Chapter

Fibroblast-Like Synovial Cell Subsets in Rheumatoid Arthritis

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Abstract

Fibroblasts like synoviocytes (FLS) play several significant roles in rheumatoid arthritis (RA) pathophysiology. This chapter will describe known roles of FLS in disease initiation, joint inflammation, disease persistence and joint destruction. It will describe the newly characterized subsets of FLS based on single cell RNA sequencing studies, and their association to specific aspects of the disease. Finally, we will discuss the future of targeting FLS in the treatment of RA. The FLS in the synovial lining layer are identified by surface complement decay-accelerating factor (CD55) along with lubricin and metallopeptidase expression. Pathological activation of this lining layer subset result in bone and cartilage damage in mice. FLS of the sublining layer are often characterized by THY1 expression, but recent studies have highlighted a heterogeneity where several distinct subsets are identified by additional markers. Sublining FLS expressing human leukocyte antigen-DRA (HLA-DRA) produce C-X-C motif chemokine 12 (CXCL12) and receptor activator of nuclear factor-κB ligand (RANKL) and seems to constitute a pro-inflammatory subset that is associated with inflammation and tertiary lymphoid structures. Another subset of FLS characterized by CD34 expression may discriminate a common progenitor fibroblast subset. Taken together, studies isolating and characterizing gene expression in synovial FLS report both associations of unknown importance and markers that may impose protective or destructive features. This supports evidence of FLS as active players in RA pathology capable of cellular recruitment, local cellular crosstalk and promotion of joint destruction. These discoveries may serve as an atlas for synovial activation in RA and have identified several potential fibroblast markers for the development of targeted treatment.

Keywords: Fibroblast like synoviocytes, Rheumatoid arthritis, Inflammation, Autoimmunity, Tertiary lymphoid structures, Fibroblast activation protein, Fibroblast targeted treatment

1. Introduction

In normal resting conditions the synovial membrane is a thin layer of well-ordered cells historically called type A and B synoviocytes. These cells form a barrier

between the articular cavity and a sublining layer, the latter being heterogeneous and composed of several cell linages. Fibroblasts, immune cells and mature vasculature (capillaries, arterioles and venules) made up of pericytes and endothelia are some of the various cell types constituting this layer [1–3].

2. Rheumatoid arthritis

Rheumatoid arthritis (RA) has a multifactorial etiology and is one among the most common systemic autoimmune diseases [4, 5]. The factors that mediate the initiation of RA is yet to be unraveled. However, the pathology of RA involves abnormalities in both the innate and the adaptive immune system, and both of these systems are implicated with the progression and persistence of the disease [6, 7]. The synovial membrane is the primary site of pathology during the synovitis stage of the disease and characterized by proliferation of tissue resident, synovial cells and the infiltration of inflammatory cells from the blood. RA is a chronic, progressive disease leading to degradation of articular cartilage and bone along with several systemic manifestations [8].

In RA, the inflamed synovial membrane undergoes hyperplasia and transforms into less structured lining layer and sublining tissues both rich in fibroblasts like synoviocytes (FLS) [9, 10]. This inflamed synovial membrane eventually begins to invade the cartilage surfaces and the underlying bone, commonly referred to as pannus [11, 12].

Present day treatment strategies for RA primarily focuses on suppression of cytokine signaling and T- and B-cell activity. These therapies have highlighted the importance of immune response in driving the progression of RA. However, they also clearly demonstrate that in a large proportion of patients these treatments are incapable of inducing disease remission [8, 13]. Synovial phenotyping of RA patients based on histology has highlighted a fibroblast dominated synovial pathotype [14]. This pathotype is believed to include a large proportion of the non-responders to conventional and biologic disease modifying anti-rheumatic drugs [15–17]. This is supported *in vitro* where anti-tumor necrosis factor alpha (TNFα) treatments were ineffective in cultures dominated by FLS [18]. Furthermore, a recently published, biopsy driven clinical trial in RA patients with inadequate response to anti-TNFα treatment, showed significantly higher response rates when patients with B-cell poor synovium were treated with IL-6 receptor inhibitor tocilizumab compared to the B-cell depleting agent rituximab [19].

