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The synovial fluid fibroblast-like synoviocyte: A long-neglected piece in the puzzle of rheumatoid arthritis pathogenesis

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Rheumatoid arthritis (RA) is a systemic autoimmune disease during which fibroblast-like synoviocytes (FLS) contribute to both joint inflammation and destruction. FLS represent the core component of the synovial membrane. Following inflammation of this membrane, an effusion of cell-rich synovial fluid (SF) fills the joint cavity. Unlikely, SF has been shown to contain fibroblasts with some shared phenotypic traits with the synovial membrane FLS. These cells are called SF-FLS and their origin is still unclear. They are either brought into the synovium *via* migration through blood vessels, or they could originate within the synovium and exist in projections of the synovial membrane. SF-FLS function and phenotype are poorly documented compared to recently well-characterized synovial membrane FLS subsets. Furthermore, no study has yet reported a SF-FLS single-cell profiling analysis. This review will discuss the origin and cellular characteristics of SF-FLS in patients with RA. In addition, recent advances on the involvement of SF-FLS in the pathogenesis of RA will be summarized. Current knowledge on possible relationships between SF-FLS and other types of fibroblasts, including synovial membrane FLS, circulating fibrocytes, and pre-inflammatory mesenchymal (PRIME) cells will also be addressed. Finally, recent therapeutic strategies employed to specifically target SF-FLS in RA will be discussed.

Abbreviations: ACPA, Anti-Citrullinated Peptide Antibodies; CD55, Complement Decay-accelerating Factor; CDH, Cadherin; DKK, Dickkopf; ECM, Extracellular Matrix; FLS, Fibroblast-Like Synoviocytes, Fzd, Frizzled; HLA, Human Leukocyte Antigen; ICAM1, Intercellular Adhesion Molecule 1; IgG, Immunoglobulin G; JAK, Janus Kinase; LRP5, Low density lipoprotein Receptor-related Protein5; MMP, Matrix Metalloproteinases; MSC, Mesenchymal Stem Cells; PRIME cells, Pre-Inflammatory Mesenchymal cells; RA, Rheumatoid Arthritis; scRNA-seq, Single cell RNA sequencing; SF, Synovial Fluid; THY1, THYmocyte differentiation antigen-1; TRAIL, TNF-Related Apoptosis Inducing Ligand; VCAM1, Vascular Cell Adhesion Molecule 1; Wnt, Wingless.

KEYWORDS

rheumatoid arthritis, synovial fluid, fibroblast-like synoviocyte, inflammation, PRIME cells, fibrocyte, joint destruction

Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disease that affects approximately 1% of the world's population (1). The etiology of RA has not yet been fully understood and genetic, environmental and stochastic factors have been contended to be involved (2). It has been reported that a combination of epigenetic modifications and environmental factors can lead to modified self-antigens such as immunoglobulin G (IgG), collagen II, fibrin and vimentin. These proteins with arginine residues can be converted to citrulline by peptidyl arginine deiminases, and this post-translational modification is called citrullination. During RA, an autoimmune response is developed against citrullinated peptides detected as anti-citrullinated peptide antibodies (ACPA) (3). RA is also characterized by persistent inflammation, frequently resulting in bone erosion and joint destruction if left untreated (4). An effusion of synovial fluid (SF), which fills the joint cavity, is also observed. SF is known to contain several immune cells and inflammatory cytokines, together entertaining inflammation in the affected area (5). RA pathology involves abnormalities of both the immune system (including T cells, B cells, plasma cells, dendritic cells, macrophages and mast cells) and fibroblast-like synoviocyte (FLS) activity (6, 7).

FLS, also known as type B synoviocytes, have been identified as important cellular players in RA pathogenesis (8). There is growing evidence that FLS activation is an early step in the development of RA (6). Indeed, RA FLS exhibit an aggressive phenotype and produce pathogenic inflammatory mediators such as cytokines. They also produce matrix-degrading enzymes that promote local inflammation and disease perpetuation. Furthermore, RA FLS are involved in antigen presentation to autoreactive T cells (1, 9–11). Their excessive proliferation and resistance to apoptosis are the cause of synovial hypertrophy. Their migratory and invasive properties exacerbate joint damage (12). Interestingly, different FLS subsets with distinct functions have been characterized in the synovial tissue (13–15). In addition to tissue-resident cells, FLS have also been found in the synovial fluid (SF) of patients with RA (16–19).

