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INVITED REVIEW

INVITED REVIEW THEMED ISSUE

Fibroblast pathology in inflammatory joint disease

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Abstract

Rheumatoid arthritis is an immune-mediated inflammatory disease in which fibroblasts contribute to both joint damage and inflammation. Fibroblasts are a major cell constituent of the lining of the joint cavity called the synovial membrane. Under resting conditions, fibroblasts have an important role in maintaining joint homeostasis, producing extracellular matrix and joint lubricants. In contrast, during joint inflammation, fibroblasts contribute to disease pathology by producing pathogenic levels of inflammatory mediators that drive the recruitment and retention of inflammatory cells within the joint. Recent advances in single-cell profiling techniques have transformed our ability to examine fibroblast biology, leading to the identification of specific fibroblast subsets, defining a previously underappreciated heterogeneity of disease-associated fibroblast populations. These studies are challenging the previously held dogma that fibroblasts are homogeneous and are providing unique insights into their role in inflammatory joint pathology. In this review, we discuss the recent advances in our understanding of how fibroblast heterogeneity contributes to joint pathology in rheumatoid arthritis. Finally, we address how these insights could lead to the development of novel therapies that directly target selective populations of fibroblasts in the future.

KEYWORDS

fibroblasts, inflammation, inflammatory disease, rheumatoid arthritis, single-cell transcriptomics, synovium

1 | INTRODUCTION

In this review, we highlight new approaches and applications of single-cell profiling techniques^{1,2} and how these data are leading to unique insights into the phenotypic and functional heterogeneity of fibroblasts in the joint.³ Finally, we will discuss how defining this previously underappreciated fibroblast heterogeneity in rheumatoid

arthritis (RA) is leading to the identification of pathological fibroblast cell states that could be therapeutically targeted in inflammatory joint disease.⁴

1.1 | Rheumatoid arthritis

RA is a prototypic immune-mediated inflammatory disease characterized by persistent synovial joint inflammation that, if untreated, leads to progressive joint damage.⁵⁻⁷ While the

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introduction of biological disease-modifying anti-rheumatic drugs (bDMARDs) targeting either leukocytes or their derived products has led to a step change in the management of RA, 30%-40% of patients do not respond to such therapies, regardless of the mechanism of action of the drug used.⁸⁻¹³ These observations suggest the existence of additional pathways of disease persistence that remain to be identified and therapeutically targeted.¹⁴ While autoimmunity and systemic immune dysregulation are fundamental underlying disease processes in RA, the primary site of pathology during the effector phase of the disease occurs in the lining of synovial joints (called the synovial membrane).⁷ As a result, the pathogenic role of cells resident in synovial tissue, most notably fibroblasts and macrophages, has gained considerable attention as potential therapeutic targets in inflammatory joint disease.¹⁵⁻¹⁸

1.2 | Synovial tissue architecture

The synovium is a thin mesenchymal tissue that encapsulates the joint cavity and provides a barrier and lubricates the joint during locomotion.^{19,20} The membrane comprises a complex cellular ecosystem of tissue-resident macrophages, fibroblasts, nerves, and endothelial cells organized into a distinct microanatomy. The synovium is compartmentalized into histologically distinct zones: the lining layer and sub-lining layer with each compartment serving as a specialized tissue niche, adapted to perform specific tissue functions and unique roles in tissue homeostasis.¹⁹

In a healthy joint, the lining of the synovium is only 1-3 cell layers thick and is composed of tissue-resident macrophages and fibroblasts.²¹ This zone of the synovium controls cellular and molecular trafficking between the synovial membrane and the joint cavity, maintaining the integrity of the joint and regulating the composition of the synovial fluid, which ensures sufficient lubrication for joint locomotion and exchange of nutrients between the synovial tissue and the synovial fluid. In contrast, the sub-lining is comprised of fibroblasts and tissue-resident macrophages distributed throughout a loose connective tissue that contains blood and lymphatic vessels to ensure effective transport of nutrients and cells to and from the systemic circulation to the joint.

The synovial membrane undergoes extensive remodeling in response to inflammation and can expand to as much as 10-20 cell layers thick.^{22,23} Infiltrating immune cells and proliferation of fibroblasts result in synovial hyperplasia, characterized by a heterogeneous population of fibroblasts and macrophages that promote tissue inflammation and damage.^{23,24} The formation of pannus tissue, a well-described architectural feature of the chronically inflamed joint,²⁵ is comprised of hypertrophic synovium, composed of macrophages and fibroblasts that produce destructive enzymes that degrade articular cartilage and bone.²⁶ The cellular components and mechanisms underlying the disease-specific remodeling of the synovium have yet to be fully elucidated.²⁷

1.3 | Definitions of tissue fibroblasts in health and disease

Fibroblasts are ubiquitous mesenchymal cells that make up the stromal compartment of organ tissues.^{4,28} Historically identified by their morphology, ability to adhere to plastic and absence of epithelial, vascular, and leukocyte lineage markers, their study has been compounded by a lack of fibroblast-specific cell markers.^{29,30} Under steady-state conditions, fibroblasts are more generally defined by their expression of collagen 1-alpha, platelet-derived growth factor receptor-alpha (PDGFR α), and THY1 (CD90),³¹⁻³³ although these markers are not fibroblast-specific and expression does not reflect the underlying heterogeneity of this cellular population. Current evidence indicates that fibroblast populations consist of diverse cellular subsets based on their developmental origin,^{34,35} anatomical location,^{36,37} and tissue function.³⁸ Emerging single-cell profiling data of the transcriptional landscape of fibroblasts across different tissues are providing a framework for a consensus definition of fibroblast phenotypes and description of their heterogeneity.^{4,39,40} This is the first crucial step in developing a universal definition of fibroblast phenotypes, but must now be extended to incorporate definitions based on cellular function that associate with the expression of specific gene programs.

1.4 | Fibroblasts in synovial joints

Synovial fibroblasts are tissue-resident cells only found in the synovium of diarthrodial joints.⁴¹ Specifically adapted to this microenvironment, these cells have an essential function in maintaining joint homeostasis and have key effector roles in inflammatory joint disease. Their true phenotypic and functional heterogeneity has only recently been fully appreciated. The molecular mechanisms leading to the functional specialization of synovial fibroblasts are an area of intense research and are leading to potentially new therapeutic avenues, including selectively targeting pathogenic subsets of fibroblasts in inflammatory joint disease.²⁶ It is hoped that targeting these cells directly in the joint microenvironment could break the therapeutic ceiling in RA and re-establish joint homeostasis.

2 | SYNOVIAL FIBROBLASTS IN THE HEALTHY JOINT

Under resting conditions, the synovial microanatomy is characterized by a well-defined thin synovial lining layer containing a high density of fibroblasts and tissue-resident macrophages.^{42,43} The sub-lining layer by contrast is less well defined, comprising a loose connective tissue, sparsely populated by fibroblasts within a collagenous extracellular matrix, interspersed with adipose cells and blood vessels. The healthy joint cavity is a fluid-containing sterile space that functions to lubricate the joint cavity and lacks immune cell trafficking.¹⁹ Lining layer fibroblasts directly contribute to the composition of the synovial fluid by producing lubricin

(proteoglycan 4) and hyaluronic acid that lubricate the joint during locomotion.²⁰ These cells also regulate ion transport and deposit extracellular matrix made up of type III, IV, V, and VI collagen and laminin allowing nutrient exchange between the synovial fluid and the synovial membrane.²⁷

The lining layer fibroblasts have traditionally been thought of as a loose association of cells that lack tight junctions but are supported by a porous basement-like membrane.⁴² A recent study in mice and humans has challenged our understanding of the cellular architecture of the synovium, demonstrating the presence of tissue-resident macrophages directly adjacent to the lining layer fibroblasts, on the outmost layer the synovial membrane⁴³ (see Figure 1). These macrophages express the protein CX₃CR1 and form an immunological barrier between the joint cavity and the synovial membrane that maintains immune privilege in the joint. This barrier is maintained by tight junctions forming a pseudo-epithelial structure that surrounds the joint. These macrophages may be similar to yolk sac-derived tissue-resident macrophages found in large cavities such as the peritoneum.⁴⁴ In the postnatal joint, these barrier macrophages are maintained through a pool of locally proliferating CX₃CR1⁺MHCII⁺ mononuclear cells that are embedded in the sub-lining tissue. An overview of synovial structure can be found in Figure 1. The function of this anatomical localization of barrier macrophages with lining layer fibroblasts has yet to be explored, but fibroblasts in this context may play a supportive role, helping to sustain an effective macrophage barrier. This reciprocal relationship is observed in large cavities, where fibroblasts act to prevent cavity lining macrophages from entering the tissue.⁴⁴ The potential for reciprocal fibroblast-macrophage relationship under steady-state and pathological conditions in the synovium requires further study.

The function of fibroblasts located in sub-lining tissue under resting conditions has yet to be fully defined; however, studies of global populations of fibroblasts isolated from the healthy joint display an immunosuppressive function similar to that observed in mesenchymal stem cells.^{45,46} While significant progress has been made in understanding fibroblast heterogeneity under pathological conditions,^{3,40,47} little is known about the true heterogeneity of synovial fibroblasts under healthy conditions. In particular, it is not known whether the subsets of fibroblasts identified in the disease context also exist under resting healthy conditions or are simply a function of disease-driven differentiation or changes in cell activation state. The study of the resting joint has been compounded by a lack of access to healthy synovium, although this is being addressed through large cell atlas consortia studies, such as the human cell atlas.^{48,49}

3 | FIBROBLASTS IN INFLAMMATORY JOINT PATHOLOGY

We will next review the diversity of synovial fibroblasts during joint inflammation and the role of these cells in arthritis. It is now well

established that synovial fibroblasts are key effectors in joint inflammation and damage.⁵⁰ They have diverse functions that impact on many disease pathways and as a result are an attractive therapeutic target.¹⁶ However, they remain difficult to study in human disease largely due to a lack of specific cell surface markers which would allow for their identification from other cell types. The application of single-cell profiling technologies such as single-cell RNA sequencing and high dimensional imaging studies has revealed the true cellular diversity of synovial fibroblasts and defined their tissue-specific niches in the joint.^{3,38,47,51} While progress has been made recently in our understanding of the phenotypic diversity of these cells, linking this phenotypic heterogeneity to cellular function and ultimately role in disease pathology is an essential part of future research.