In the following sections, we will first describe RA FLS in general before the era of single cell RNA sequencing (scRNA-seq). We will summarize the known and proposed roles of FLS in RA initiation, joint inflammation, disease persistence and joint destruction. Finally, we will describe the newly characterized subsets of FLS based on scRNA-seq studies their connection to specific aspects of clinical disease, future outlooks in the context of RA diagnosis, RA tissue phenotyping and therapy targeting FLS.

3. Fibroblast like synovial cells in rheumatoid arthritis

3.1 Disease initiation

The central role of FLS in RA pathology is highlighted in murine studies demonstrating that activation of FLS is sufficient to initiate local joint inflammation leading to persistent arthritis [20, 21].

Furthermore, FLS greatly contribute to the transformation of the thin synovial membrane into a multi-layered invasive hyperplastic pannus [22]. This expansion of FLS in the inflamed synovium is likely a result of at least one of the following processes. First, pathological subsets of FLS seem to proliferate to some extent and develop a local resistance to apoptosis [23–25]. Secondly, pluripotent mesenchymal stem cells may migrate into the synovium from the circulation, where they differentiate into mature pathological subsets of FLS [26]. Lastly, a local mesenchymal progenitor cell population may undergo activation and differentiation into distinct phenotypes of FLS [27]. Collectively, this leads to a local increase in pathological FLS in the RA synovium.

3.2 Joint inflammation

Pathogenic FLS constitute the majority of cells found in the inflamed synovial tissues, and play an important role in the inflammatory cascade, linking innate and adaptive immunity [6, 10]. FLS are capable of significantly affecting the local inflamed environment through production of cytokines and chemokines leading to recruitment and activation of immune cells [9, 28]. Specifically, pathogenic FLS are able to provide an adequate survival signal for synovial T-cells [29], a signal that is superior to the one produced by non-inflammatory fibroblasts [30]. This interaction between FLS and lymphocytes can inhibit the resolution of local inflammation [30, 31] through both paracrine and direct cell–cell interactions [32]. This pathogenic role of the FLS is facilitated by the up-regulation of several adhesion molecules such as intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) [6, 33]. In addition to recruitment and co-activation of T-cells in the inflamed joint, FLS have been shown to be able to present antigens on class II major histocompatibility complex (MHC-II) to CD4+ T-cells [34].

Furthermore, FLS are involved in the formation of tertiary lymphoid structures (TLS) in the RA synovium. Stromal cell populations such as the fibroblastic reticular cell support organization of these lymphocyte aggregates similarly to that of secondary lymphoid organs with distinct T- and B-cell niches [35]. Thymocyte differentiation antigen 1 (THY1, also known as CD90) and podoplanin (PDPN) positive fibroblast associated with TLS in RA (**Table 1**) produce several chemokines such as C-X-C motif ligand (CXCL) 13 and C-C motif ligand (CCL)21 implicated with lymphocyte recruitment and organization [47, 48]. Another marker associated with the TLS associated fibroblast is the receptor activator of nuclear factor kappa-β ligand (RANKL), which is important in both bone homeostasis and lymph node development [35, 49].

Collectively, FLS may be involved in both the pro-inflammatory initiation in the synovium, lymphocyte recruitment and the organization of TLS. A fibroblast driven RA phenotype resulting in persistent inflammation and a lymphoid rich synovium similar to what have been shown by histology.

3.3 Disease persistence

The highly proliferating and pathogenic RA FLS are very different from their quiescent state during non-inflamed conditions where FLS control the structural integrity of the joint lining and sublining layer [22]. The immunological events initiating a pathogenic state of RA FLS is still not fully understood, but proliferation and transformation of the FLS may occur prior to immune infiltration [50].