There is an increasing interest within the research community in analyzing tissue-resident FLS and their role in the development of RA. Recent data confirm the importance of these cells in synovial inflammation, joint destruction and even disease spread into distal sites (2, 7, 8).

This mini review will cover recent insights about the cellular characteristics and involvement of SF-FLS in the RA

pathogenesis. The eventual relationship between RA SF-FLS and the other fibroblast populations, including tissue-resident FLS, fibrocytes and pre-inflammatory mesenchymal (PRIME) cells will be focused on. Finally, recent therapeutic strategies employed to specifically target SF-FLS in RA will be discussed.

Origin of SF-FLS

The exact origin of SF-FLS has not yet been determined. However, there are different opinions on the possible source of these cells (16–20). The first one suggests that SF-FLS arise from circulating progenitors introduced into the synovium in response to cytokines produced in the inflamed joint (17). This hypothesis was proposed after the identification of circulating cells with fibroblastic characteristics in the peripheral blood of patients with RA (21–23).

Circulating human fibrocytes, which have already been shown to differentiate into mature fibroblasts, are the best candidates to be the origin of SF-FLS (22).

Fibrocytes are circulating progenitor cells of the mesenchymal lineage and represent approximately 0.5% of peripheral blood leukocytes (24, 25). They express CD34 and produce components of the connective tissue matrix, including collagen I (col I) and fibronectin (26, 27). Fibrocytes are among the earliest responding cells in the innate response to injury or tissue invasion (21, 28). Galligan et al. provided evidence that circulating fibrocyte cells migrate into the joints during the inflammatory process and that an adoptive transfer of activated fibrocytes in recipient mice enhances the disease process (29). In addition, high percentages of fibrocytes have been found in both blood and SF of patients with RA. Interestingly, SF-fibrocytes have low CD34 expression which reflects an eventual cell differentiation (30). While SF-FLS reappear in joints after synovectomy indicating possible migration and differentiation of progenitors into FLS (17), the possibility that these cells may emerge from the synovial membrane after desquamation of lining or sub-lining FLS cannot be excluded (31).

Culture of SF-FLS

SF-FLS can be isolated from synovial fluid and grown in culture for several prolonged passages. SF is generally aspirated from the knee, wrist or elbow during an RA flare-up (16–19, 31). Centrifugation of SF gives a cell pellet, and some cells are able to

adhere to tissue culture dishes. Non-adherent cells are eliminated, leaving a mixture of two major cell populations: SF-FLS and SF-macrophages (16, 17). SF-macrophages are terminally differentiated cells with a limited lifespan *in vitro*, and rarely survive more than a few weeks in culture. SF-FLS proliferate rapidly in passages 1 through 4, generally reaching confluence within 10 to 20 days, but grow more slowly in later passages (16–19, 31, 32). Several cultures were maintained for over one year, reaching passage 9 or 10 (16, 17). The culture of SF-FLS is considerably longer than that of synovium FLS (33). The prevalence of positive cultures varies from a study to another. Generally, SF-FLS are obtained from 60% to 80% of SF from RA patients during the disease flare-up (16, 18, 19). Neidhart et al. demonstrated that the presence of RA FLS in SF can be affected by many clinical factors (16). However, Stebulis et al. found that patient's treatment, initial fluid volume, or cell counts are not associated with the presence of RA-FLS in SF (17).

Morphology of cultured SF-FLS

SF-FLS from patients with RA display multiple changes in cell morphology during culture (17, 18, 34). Under light microscopy, SF-FLS appear elongated with an irregular stellate cell shape during the first few days of culture (16, 17). In primary cultures, SF-FLS are frequently observed close to cells with macrophage morphology (17). Less frequently, SF-FLS start proliferating only after separation from macrophages (16). After the first weeks of culture, SF-FLS became larger and actively proliferative. In most cultures, spindle-shaped cells appear to develop from dense cellular clusters, but isolated cells are also observed (17). Similar to the changes observed in

FLS isolated from RA synovium, SF-FLS from RA patients grow in an anchorage-independent mode and form villous projections that float freely in the culture medium. Furthermore, SF-FLS exhibit the ability to form a tissue-like structure in culture (17, 18). The tissue border is composed of multiple layers of SF-FLS aligned longitudinally. Non-RA cultures of SF-FLS are unable to form any tissue-like structures (17).