3.1 | Inflammatory remodeling of the synovium

During a chronic inflammatory insult, the synovial microenvironment is remodeled with the expansion and formation of a pathological, highly organized sub-lining tissue with diverse cellularity, including a significant expansion of tissue-resident synovial fibroblasts and the formation of an invasive hyperplastic pannus tissue (see Figure 1). The synovial membrane expands to 10-20 cell layers thick, with remodeling involving selective expansion of distinct cellular populations both immune and non-immune and organization of these cells into specialized compartments within the synovial tissue.⁵¹ These changes in the synovial microenvironment also lead to changes in the phenotype of both tissue-resident synovial cells and infiltrating inflammatory cells. These individual features of synovial remodeling all involve synovial fibroblasts which have a critical role in mediating inflammatory joint pathology, as emphasized by early studies of cadherin 11 (CDH11), an adhesion protein expressed by synovial fibroblasts during inflammation.⁵² CDH11 determines adhesion between fibroblasts and is critical for the formation of synovial tissue hyperplasia, also increases fibroblast migration and invasion, and synergizes in the activation of fibroblasts to produce matrix metalloproteinases (MMPs), cytokines, and chemokines.^{42,53-55} The genetic deletion of *Cdh11* in mice results in the impaired formation of a hyperplastic synovium leading to attenuation of synovial inflammation in mice.⁴² These studies emphasize the critical role of the synovial remodeling in driving inflammatory joint pathology and its importance as a potential site for modulating the disease process.

3.1.1 | Cellular expansion

The mechanism of pathological cellular expansion in the synovium is multifactorial, including (a) active proliferation in response to growth factors and cytokines produced by immune cells⁵⁶; (b) a low rate of apoptosis due to increased pro-survival factors and resistance to stress induced apoptosis (compared to other cell types); and (c) an increased accumulation of senescent cells.⁵⁷⁻⁵⁹ The differential effects of these processes on different subsets of synovial fibroblasts

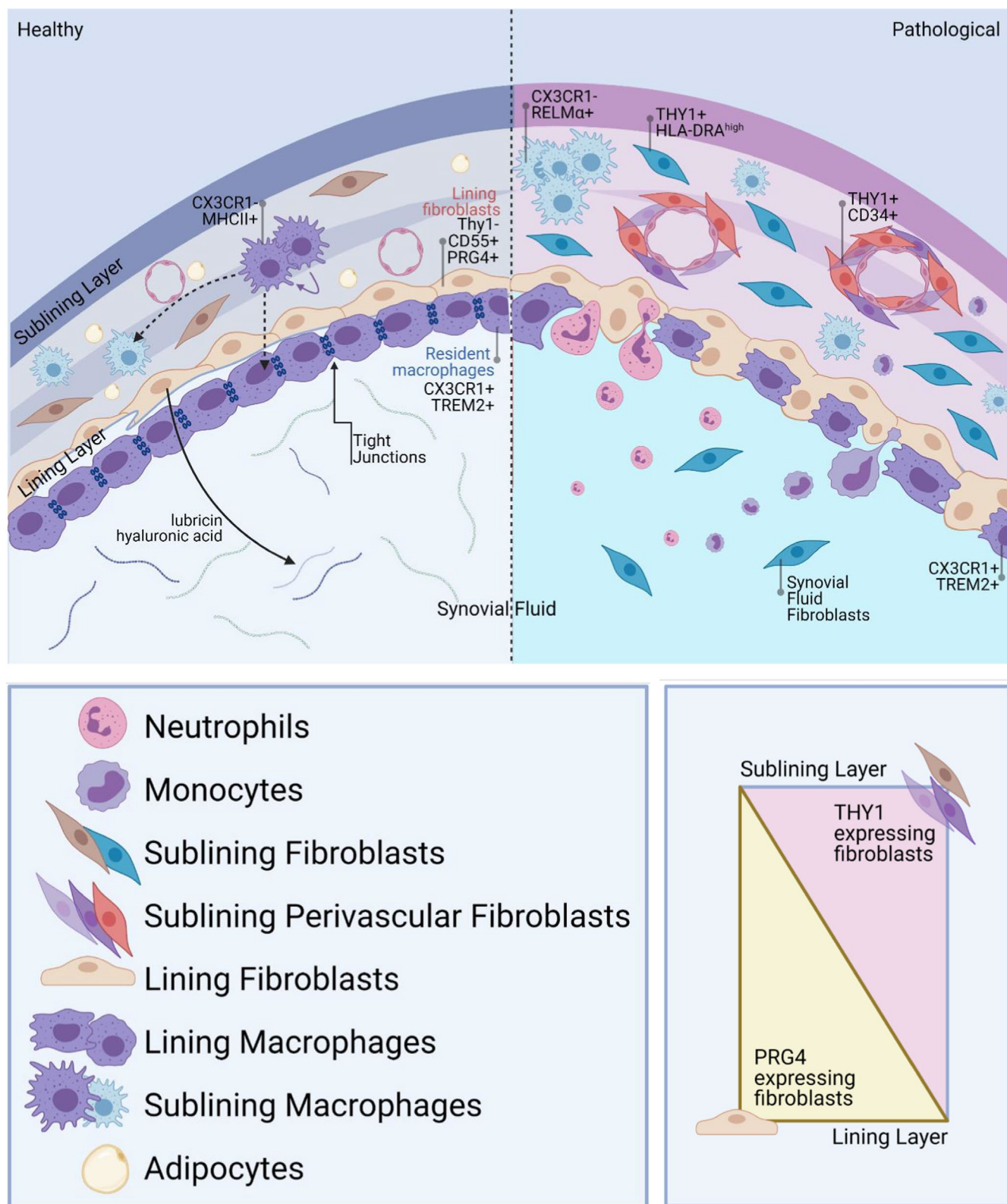


FIGURE 1 Synovial structure in health and disease. The healthy synovium consists of a lining layer containing lubricin and hyaluronic acid secreting fibroblasts (THY1⁺ CD55⁺ PRG4⁺) adjacent to an outer lining of tissue-resident barrier macrophages (CX₃CR1⁺ TREM2⁺) that maintain immune privilege in the joint.^{43,47} This macrophage population is replenished from CX₃CR1⁺ MHCII⁺ macrophages embedded within the sub-lining layer. Under resting conditions, the sub-lining layer and the synovial membrane are not well defined. During chronic joint inflammation, the macrophage barrier is lost and there is pathological expansion and remodeling of the synovial sub-lining layer leading to extensive fibroblast heterogeneity.^{3,47} Some fibroblasts are detached from the synovium and found free-floating in the synovial fluid²⁰²

and how this contributes to quantitative changes of different populations of fibroblasts during pathology are yet to be elucidated. In addition, the contribution of migrating cell populations from other tissue sources is still under active debate (see “Migratory potential of synovial fibroblasts” and Figure 2).

3.1.2 | Cellular organization

The synovial microanatomy also undergoes an organizational response to inflammation, resulting in well-defined synovial tissue niches that compartmentalize pathogenic synovial cells. Lining and sub-lining cell zones are present, but joint inflammation is associated with the loss of the CX₃CR1-expressing barrier macrophages⁴³ and the development of a more defined sub-lining tissue characterized by replacement of adipose tissue with dense cellular infiltrates of inflammatory cells and expansion of tissue-resident macrophages and fibroblasts.⁶⁰ Fibroblasts define this sub-lining cellular landscape, providing an address code to localize immune cells in this tissue compartment by contributing to chemokine gradients.⁶¹ The positional identity of fibroblasts in the synovial tissue is mediated

by microenvironmental instructive signals that polarize fibroblast phenotype, depending on tissue location, by regulating the expression of specific gene expression programs.⁶² Using single-cell RNA sequencing followed by trajectory analysis and spatial localization of fibroblast subsets within human RA synovial tissue, it has been shown that human synovial fibroblasts exhibit positional identity along a spatial axis that extends from the perivascular space in the sub-lining tissue to the synovial lining layer. This is mediated by endothelium-derived NOTCH3 signaling, which is a key positional determinant of perivascular fibroblast identity.⁶² Other positional cues and polarizing signals that facilitate fibroblast specialization and positional identity have yet to be defined, but these could represent potentially important therapeutic avenues. In summary, the synovial architecture also has an organizational response to inflammation co-ordinated by synovial fibroblasts whose phenotype defines compartmentalized cellular niches within the synovial tissue that contain pathogenic cells.²⁷

3.1.3 | Tissue pathotypes

Synovial histopathology in RA is heterogeneous and has been defined as pauci-immune (scant infiltration of immune cells and prevalence of tissue resident fibroblasts, diffuse-myeloid (predominant macrophages within the sublining tissue and lacking B/plasma cell aggregates) and lympho-myeloid (characterized by well-organized B or plasma cell aggregates and rich in macrophages).^{63,64} Synovial tissue transcriptomic profiles from untreated RA patients suggest that synovial tissue pathotype signatures are associated with specific disease trajectories, treatment response, and disease outcomes.^{65,66} Patients with a fibroid (pauci-immune) pathotype have an enriched fibroblast gene signature and tend to have the poorest response to treatment.⁶⁷ These findings support a role for fibroblasts as effector cells in treatment-refractory disease pathways. Understanding how fibroblast phenotype and heterogeneity contribute to treatment response will therefore be vital in our understanding of how and when to target fibroblasts in RA.