Classical synovial subsets in RA	**Markers**		
Fibroblast like synoviocytes [36–38]	Vimentin, THY1, prolyl-5-hydroxylase, CDH11, CD45, HLA-DR, α-SMA, CD55		
Macrophage like synoviocytes [36, 39–41]	CD14, CD68, RFD7, CD163, CD206, HLA-DR, CD97		
Tertiary lymphoid structure associated fibroblast [35]	PDPN, THY1, FAP, CXCL13, CCL21, RANKL, CD21		
Fibrocyte [42]	CD34, CD45, CD14, CD11, MHC-II		
Single cell analysis of synovial fibroblast subsets in RA			
Published studies and subsets	**Cluster markers**	**Associated transcription profile**	
Stephenson et al. 2018 [43]	Fibroblast sorting strategy: CD45 - Propidium iodide - PDPN+		
Sublining fibroblast	THY1+		
Lining fibroblast	CD55+	HAS1	
Mizoguchi et al. 2018 [44]	Fibroblast sorting strategy: CD45- CD31- CD235a- CD146- PDPN+		
Perivascular fibroblast	THY1+ CD34-	$RANKL^{high}$, OPG^{low}	Migration factors: CTHRC1, TWIST1, POSTN, LOXL2, PDGFBB, MMP14
Sublining fibroblast	CD34+	IL6, CXCL12, CCL2, OPG	
Lining fibroblast	THY1- CD34-	CD55, PRG4, HAS1, MMP1, MMP3	
Zhang et al. 2019 [10]	Fibroblast sorting strategy: CD45- CD31- PDPN+		
SC-F1 (sublining)	THY1+ CD34+	C3, FOS	
SC-F2 (sublining)	THY1+ $HLA\text{-}DRA^{high}$	IL6, CXCL12	
SC-F3 (sublining)	THY1+ DKK3+	CADM1, COL8A2	
SC-F4 (lining)	THY1- CD55+	PRG4, HBEGF, CLIC5	
Croft et al. 2019 [45]	Reanalysis of human data from Zhang et al. [10].		
F1 (sublining)	THY1+	DKK3, OGN, CD9,	
F2 (sublining)	THY1+	MDK, COL8A1, AEBP1	
F3 (sublining)	THY1+	IRF1, EGR1, JUNB	
F4 (lining)	THY1-	CLIC5, CD55, HBEGF	
F5 (sublining)	CD34+	C3, APOD	
Single cell analysis of circulating mesenchymal cells in RA:			
Published study and subset	**Associated transcription profile**		
Orange et al. 2020 [46]	Fibroblast sorting strategy: CD45- CD31- PDPN+		
AC3 (sublining fibroblast phenotype)	CD34, HLA-DR, DKK3, FAPα, CDH11		

The table contains a list of surface and transcriptional profiles of fibroblast subsets, fibroblast like cell subsets and macrophage subsets (pre-scRNA-seq) related to rheumatoid arthritis. For scRNA-seq studies, fibroblast subset names refer to the original articles. "+" and "- "shows whether the cells of interest are positive or negative for the cellular markers. The cellular markers which are discussed in the text are also listed under abbreviations.

Table 1.
Surface and transcriptional profiles of FLS subsets (and related cellular subsets) in rheumatoid arthritis.

In RA, subsets of FLS can differentiate to become inflammatory, migratory, and invasive, thus collectively fostering disease aggravation in various animal models of RA [45, 51, 52]. Constitutive activation is a hallmark of RA FLS and leads to production of several inflammatory cytokines, such as interleukin (IL)-1β, TNFα and IL-6

and chemokines such as monocyte chemoattractant protein 1 (MCP-1/CLL2) [9] and CXCL12 [53]. Even though the activation of RA FLS is greatly affected by pro-inflammatory factors in the local environment, epigenetic changes are also important [54]. Epigenetic changes lead to constitutive activation even when the cells are removed from the inflamed environment and remain without addition of proinflammatory stimuli [52]. Moreover, a recent study suggests a link between epigenetic-driven positional identity of FLS (e.g. small versus large joints and proximal versus distal joints) and clinical disease patterns [55]. This link is further supported by the finding of oncogenes at sites of tissue destruction [56, 57] together with a highly activated nuclear factor κ beta pathway in RA FLS [58].

Altered metabolic activity with increased glycolysis is another hallmark of RA FLS [59]. Metabolic reprogramming of FLS were recently connected to complement C3 and C3a receptor-activation. Here repeated inflammatory challenges resulted in a distinct pro-inflammatory phenotypic priming of FLS in mice models of arthritis [60].

On the opposite side, several factors attempt to facilitate remission of proinflammatory FLS. One such potential immune regulator is the MerTK expressing synovial macrophage which *in vitro* reduce matrix metalloproteinase (MMP) production by lining layer FLS [61].

Thus, even though FLS are responsive to their inflammatory context they may possess a distinct positional identity which enables a cytokine-independent intrinsic activation contributing to disease persistence in RA.

