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Utilizing 3D cultures to study the role of fibroblast-like synoviocytes in Rheumatoid Arthritis.

Degree Project in Medicine

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Gothenburg, Sweden 2021

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List of abbreviations

RA - Rheumatoid Arthritis

3D-culture - Three-Dimensional Culture

FLS - Fibroblast-Like Synoviocytes

DMARDS - Disease-Modifying Anti Rheumatic Drugs

AREG - Amphiregulin

IL6 - Interleukin 6

TNFα - Tumor Necrosis Factor -α

mRNA - messenger RNA

cDNA - complementary DNA

qPCR- quantitative Polymerase Chain Reaction

EGFR - Epidermal Growth Factor Receptor

MMP - Matrix MetalloProtein

NK - Natural Killer Cells

DC - Dendritic Cells

PRR - Pattern Recognition Receptors

PAMP - Pathogen Associated Molecular Patterns

DAMP- Danger-Associated Molecular Pattern

APC - Antigen Presenting Cells

MHC- Major Histocompatibility Complex

BCR - B-cell Receptor

IFN-γ - Interferon-γ

IgE - Immunoglobulin E

SLE - Systemic Lupus Erythematosus

HLA-DR4 - Human Leukocyt Antigen, with subgroup DR4

CMV - Cytomegalovirus

EBV - Epstein-Barr Virus

ECM - Extracellular Matrix

TGF-β - Transforming Growth Factor-β

CCL2 - Chemokine Ligand 2

CXCL - CXC Ligand

RF - Rheumatoid Factor

ACPA - Anti-Citrullinated Protein Antibodies

PDGF - Platelet Derived Growth Factor

PGE2 - Prostaglandin E2

HC-FLS - Healthy Control-Fibroblast-like synoviocytes

RA-FLS - Rheumatoid Arthritis-Fibroblast-like synoviocytes

ILC – Innate lymphoid cells

EGF - Epidermal Growth Factor

ERBB2 - Receptor Tyrosine-Protein Kinase erbB-2

CSF2RB - Colony Stimulating Factor 2 Receptor Subunit Beta

TSLP - Thymic Stromal Lympopoietin

ACTA2 - Actin Alpa 2

COL1A1 - Collagen type 1, Alpha 1

COl1A2 - Collagen typ 1, Alpha 2

BIRC5 - Baculoviral Inhibitor of Apoptosis Repeat Containing 5

MKI67 - Marker of proliferation Ki-67

HB-EGF - Heparin-Binding Epidermal Growth Factor

Abstract

Title: Utilizing 3D cultures to study the role of fibroblast-like synoviocytes in Rheumatoid Arthritis.

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Degree project, Programme in Medicine at University of Gothenburg, Department of Rheumatology and Inflammatory research. Sweden, 2021.

Introduction: Rheumatoid arthritis (RA) is an autoimmune disease affecting multiple joints in the body, characterized by chronic inflammation and destruction of cartilaginous structures. The key effector of the disease is activated fibroblast-like synoviocytes, (FLS), cells that are found in the synovium of diarthrodial joints. As a response to proinflammatory cytokines, cellproliferation, production of inflammatory cytokines and degrading enzymes occurs to a greater extent than in healthy individuals causing damage to the joints.

Aim: To evaluate if FLS from patients with RA differs regarding growth, gene expression and response to stimulation with cytokines, such as Tumor necrosis factor-α (TNFα) and Amphiregulin (AREG) compared to fibroblast-like synoviocytes from healthy individuals after being cultured in 3D.

Methods: This study processes 12 primary cell lines of FLS, collected from synovial tissue from healthy individuals and patients with RA. The cells were cultured in 3D and stimulated with TNFα, AREG, a combination of both, or left unstimulated. Extraction of mRNA and generation of cDNA was performed to be able to do qPCR to study the expression of different genes as well as histological differences.

Results: Results showing for examples increased gene expression of EGFR in HC-FLS (healthy control) after stimulated with TNFα, together with increased gene expression of MMP3 after stimulated with TNFα in RA-FLS, which all is involved in key events in pathogenesis of the disease.