Ectopic lymphoid structures

It has also been shown that aggregates of lymphocytes can form structures in the synovial tissue that resemble secondary lymphoid organs. They display varying degrees of organization, but are characterized by a T cell-rich zone enclosing a central B cell-rich zone served by a network of high endothelial venules.⁶⁸ These ectopic lymphoid follicle-like structures, resembling germinal centers, are characterized by a follicular dendritic cell network (specialized stromal cell), essential for affinity maturation of B cells.^{69,70} These structures have been observed with variable prevalence in RA^{64,71} and are associated with severe disease, T cell priming and are surrounded by plasma cells producing autoantibodies locally in the tissue.^{69,72,73}

The pathophysiological role of these structures in promoting chronic joint inflammation is still uncertain, but their presence characterizes a distinct pathotype of synovitis that can be detected by

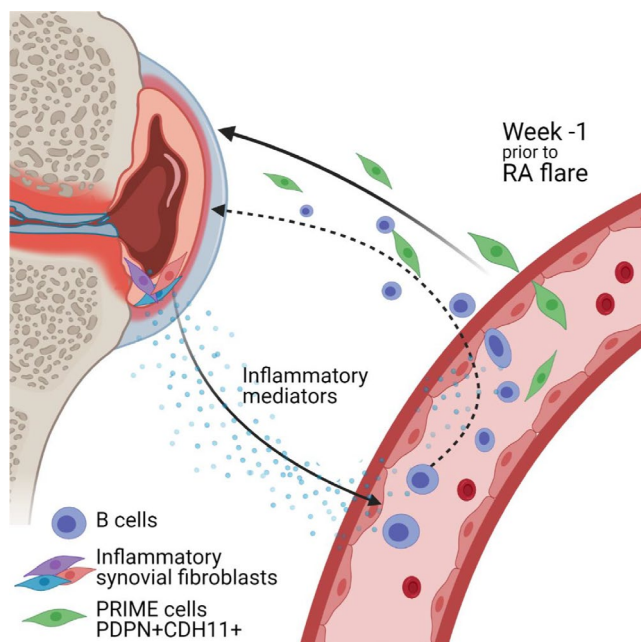


FIGURE 2 Migratory potential of synovial fibroblasts. It has been proposed that fibroblasts have a migratory potential with the capacity to leave the joint and travel in the bloodstream to distal sites of engraftment.⁹⁵ A recent study of the RNA transcriptional profile of peripheral blood samples obtained 1 week prior to an arthritis flare revealed a gene transcript signature consistent with a mesenchymal cell phenotype.¹⁷⁷ These cells were referred to as pre-inflammatory mesenchymal cells (PRIME cells). PRIME cells are thought to have a sub-lining layer phenotype, and the authors propose that peripheral blood B cells lead to the activation of PRIME cells in the blood and the migration of these cells into the synovium. This engraftment then leads to the onset of an arthritis flare. This working model however is yet to be proven

histology.⁷² The cytokine expression profile of synovial fibroblasts suggests these cells are able to contribute to the formation of ectopic lymphoid structures. RA synovial fibroblasts have been found to express high levels of CCL19, CCL21, receptor activator of nuclear factor kappa B (NF- κ B) ligand, and IL-7 (factors critical for ectopic lymphoid structure formation^{68,74,75}), when compared to synovial fibroblasts isolated from non-inflammatory conditions.⁷⁶ Synovial fibroblasts are also able to promote the retention and survival of plasma cells that either mature in the ectopic lymphoid structure or infiltrate the synovial tissue.⁷⁷⁻⁷⁹ The role of joint fibroblasts in supporting the development and maintenance of these structures is still uncertain and warrants further investigation.⁸⁰

Pannus tissue

It represents another distinct cellular niche in the synovium, and pannus-resident fibroblasts may represent a distinct population of fibroblasts with specialized functions.²⁷ Galectin 3, a secreted β -galactoside-binding protein, is present almost exclusively in the pannus of the inflamed RA synovium.^{81,82} Its expression is upregulated at sites of cartilage invasion after the attachment of synovial fibroblasts to cartilage oligomeric matrix protein.⁸³ Galectin 3 is then able to directly activate pannus-resident fibroblasts, stimulating the secretion of pro-inflammatory cytokines, chemokines, and MMP3, leading to the recruitment of inflammatory cells to the pathogenic tissue site.⁸⁴ At the pannus-cartilage interface, fibroblast-mediated production of MMPs, such as MMP1, MMP3, and MMP13, damages the collagen-rich structures of cartilage and enables fibroblasts to invade.^{85,86}

3.1.4 | Cellular phenotypes

The proliferative and organizational response to inflammation, observed at the tissue level in the synovium, is also associated with changes in cellular phenotypes leading to a diverse repertoire of tissue-resident cells.⁸⁷ There is also a global shift toward pro-inflammatory cell states with active suppression of pro-resolving cell states, resulting in a diverse cellular ecosystem that supports tissue inflammation and inhibits resolution.^{22,23,88} Synovial fibroblasts can acquire a destructive phenotype, forming part of the invasive pannus tissue and along with macrophages, and release tissue destructive enzymes responsible for degradation of cartilage and bone. RA synovial fibroblasts attach to and invade cartilage directly, an effect that is independent of an intact adaptive immune system.^{85,89} The pannus microenvironment becomes hypoxic, and fibroblasts within this tissue niche up-regulate tissue-degrading enzymes and apoptosis-inhibiting factors.⁹⁰⁻⁹⁴ These destructive fibroblasts have been found to have an epigenetically imprinted phenotype that is maintained even when these cells are removed from the joint and cultured for prolonged periods of time, before being re-introduced into the cartilage implant.^{85,95,96} This re-programming of fibroblast phenotype might explain why joint destruction and damage in RA do not always directly correlate with the severity of inflammation.

In summary, joint inflammation is associated with significant remodeling of the synovial microenvironment observed at a spatiotemporal and phenotypic cellular level. These processes direct site-specific aspects of pathology and may underlie the clinical heterogeneity of RA. Synovial fibroblasts are critical cells in this process and orchestrate the synovial microanatomy into distinct anatomical compartments and drive the spatial organization of synovial cells to develop functional cellular networks. As detailed cellular atlases emerge of the developing, healthy, and inflamed joints, we will gain further critical insights into the cellular ecosystem of the joint and its role in disease.

3.2 | Synovial fibroblast phenotypic and functional diversity

Tissue fibroblasts have historically been considered as functionally homogeneous cells, involved primarily in extracellular matrix production. However, it is now widely accepted that these cells perform a number of specialized functions.^{97,98} In the joint, the lining and sub-lining of the synovial membrane are anatomically separated and contain morphologically distinct populations of fibroblasts.^{53,99} Numerous human studies have demonstrated potential heterogeneity in the expression of cell surface marker proteins,¹⁰⁰ proteoglycans,¹⁰¹ and various chemokines³⁸ between different compartments of the synovial membrane, consistent with the existence of different synovial fibroblasts within each of these tissue compartments. Early studies also implied a stratification of function between the lining and sub-lining layer fibroblasts, with a more pro-inflammatory function attributed to the sub-lining layer fibroblasts.^{100,102} For example, a single-cell RNA-sequencing analysis of the human RA synovial tissue initially identified two main fibroblast phenotypes: a CD55⁺ population in the lining and a THY1⁺ population in the sub-lining.¹⁰² The CD55⁺ fibroblasts were enriched for *HAS1* (encoding a hyaluronan synthase), and the THY1⁺ fibroblasts were enriched for genes related to MMP expression and organization of the extracellular matrix. The phenotypic diversity of synovial fibroblasts has, however, only recently been addressed comprehensively using single-cell profiling technologies from enzymatically digested synovial tissue in mice and humans.^{38,62} These studies have clearly defined transcriptomically distinct populations of fibroblasts under inflammatory conditions. Initially, Mizoguchi *et al*³⁸ explored this diversity of human RA synovial fibroblasts by using flow cytometry to sort purify putative fibroblast subsets based on the expression of stromal markers PDPN, CDH11, CD34, and THY1. These proteins were chosen as they were expressed at low levels under resting conditions and significantly upregulated during joint inflammation.^{42,55,103-107} Following analysis by microarray and low-input, bulk-cell RNA sequencing followed by principal component analysis, three putative synovial fibroblast subsets were identified with unique transcriptomic profiles: PDPN⁺ CD34⁺ THY1⁺ lining fibroblasts, PDPN⁺ CD34⁺ THY1⁺ sub-lining fibroblasts, and PDPN⁺ CD34⁺ THY1⁺ sub-lining fibroblasts.³⁸ Of these, the THY1⁺ fibroblast subsets were expanded

in the inflamed synovium in RA and correlated with the severity of joint inflammation. In contrast, in the synovia of patients with osteoarthritis (OA), a non-inflammatory joint disease, the $PDPN^+ CD34^- THY1^-$ fibroblasts were expanded. Although these populations differed in their location within the joint, almost all subsets were positive for protein CDH11, which was previously shown to be associated with pathological behavior of fibroblasts in vitro and in experimental arthritis mouse models.⁴²