3.4 Joint destruction

The severe joint destruction of late-stage RA is in part attributed to the pannus tissue which is rich in FLS. RA FLS are identified as invaders of the joint cartilage *in vivo* [62, 63], an invasive behavior that has been confirmed *in vitro* [64] and in mice [52]. FLS mediate cartilage degradation which is attributed to a combination of facilitating adhesion factors and production of proteases, here among several well-known matrix metalloproteinases (MMPs) [9, 52, 64]. Cartilage degradation is ameliorated when fibroblast activation protein (FAP) deficiency is induced in the human TNFα transgenic mice model of arthritis [65]. The invasiveness of pathological RA FLS is further emphasized by human FLS migrating to other joints in mouse models of RA and degrading the implanted human cartilage [51]. Migration that may be facilitated by specific anticitrulinated protein antibodies [66]. Notably, the *ex vivo* invasiveness of FLS correlates with joint erosions [67].

Increased osteoclastic activity leading to bone erosions in RA is another major factor in joint destruction. Here FLS produce CXCL12, RANKL, dickkopf related protein (DKK) 1, etc. which may increase both osteoclast migration, differentiation, proliferation/activation and inhibit osteoblast function [53, 68, 69].

4. Single cell analysis of synovial fibroblast subsets in rheumatoid arthritis

4.1 Phenotyping of fibroblast like synovial cells

Increasing spatial and molecular resolution in present day cellular analysis are changing our view of the synovial membrane in RA. Most notable is the identification of different fibroblast subsets within the inflamed synovial membrane. Recent work

and ongoing studies are utilizing scRNA-seq, CyTOF and flow cytometry cell sorting to further investigate and distinguish these subsets and their role in disease pathology.

Recent scRNA-seq studies have identified several distinct disease-associated subsets in the inflamed synovial membrane, often grouped as lining layer or sublining layer FLS [10, 43, 44], **Figure 1**. The present studies utilize flow cytometry assisted cell sorting and transcriptomic clustering strategies based on exclusion of hematopoietic lineage cells (CD45), endothelial cells (CD31), red blood cells (CD235a), and pericytes (CD146) while using PDPN or collagen production as a positive marker (**Table 1**).

4.2 Lining layer fibroblasts

A common finding in scRNA-seq studies confirms the presence of complement decay-accelerating factor (CD55) and absence of THY1 expression in FLS of the lining layer (**Table 1**). Of note, Mizoguchi et al. [44] did not report histological data of CD55 distribution, but a high level of CD55 gene expression in CD34- THY1- lining layer fibroblasts. All scRNA-seq studies (**Table 1**) of joint tissue reported lubricin (PRG4) expression in the lining layer subset [10, 43–45]. All present studies showed similar patterns of gene expression pertaining to the potential markers of FLS presented in the following section.

Zhang et al. [10] and the reanalysis of the same human data by Croft et al. [45] both reported a distinct lining fibroblast subset, SC-F4 and F4 respectively. This lining fibroblast subset was associated with expression of chloride intracellular ion channel 5 (CLIC5) and heparin binding epidermal growth factor-like growth factor (HBEGF). Mizoguchi et al. [44] and Stephenson et al. [43] also reported increased hyaluronan synthase 1 (HAS1) and metallopeptidase expression.