SF-FLS versus other fibroblasts/mesenchymal stem cells

Recent studies support the idea that FLS constitute a heterogeneous population and that distinct subtypes of FLS do exist. These cells are involved differently in the pathogenesis of RA. They differ in their gene expression patterns, cellular markers and epigenetic signature (14, 15, 33). The synovium also houses mesenchymal stem cells (MSC). While both SF-FLS and MSC are part of the synovium their connection remains unclear. Possible relationships between SF-FLS and the other fibroblasts/MSC will be discussed below and summarized in Table 1.

SF-FLS versus lining and sub-lining FLS

The term synovium refers to a thin membrane that encapsulates the joint cavity (39). This membrane is divided into two regions: the intima or synovial lining composed of intimal lining FLS and macrophages, and the sub-lining layer or sub-intima that contains principally sub-lining FLS, macrophages and adipose cells (40, 41). During RA, FLS hyperplasia occurs in both locations (42).

TABLE 1 Profile of SF-FLS surface markers.

SF-FLS marker	Other fibroblast/MSC in which it is found	Functions	References
CD55	Lining FLS	Oncogenic properties such as invasion and migration	(16, 35, 36)
PDPN	Sub-lining FLS PRIME cells	Mediates tumor cell migration and invasion Play a role in severe RA	(19, 23, 35)
THY1 (CD90)	Sub-lining FLS SF-MSC	Involved in tissue destruction by highly expressing genes related to osteoclast differentiation or activation, such as receptor activator of nuclear factor- κ B ligand (RANKL)	(15, 19, 35)
HLA-DR	Fibrocytes Sub-lining FLS	Antigen presenting molecule	(19, 37)
CDH11	Lining FLS PRIME cells	Critical for homotypic aggregation of FLS <i>in vitro</i> and <i>in vivo</i>	(20, 23, 27)
CXCR3	Fibrocytes	Mediates cell invasion	(27, 38)
IL1RI	Fibrocytes	Involved in IL1 induced inflammatory responses	(27)
Wnt receptors : Fzd4, Fzd5, Ror2, Ryk, LRP5	Fibrocytes	Mediate Wnt signaling Involved in cell migration and Inflammation	(27)
ICAM1	Sub-lining FLS	Expressed by FLS and linked to their inflammatory properties	(19)

Tissue-resident FLS have long been considered as functionally homogeneous cells. However, it is now widely accepted that these cells are heterogeneous groups that perform a number of distinct and specialized functions (13).

Recently, Stephenson et al. reported two distinct human FLS subsets in RA synovium using single cell RNA sequencing (scRNA seq) transcriptomics. The first subset is localized in the intima and expresses complement decay-accelerating factor or CD55 molecule (*CD55*⁺ lining FLS) while the second subset is found in the sub-intima and expresses thymocyte differentiation antigen1 (THY1) or CD90 marker (THY1⁺ sub-lining FLS) (35).

Lately, Zhang et al. described some heterogeneity in the sub-lining layer FLS and defined three THY1⁺ groups with additional subset markers: CD34 specific to the first group, human leukocyte antigen (HLA)-DRA-high that characterized the second group and Dickkopf 3 (DKK3) that defined the third group (43) (43).

In order to phenotypically characterize the SF-FLS of patients with RA, Koster et al. used three surface markers: podoplanin (PDPN), THY1 and CD34 (19). After a few passages, the SF-FLS cultures consist of primarily PDPN⁺THY1⁺CD34⁻ cells. Furthermore, following co-cultures with immune cells or INF γ , SF-FLS express HLA-DR.

These results demonstrate that SF-FLS from patients with RA share phenotypic characteristics with a pathogenic subset of sub-lining RA-FLS (19). However, Neidhart et al. showed by flow cytometry the presence of common markers between SF-FLS and intimal lining FLS during RA including the intimal lining marker CD55 (16).