The observation that sub-lining fibroblasts are expanded in inflamed synovia in RA has been confirmed in several studies including Zhang *et al*.³ The authors used mass cytometry and unbiased single-cell RNA-sequence clustering to identify four synovial fibroblast populations with distinct transcriptomic profiles in a collaborative study, as part of the Accelerating Medicines Partnership-Rheumatoid Arthritis/Systemic Lupus Erythematosus (AMP RA/SLE) Consortium. These subsets included a $CD34^+$, $THY1^+$, and $HLA-DR^{high}$ -expressing subset, found in both the sub-lining layer and a $PRG4^+$ (gene encoding lubricin) lining layer population of fibroblasts. $THY1^+ HLA-DR^{high}$ sub-lining fibroblasts were >15-fold expanded in RA synovium and are considered pro-inflammatory, expressing high amounts of *IL-6*, *CXCL12*, and *CCL2*. In addition to the above, Zhang *et al*.³ identified a novel sub-lining fibroblast subset with high expression of *DKK3* encoding Dickkopf3, a protein upregulated in OA that prevents cartilage degradation in vitro.¹⁰⁸ *DKK3* overexpression in fibroblasts suppresses cell proliferation and promotes apoptosis via $TGF\beta 1/SMAD$ signaling¹⁰⁹ as well as impairing angiogenesis and inhibiting tumor growth through induction of endoplasmic reticulum stress.¹¹⁰ Interestingly, Zhang *et al*.³ demonstrated that *DKK3*⁺ sub-lining fibroblasts also expressed high levels of *OPG*, *CADM1*, and *MFAP2* which are involved in bone formation,¹¹¹ enhanced intestinal barrier function,¹¹² and elastic fiber structural formation.¹¹³ Thus, *DKK3*⁺ fibroblasts may play a role in immuno-regulation and/or restoring joint homeostasis. Finally, Zhang *et al*.³ demonstrated lining layer subsets consisting of (species-specific) $CD55^+ CD34^- THY1^-$ fibroblasts and express high levels of genes *MMP1*, *MMP3*, *PRG4*, *HAS1*, and *CD55*.^{3,38,47,62} See Table 1 for a summary of fibroblasts subsets, along with evidence of the proposed function.

Our work has defined the synovial fibroblast heterogeneity in both human and mouse arthritis.⁴⁷ We found that stromal markers were highly expressed in the inflamed synovia (tissue biopsy studies) in those patients who developed RA, compared to those in which joint inflammation resolved.⁹⁸ Of these markers, high expression levels of fibroblast activation protein- α (FAP α), a cell membrane dipeptidyl dipeptidase expressed by synovial fibroblasts,¹¹⁴ were the strongest predictor of chronic joint inflammation.⁹⁸ This led us to develop the hypothesis that FAP α may be a biomarker of a pathogenic subset of synovial fibroblasts. Deletion of FAP α^+ cells attenuated joint inflammation and bone erosion in serum transfer-induced arthritis in mice, suggesting that FAP α -expressing fibroblasts play an important pathological role in arthritis. Flow cytometry confirmed FAP α^+ fibroblasts were split into two discrete subsets: $THY1^+$ - and $THY1^-$ FAP α -expressing cells. Bulk population RNA-seq of sorted purified populations confirmed that $Fap\alpha^+ Thy1^-$ cells

displayed a transcriptional signature of lining fibroblasts, whereas $Fap\alpha^+ Thy1^+$ cells expressed a sub-lining phenotype. The most significant differences in transcriptional profiles were observed between the lining and sub-lining fibroblast populations, suggesting that anatomical location in the tissue was a major determinant of phenotype and gene expression programs.

Using single-cell transcriptomics of enzymatically digested mouse synovia, we confirmed the presence of several transcriptionally distinct populations of fibroblasts with one $Thy1^-$ lining layer population (also expressing *Cd55*, *Prg4*, and *Clic5*) and three $Thy1^+$ sub-lining layer subsets. A cross-comparison between human and mouse single-cell datasets revealed two $Thy1^+$ subsets that are shared between human and mouse, including a $CD34^+ C3^+$ (this subset is localized within the perivascular zone around blood vessels and involved in immuno-inflammatory processes and stromal memory) and a $COL8A1^+$, $COL1A1^+ MDK^+$ (relating to bone, cartilage, and ECM remodeling). In addition, a single lining layer fibroblast phenotype was identified by a cassette of gene expression markers *CLIC5*+, *TSPAN15*+, and *PRG4*+. Re-analysis of the Zhang *et al*.³ human synovial tissue dataset revealed *FAP α* expression within the pathogenic subset of fibroblasts ($THY1^+ HLA-DR^{high}$) identified in this dataset and predominately within the sub-lining cell fibroblast populations.⁴⁷

The transcriptional gene expression programs that define these fibroblast subsets correlate with different effector cell functions. As mentioned above, Zhang *et al* showed in human synovial tissue that $THY1^+ HLA-DR^{high}$ fibroblasts express the highest level of *IL-6*, *CXCL12*, *CCL2*, and interferon-stimulated genes, suggesting a pro-inflammatory phenotype.³ In mouse studies, the $Fap\alpha^+ Thy1^+$ subset is located in the sub-lining and has an immune effector profile characterized by high expression of a number of cytokines and chemokines, including *IL-6*, *IL-33*, and *IL-34*.⁴⁷ The $Fap\alpha^+ Thy1^-$ subset is located in the lining and has a damage effector profile that includes high expression of inducers of osteoclast activity (*CCL9* and *TNFS11*) and MMPs involved in cartilage degradation (*MMP3*, *MMP9*, and *MMP13*), indicating that the cells mediate bone and cartilage damage.⁴⁷ Consistent with these findings, subsets of synovial sub-lining fibroblasts have been found to have a secretory profile consistent with an inflammatory state in both mouse and human fibroblasts, secreting chemokines and cytokines (eg, *IL-6*, *CXCL12*, and *CCL2*) in response to $TNF\alpha$ stimulation in vitro, that have the potential to modulate the inflammatory response in the joint.³⁸ The proportion of $CD34 THY1^+$ cells in the joint correlated positively with the proportion of leukocytes and the extent of synovitis in the human RA synovium.³⁸ In contrast, lining fibroblasts are the pre-dominant source of *MMP1* and *MMP3* and are able to stimulate osteoclastogenesis through the production of *RANKL*, and these findings are consistent with the lining fibroblasts having a destructive phenotype with the ability to invade and degrade articular cartilage and bone in mice.⁴⁷ We have previously shown that $PDPN^+$ human synovial fibroblasts attached to, invaded, and degraded articular cartilage following implantation to immunodeficient mice.¹⁰⁰

The definitive evidence that these transcriptionally distinct populations of fibroblasts have non-overlapping effector cell functions

TABLE 1 Major fibroblast subsets and proposed function in the inflamed synovium

Subsets	Tissue location	Proposed function	Mouse homologous Subset	Disease pathology	Common markers
DKK3 ⁺ Croft et al ⁴⁷ Zhang et al ³	Sub-lining layer	Express genes related to the extracellular matrix. Bone formation, cell junction formation, and elastic fiber structural integrity	Not yet defined	Not yet defined—may have a role in tissue remodeling.	DKK3, CADM1, Mfap2, OPG, Col8a2,
Thy1 ⁺ HLA-DR ^{hi} Zhang et al ³	Sub-lining layer (perivascular and interstitial)	Immunoregulatory, pro-inflammatory effect, NOTCH3-mediated differentiation.	Yes	Expanded by >15-fold in RA in synovial tissue containing high levels of leukocyte infiltration compared with synovial tissue in OA. IL-6 expression. Express genes related to MHC class II presentation and the IFN γ -mediated signaling pathway.	Col11a1, Mdk, Col8a1, Postn, MMP13, Col1a1,
CD55 ⁺ PRG4 ⁺ Croft et al ⁴⁷ Zhang et al ³ Stephenson et al ¹⁰²	Lining layer	Lining layer function under resting conditions—secretes lubricin and hyaluronic acid	Yes	Increased osteoclast activity and structural joint damage. Less abundant in RA in synovial tissue containing high levels of leukocyte infiltration than in synovial tissue in OA	CLIC5, Tspan15, PRG4, Hbegf, Htra1, Sema3a
Thy1 ⁺ CD34 ⁺ Croft et al ⁴⁷ Friščić, J. et al ¹⁶³ Mizoguchi et al ³⁸	Sub-lining layer (perivascular)	Immunoregulatory, tissue priming via C3 activation, pro-inflammatory effect, perivascular location, NOTCH3-mediated differentiation.	Yes	Expression of genes involved in complement activation, responsible for tissue priming response that underlies progression of arthritis. Express genes related to the extracellular matrix. Increased invasive and migratory properties in vitro. Increased ability to recruit peripheral blood monocytes in vitro	Apod, C3, CD34, Mfap5, Clip, CXCL14

Note: Recent studies^{38,102} have identified different fibroblast subsets in the joint defined by their transcriptomic signatures, anatomical location, and potential function and role in disease pathology.

came from our adoptive transfer studies of selected fibroblasts in vivo using mouse models of arthritis.⁴⁷ We were able to demonstrate that the intra-articular injection of murine FAP α ⁺ THY1⁺ immune effector sub-lining fibroblast subsets into the inflamed mouse synovium exacerbated joint inflammation, whereas the injection of FAP α ⁺ THY1⁺ subsets did not affect the severity of inflammation but resulted in more joint damage. These data collectively demonstrate that the immune effector functions of synovial fibroblasts are conferred by sub-lining subsets, whereas damage is mediated predominately by the lining layer fibroblasts. The co-independence of these two cell populations remains uncertain as lining fibroblasts only had a destructive phenotype when stimulated ex vivo with

pro-inflammatory cytokines, perhaps suggesting that damage also requires an intact pathological sub-lining layer in vivo. We further demonstrated an expansion of immune effector fibroblasts expressing FAP α and THY1 in the synovial tissue of individuals with RA, compared to patients with OA, consistent with previous studies demonstrating an expansion of sub-lining fibroblast subsets in RA.³⁸ The differential expansion of these two fibroblast populations in the synovium could explain the inflammatory pathotype of RA, in contrast to a more destructive pathotype observed in OA.