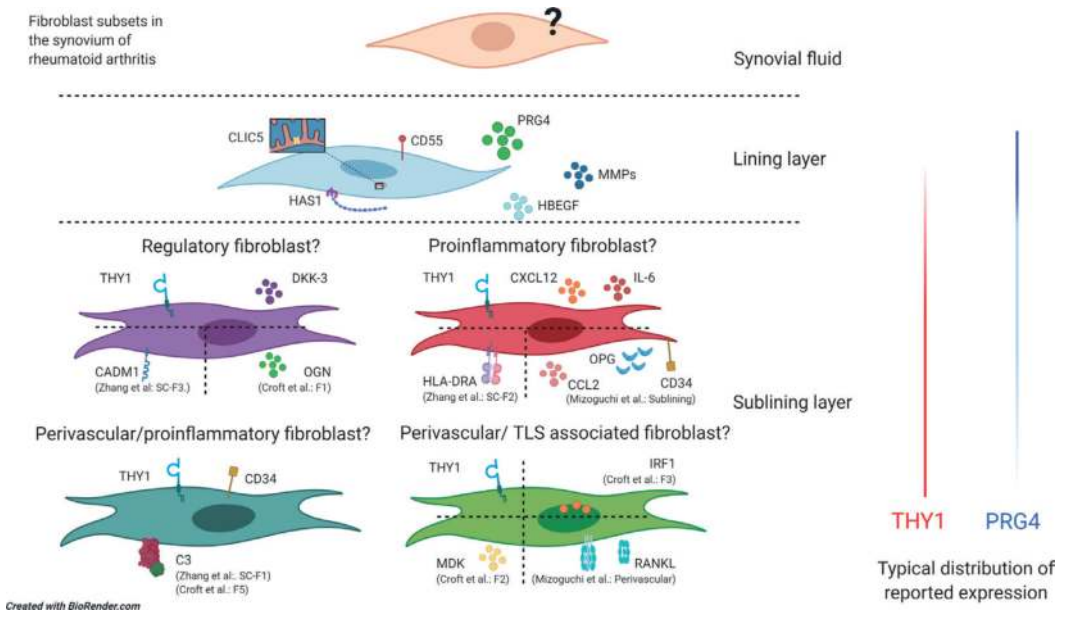

Figure 1.
The figure is a schematic presentation of fibroblast subsets identified by scRNA-seq studies of synovial tissue from patients with rheumatoid arthritis. The subsets have been divided into lining layer FLS and sublining layer FLS. No scRNA-seq studies yet have examined fibroblast subsets from the synovial fluid. Based on grouping markers and transcription profiles listed in ***Table 1****, we propose 4 sublining phenotypes. Cells have been divided by dashed lines when the cellular markers were not listed in all the original studies. THY-1 and PRG4 expression gradients from the lining layer to the sublining layer is shown by the color density of the red and blue bars. The cellular markers are discussed in the text and listed under abbreviations. TLS: Tertiary lymphoid structures.*

HAS1 is important for hyaluronan production and is a response to pro-inflammatory stimuli in RA synoviocytes. This activation results in hyaluronan cell coating, leukocyte/monocyte recruitment and facilitation of fibroblast-monocytes binding [70].

CD55, a C3 convertase inhibitor, has received increasing interest in cancer, where CD55/CD97 binding is associated with several oncogenic properties such as invasion and migration [71]. In RA, CD55 positive FLS are exclusive to the lining layer and in proximity to CD97 positive macrophages, suggesting a possible mechanism of crosstalk [36]. CD55 is not exclusive to RA [72], but it has been suggested as a protective factor in a mice model of immune complex mediated arthritis [73].

In the context of joint tissue, the mucin-like glycoprotein, PRG4, has been proposed as having a dual role comprising of well-known lubricating property and as a moderator of inflammation via NF-κβ pathways through interaction with both CD44 and toll-like receptors [74].

CLIC5 is present in several intracellular organelles, but predominantly located at the mitochondrial inner membrane, where it has been associated with modulation of reactive oxygen species [75]. However, no functional studies have been published regarding CLIC5 in RA.

The epidermal growth factor family member, HBEGF, is present and involved in several physiological processes such as wound healing, tumor formation and angiogenesis. One common topic is its association with cell migration, as seen in keratinocyte/fibroblast models and in enterocytes in necrotizing enterocolitis [76]. In RA, HBEGF positive macrophages have recently been shown to increase synovial fibroblast invasiveness in an *in vitro* model [77].

Several matrix metalloproteinases, MMP1, MMP3 and MMP14 was connected to a specific subset of FLS by Mizoguchi et al. [44]. These destructive enzymes have previously been connected to cartilage degradation in RA, but MMP14 was also noted by Mizoguchi et al. as a migratory factor [44].

Taken together, studies isolating and characterizing gene expression in lining layer fibroblasts report both associations of unknown importance and markers that may impose protective and destructive features. This suggests that the lining layer fibroblast subset is an active subset in RA pathology capable of cellular recruitment and significant local cellular crosstalk.

4.3 Sublining layer fibroblasts

The scRNA-seq studies have reported several distinct sublining subsets presented in **Table 1**. The initial study by Stephenson et al. [43] identified THY1 as a marker of sublining fibroblasts and the subsequent scRNA-seq studies confirmed THY1 as a specific, albeit not universal marker of sublining fibroblasts [10, 44, 45].