SF-FLS versus SF-mesenchymal stem cells

MSC were initially isolated from bone marrow (44). *In vitro*, they can adhere to plastic culture-dishes and undergo differentiation into mature mesenchymal cells especially osteoblasts, chondrocytes and adipocytes (44, 45).

Moreover, MSC express the surface markers CD73, THY1, and CD105. They are negative for CD45, CD34, CD14, CD11b, CD79 α , CD19, and HLA-DR surface molecules (46).

De Bari et al. characterized multipotent MSC from adult human synovium (47). In this context, it has been shown that MSC can be isolated from the synovial fluid of patients with RA (48, 49). The precise role of MSC in RA pathology remains unclear. In experimental arthritis, joint inflammation is preceded by infiltration of MSC, which may contribute to synovial membrane hyperplasia (50). SF-MSC are obtained *in vitro* according to the same protocol used for the SF-FLS culture. After SF culture, SF-MSC are maintained in culture with the plastic-adherent mononuclear cell fraction and can be cultured as fibroblast-like cells (48). Meanwhile, the relationship between the SF-MSC and SF-FLS remains unclear (39).

It has been proven that SF-FLS express several adhesion molecules, such as the InterCellular Adhesion Molecule 1 (ICAM1) and fibroblast makers, such as collagen and vimentin (19, 20, 27). Similarly, SF-MSC are reported to express the majority of these markers (37).

SF-FLS versus pre- inflammatory mesenchymal cells

Recently, Orange et al. reported the presence of circulating fibroblast-like cells in the blood of patients with RA a few weeks before the disease flare-up (23). These cells are identified as Pre-inflammatory Mesenchymal (PRIME) cells. PRIME cells share some markers with SF-FLS such as cadherin 11(CDH11) and HLA-DR. Furthermore, these cells display cellular characteristics of certain sub-lining FLS subsets (23).

SF-FLS versus circulating fibrocytes

During RA, the increased number of FLS in the synovium contrasts with the relatively low number of mitotic cells (51). This observation led Galligan et al. to study the involvement of circulating fibrocytes in the pathogenesis of RA. They provided evidence that circulating fibrocyte cells migrate into the joints during the inflammatory process (22). They also hypothesized that circulating fibrocytes may be precursors of FLS.

A recent work by Elhaj Mahmoud et al. showed that SF-FLS and fibrocytes from patients with RA express the same levels of IL1receptor I (IL1RI). The expression of IL8 receptors (CXCR1 and CXCR2) is restricted to fibrocytes while the expression of CXCR3 is reported in both cell types (27).

This chemokine receptor and its ligands CXCL9, CXCL10 and CXCL11 are involved in various inflammatory diseases, such as RA, multiple sclerosis, transplantation rejection, atherosclerosis, and inflammatory skin diseases (38).

Additionally, SF-FLS and fibrocytes from patients with RA express the Wingless (Wnt) receptors: Frizzled (Fzd) 4, Fzd5, Ror2, Ryk and the low density lipoprotein receptor-related protein 5 (LRP5). These receptors play an important role in FLS-mediated inflammatory response and cell migration (34).

Involvement of SF-FLS in the pathogenesis of RA

The transformed phenotype of RA SF-FLS

RA FLS display certain unique features of transformed cells. Once activated, RA FLS have their phenotype significantly modified. They exhibit the characteristics of transformed cells,

including loss of contact inhibition (52, 53). Indeed, it has been demonstrated that SF-FLS proliferation in culture continued despite the formation of a confluent cell layer (17). This characteristic was initially described in the cultured lining and sub-lining FLS from patients with RA (54).

The transformation of SF-FLS phenotype during RA suggests specific alterations in the transcription and the epigenetic profile of disease-relevant genes as well as intracellular signaling pathways, including alterations in apoptotic cascades (52–54).

These changes comprise upregulation of several proto-oncogenes as well as downregulation of potentially protective tumor suppressor genes (52–54). Numerous oncogenes involved in cell cycle regulation or acting as transcription factors such as c-Fos, Cyclin E and Wnt1-inducible-signaling pathway protein 1 (WISP1) are expressed at high levels in RA SF-FLS (18).