The molecular mechanism driving the expansion of sub-lining layer fibroblasts in RA has recently been elucidated. Wei *et al*⁶² recognized that expansive fibroblast populations in the sub-lining were

located around the endothelium in the perivascular space, suggesting that vascular endothelial-derived signals could be important in modulating fibroblast phenotype. The investigators determined that the pathogenic sub-lining fibroblast phenotype and the expansion of this population are driven by endothelial-derived NOTCH3 signaling. The critical role of NOTCH3 signaling in arthritis was confirmed by the genetic deletion of the *Notch3* gene and antibody blockade of NOTCH3 signaling during experimental arthritis in mice, with both approaches leading to attenuated synovial inflammation and damage. These studies demonstrate that the inhibition of NOTCH3-mediated induction of pathogenic fibroblast differentiation could be an effective therapeutic approach in RA.

It is interesting that targeting a sub-lining layer fibroblast is also effective at inhibiting joint damage, given the observation that immune regulatory and joint destructive functions may be carried on two different effector fibroblast subsets. These findings suggest that an intact pathogenic synovial sub-lining may be required to mediate the destructive abilities of lining fibroblasts or that redundancy in function exists between fibroblast subsets in the synovium. Indeed, we only observed a destructive phenotype in lining layer fibroblasts in vitro following stimulation with TNF α .³⁸ The genetic deletion of *Cdh11* gene which encodes the CDH11 protein that mediates cell-to-cell contact between synovial fibroblasts in mice disrupts the synovial membrane architecture, inhibiting synovial hyperplasia, and protects the joint against both damage and inflammation.⁴² Collectively, these data suggest that the architectural reorganization or function of cells in this compartment in response to inflammation can be protective.

In conclusion, recent studies have described a diverse repertoire of fibroblast phenotypes with effector functions mapped to individual subsets, summarized in *Table 1*. However, several challenges and uncertainties still remain. Firstly, a detailed functional analysis of individual fibroblast subsets is still needed. Secondly, in order to understand the contribution of fibroblast heterogeneity to inflammatory joint pathology, it is important to understand the spatiotemporal changes of these fibroblast populations as disease progresses and in response to treatment. Thirdly, it is vital that we understand how changes in fibroblast phenotype and differentiation are regulated and whether pathogenic fibroblast subsets defined by single-cell analysis represent reversible changes in cell status. Collectively, this knowledge will allow us to determine the role of synovial fibroblasts in driving specific disease pathologies and determine how and when to utilize anti-fibroblast therapy in the future.

3.3 | Synovial fibroblasts as immune effector cells

Two important functional changes occur during the progression of RA. Firstly, synovial fibroblasts lose their immunosuppressive capability and secondly become actively immune-stimulatory.^{45,46,98,115} The persistent pathological phenotype of fibroblasts in established RA demonstrates that synovial fibroblasts are not merely “passive

responders” to the inflammatory milieu, but can independently influence the inflammatory status of the joint.⁵¹

3.3.1 | Fibroblast/endothelial cross talk determines immuno-phenotype

The interaction between leukocytes and synovial fibroblasts during an acute inflammatory response ultimately leads to resolution of the inflammatory focus. However, such interactions at sites of chronic inflammation lead to sustained leukocyte survival and retention within the joint, leading to the persistence of the inflammatory lesion.^{15,116–118} For example, upon coculture with endothelial cells (EC), fibroblasts isolated from patients with resolving synovitis and non-inflamed joints suppressed lymphocyte adhesion in response to TNF α stimulation.⁴⁵ This immune-protective effect was lost in fibroblasts isolated from patients with very early RA (≤ 3 months since disease onset), allowing increased lymphocyte recruitment. This stimulatory phenotype was characterized by high levels of IL-6 and TGF- $\beta 1$ production. Hence, fibroblasts cultured from the synovial tissues of patients with divergent disease outcomes (resolving vs persistence) are also functionally distinct. Moreover, fibroblast-EC interactions evolve with RA progression. In contrast, fibroblasts from very early RA have not yet acquired the ability to autonomously activate EC in the absence of exogenous cytokines.

IL-6 and TGF- $\beta 1$ are each able to induce divergent pro-inflammatory or anti-inflammatory effects depending on the inflammatory context or cell type.¹¹⁹ Emerging evidence reveals complex cross talk between IL-6 and TGF- $\beta 1$ signaling pathways, in which each cytokine can positively or negatively regulate the expression or activity of the other depending on the inflammatory context.^{120,121} IL-6 and TGF- $\beta 1$ were identified as the bioactive agents required for the inhibitory effects on recruitment of cocultured resolving fibroblasts.⁴⁵ Furthermore, neutralization of IL-6 and TGF- $\beta 1$ inhibited the recruitment effect of cocultured very early RA fibroblasts. This suggests that in very early RA, IL-6 and TGF- $\beta 1$ have not simply lost efficacy, but trigger stimulatory rather than inhibitory downstream events.

Synovial fibroblasts are a major source of IL-6 in RA.¹²² Recent evidence suggests synovial fibroblasts isolated from patients with RA sustain IL-6 production (as well as heightened production of other chemotactic mediators such as IL-8) by creating a positive feedback loop involving autocrine LIF, LIF receptor, and STAT4 signaling.¹²² IL-6 released during coculture with EC signals through CD126 expressed by EC, but not fibroblasts.⁴⁵ Given that fibroblasts cannot respond to IL-6 generated during coculture, distinct fibroblast-EC interactions must regulate EC responses to IL-6 and produce the discrete patterns of lymphocyte recruitment. High SOCS3 expression (ie, negative regulation of STAT activation), as seen in the EC from cocultures with fibroblasts isolated from patients with resolving synovitis, triggers an immuno-protective IL-6 response. Conversely, failure to induce SOCS3 was associated with loss of immunosuppressive responses in the EC from cocultures very early

RA disease fibroblasts. In murine adjuvant-induced arthritis, low endothelial SOCS3 levels, with negative regulation of IL-6 signaling, have been linked with more severe arthritis.¹²³ In summary, there are two distinct IL-6 signaling pathways in ECs, which are induced in a disease outcome-specific manner and elicit different functional consequences in EC.

3.3.2 | Fibroblasts as innate immune cells

In addition to loss of immune-protective function, synovial fibroblasts also play an important role in RA pathology through cross talk with other cell types. Historical evidence has shown synovial fibroblasts can act as innate immune cells through the expression of pattern recognition receptors (PRRs) such as TLR2, TLR3, TLR4, TLR7, and TLR9. TLR4 recognizes anti-citrullinated protein antibodies (ACPAs) and has been demonstrated to play an important role in RA pathology.^{124,125} Recently, it has been shown that soluble CD14 can also induce the expression of inflammatory cytokines such as IL-6, TNF α , IL-8, adhesion molecules, MMPs, and RANKL through TLR4.¹²⁶ TNF α has been shown to activate TLR3 and induce the production of pro-inflammatory mediators through NF κ B signaling.¹²⁷ Silencing of regulator of G-protein signaling (RGS1) inactivates TLR3 on synovial fibroblasts and suppresses inflammation and angiogenesis in collagen-induced arthritis in mice.¹²⁸ In addition, when cocultured with T cells, synovial fibroblast TLRs induce cell-cell contact and cytokine-mediated TH1 and TH17 expansion resulting in IFN γ and IL-17 production.¹²⁹ Thus, like innate cells, synovial fibroblasts are capable of recognizing pathogen-associated molecular patterns and endogenous ligands which contribute to the pathogenesis of RA.

3.3.3 | Fibroblast/B cell interactions

B cells support RA pathology through the production of autoantibodies.¹³⁰ Human RA synovial fibroblast TLR3 stimulation has also been shown to induce production of B cell-activating factor (BAFF) and a proliferation-inducing ligand (APRIL) resulting in enhanced maturation and survival, and class switching of antibodies and survival of plasma cells, respectively.⁷⁷⁻⁷⁹ Production of IL-6 by synovial fibroblasts isolated from patients with RA also promotes maturation, expansion, and survival of B cells.¹³⁰

3.3.4 | Fibroblast subsets promote T cell recruitment

It is well documented that synovial fibroblasts can present antigens to CD4⁺ T cells during RA via MHCII.^{131,132} In addition, the production of chemokines such as CXCL10 and CXCL19 by RA synovial fibroblasts is key promoters of lymphocyte recruitment.¹³³ A recent cross-disease analysis which included human fibroblasts isolated from the RA synovium has demonstrated SPARC⁺ COL3A1⁺ synovial fibroblasts interact with blood vessels via NOTCH signaling to

alter the EC compartment, priming it to promote cellular infiltration which is further supported through the differentiation of CXCL10⁺ CXCL19⁺ synovial fibroblasts.⁴⁰ CXCL10⁺ CXCL19⁺ fibroblasts interact directly with T cells and are likely analogous to Zhang *et al*³ defined HLA-DRA^{high} pathogenic subset that displays a strong response to IFN γ (a pro-inflammatory molecule abundantly secreted by CD8⁺ T cells). The expression of RA synovial fibroblast CX₃CL1 and CX₃CR1 by CD8⁺ and CD4⁺ T cells has also been shown to positively correlate with RA pathology. In support of this, treatment targeting CX₃CL1 production has shown efficacy in early phase clinical trials in RA.¹³⁴

While the global effect of depleting sub-lining layer synovial fibroblast subsets has been determined to have an anti-inflammatory effect in mice,⁴⁷ the individual contribution of distinct sub-lining fibroblast subsets in regulating differential aspects of inflammation has yet to be determined. In RA, THY1⁺ HLA-DRA^{hi} fibroblasts express an interferon-stimulated phenotype suggesting that IFN γ -producing lymphocytes that infiltrate the tissue may stimulate an interferon-activated gene signature in this fibroblast population and drive their differentiation into a pro-inflammatory phenotype.³ It is likely that other critical interactions between synovial fibroblasts and other infiltrating inflammatory cells result in the modulation of the tissue fibroblast phenotype. It will be vital to define the effect of these cellular interactions on the regulation of individual fibroblast subset phenotypes in the future, since these interactions are likely to evolve as disease progresses, and in response to treatment with specific immunomodulatory therapies.