Zhang et al. characterized this heterogeneity of the sublining layer fibroblasts and defined three THY1+ groups with additional subset markers; CD34 defined the SC-F1 cluster, human leukocyte antigen (HLA)-DRA^{high} defined the SC-F2 cluster and DKK-3 defined the SC-F3 cluster. The SC-F2 in particular was significantly increased in leukocyte-rich RA ssynovium compared to leukocyte-poor RA synovium and osteoarthritis (OA) synovium [10], suggesting these to encompass TLS-associated fibroblast subsets. Reanalysis of these human data by Croft et al. [45] enabled the distinction of four sublining layer fibroblast groups (F1–3,-5, **Table 1**).

As with the lining layer, large sets of multiomics data are available. Several markers connected to joint inflammation and destruction have been identified in these subsets. However, the markers most consistently reported are THY1, HLA-DRA, CD34, DKK3.

THY1 is a glycoprotein present on the membrane of several different cells including endothelial and mesenchymal cells [78]. Among the functions associated with THY1 expression is cellular contact, CD97 binding, integrin binding, trans-endothelial migration and MMP-9 and CXCL8 secretion after binding to neutrophiles [78].

As with THY1, CD34 is an established marker in different cell types including several stromal cells, epi/endothelial cells and fibrocytes [79]. Its function is largely unknown but has been linked to proliferation, adhesion, differentiation and is proposed as a marker of progenitor subsets in both mesenchymal, epithelial and endothelial cells [79].

MHC molecule (both class I and II) functions are typically attributed to antigen presentation. Several MHC molecules have been associated with autoimmune disease. Examples are the association of the MHC-I molecule HLA-B27 with ankylosing spondylitis, reactive arthritis and juvenile idiopathic arthritis subsets [80], and the association of MHC-II molecules HLA-DR1 and DR4 association with RA [81]. The specific function of HLA-DRA in RA FLS is yet to be investigated.

The DKK family of glycoproteins are well known modulators of WNT pathways connected to embryogenesis, bone formation and eye and skin development [82]. DKK-1 has been extensively described in fibroblasts from RA patients and is a key player in joint remodeling [69]. DKK-3 has been reported as a chondroprotective factor in OA [83] and suggested as a B-cell modulator whose absence aggravates autoimmune symptoms in a murine systemic lupus erythematosus model [84] and a CD8 T-cell modulator involved in antigen tolerance [85].

Enrichment of several genes related to pro-inflammatory cytokines and proteins related to bone metabolism in RA have been reported in sublining fibroblasts including IL-6, MCP-1/CCL2, CXCL12 and RANKL. Two proteins not mentioned above is the RANKL decoy receptor osteoprotegerin (OPG) which inhibit osteoclastogenesis in synovial macrophages [86] and the relatively new osteoglycin (OGN) that may both be part of the vascular system and may affect osteoblast differentiation [87].

The interferon regulatory factor 1 (IRF1) is a significant component of the interferon signature/inflammation pathway, through which TNF induces production of CXCL9–11 and in its absence diminishes B-cell activating factor expression [88].

The heparin-binding growth factor midkine (MDK) is less investigated than the above-mentioned cytokines but has been identified in human synoviocytes and associated with leukocyte migration to the synovium and osteoclastogenesis in mice [89].

C3, a unifying step for all three complement activating pathways has previously been located around microvasculature in the sublining of the RA synovium [90].

The cellular adhesion molecule 1 (CADM1) is a transmembrane member of the immunoglobulin superfamily with no known relation to RA. It has been identified as a tumor suppressor gene in solid cancers such as squamous cell carcinoma a, but may contribute to infiltration in adult T-cell leukemia/lymphoma [91].

To summarize, the sublining layer is a heterogeneous compartment of the inflamed RA synovium, regarding both cell linages and especially fibroblast subsets (**Table 1** and **Figure 1**). Several distinct fibroblast subsets have been identified, but recuring markers such as HLA-DRA, CD34 and DKK-3 are relatively unknown in the RA context. Results from scRNA-seq studies propose that the sublining layer fibroblast subsets are significantly involved in cellular crosstalk, leukocyte recruitment, para- and autocrine pro-inflammatory stimulation, and joint tissue destruction. Notably, some distinguishing factors such as DKK-3 may be enriched to form a regulatory anti-inflammatory and pro self-tolerance subset with similar chondroprotective effects and immune modulation of antigen tolerance mentioned in the previous

section. An HLA-DRA^{high}/CXCL12/RANKL^{high} associated subset may constitute the pro-inflammatory TLS associated fibroblast subsets and CD34 may discriminate a common progenitor fibroblast subset. Together, the presence of both pro-inflammatory subsets and potential anti-inflammatory and progenitor subsets suggests an ongoing cellular balancing throughout the sublining layer, which may open avenues for new research in treatment strategies targeting FLS.