Epigenetic modifications in SF-FLS during RA are also important. Gene-specific methylation changes are detected in RA SF-FLS (55). Glossop et al. identified a total of 195 genes that display statistically significant methylation changes in the SF-FLS from patients with RA. A hypomethylation of multiple genes has been described, such as A Disintegrin And Metalloproteinase with Thrombospondin Motifs 14 (ADAMTS14), a member of the ADAMTS family of proteinases and Methionine Sulfoxide Reductase A (MSRA), an enzyme involved in the response to oxidative damage (55). SF-FLS from patients with RA present a hypomethylation of MIR155HG, a host gene of the miRNA miR-155, involved in arthritis and cancer (55, 56). This aberrant methylation change of MIR155HG is observed in SF-FLS but not in synovium FLS (55). In addition, SF-FLS express an adhesion molecule called CDH11 crucial for the establishment of synovial architecture (20, 27). CDH11 promotes FLS migration and invasion. Furthermore, this protein enhances the production of matrix metalloproteinases (MMP) and cytokines by RA FLS (57–59). SF-FLS express fibronectin which enhances cell survival and facilitates cell adhesion to the surface of cartilage. Fibronectin is also a potent chemoattractant for fibroblasts and may facilitate chemokine signaling (18, 60, 61).

SF-FLS and inflammation

During RA, SF-FLS actively participate in synovial inflammation. It has been shown that RA SF-FLS release multiple inflammatory cytokines dominated by CCL2 and IL6 without exogenous stimulation (19). This corroborates the findings of previous data showing that RA SF-FLS secrete IL6, IL8, IL1 β and CCL2 (17, 34). In addition, RA FLS from the intimal lining have been considered primary sources of IL6, as shown by *in situ* hybridization and immunohistochemistry

studies (6). However, recent data showed that SF-FLS express higher levels of IL6 than tissue FLS and fibrocytes from patients with RA (34). The release of these cytokines contributes to joint inflammation and cell recruitment into the inflamed synovium (1, 6). The inflammatory profile of RA SF-FLS reflects the activation of numerous signaling systems, including the Wnt pathway (62). Functionally, aberrant expression of Wnt signaling components has been observed in RA FLS, including SF-FLS (34, 62). Wnt5a is particularly overexpressed in RA SF-FLS (62). This Wnt ligand is involved in synovial inflammation and induces the production of numerous pro-inflammatory cytokines (62, 63) (Figure 1).

RA SF-FLS and cartilage invasion/destruction

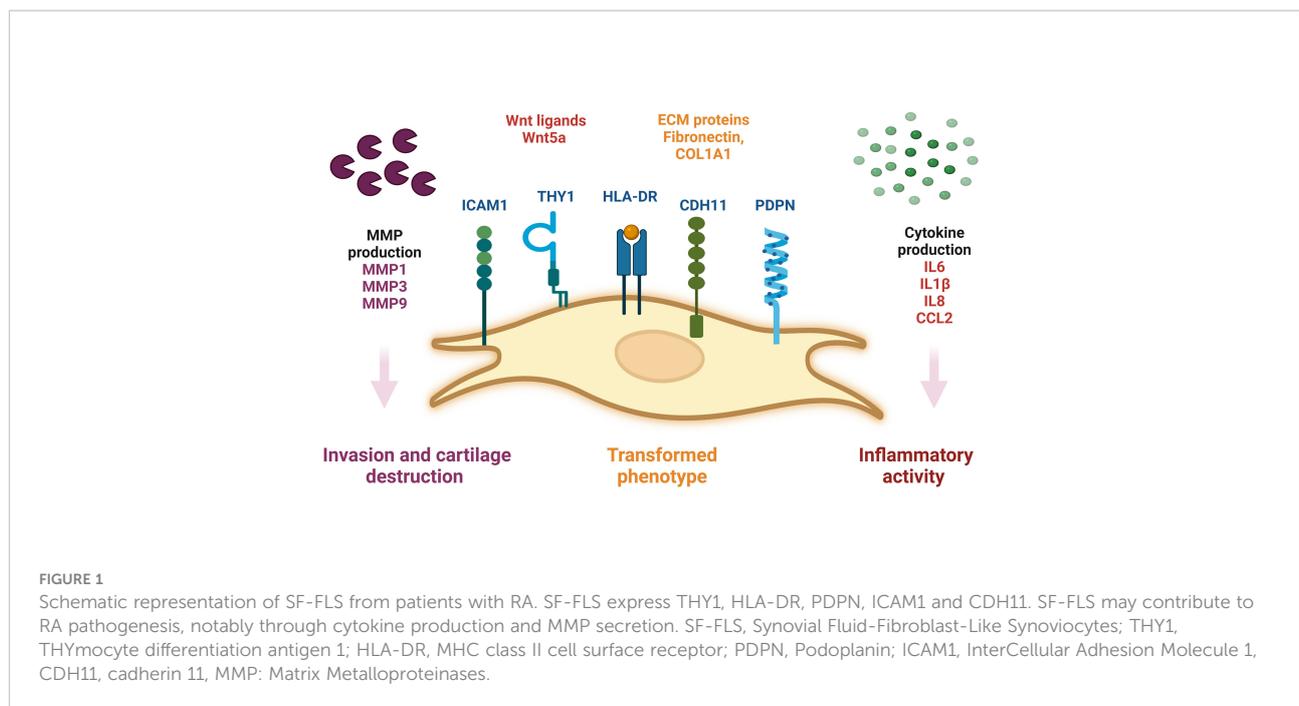
One of the first described and most characteristic features of RA SF-FLS is their ability to invade and destroy cartilage (16). It has been demonstrated that RA SF-FLS mediate cartilage degradation independently of the hyperplastic synovial tissue (16, 19). This potential is attributed to a combination of adhesion-facilitating factors and production of proteases especially the well-known MMP (16, 20).