3.4 | Fibroblast-macrophage coupling serves as an immune-regulatory checkpoint in the synovium

Under steady-state conditions, the other major cellular component of the synovial membrane is macrophages. Macrophages derive from two main cellular lineages: bone-marrow-derived monocytes and monocyte-independent macrophages derived from cells that disperse into the tissues during embryonic development. The tissue-resident macrophages from the latter lineage have distinctive gene expression profiles that depend on the particular tissue in which they reside.¹³⁵ Under resting conditions in mice, 40% of synovial tissue macrophages are CX₃CR1⁺ macrophages that constitute a barrier layer adjacent to lining layer fibroblasts.⁴³ These macrophages have an immunoregulatory phenotype expressing *Trem2* and genes encoding TAM receptors such as *Axl* and *Mfge8* that mediate the clearance of apoptotic cells. These barrier CX₃CR1⁺ macrophages are derived from CX₃CR1⁺MHCII⁺ cells situated deep within the sub-lining layer. These MHCII⁺ synovial macrophages also give rise to CX₃CR1⁺ RELM α ⁺ sub-lining macrophages which are found to be expanded during experimental arthritis in mice and in synovial tissue in RA. Depletion of CX₃CR1⁺ macrophages during experimental arthritis in mice leads to synovial barrier breakdown and cellular infiltration of neutrophils and monocyte-derived cells into the synovium. Although CX₃CR1⁺ lining macrophages do not expand during

inflammation, they do, however, maintain an immunoregulatory phenotype and actively clear apoptotic cells.

These data suggest that CX₃CR1⁺ macrophages are the first immune-regulatory checkpoint that attempts to suppress or limit synovial inflammation and the failure of this checkpoint leads to established joint inflammation. The functional purpose of the anatomical coupling of lining macrophages with lining layer fibroblasts (which are functionally, spatially and anatomically distinct from sub-lining fibroblasts) is currently unknown. We hypothesize that the loss of this spatial coupling during inflammation may lead to the emergence of a pathogenic lining layer fibroblast phenotype or a compensatory repair like phenotype.

In the sub-lining tissue, MHCII⁺ interstitial macrophages replenish the end-stage CX₃CR1⁺ macrophages, although the exact role of end-stage interstitial macrophages is currently unclear. However, as MHCII⁺ macrophages also give rise to pro-inflammatory sub-lining RELM α -expressing macrophages, it is important to understand the mechanism in which CX₃CR1⁺ and RELM α ⁺ macrophage differentiation is determined. As lining and sub-lining macrophages are long-lived, it is also possible there is a pool of MHCII⁺ macrophages which is rapidly depleted in response to an inflammatory stimulus. Further studies are required to explore the interactions between lining layer macrophages and fibroblasts and the molecular mechanisms that lead to disruption of the synovial macrophage barrier and expansion of the invasive fibroblast population seen in RA.

The second fibroblast-macrophage regulatory checkpoint is observed between fibroblasts and sub-lining macrophages. Sub-lining macrophages comprise heterogeneous populations of monocyte-derived and tissue-resident macrophages. A recent comprehensive analysis of their heterogeneity has revealed the transcriptional and functional diversity of synovial tissue macrophages.⁸⁷ The investigators isolated macrophages from patients with early/active RA, treatment-refractory/active RA, and RA in sustained clinical remission. The analysis revealed four distinct subsets that comprised nine discrete phenotypic states. Two of these subpopulations (MERTK⁺ TREM2^{high} macrophages and MERTK⁺ LYVE1⁺ macrophages) were enriched in the healthy synovium and in patients with RA whose disease was in remission compared with those with active disease. A reduction in these cellular subsets in the tissue was associated with an increased risk of future disease flare. In vitro evidence showed MERTK⁺ macrophages produced high amounts of pro-resolving lipids and when cocultured with synovial fibroblasts induced a repair response in fibroblasts. In contrast MERTK⁺ macrophages are expanded during active disease compared to remission. This population comprises subsets of macrophages expressing alarmins (CD48⁺ S100A12⁺), bone remodeling molecules (CD48⁺ SPP1⁺), an interferon signature (HLA⁺ ISG15⁺ cluster), and an antigen-presenting cell signature (HLA⁺ CLEC10A⁺). MERTK⁺ macrophages produce TNF α and IL-6 and induce pathological activation of fibroblasts.

As a result, MERTK⁺ macrophages may act as an off switch by negatively regulating pro-inflammatory fibroblast phenotypes. We hypothesize the failure of this checkpoint could lead to the

emergence of persistently activated, pathogenic fibroblast subsets. In addition, another study has revealed the existence of macrophages in the RA synovium that are positive for the growth factor HB-EGF.¹³⁶ These macrophages promoted fibroblast invasiveness in an epidermal growth factor receptor-dependent manner. Thus, indicating that intercellular networks between these pathogenic populations of synovial tissue cells can contribute to inflammatory joint pathology.

In summary, these studies demonstrate that fibroblast phenotypes can be regulated by synovial tissue macrophages. The coupling of these tissue-resident cell types appears to be critical in determining if joint inflammation either persists or resolves. If these checkpoints fail to limit or restrict inflammation, then disease persists. Certain macrophage subsets may therefore act as critical "off" switches, resulting in the suppression of pro-inflammatory fibroblast phenotypes. It is not yet known, however, how inflammation-induced epigenetic changes and metabolic re-programming of fibroblasts observed in chronic disease might impact on their ability to respond to these regulatory changes in their phenotype induced by macrophages. In addition, quantitative changes in these two cell populations may underpin disease progression and treatment response. Understanding the nature of the functional networks established by these tissue-resident synovial cells at different stages of disease and in response to treatment will be critical in developing novel therapeutic approaches that aim to restore joint homeostasis.

3.5 | Synovial fibroblasts—passive responders or transformed aggressors

Current evidence suggests that the phenotype (based on positional identity) of synovial fibroblasts is acquired from microenvironmental instructive signals from within the synovial tissue, rather than a pre-determined phenotype. One instructive signal of fibroblast phenotype and positional identity in the tissue has been identified as endothelial-derived NOTCH3 signaling which drives a sub-lining pathogenic fibroblast phenotype, responsible for driving arthritis pathology in the joint.⁶² The removal of these fibroblasts from the joint resulted in the rapid loss of their tissue phenotype and the acquisition of a transitional fibroblast phenotype. These findings are consistent with observations that culture of synovial fibroblasts can lead to alterations in their phenotype over time.^{137,138} These observations suggest a high degree of cellular plasticity in fibroblast phenotype that is dependent on external signals that regulate specific programs of gene expression within these tissue-resident cells.

However, a large body of evidence supports the concept that disease-associated fibroblast phenotypes evolve with the chronicity of disease, resulting in a stably activated and aggressive phenotype in established disease.⁵¹ The strongest evidence that an autonomous destructive fibroblast phenotype is established in chronic disease is the finding that invasive behavior is retained by human RA synovial fibroblasts in ex vivo invasion assays and in human RA fibroblast-cartilage co-implantation assays in mice.^{26,51,139} Thus, the

invasive phenotype of fibroblasts in RA is dependent on both autonomous and local microenvironmental signals and is highly likely to be epigenetically imprinted in chronic disease.^{26,51} Epigenetic mechanisms can induce stable changes in gene expression without altering the genome, and have been extensively reviewed elsewhere.¹⁴⁰⁻¹⁴² These mechanisms are essential during embryonic development and define tissue- and cell-specific gene expression.¹⁴³ In postnatal tissues, these mechanisms operate to allow cells to adopt to microenvironmental changes.¹⁴³

However, changes in the epigenome can lead to stably, imprinted, and pathologically driven changes in gene expression.¹⁴¹ These pathogenic programs of gene expression could lead to the emergence of disease-associated effector cell phenotypes that ultimately fail to respond to negative regulation by immunosuppressive signals, resulting in the persistence of joint inflammation.⁵¹

There are three main types of epigenetic modifications reported in RA fibroblasts: methylation, histone modifications, and microRNAs (miRNAs).¹⁴⁴⁻¹⁴⁶ Such modifications are disease-specific displaying differences between RA and OA synovial fibroblasts.¹⁴⁷ This suggests epigenetic modifications can determine disease phenotype.¹⁴⁸ Hypo- and hypermethylation (unmethylated or methylated CpG site that is normally or not normally methylated, respectively) are associated with an aggressive fibroblast phenotype, inducing expression of pro-inflammatory genes which favor RA synovial fibroblast proliferation and survival as well as destructive endogenously activated pathways which activate MMPs.^{149,150} Changes in fibroblast methylome can occur in early RA and may be useful as potential prognostic biomarker of disease progression.¹⁵⁰ Histone degradation, acetylation, and methylation have also been implicated in RA fibroblast phenotype where prolonged exposure to TNF α in vitro can increase chromatin accessibility in RA fibroblasts by decreasing total histone4 (H4) and hyperacetylating the remaining H4, priming the cell for enhanced pro-inflammatory cytokine and chemokine production.¹⁵¹ Hyperacetylation of synovial fibroblast H3 also causes the chromatin region of the IL-6 promoter to loosen resulting exacerbated expression.¹⁵² The inflammatory milieu also deregulates the expression of fibroblast miRNA.¹⁵³ For example, inhibiting histone acetylation in mouse synovial fibroblasts enhances miR-124 production and resolves synovial hyperplasia and inflammation in experimental arthritis by suppressing JAK/STAT signaling.¹⁵⁴ Furthermore, a recent study demonstrated plasma levels of miR-23a-5p are significantly lower in RA blood.¹⁵⁵ Overexpression of miR-23a-5p suppressed IL-6 and IL-1b production by inhibiting TLR4/NF κ B signaling.