Conclusion: 3D-cultures of FLS, is affected by TNFα and AREG by promotion of expansion, increased expression of degrading enzymes and increased proliferation of cells. TNFα promotes an aggressive phenotype of FLS.

Key words: Rheumatoid Arthritis - Fibroblast-like Synoviocytes (FLS) - 3D-culture - Amphiregulin

1. Background

Rheumatoid arthritis is known as one of the most common autoimmune systemic diseases (*1*). Rheumatoid Arthritis affects around 1% of the population (*2*) and if left untreated it causes severe complications as well as early death. The disease predominates in women, with female: male ratio about 2:1 to 3:1 (*3*). The characteristics is the chronic inflammation in specifically diarthrodial joints, pannus, (tumor-like enlargements of the synovial membrane) and destruction of cartilage and bones of the joints (*1*).

The immune system is a complex system, which main function is to protect our body from potentially harmfully pathogens, as well as recognize virus infected cells and cancer cells. It is based upon several control mechanism and cells to keep us safe from pathogens and eliminate any potentially harmful bacteria or viruses and limit the inflammatory response. When the control mechanisms fail, it leads to autoimmune disorders, which basically means that the body attacks itself (*4*). Why this happens is still unknown but it is believed to be caused by a complex interaction between environment and genetic factors and although Rheumatoid Arthritis causes a systemic dysregulation and autoimmunity, it is primarily the joints that are the clinical manifestations (*5*).

The immune system

Our immune system consists of both innate-and adaptive immune system, with two different functions. The innate system is very fast and we rely on it to act identically on different kind of pathogens every time something encounters our body. The cells of the innate immune system are natural killer-cells (NK), innate lymphoid cells (ILC), dendritic cells (DC), granulocytes, monocytes, macrophages and mast cells (*6, 7*). These cells have pattern recognition receptors

PRR, that recognize pathogen associated molecular patterns, PAMPs, which is expressed on the pathogens and endogenous danger signals, danger-associated molecular pattern, DAMPs are released during cellular stress (*4*).

The link between the innate and adaptive immune system is provided by dendritic cells. Whereas the innate immune system is quite general, the adaptive immune system is very specific and responds to a specific pathogen that induces them and gives a long-lasting protection. The main cells of the adaptive immune system are T cells and B cells, which are classes of different types of lymphocytes (*4*).

Dendritic cells and macrophages, so called antigen-presenting cells (APC), ingest pathogens and present antigens on their MHC proteins. A B cell present only peptides that is derived from an antigen that's a specific match for its B cell receptor (BCR), to be able to start the B cell activation. The T-helper cell activates only the B cells with antigens that match the BCR, which is a requirement for the linked recognition. After a T-helper cell has become activated to an effector cell, the T cell secretes pro-inflammatory cytokines that activates B cells and other immune cells to induce proliferation and differentiation. E.g. T cells secrete TNFα and IL-6 to activate macrophages and endothelial cells (*4*).

TH_1, TH_2 and TH_{17} are all derived from $CD4^+$ cells and serves a specific purpose in our immune system. The TH_1 cells secrete IFN-γ and is involved in activation of macrophages and promotes production of IgG by B cells. It serves as a defense host against intracellular microbes and is a driven part of autoimmune diseases with tissue damages and association to chronic infections. The TH_2 cell secrete Il4, IL5 and IL13 and is involved in the activation of eosinophils and mast

cells as well as production of IgE and alternative activation of macrophages. The TH_2 plays an important part in the allergic reactions and diseases.

The TH_{17} secrete IL17 and IL22 and is involved in neutrophilic and monocytic inflammation. It serves as host defense against extracellular bacteria and fungi and is active in autoimmune inflammatory diseases (*8*).