MMP are a family of proteinases that degrade and remodel multiple components of the extracellular matrix (ECM), including collagens, fibronectin and hyaluronan (64, 65). Their enzymatic activity controls significant cellular functions such as proliferation, adhesion and migration (66, 67). MMP3 plays a pivotal role in cartilage destruction (68, 69). Previous studies have shown that SF-FLS produce a high level of MMP3 (20, 27). During RA, baseline levels of MMP3 serve as a biomarker of progressive cartilage damage. MMP3 itself may be responsible for the activation of other MMP resulting in the degradation of ECM proteins in cartilage (68). RA SF-FLS secrete MMP1 and MMP9, which are also involved in cartilage destruction (16, 20, 27). Adhesion molecules including, ICAM1 and Vascular Cell Adhesion Molecule1 (VCAM-1), facilitate the anchoring of RA FLS to the cartilaginous ECM components (6). A recent study showed that ICAM1 is highly expressed in cultured SF-FLS from patients with RA (19). Furthermore, CDH11, which is expressed by SF-FLS during RA, is relevant in cartilage destruction (6).

RA SF-FLS as potential migratory cells

Migration and invasion are complex processes that require dynamic interactions between cells and the surrounding matrix.

Fibroblast migration in human disease has only recently been reported (13). The first cell with fibroblastic properties described to migrate from the circulation into joint was



fibrocytes (70). The discovery of detectable pre-inflammatory PRIME cells in the blood of patients prior to an arthritis flare-up has revived the debate on synovial fibroblasts migration (13, 23).

Previously, some authors supposed that SF-FLS have the ability to migrate and “metastasize” from one joint to another, and potentially spread the disease (16, 19). However, this idea remains unproven. Interestingly, SF-FLS and a subset of sub-lining FLS share properties with recently described PRIME cells (19, 23). PRIME cells are thought to have a sub-lining layer phenotype. The authors proposed that peripheral blood B cells lead to the activation of PRIME cells in the circulation and the migration of these cells into the synovium (23). The question remains: what is the relation between SF-FLS, sub-lining FLS and PRIME cells? It is tempting to speculate that SF-FLS, sub-lining FLS and PRIME cells are the same cell type. SF-FLS may derive from the sub-intimal lining and fall into the SF. These cells may migrate through the circulation, increase in blood before a flare-up and then decrease just after symptom onset (Figure 2).

Targeting SF-FLS in RA

Despite great advances in RA therapy, numerous patients with RA have persistent disease. Current daytime treatment strategies for RA focus on suppressing cytokine signaling and T- and B-cell activity (1).

