vascular endothelial growth factor (VEGF).[134,135] To better mimic in vivo mechanical forces and osteogenic environment, bioreactor technology has been adapted to mold these 3D bone models.[136] Just as the synovium model is incomplete without the physiological relevance of the lymphatics, new bone in vitro models has begun to incorporate the nutrient perfusion of a blood vasculature system.[137–139]

An ideal successful model of RA pathogenesis within the joint would require the amalgamation of all three aspects (synovium, articular cartilage, and subchondral bone) to fully mimic all the inflammatory consequences of the disease, including pannus formation, cartilage degradation, and bone erosion.[140] A myriad of in vitro models has utilized scaffold-based bone and scaffold-free cartilage,[141] differing scaffolds for bone and cartilage, bi-layered scaffolds, and homogeneous scaffolds for both bone and cartilage.[142] In another method, Lin and colleagues created separate regions of chondrogenic and osteogenic differentiation on iPSCs-derived MSCs encapsulated in a gelatin scaffold using a dual-flow bioreactor.[143] In one of the most advanced models, Damerau and colleagues created the synovial, cartilage, and bone components of the joint in a 3D model differentiated entirely from the MSCs of a single donor.[144] RA inflammation was modeled with inflammatory cytokines and relevant immune cells, and the model was even tested as a preclinical tool for drug evaluation.[140,145]

3.4 | Tissue explant models ex vivo

By nature of their in vivo proximity and source from affected patients, ex vivo culture models and tissue explants offer some of the most accurate and physiologically complete models of joint inflammatory disease. When ethically sourced and available, joint explants, including synovium, cartilage, bone, and other connective tissue, can be obtained from joint replacement surgery and biopsy for extensive immunohistological and molecular analysis to understand the pathophysiology of OA and RA.[146] For instance, Anderson and colleagues found a correlation of certain synovial cytokines with imaging pathology and disease activity in MRI of Doppler ultrasound on joint explants.[147] Ex vivo models have been particularly useful in osteochondral research, as explants retain native bone cellular communication and ECM structure.[148] However, tissue explants are

often varied by individual health and medication of the donors, and are limited by necrotic cell death at wound edges due to deprival of nutrient supply from the native vasculature.[149] Both synovial[150] and bone[151] explants have been utilized for therapeutic screening to curb pro-inflammatory cytokine and matrix degradative enzymes in RA pathogenesis. Certain therapies have achieved synergistic effects in these models, such as anti-TNF-α antibodies and interleukin 1 receptor antagonist (IL-1Ra) resulting in significantly decreased IL-6 and MMP-3 production in synovial explants.[152] Even herbal components, such as kirenol have been shown to inhibit FLS proliferation and IL-6 secretion in explants.[153] Research into RA-associated expression profiles with knee arthroplasty samples have found that interaction of CD40 with CD154 increased the expression of inflammatory cytokines and MMPs.[154] Inspired by these knee explant models, Schultz et al. developed a 3D in vitro model to investigate destructive processes in RA, studying the role of FLS in joint degradation.[155] In a more recent model 10 years later, Pretzel et al.[156] mimicked the early degradative processes of synovial fibroblasts similar to tissue explant models.

3.5 | Microfluidic chip models in vitro

One of the most promising and evolving areas of in vitro RA joint research is microfluidic chip technology, which entails co-culturing multiple different cell types in customized, spatially distinct patterns often determined by lithography etching on flexible materials such as polydimethylsiloxane (PDMS) connected by microfluidic channels to mimic cell and nutrient transport as in a full physiological system. The microfluidic channels enable constant perfusion of nutrients and real-time monitoring or control of factors such as pH, temperature, and oxygen concentration.[157,158] With fluid perfusion technology, specific concentration gradients, cellular architectures, and fluid shear force can be controlled.[159] Only very few attempts to date have been made to mimic the subchondral bone and articular cartilage interface, as well as the synovium, with microfluidic chip modeling.[160,161] For example, Rosser et al.[160] created 3D cartilage constructs from equine chondrocytes to simulate a physiological nutrient gradient across a matrix, driving native cartilage tissue behavior (Figure 2B–C). In terms of synovium, Rothbauer et al.[161] created one of the only known synovium-on-chip system, constituting synovial organoids composed of primary human FLS within a Matrigel micromass, to study the effect of TNF-α inflammation on synovial remodeling (Figure 2D–E). Migration and remodeling of synoviocytes were monitored non-invasively with light scattering. However, the aforementioned model used isolated synovial organoids to model the synovium, in the absence of relevant immune cells and blood vasculature. While other organ systems such as liver, kidney, or heart have been incorporated into microfluidic chip systems for disease study and drug screening, relatively little research has focused on joint-on-chip or synovium-on-chip systems, presenting a promising area for in vitro RA research to understand the multiple factors in the disease.[162]