In addition to the above, the role of long noncoding RNAs (lncRNAs) has recently been addressed and has shown they are key players in regulating epigenetic and chromatin remodeling and transcriptional and post-transcriptional modifications. Over the last five years, there have been an increasing number of publications demonstrating the important role in which lncRNAs play in RA pathology (reviewed in Lao *et al*¹⁵⁶). In RA, lncRNAs have both suppressive and pathological effects in synovial fibroblasts, respectively, sequestering or activating NF κ B signaling and ultimately affecting fibroblast

proliferation, migration, invasiveness, and apoptosis (key characteristics of fibroblasts within chronically inflamed pannus tissue).¹⁵⁷ For example, a recent publication shows lncRNA *NEAT1* is upregulated in RA fibroblasts and by targeting and repressing miR-204-5p exacerbates IL-1 β and IL-6 production in a TNF α -dependent manner.¹⁵⁸ In contrast, plasma concentrations of lncRNA *DILC* negatively correlate with IL-6 production in RA fibroblasts and therefore disease severity.¹⁵⁹ Overexpression of *DILC* in RA fibroblasts induces apoptosis and inhibits IL-6 production.

In summary, synovial fibroblasts are plastic and local signals from within the joint microenvironment determine the induction of specific gene expression programs that determine their phenotype. During chronic inflammation, it is highly likely that pathogenic gene expression programs become imprinted as a result of persistent epigenetic modifications, in response to the inflammatory microenvironment. As a result, therapeutic interventions aimed at restoring physiological fibroblast cell states will need to either prevent the acquisition of these epigenetic modifications or reverse the process in established disease. Characterizing the epigenetic landscape of synovial fibroblasts and the disease-driven alterations in the gene expression programs that underlie specific changes in their phenotype may open new therapeutic avenues for modulating pathogenic fibroblast phenotype in disease.

3.6 | Metabolic priming of synovial fibroblasts underpinning persistent disease

Macrophages can have a protective role in inflammation by entering a transient state of non-responsiveness.^{43,160} Synovial fibroblasts, however, develop increased pathogenicity as disease progresses leading to amplified production of inflammatory mediators,^{122,161,162} proliferative and invasive behavior,⁵¹ and promotion of leukocyte trafficking into the joint.^{15,45}

Recent work has demonstrated that the expanded THY1⁺ CD34⁺ fibroblast subset is responsible for local, inflammation-driven tissue priming via a complement 3-dependent mechanism.¹⁶³ This subset of fibroblasts was found to express high levels of complement proteins and undergo metabolic rewiring after repeated inflammatory challenges. While physiologically, cellular priming functions to allow a site-specific, amplified, and robust tissue response to a repeated inflammatory episode, pathologically tissue priming may underpin the switch from resolving to persistent inflammatory arthritis.¹⁶⁴ The observation that inflammatory tissue priming is mediated by tissue-resident fibroblasts may be of relevance to why joint inflammation occurs in specific patterns in RA and tends to relapse in previously affected joints.¹¹⁶ This mechanism may also contribute to the tissue tropism observed in other immune-mediated inflammatory diseases, particularly if a common tissue-resident fibroblast population exists within specific tissues affected in these diseases.

The underpinning mechanism of fibroblast-mediated tissue priming is also of interest because it may be therapeutically targetable. Synovial fibroblasts undergo metabolic adaptation in order

to maintain viability and function in the hypoxic and inflammatory environment of the joint.^{165,166} An essential component of this adaptation is a glycolytic shift in synovial fibroblast metabolism that correlates with the acquisition of a pathogenic phenotype. Hexokinase-2, an enzyme that phosphorylates glucose to produce glucose-6-phosphate resulting in an increase rate of glycolysis in rapidly proliferating cells, has been shown to mediate synovial tissue hypertrophy underpinning the severity of joint inflammation and damage during arthritis.¹⁶⁷ Targeting these metabolic pathways could be a viable therapeutic approach in RA.¹⁶⁸

The mechanism driving the metabolic re-programming of synovial fibroblasts in response to repeated inflammatory insults have been shown to be the result of an amplified production of C3 and its receptor activation in response to inflammation.¹⁶³ This results in inflammasome activation and protection from cell senescence via mammalian target of rapamycin (mTOR) and hypoxia-inducible factor (HIF) 1 α signaling. This mechanism of inflammatory re-programming operating in a selective population of pathogenic fibroblasts underpins tissue priming and the persistence of joint inflammation. It is highly likely that similar fibroblast subsets exist in other tissues and can be primed, promoting site-specific recurrence of inflammation. Importantly, these findings further support the rationale for therapeutically targeting the complement system in RA.^{169,170}

3.7 | Migratory potential of synovial fibroblasts

Studies in mice have previously suggested that synovial fibroblasts also have migratory potential and can leave the joint and migrate to distant joints in vivo, potentially spreading disease from one joint to another.^{95,96,171} Several studies in mice have supported this hypothesis; firstly, migration of mesenchymal precursors from the blood into the joint prior to the onset of inflammatory arthritis has been observed in experimental arthritis models¹⁷² and secondly the observation that fibroblasts are indispensable for triggering arthritis in joints and can be directly arthrogenic.^{173,174} Furthermore, it has been shown that human RA synovial fibroblasts implanted with a cartilage/sponge matrix into an immunodeficient mouse can travel from the site of engraftment (cartilage/cell implant) to a distant cartilage implant.⁹⁶ Cartilage damage stimulates fibroblasts to leave the vasculature and enter the joint, before they attach and invade articular cartilage.¹⁷⁵ For example, in TNF α transgenic mice, loss of cartilage proteoglycan is a prerequisite for the destruction of cartilage by synovial fibroblasts.¹⁷⁶ Furthermore, collagenase or IL-1 injection (IL-1 results in the loss of cartilage proteoglycan) into mouse synovial joints attracts intravenously injected human RA synovial fibroblasts to the joint and promotes attachment and degradation of cartilage.^{96,175} The exact phenotype of fibroblasts that have this migratory potential is not fully known. Our own work has shown that migrating cells that invade a distal cartilage implant in vivo, expressed PDPN⁺ and not, initially sub-lining layer fibroblast markers.¹⁰⁰ However, as the implant matures and vasculature became established, the organization of fibroblasts at the site

represented the synovial tissue architecture, in which cells were organized into lining and sub-lining layer zones with the differential expression of cell surface markers. Although these findings have not been confirmed in the context of newly characterized synovial fibroblast subsets,^{3,47} the data are consistent with the observations of fibroblast phenotype plasticity in response to microenvironmental cues.⁶²

Despite the importance of these findings, fibroblast migration in human disease has only recently been reported. The discovery of pre-inflammatory mesenchymal cells (PRIME cells) that are detectable in the blood of patients prior to an arthritis flare has re-opened the debate on “migrating” synovial fibroblasts. Orange *et al*¹⁷⁷ identified PRIME cells as potential cellular biomarkers that predict disease flare in patients with RA and demonstrated that these cells have a mesenchymal cell phenotype and are recruited by naive B cells leading up to the flare and then decrease upon symptom onset (see Figure 2). The authors suggest that a comparison with single-cell fibroblast data generated by Zhang *et al*⁴⁷ revealed the gene expression signature of PRIME cells to be most closely related to sub-lining layer fibroblasts in the joint. Deep phenotyping and functional analysis of these cells has yet to be performed, and as a result, their direct pathogenic role in disease is yet to be determined. Importantly, it is assumed by the authors that these cells have the ability to enter the joint and directly contribute to inflammatory joint pathology, triggering an arthritis flare. However, the exact mechanism by which these cells contribute to arthritis flare currently remains unproven and is speculative.¹⁷⁸

3.8 | Joint-specific features of synovial fibroblasts

It has long been recognized that pathognomonic disease patterns are associated with different types of inflammatory arthritis. The cellular and molecular mechanisms underlying this tissue tropism are poorly understood. There is now emerging evidence of significant differences in the transcriptional and epigenetic profiles of synovial fibroblasts isolated from different joint locations.³⁷ This anatomically driven transcriptional diversity follows a pattern imprinted in embryonic development and translates to joint-specific phenotypes of synovial fibroblasts.¹⁷⁹

The spatial and temporal expression of key developmental genes, such as the homeobox (*HOX*) family of transcription factors, tightly controls the correct establishment of the body axis during embryonic development.¹⁸⁰⁻¹⁸² Studies analyzing synovial fibroblasts isolated from specific human joints have detected differences in transcriptional expression of *HOX* genes and WNT signaling pathways¹⁷⁹ (involved in both cellular differentiation and development¹⁸³). Hierarchical cluster analysis of transcriptional data from synovial fibroblasts isolated from the knees, hands, and shoulders found that the cells segregate according to anatomical joint location and that *HOX* gene signature detected in these fibroblasts shared similar features to the embryonic positional *HOX* gene expression pattern during limb development. Investigators have further

identified that DNA methylation and histone marks tightly regulate joint-specific *HOX* gene expression in synovial fibroblasts.^{184,185} It is not yet known whether the epigenetic imprinting of synovial fibroblasts occurs during embryonic development at sites distant to the joint or during postnatal development when supporting the unique biomechanical features of a specific joint.