Certain molecules including Janus Kinase (JAK) inhibitors have been shown to reduce FLS aggressiveness. However, therapies that selectively target FLS are still lacking (71).

FLS-targeted therapies present a nice advantage as they could potentially be used in combination with immune suppressors with limited side effects on host defense. So far, only CDH11 has emerged as a potential target for selective anti-FLS therapy. Indeed, CDH11 is expressed by SF-FLS during RA (27) and is involved in RA pathogenesis. However, a phase II study of a monoclonal antibody directed against CDH11 (RG6125) was discontinued in 2019 due to a lack of efficacy (72).

Several observations have suggested that targeting the TNF-related apoptosis inducing ligand (TRAIL), a member of the TNF family or its death receptors TRAIL-R1 (DR4) and TRAIL-R2 (DR5) may have a therapeutic role in RA. A previous study by Miranda-Carús et al. demonstrated that SF-FLS from patients with RA express DR5 and undergo apoptosis when exposed to an agonistic anti-DR5 antibody (31).

Recently, Notch3, a transmembrane receptor of the Notch family, has been described as a new therapeutic FLS target. Indeed, Wei et al. showed that the injection of Notch3-neutralizing monoclonal antibody attenuates the severity of arthritis in mouse model. This receptor is principally expressed by THY1+ FLS and transduces signals from endothelial cells to FLS to expand during RA (73). To the best of our knowledge, no study has yet reported Notch3 expression in SF-FLS.

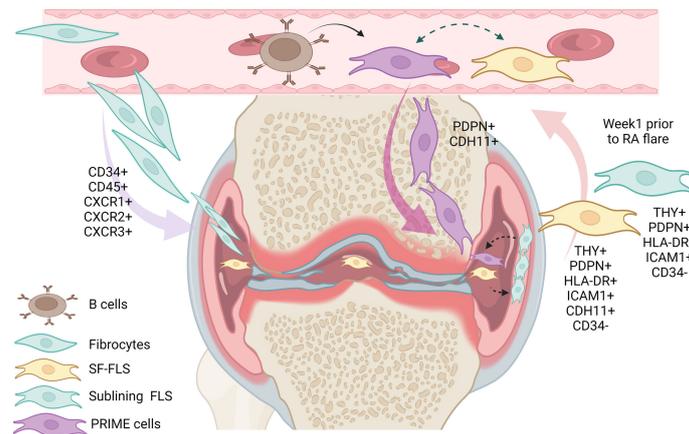


FIGURE 2

Illustration of the eventual relation between SF-FLS, fibrocytes, sub-lining FLS and PRIME cells. It has been shown that fibrocytes from patients with RA have a migratory potential with the capacity to leave the blood and reach the inflamed joint. Within the inflamed joint, fibrocytes differentiate into FLS. According to this model, SF-FLS may constitute a fibrocyte differentiation state. New data confirm that SF-FLS have a sub-lining layer phenotype. PRIME cells are also thought to have a phenotype similar to both SF-FLS and a sub-lining FLS subset. PRIME cells are activated by B cells and their number increases in the blood prior to a flare-up and migrate into the joint to enhance inflammation. SF-FLS, Synovial-Fluid Fibroblast-Like Synoviocytes; PRIME cells, PRe-Inflammatory MEsenchymal cells.

Conclusion

The heterogeneity of FLS subsets strengthens their pathogenic role in the pathogenesis of RA. Among the different FLS subsets, SF-FLS remain poorly studied and characterized. These synovial fluid-derived cells exhibit the same cellular properties as those of transformed cells, including loss of contact inhibition when cultured. During RA, SF-FLS secrete pro-inflammatory cytokines and MMP and actively participate in cartilage destruction. Phenotypically, SF-FLS share cellular characteristics with a pathological sub-lining FLS subset. SF-FLS also share properties with the recently described PRIME cells, found in increased numbers in patients with RA preceding disease flares. A thorough characterization of SF-FLS will not only help to identify new therapeutic targets, but also to understand the pathogenesis of a disease that remains a major public health problem.

Author contributions

DM, conceived the study, wrote the manuscript and prepared the figures. WK and LL, wrote the manuscript and prepared the table. NS, LT, SR, SJ, HS, MK-S, EC contributed to the layout organization and writing. All authors approved the final manuscript

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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