4 | IN VITRO MODELS OF LYMPHATICS

However, considering the recently discovered importance of the lymphatic system and draining lymph nodes in both mediating and being functionally affected by RA inflammation in the synovium, a more insightful in vitro model of the RA-afflicted synovium should include the immune cell and cytokine flow from the lymphatic vasculature. As of yet, there have been no reported models that combine the synovial membrane with supplying lymphatics, but considerable progress has been made in terms of modeling both separately (Figure 3). Utilizing advanced techniques for microchannel fabrication and tunable fluid dynamics, modeling lymphatic vasculature has arisen as a relevant technique to find therapeutic targets for disease and study the lymphatic structural and functional change in response to maladies such as cancer, obesity, or autoimmune diseases.[15,163–166]

4.1 | Establishing lymphatic barrier function and luminal flow

Cell sources for LECs in modeling lymphatic networks in vitro have ranged from vendors to primary cells isolated from humans or mice to stem cell-derived LECs. Culturing methods have differed from standard cell culture plates or Transwell inserts for 2D to spheroids and thick matrices for 3D. Regardless, the consensus found is that physiologically relevant fluid flow is essential for lymphatic vessel formation and function.[167] Tunable luminal flow along the axis of lymphatic vessels is greatly amenable to microfluidic organ-on-chip technology, which has been successful in replicating not only vessel architecture but the supporting ECM and microenvironment around the lymphatics, including nearby tumors and extracellular fluids uptaken in lymphedema. For example, Gong et al. leveraged both luminal flow and lymphatic barrier function in a tubular lymphatic vessel model embedded in a collagen gel mimicking ECM. The model helped in demonstrating the defective lymphatic junctions and therefore drainage in a tumor microenvironment, showing promise as a system for controlled disease mechanism studies.[167] Henderson et al. utilized a 3D lymphatic vessel model to understand lymphatic junction remodeling and permeability in different matrices, showing lymphatic zippering and reduced permeability in fibronectin via activated integrin alpha 5[165] (Figure 3A).

4.2 | Controlling vessel geometries and throughputs

Lymphatic vasculature in microfluidic chip devices has also been bolstered by the development of advanced bioprinting technologies. Whether by extrusion-based, inkjet-based, laser-assisted, or other techniques, bioprinting has the capability of combining polymers with live cells to create precise 3D geometries and patterns for tissue engineering, drug screening, and in vitro disease models.