4.4 Fibroblast like synoviocytes in rheumatoid arthritis compared to other arthritides

In RA, synovial division into lining/sublining layers suggests differentiated roles of subsets of FLS regarding cytokine production, joint destruction, and possible regulatory mechanisms.

The expansion of these distinct subsets is different in RA compared with OA. Mizoguchi et al. reported a greater fraction of the THY1$^+$ CD34$^-$ (perivascular) subset but less of the THY1$^-$ CD34$^-$ (lining) subset in RA compared with OA [44]. Notably, here the proportion of THY1$^+$ CD34$^-$ (perivascular) FLS correlated with leukocyte infiltration and ultrasonic and histological synovitis [44].

Similarly, Zhang et al. reported an overabundance of the THY1$^+$ CD34$^-$ HLA-DRA^{high} (SC-F2) subset with upregulated expression of CXCL12 and IL-6 and a THY1$^+$ CD34$^+$ (SC-F1) subset in RA. In contrast, lining FLS (SC-F4) were more fabundant in OA [10].

The causal link between distinct subsets and RA pathogenesis was investigated in mice by Croft et al. Here the mouse thy1$^-$ subset homologous to human lining FLS (F4) were correlated to joint damage and mouse thy1$^+$ sublining FLS correlated to inflammation [45]. Notably, the elimination of FAP expressing subsets reduced pannus formation and joint destruction [45]. This suggests that FAP is a marker of pathologically active FLS in RA [45, 92, 93].

Comparison of subsets of FLS in RA and psoriatic arthritis are underway [94] and may potentially assist in discriminating these arthritides.

5. Fibroblasts derived from synovial fluid versus synovial tissue

Arthrocentesis is a common therapeutic procedure in treatment of RA. Fibroblast cultured from synovial fluid aspirates initially express similar phenotypical traits compared to tissue derived synovial fibroblast cultures [95, 96]. Despite these similarities, synovial fluid derived fibroblasts are likely a proxy regarding changes in the synovium and results must be interpreted as such. In both research and clinical settings synovial biopsies are both economical and well tolerated [97–100]. However, synovial fluid analysis of both cellular and soluble components is very useful in clinical settings where the length of consultations/sterile procedural environments/analytic facilities may limit the use of synovial biopsies. To the authors knowledge, no studies have yet reported scRNA-seq analysis of synovial fluid fibroblasts.

6. Circulating mesenchymal fibroblast like cells in rheumatoid arthritis

In excess to tissue resident FLS, Orange et al. recently highlighted the presence of circulating fibroblast-like cells in the blood of RA patients shortly before

symptomatic disease flare [46]. Interestingly these pre inflammatory mesenchymal (PRIME) cells show enrichment of previously reported markers of distinct sublining subsets of FLS e.g., DKK-3, CD34 and HLA-DR. This suggests that PRIME cells may constitute a heterogeneous pool of circulating FLS-like cells with distinct functions. Subsets of FLS migrating from the RA affected synovium, or a common homogeneous pool of circulating progenitor FLS awaiting recruitment signals from local sites of inflammation could potentially be the origin of these cells, although this remains to be investigated. Regardless, PRIME cells may not only be a useful marker predicting disease flares in RA, but also potentially explain how synovitis is transmitted from joint to joint [51].

7. Future therapeutic perspectives

The insights recently generated through high resolution scRNA-seq have revolutionized our understanding of specific subsets of FLS in RA and their involvement in driving different aspects of RA pathobiology. This understanding has also provided the basis for generating specific targetable markers of pathological subsets of FLS in RA. Targeting strategies that could be used as either monotherapy or as an add-on treatment to present day cytokine or lymphocyte inhibitors [101].

FLS could be targeted by drugs used in fibrotic conditions such as nintedanib or pirfenidone [102]. However, these drugs are likely affecting a completely different aspect of fibroblast functions. Therefore, new drugs are needed. An example is the addition of the cyclin-dependent kinase inhibitor, Seliciclib, which is currently being evaluated [103].