Autoimmunity

The common feature for autoimmune diseases is a dysregulation of the inflammatory mechanisms that causes the condition. The dysregulation can be the inability to terminate or down regulate an immune response or that the immune response is triggered more easily. The chronic inflammation that is caused by a constant activated immune response is due to the presented antigen (*9*). Autoimmune disease can be either systemic, like SLE or organ-specific, like type I diabetes. The systemic autoimmune diseases target omnipresent expressed auto-antigens and commonly an end-organ injure is mediated by autoantibodies. In organ-specific autoimmune diseases the autoantigens are tissue- or cell-specific in location or availability and the end-organ injury can be mediated by T cells and/or autoantibodies (*8*).

Autoimmunity is also believed to be developed due to normal T cells activation and inaccurate deletion in thymus. These T cells can be activated by APC and once activated they can produce pro-inflammatory factors and cause damage to surrounding tissue (*8*).

Etiology/pathogenesis of Rheumatoid Arthritis

The etiology of Rheumatoid arthritis is believed to be multifactorial, involving different cell types such as T cells, B cells, neutrophils, macrophages, fibroblast, dendritic cells and chondrocytes. Several genes are associated with RA and is decisive to the severity of the disease

(*10*). Another crucial factor for the severity of the disease, which accounts for about 40% of the genetic influence is the structure of Class II MHC molecules on antigen-presenting cells. The association is found on a variable region of DR-β chain on HLA-DR4 (*10*).

Currently, there is no cure for Rheumatoid Arthritis but treatments are available. The treatments include T cell regulations, B cell depletion, and cytokine antagonists but many patients are left without the striving effect (*11*).

The pathogenesis of RA involves an interplay between environmental factors, epigenetic modifications, susceptible genes and loss of tolerance to self. The loss of tolerance to self-proteins leads to T- and B cells activation, causing inflammatory responses. The inflammatory response involves both adaptive and innate immunity, causing tissue response, cartilage degradation and bone erosion primarily by feedback loops. Smoking, bronchial stress increase the risk of RA among persons with susceptibility HLA-alleles. Infectious agents, such as CMV, EBV and E-coli have long been linked with RA due to the formation of immune complexes during ongoing infection including the autoantibody Rheumatoid factor, RF.

Infiltration of leukocytes into the synovial compartment cause synovial inflammation, synovitis. Endothelial activation in synovial microvessels enables cell migration, causing increased expression of adhesion molecules and cytokines which, combined with reorganization of synovial architectural and local fibroblast activation allows an increased buildup of inflammatory tissue in RA.

Another crucial factor for RA is the cytokine production that comes from many cell populations in the synovial lining. The pattern of cytokines varies over time in the disease and early RA differ from chronic disease. TNFα plays an important and fundamental role as it activate cytokine and

chemokine expression, expression of endothelial adhesion molecules, suppression of regulatory T cells and induction of pain. IL6 is another important cytokine as it is involved in leukocyte activation and antibody production.

The important and central role of these two cytokines has been identified by therapeutic blockades in patients with RA (*11*).

Physiology of a healthy joint

In the healthy joint both fibroblast-like synoviocytes (FLS) and macrophage-like synoviocytes is found on a bed of thin connective tissue called the sublining layer. This layer also contains fibroblast, macrophages, fat cells and blood vessels (*8*). FLS contributes to the synovial fluid by producing hyaluronic acid and proteoglycan 4, which nourishes the underlying cartilage. The synovial fluid contains leukocytes, constituents of blood plasma and proteins (*12, 13*). FLS is also involved in the making and maintaining of extracellular matrix (ECM) by producing components, such as proteoglycans and collagens as well as degrading enzymes, such as cathepsins and matrix metalloproteinases (MMPs) (*14*).

Physiology of a RA-joint

FLS is a great contributor to the destruction of cartilage and non-osseous support structures of the joint (*15-18*). The synovial lining transforms into invasive hyperplastic tissue known as pannus (tumor like structure) (*19*). FLS proliferate as a response to TNFα, IL-1 or TGF-β, produced by immune cells in the inflamed joint (*20*).

In the pannus, FLS-mediated overproduction of MMPs causes damage to the collagen-rich structures and enabling FLS to invade cartilage. This is primarily mediated by MMP1, MMP3 and MMP13 (*15-18, 21, 22*).