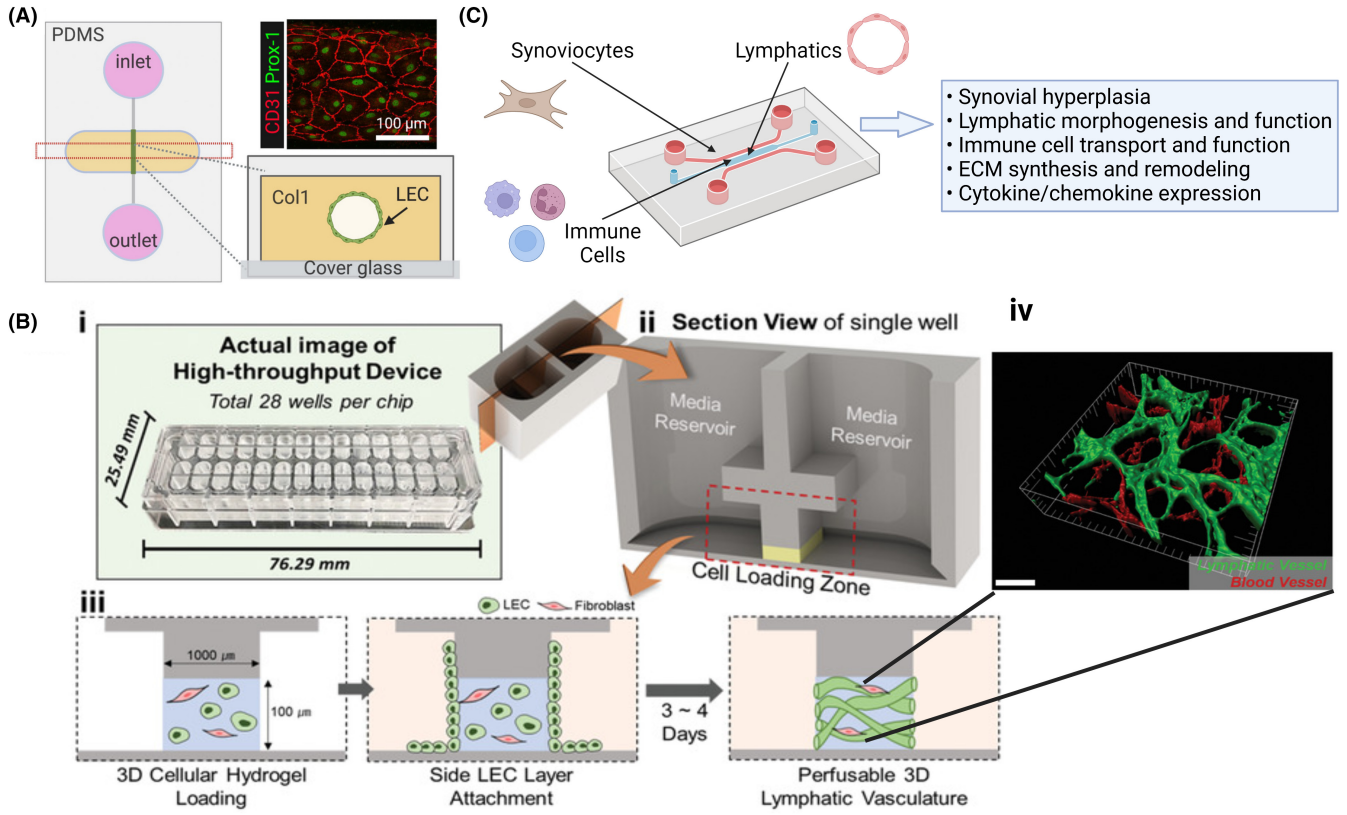

FIGURE 3 Examples of microfluidic chip in vitro models of lymphatic vasculature. (A) A schematic of an organotypic 3D lymphatic vessel model (LV-on-chip). Prox-1 (green) and CD31 (red) expression confirm the lymphatic endothelial identity and cell morphology in the channel.[165] (B) Schematic of the high-throughput model of tumor lymphatic vessel network[166] (i) Design of injection-molded high-throughput device. (ii) Section view of a single well, representing channel configuration. (iii) Stepwise protocol of 3D cellular hydrogel and side LEC attachment for reconstituting 3D human LV network in vitro. (iv) 3D reconstruction of the representative confocal image of LV-BV co-culture condition. Scale bar = 100 μm. (C) Demonstration of how synovial membrane cells and synovial-draining lymphatics might be combined into an in vitro microfluidic chip model, along with the necessary tests and benchmarks to determine the effect of cytokine or immune cell-induced inflammation on lymphatic function and synovial microenvironment. Figures were adapted with permission from each indicated reference as follows: (A) Henderson et al.[165] and (B) Lee et al.[166]

Improvements in bioprinters have enabled more precise control over cellular construct architecture for optimum cell culturing, up to even the nanometer scale. Utilizing this technology, Zhang et al. fabricated hollow lymphatic vessel tubes for an in vitro model and were able to adjust wall thickness via bioink flow rate.[168] The group was even able to mimic the one end-blinded characteristic of lymphatic capillaries. Even more advanced work such as a high-throughput model of tumor lymphatic vessel network by Lee et al. has been able to recapitulate perfusable, self-organized lymphatics vessels in the tumor microenvironment through spontaneous capillary flow-driven patterning of a 3D cellular hydrogel mold (Figure 3B).