The well-known FAP marker of activated stromal cells has a diagnostic and prognostic potential through precise and low background positron emission tomography tracers developed in cancer-immunology [104]. The recent development of specific quinoline-based positron emission tomography tracers that act as FAP inhibitors have demonstrated promising results both preclinically and clinically in different cancers but could also be promising as diagnostic and prognostic markers of RA [105]. Further, the clinical potential of targeting FAP expressing FLS would be a targeted treatment eliminating pathologically activated RA FLS, in both the lining and the sublining layer [45, 93].

Among other interesting targets, NOTCH3 is one of the most recently *in vivo* validated pathological targets. NOTCH3 is expressed on the surface of RA FLS and linked with THY1 expression. NOTCH3 may also be a useful target in a therapeutic senescence strategy through selective activation of the g-protein coupled receptor melanocontin type 1 receptor [106]. Furthermore, in an animal model of RA injection of NOTCH3-neutralizing monoclonal antibody attenuated the severity of arthritis. Taken together, the *in vitro* studies on NOTCH3, including its connection to spatial distribution of FLS and the above-mentioned animal study underline NOTCH3 as a promising therapeutical target in RA [106, 107]. Targeting the complement C3 - C3a receptor axis may serve as another preventive or complementary strategy, where metabolic priming of FLS can be avoided or reduced [60]. Another possible strategy of targeting FLS is drug delivery via the extra domain A fibronectin splice variant identified in OA and RA [108, 109] and utilized in cancer [110].

Several other reagents targeting FLS are currently being tested ranging from metabolite modulators to treatments targeting intracellular signal transduction or epigenetic changes [111].

Collectively, these therapies targeting subsets of FLS are emerging as promising diagnostic and therapeutic tools. Tools for optimized and stratified treatments in RA based on which cellular mechanisms and which fibroblast subsets are pathologically activated in the individual patient.

8. Conclusions

Collectively, pathological FLS presented in this chapter are deeply connected to the RA pathophysiology of disease initiation, joint inflammation, disease persistence and joint tissue destruction.

Recent scRNA-seq studies have identified several distinct subsets of FLS causally linked to major elements of RA pathogenesis e.g., inflammation and joint destruction, while other subsets may present regulatory, pro-inflammatory TLS associated or common progenitor FLS.

These first steps in a scRNA-seq era of RA research warrants both rejoice and due diligence. Due diligence because we henceforth must appreciate the cellular diversity and the complex cellular crosstalk of the RA synovium. Like FLS, monocytes/macrophages and lymphocytes exhibit distinct subsets in RA, which may be as important in understanding the spectrum of RA disease, e.g., lymphocyte dominated vs. lymphocyte poor synovium and erosive vs. non-erosive disease. Furthermore, we must appreciate the heterogeneity of FLS and cellular organization (here among TLS formation) of the sublining layer.

Rejoice because the recent subset studies have produced a language and knowledge and a novel nomenclature for FLS in future research. A breakthrough that might enable clinicians in the future to modulate specific aspects of RA through fibroblast subset targeted treatment.

Conflict of interest

The authors declare no conflict of interest.

Abbreviations

RA	Rheumatoid arthritis
FLS	Fibroblast like synoviocytes
TNFα	Tumor necrosis factor alpha
scRNA-seq	Single cell RNA sequencing
ICAM-1	Intercellular adhesion molecule 1
VCAM-1	Vascular cell adhesion molecule 1
MHC-II	Type 2 major histocompadability complex
TLS	Tertiary lymphoid structures
THY1	Thymocyte differentiation antigen 1
PDPN	Podoplanin
CXCL	C-X-C motif ligand
CCL	C-C motif ligand
RANKL	Receptor activator of nuclear factor kappa-β ligand
IL	Interleukin

MCP-1/CLL2	Monocyte chemoattractant protein 1
MMP	Matrix metalloproteinases
FAP	Fibroblast activation protein
DKK	Dickkopf related protein
CD55	Complement decay-accelerating factor
PRG4	Lubricin
CLIC5	Chloride intracellular ion channel 5
HBEGF	Heparin binding epidermal growth factor-like growth factor
HLA	Human leukocyte antigen
OA	Osteoarthritis
OPG	Osteoprotegerin
OGN	Osteoglycin
IRF1	Interferon regulatory factor 1
MDK	Midkine
CADM1	Cellular adhesion molecule 1

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