In RA, FLS attracts monocytes from vascular structures by secreting CC-chemokine ligand 2 (CCL2), CXCL1, CXCL8, and CXCL10. The recruited monocytes, when following the differentiation into macrophages, are a prominent source of IL-1β and TNFα in the synovium. This in turn activates FLS to produce pro-inflammatory cytokines, tissue-destructive factors and chemokines, such as MMPs, IL-6 and CXCL8 (*23-27*).

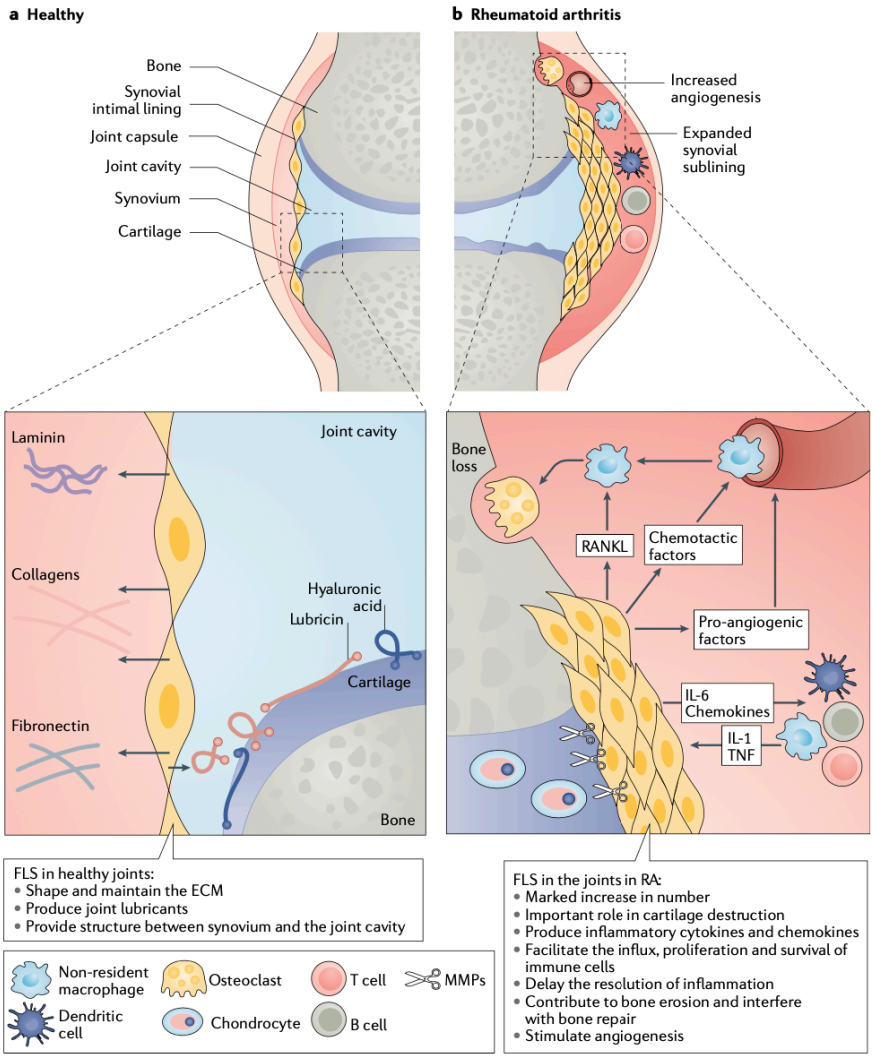

Fig 1. In a healthy joint, the intimal synovial layer is one or two cell layer deep, organized in a loose manner. The FLS produce hyaluronic acid, lubricin and other joint lubricant. It is also involved in creating components to ECM, such as type IV collagen.
In RA, the intimal synovial layer is expanded greatly, consisting of a layer with multiple cells, transforming it into a hyperplastic pannus. The increased production of MMPs contributes to the destruction of cartilage and bone. The inflammatory cells promote and maintain inflammation in the joint by production of various cytokines (14).