4.3 | Establishing interstitial flow and transport functionality

In addition to luminal flow, the interstitial flow of extracellular lymphatic fluid between the lymphatic vessel wall and the extracellular space or ECM in the body is essential for the draining capacity of lymphatic vessels and lymph nodes. The initial lymph vessels in particular uptake excess extracellular fluid, immune cells, and foreign antigens for recirculation through the blood or transport to lymph nodes for adaptive immune cell education. It has been shown that faulty lymphatic drainage function is indicative of disease pathology, along with other changes in LEC structure and function. Kim et al. created interstitial flow in a microfluidic platform to test its effect on lymphatic sprouting in lymphangiogenesis. A central lymphatic channel was separated from two fibroblast channels on the sides by two fluidic channels controlling the interstitial flow pressure gradient and biochemical stimulation.[169]

5 | MODELING THE ROLE OF LYMPHATICS IN RA

Though the role of lymphatics in mediating RA pathogenesis has only been recently explored, the progress thus far in validating and characterizing processes such as immune cell retention and lymphatic vessel swelling has been prolific by select groups in animal

TABLE 1 Advantages and disadvantages of potential strategies for synovium and lymphatics in vitro models

	Organoid	3D Bioprinting	Organ-on-chip
Advantages	• Recapitulates native organ architecture and cell types unlike spheroids and 2D co-culture • Suitable for long-term maintenance • Derived from stem or progenitor cells to model in vivo cell and tissue development	• Scalability, reproducibility, and multi-dimensional controls[172] • Ability to create complex vessel architecture[173,174] • Ideal for multi-scales of vessel architecture[168] • Inclusion of cell signals through bioactive bioink	• High tunability of luminal fluid shear stress, interstitial flow rate, and cell micropatterning[175] • Constant perfusion of nutrients • Co-culture of multi-cell types in multi-compartments • Real-time monitoring and control of pH, temperature, and oxygen concentration
Disadvantages	• Requires sources of donor progenitor cells • Requires complex bioreactors and culture maintenance • Fully vascularized organoids are still in need (brain, heart, liver, etc.)[170,171]	• High instrumentation cost • Precision depends on the capability of the instrument • Limited biomaterials available for biocompatible and printable bioink	• Difficult to standardize and scale-up • Increasing difficulty combining multiple organ systems in one model • Requires external pumps, connectors, and flow to operate[162]

models, primarily due to advances in dynamic imaging technologies such as MRI and NIR-ICG.

In discussing the potential incorporation of lymphatics into RA, we review co-culture models of vasculatures in different disease conditions. For instance, Wörsdörfer and colleagues have successfully demonstrated that complex vascularized tumor and neural organoids can be developed with mesodermal progenitor cells.[170] The generated blood vessels display functional endothelial cell–cell junctions as well as the hierarchical organization and respond to pro-angiogenic or anti-angiogenic signals. Most impressively, the vessels within the tumor organoids were capable of connecting to host vessels ensuing transplantation. Other organs such as the brain, heart, liver, and gastrointestinal systems have been successfully developed and are beginning to benefit from the inclusion of physiologically relevant vasculature for nutrient transport, as many other diseases are linked to the dysfunction of the blood of lymph vessels.[171] With adaptations to include LECs, such models can be easily converted to diseased tissue models with lymphatic vasculature.

3D bioprinting technologies benefit from scalability, reproducibility, and multi-dimensional control that are highly amenable to incorporating vasculature into tissues for disease modeling and tissue engineering applications.[172] Highly vascularized tissues such as heart, liver, and kidney have benefited from this technology, which is particularly useful for recapitulating the tumor microenvironment.[173] Maiullari et al.[174] demonstrated such potential with an iPSC-derived cardiomyocyte (iPSC-CM) cardiac tissue model developed with 3D bioprinting that contained human umbilical vein endothelial cells forming vessel structures. Utilizing LECs, 3D bioprinted models could be modified to form an inflammatory microenvironment, relevant not to just RA but other autoimmune diseases.