This epigenetically driven anatomical diversity of synovial fibroblasts also translates into joint-specific fibroblast phenotypes and functional specialization that is dependent on joint location.³⁷ For example, fibroblasts isolated from the hand joints have differential responses to cytokine stimulation and produce higher levels of matrix-degrading enzymes. They are more proliferative and have distinct chemotactic properties when compared to synovial fibroblasts from other joints.¹⁷⁹ As a result, these differences in cellular phenotype may underlie the specific patterns of joint involvement observed in different types of arthritis by creating unique tissue microenvironments that confer a susceptibility of specific joints to certain types of arthritis.^{3,38} The existence of developmentally determined, positionally imprinted risk signatures of synovial fibroblasts may provide important mechanistic insights into the tissue tropism observed in immune-mediated inflammatory diseases and the disease pathways underlying the persistence of inflammation at distinct anatomical sites.³⁷

Implications for therapy have also been described due to differences in synovial fibroblasts isolated from different affected joints (eg, hip versus knee joint) in patients with RA.^{179,185,186} Differences in expression of cytokine signaling pathways and differential sensitivity to cytokine neutralization or signaling blockade may contribute to variable treatment responses at different joint locations. It will now be important to integrate joint-specific differences in fibroblast function with the recent description of specific fibroblast subset heterogeneity defined by single-cell profiling technologies. This may allow for the identification of shared and unique fibroblast subsets that confer specific functions and pathogenic roles across different joints within the same disease.

4 | THERAPEUTIC TARGETING OF FIBROBLASTS

There are several potential therapeutic approaches to targeting fibroblast-mediated pathology in inflammatory joint disease, including both direct and indirect approaches.^{16,26} Indirect approaches include the neutralization of fibroblast-specific activating factors such as cytokines or growth factors (including those that drive autotrophic, primary cell amplification loops that sustain specific fibroblast phenotypes or activation status) and the blockade of fibroblast derived factors, such as chemokines and cytokines that mediate inflammatory joint pathology.^{26,12216} However, such indirect approaches are already a component of some currently available treatments such as anti-cytokine therapies or signal transduction inhibitors.¹⁸⁷ These therapies have not been sufficient to break the current ceiling of therapeutic response in RA, and as a result, new therapeutic

approaches are needed.¹⁸⁸ There are several potential approaches to directly target fibroblasts described below.

4.1 | Direct targeting of pathogenic subsets of fibroblasts

Linking the transcriptionally defined cellular phenotype of fibroblasts more clearly to cellular function and pathogenic role in disease makes targeting distinct subsets of fibroblasts increasingly possible.²⁶ Targeting pathological immune cells by selective depletion has proved highly efficacious in RA and other immune-mediated inflammatory diseases, such as the use of rituximab (targeting CD20 on B-cells),¹⁸⁹ for example. A prerequisite for such a therapeutic approach to be effective in targeting fibroblasts is the ability to selectively deplete a population of pathogenic fibroblasts accurately defined by a cell surface marker, expressed only by the pathogenic population of cells to be targeted. In the case of fibroblasts, we have used a diphtheria toxin system to selectively deplete a pathogenic population FAP α -expressing fibroblasts leading to attenuated synovial inflammation and joint damage in experimental arthritis in mice.⁴⁷ These findings provide a therapeutic rationale for the selective targeting of FAP α -expressing fibroblasts in human inflammatory joint disease. However, for such an approach to be effective in humans, we must be able to identify fibroblast- and tissue-specific markers to localize these cytotoxic therapies to the joint and reduce the risk of "off" target effects in other tissues. Targeting FAP α -expressing fibroblasts with chimeric antigen receptor (CAR)-specific T cells has proven to be efficacious in reducing cardiac fibrosis, as well as restoring function and improving recovery following cardiac injury in mice.¹⁹⁰ Furthermore, a phase I clinical trial has proven that local delivery of FAP α CAR T cells is safe in patients with pleural mesothelioma.¹⁹¹ These findings are promising, prompting development of such therapeutic approaches in RA and other autoimmune diseases.¹⁹²

Strategies for targeting fibroblasts based on specific cell surface receptors such as CDH11 have previously been evaluated. However, a phase II clinical trial was discontinued due to inefficacy.¹³⁴ Many of the markers expressed by immune effector and synovial fibroblast subsets, such as THY1, CD34, and PDPN, are also expressed by other cellular lineages.^{107,193,194} Comprehensive analysis of the cell surface expression profile of pathogenic fibroblast subsets mapped to their transcriptional phenotype, coupled with the development of bivalent or trivalent antibody targeted cell depletion strategies and novel cellular drug delivery systems (eg, liposomes), may allow for more accurate fibroblast targeting in the future.

4.2 | Targeting the differentiation of synovial fibroblasts

Another potential therapeutic avenue involves targeting the underlying molecular mechanisms driving the differentiation of

fibroblasts toward specific effector cell phenotypes. This has been demonstrated by the inhibition of NOTCH3 signaling in experimental arthritis in mice. Wei *et al*⁶² reported significant attenuation of joint inflammation and damage in mice, following the genetic deletion of the *Notch3* gene or therapeutic blockade of NOTCH3 signaling. The cellular mechanism underlying this effect was the inhibition of fibroblast differentiation toward a pathogenic sub-lining phenotype, preventing the expansion of perivascular fibroblasts. NOTCH3 signaling blockade also significantly diminished the fraction of SPARC⁺ COL3A1⁺ but not CXCL10⁺ CXCL19⁺ fibroblasts.⁴⁰ As SPARC⁺ COL3A1⁺ fibroblasts potentially remodel the synovial vasculature to promote cellular infiltration and CXCL10⁺ CXCL19⁺ directly communicate with T cells to maintain activation and recruitment,⁴⁰ NOTCH3 inhibition could be more effective in early disease.

4.3 | Modulating the pathogenic behavior of fibroblasts

Cellular metabolism is a major regulator of fibroblast behavior.¹⁶⁵ Targeting dysregulated metabolism in synovial fibroblasts using metabolism modifiers may be another therapeutic option.¹⁶⁸ Examples include targeting hexokinase-2—the glycolytic enzyme that is a critical driver of synovial hyperplasia in response to joint inflammation.¹⁶⁷ This enzyme is modulated by currently available treatments such as tofacitinib that reduces the expression of this enzyme¹⁹⁵ in synovial tissue. More targeted interventions could include the therapeutic blockade of the complement system preventing fibroblast-mediated synovial tissue priming.¹⁶³ While complement targeted therapies in RA have not yet been shown to be effective,^{169,170} possible therapeutic interventions could include the direct targeting of C3¹⁹⁶ or reversal of metabolic re-programming in CD34⁺ fibroblasts in the joint using inhibitors of glycolysis^{163,197} or small molecular inhibitors of the inflammasome.¹⁹⁸

Therapeutic modulation of the epigenome has the potential to re-establish a normal cell state in previously imprinted fibroblast phenotypes.¹⁹⁹ For example, a recent study on fibroblasts isolated from patients with RA demonstrated that 280 TNF α -inducible genes escape repression owing to persistent H3K27 acetylation and increased chromatin accessibility of their regulatory elements.²⁰⁰ Analysis revealed that these regulatory elements were enriched for binding motifs for NF κ B, interferon regulatory factors, and AP-1 transcription factors. As a result, targeting histone acetylation reader proteins such as BET proteins could modulate the persistently activated fibroblast phenotype. For example, BET inhibitor I-BET151 has been shown to suppress the production of cytokines and MMPs by synovial fibroblasts in vitro.²⁰¹ A comprehensive analysis of the gene regulatory pathways that modify fibroblast subset phenotype may reveal other specific therapeutic targets.

5 | CONCLUDING REMARKS

Synovial fibroblasts are specially adapted for their role in joint homeostasis, and this functional specialization is retained within distinct anatomical compartments of the synovial membrane. These specialized synovial tissue niches undergo cellular remodeling in response to inflammation and contain specific subsets of fibroblasts.²⁷ The spatial and temporal heterogeneity of synovial fibroblasts underpins their pathogenic role in inflammatory joint disease.⁴⁷ Fibroblast heterogeneity is driven by anatomical location in the tissue,¹⁰⁰ microenvironmental instructive signals,⁶² and interaction with tissue-resident leukocytes.⁸⁷ These regulatory signals and cellular interactions result in both subset differentiation and changes in cell activation status. This phenotypic heterogeneity gives rise to functional cellular diversity reflected in the existence of synovial fibroblast subsets with different effector cell functions.³⁸

The link between phenotypic heterogeneity and cellular function needs to be further explored in order to provide a clear link between transcriptional diversity and functional role in health and disease. Comprehensive analysis of synovial fibroblast subsets at different stages of disease in response to therapeutic intervention will be vital in exploring the role of specific fibroblast subsets in determining the response to treatment and disease progression and in dictating the most effective timing for the use of fibroblast targeted therapies. As yet, the impact of chronic disease on fibroblast heterogeneity is unknown. The identification of key positional cues within the synovial tissue that regulate fibroblast identity/phenotype suggests a large degree of residual plasticity in phenotype. However, this plasticity may be modulated in chronic disease, as suggested by numerous previous studies that demonstrate the development of an epigenetically imprinted fibroblast phenotypes with chronicity of joint inflammation.⁵¹ This raises the possibility that the success of fibroblast targeted therapies is dependent on the stage of disease. For example, early changes in fibroblast phenotype may underpin a treatment-refractory disease trajectory and therefore targeting these cells in early disease may be more effective at altering the course of arthritis.

Future research strategies should focus on linking single-cell transcriptomic analysis of cellular heterogeneity obtained from different pathogenic tissues to fibroblast function and build a consensus on the definition of fibroblast identity and heterogeneity, based on function rather than gene expression profiles. As we now define the cellular landscape of the joint, at different stages of disease and in different sub-groups of patients, it is vital to link these findings to function and this requires comprehensive in vitro and in vivo functional studies linked to the cell atlas of the tissue. Furthermore, the exploration of shared and distinct fibroblast phenotypes across different tissues and inflammatory diseases will be vital for developing cross-indication therapeutic targeting of specific pathogenic fibroblasts in the future, perhaps as an adjuvant to existing biologic therapies such as anti-TNF α treatment, an approach that will hopefully break the ceiling of therapeutic response in RA.

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CONFLICT OF INTEREST

The author(s) declare no conflicts of interest.

AUTHOR CONTRIBUTION

All authors contributed equally to the manuscript.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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