Autoantibodies

A common feature of the disease is the production of autoantibodies that is found in about 80% of RA patients. These autoantibodies are called Rheumatoid Factor, RF and anti-citrullinated protein antibodies (ACPA). RF is an antibody directed towards the Fc-region of IgG, whereas ACPA is antibodies directed against citrullinated proteins.

RF in combination with ACPA are used as diagnostic markers since they are to be found in the sera of RA patients years before the symptoms arise (*11*). The combination of these two markers are associated with increased disease activity and poor outcome of the disease (*28*).

Fibroblasts-like synoviocytes

Fibroblast-like synoviocytes (FLS) are highly specialized mesenchymal cells which forms the lining of the joint and is to be found in the synovium of diarthrodial joints. Normally, in a healthy joint, the synovium consists of two layers of cells, the intimal layer and the sublining layer, and FLS is primarily found in the intimal layer (*29*) The intimal layer, however, lacks tight junctions and a basement membrane for enabling passage of proteins and cells to move in to the synovial fluid (*19*).

FLS is a contributor to the pathogenesis of Rheumatoid Arthritis and are epigenetically imprinted with a more aggressive phenotype when the disease is present (*30*). In arthritis, synovial fibroblast do not respond to inflammation driven by leukocytes passively, but instead they contribute actively (*30*). FLS display different properties, among them it releases several effector molecules that act on a variety of cells in our immune system, among them are IL-1, IL-6 and IL-8 which act pro-inflammatory, EGF, PDGF that induces angiogenesis and collagenases that induces matrix degradation (*31*). FLS also display Cadherin-11, an adhesion molecule selectively specific for FLS, Cadherin-11 is responsible for aggregation of FLS in vitro, it also regulates secretion of IL-6 and other pro-inflammatory properties (*19*).

As a response to different growth factors and inflammatory cytokines produced by activated immune system FLS can divide. These growth factors (i.e. platelet derived growth factor (PDGF)) and cytokines (i.e. TNFα, IL-1, TGF-β) induce proliferation and division of FLS.

Unlike FLS in healthy individuals, RA-FLS, can migrate from one site to another presumably by the blood stream and the lymphatics. When these aggressive cells migrate and "metastasize" from joint to joint it is believed to be a contributor to the polyarticular nature of Rheumatoid arthritis (*10*).

As a combination of some properties of FLS mentioned above, inflammation induced changes promotes pannus growth by reduced ability to undergo apoptosis, protease-production causes degradation of ECM and invasion into cartilage. Further on the secretion of proinflammatory molecules modulates inflammation, cell recruitment, angiogenesis, and induction of cytokine production by immune cells explain FLS role in RA. (*19*)

3D Culture

In a recent study, it was shown that culturing FLS in three dimensional cultures instead of in a primary monolayer of cells, autonomously recreate functional characteristics of the synovial lining, such as remodulation of ECM and the production of lubricin. Lubricin is found in normal synovial fluid and is a necessary component, which is contributing to the lubricating properties. Culturing FLS in 3D cultures have proven to be a good way to recreate the functional characteristics that FLS possesses in vivo (*30*).

By culturing FLS in 3D, in different from in a monolayer, reproduces the functional characteristics, such as organization of constituents of the synovial fluid as well as organization of the dens lining ECM. As the 3D-structure makes the cells grow in an elongated spindle-shaped pattern, the cells accumulate and forms a lining-layer-like structure, at the interface between the matrix and the fluid phase. This resembles the in vivo synovial layer, causing the cultivation in 3D to resembles normal human synovium (*32*).

TNFα

Tumor Necrosis factor-α, TNFα is a pro-inflammatory cytokine that is produced by immune cells, such as B-cells and macrophages (*33, 34*) and is one of the major cytokines in the pathogenesis in Rheumatoid Arthritis (*10*).

TNFα is one of the immunomodulatory cytokines that is involved in cartilage metabolism as it, together with other cytokines such as IL-1, stimulates chondrocytes to synthesize cartilage matrix-degrading proteinases and it is also involved in the regulation of matrix protein synthesis and cell proliferation (*8*).