The high tunability of fluid shear stress and cell micropatterning in microfluidic chip systems makes them particularly useful for modeling lymphatic or blood flow through various tissue types for modeling cancer metastasis and inflammatory diseases. Nguyen et al.[175] demonstrated how the microfluidic chip platform could be used to create a pancreatic ductal adenocarcinoma-on-a-chip model to examine tumor-blood vessel interactions that facilitate metastasis. With the lymphatic chip technology previously highlighted and the tissue composition for the affected disease of interest, a feasible synovial model is possible (Figure 3C).

The circumvention of ethical issues, ease of processing, and potential for high-impact drug screening and testing are significant benefits of developing in vitro models of RA-associated lymphatics. In this area, models of the synovium microenvironment and joint-draining lymphatics have progressed separately but have yet to be combined. Numerous technological platforms such as organoids, 3D bioprinting, and organ-on-chip platforms are amenable to incorporating both synovium and lymphatic elements to build a comprehensive model of RA (Table 1).

5.1 | Challenges for in vitro models

However, as RA is by nature a physiologically complex autoimmune disease, several special complications and challenges must be considered for such a model. Various immune compartments, timescales, and biophysical or biochemical inputs contribute to the pathophysiology of RA, which will thus require careful consideration of adaptive or innate immune cells and modes of inflammation used. Not to mention that the immune cells themselves respond to cues of receptor-ligand binding, matrix stiffness, flow or shear, and cellular contact that should be modulated to mimic in vivo conditions.[176] Ideally, the immune cells used and synoviocytes in an RA model would be obtained from the same human donor to accurately show autoimmune activity, and the autoimmune inflammation would be induced by activated self-reactive T cells and autoantibodies rather than downstream inflammatory cytokines such as TNF-α. Another issue is that LEC morphology varies across different tissues including the synovium, so the gene expression profile and characteristics of lymphatic vessels in vitro within the synovium must be examined

and matched with in vivo findings. The synovial subintima is drained by initial lymphatic vessels with button-like junctions, but the most prominent expansion and collapse occur in the lymph nodes with interstitial fluid carried by the collecting lymphatics with zipper-like junctions. Therefore, a relevant in vitro model could perhaps include two separate components, with one for the initial drainage of the synovium and another for the downstream fluid movement into the collecting lymphatics and LNs.[177] That being said, processes such as the expansion and collapse of LNs with such rapid changes within a short time period may be difficult to model with microfluidic chips or organoids. To assist this, dynamic mechanical stimulation and loading representative of the forces on the synovial joint in vitro can be applied to mediate the phases of lymphatic changes, as dynamic loading is important for elements such as chondrocyte development and is even shown to affect lymphatic drainage. Recent joint-on-chip constructs focusing on the cartilage unit of the joint have used methods such as multi-axial mechanical stimulation and pneumatic cell compression consisting of deformable membranes (balloons) to apply loading to 3D cell-laden hydrogels and can be similarly used for synovial units.[178–180]

6 | CONCLUSIONS

While standard-of-care biologics have been successful in delaying joint degradation and mitigating local inflammation, few therapies have attempted to eradicate the fundamental issue of autoimmunity or target organs involved in RA pathogenesis. Thus, the emerging studies of synovial lymphatic alteration in RA can potentially help in identifying the precise mechanisms of autoimmune inflammation for numerous other diseases which have been reported to interface with the lymphatic and vascular systems. However, animal models of RA do not fully reflect pathogenesis, and in vitro models designed to isolate causative biological factors have yet to include all the cellular and biochemical components of the synovium microenvironment, especially the synovial-draining lymphatics. Given the promise of microfluidic chip systems and organoids for producing functional lymphatic vasculature and associated tissues in vitro, a model of the RA-inflamed synovium and draining lymphatics is entirely within the realm of possibility and would be beneficial for drug screening, studies of immune cell trafficking, and tissue engineering.

7 | PERSPECTIVES

1. The pathophysiology of RA is associated with changes in the function and structure of the synovial lymphatic vasculature, including an expansion and collapsed phase.
2. Synovium in vitro models coupled with associated lymphatic vasculature have yet to be developed but could allow observation of lymphatic changes that can be used for lymphatic-targeting therapeutics for RA.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

DATA AVAILABILITY STATEMENT

Not applicable.

CONSENT FOR PUBLICATION STATEMENT

The corresponding author ensures that all authors have seen and approved the final version of the paper, and all are aware of the submission of the paper.

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