TNFα is found in both rheumatoid synovial fluid and in serum. It is primarily produced by synovial macrophages and is a membrane bound protein. Functions of TNFα includes the ability to enhance cytokine production, MMP production, adhesion-molecule expression and proliferation. Since TNFα induces proliferation in FLS it causes further activation of cells and initiate degrading properties of the joint (*14*). As TNFα stimulates collagenase and PGE_2 production, induces bone resorption, stimulates resorption of proteoglycans, inhibit bone formation in vitro and inhibits the proteoglycans biosynthesis in explants of cartilage, it is a key effector in the disease (*10*).

Amphiregulin (AREG)

AREG is a protein secreted by several types of immune cells including innate lymphoid cells, (ILC) (*35*) and it is one of the seven members of the EGF family involved in RA pathology (*36*). It is an autocrine growth factor which targets astrocytes, Schwann cells and fibroblasts (*37*). Recent studies show that AREG is found in synovial tissues of RA-patients. It stimulates FLS to produce several inflammatory cytokines as well as inducing proliferation, causing synovial

hyperplasia which is one of the key events of the disease. For FLS, AREG possesses a growth-promoting property due to signaling of EGFR, causing the cells to increase in number and size. Further on, increased FLS leads to more activation of macrophages and bigger cascade of cytokines and triggers the disease (*36*).

FLS is greatly affected by AREGs function and it plays a major part in the pathogenesis of Rheumatoid Arthritis (*36*). Therapeutic targeting of FLS activation would therefore have the potential to block inflammation, by inhibiting the additional stimuli. If combined with anti-cytokine therapy, this would decrease the long term-damage and open for the possibility of long-term remission of the disease. As treatments today, specifically immunosuppressant DMARDs, in many cases causes incomplete response and puts patients at risk for infections as the immune system is downregulated, targeting FLS would focus the therapy locally in the joints (*1*).

2. Aim

If FLS from patients with RA differs regarding growth, gene expression and response to stimulation with cytokines TNFα and AREG, compared to FLS from healthy individuals after being cultured in three-dimensional cultures.

3. Materials and Methods

3.1 FLS cell lines.

Primary FLS cell lines were received from a biobank maintained by Dr. Anna-Karin Hultgård Ekwall and Dr. Anna Rudin, Sahlgrenska university Hospital. Diary number for ethical approval is 573-07 and 1081-16. Total number of cell lines use N=12, HC-FLS N= 6. RA-FLS N=6. Four cell lines was discarded due to non desirable formation of a 3D culture. RA-FLS were generated previously from synovial tissues obtained during joint replacement surgery (*38*). HC-FLS were generated previously the synovial tissue from healthy individuals obtained during orthopedic surgeries performed at least three months after joint trauma. Tissues from healthy individuals showed no visible histological sign of inflammation.

3.2 Culturing fibroblast:

Fibroblast were cultivated initially to ensure enough cell count for creating of 3D cultures. FLS medium with 10% FBS, 1% PenStrep and 1% Glutamax were changed every third day for additional growth. FLS in passage 4-8 were used for experiments.

3.3 Creating 3D-cultures:

Initial coating of wells with Poly-Hema dissolved in 20mg/ml ethanol. This to make sure the fibroblast stayed in the 3D culture and does not attach and grow on the plate. Cell count was made using Trypan Blue (Tryphan Blue Solution, T8154, SIGMA). The cells were dissolved in 450 µl Matrigel Matrix™ (Ref. 356255, Corning) and approximately 100,000 cells/drop were disposed into each well. The cells were cultivated initially for one week to allow for 3D

formation. Cultivation in 3D-formation is often referred to organoids and the 3D-cultures generated in this study is therefore equivalent to organoids.

3.4 Stimulating 3D cultures:

After one week of cultivation, the cultures were stimulated with TNFα, AREG, TNFα+AREG or left unstimulated. The plates were further incubated for additionally two weeks and the medium, with stimulation, was changed every third day. Recombinant human TNFα were obtained from Biolegend and recombinant human AREG from R&D Systems.

3.5 Preparing 3D cultures for cutting and staining.

The cultures were placed in Optimal Cutting Temperature (OCT) media. The samples were the frozen in liquid nitrogen and stored in -80°C until cutting. The slides were cut on a cryostat, fixed in ice cold acetone and the stained with Hematoxylin and Eosin, making it possible to study the histological features of FLS in 3D culture.

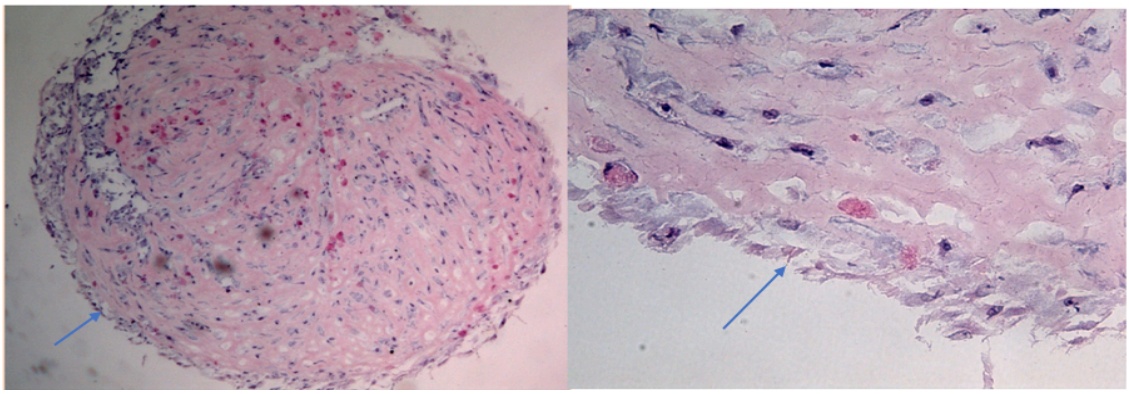

Fig.2. HC-FLS three dimensional cultures stained with hematoxylin and eosin. Blue arrows pointing at FLS.

3.6 Extraction of RNA

For extraction of RNA, Total RNA purification Plus Micro Kit (product # 48500, Norgen Biotek Corp) was used, following manufactures protocol. This kit extracts all types of RNA, but mRNA was analyzed with qPCR.

3.7 Generation of cDNA

For generation of cDNA, iScript cDNA Synthesis Kit (Bio Rad) was used following manufactures protocols. The concentration of RNA was measured with the help of Nanodrop.

3.8 qPCR

For quantitative PCR, SsoAdvanced Universal SYBR Green Supermix (Bio Rad) was used following manufactures protocols. Pre-designed primers for qPCR was obtained from Sigma Aldrich.

3.9 Statistical data

The data from qPCR was compiled in a EXCEL-document. The CT-value, which answers to gene expression. Lower CT value gives a higher and more stable gene expression and higher CT-value gives less certainty of the gene expression and might be unspecific. We also calculated Delta-CT (ΔCT), which normalizes the expression of target gene against housekeeping gene, as in our case was GAPDH. After every step, RQ was calculated which tells us how many times the target gene is expressed compared to the housekeeping gene.

Delta-Delta-CT (ΔΔCT), tells us how much the gene is expressed after stimulation compared to unstimulated. We also compared the average ΔCT as the unstimulated target gene for both HC-

FLS and RA-FLS was compared to their housekeeping gene, followed by calculations of gene expression (ΔCT) compared to unstimulated target genes as well as housekeeping gene.

The data was the calculated in Graphpad Prism Analytical Software Version 9.0.0, using 2way ANOVA tests followed by Dunnett's multiple comparisons test showing. Significant results are defined as $P<0.05$.

4. Ethics

The ethical aspects for this study are regarding approval of using fibroblast-like synoviocytes, purified from synovial tissue from either healthy individuals or patients with Rheumatoid Arthritis.

Ethics approval number: 573-07 and 1087-16.

5. Results

5.1 TNFα promotes expression of EGFR in FLS

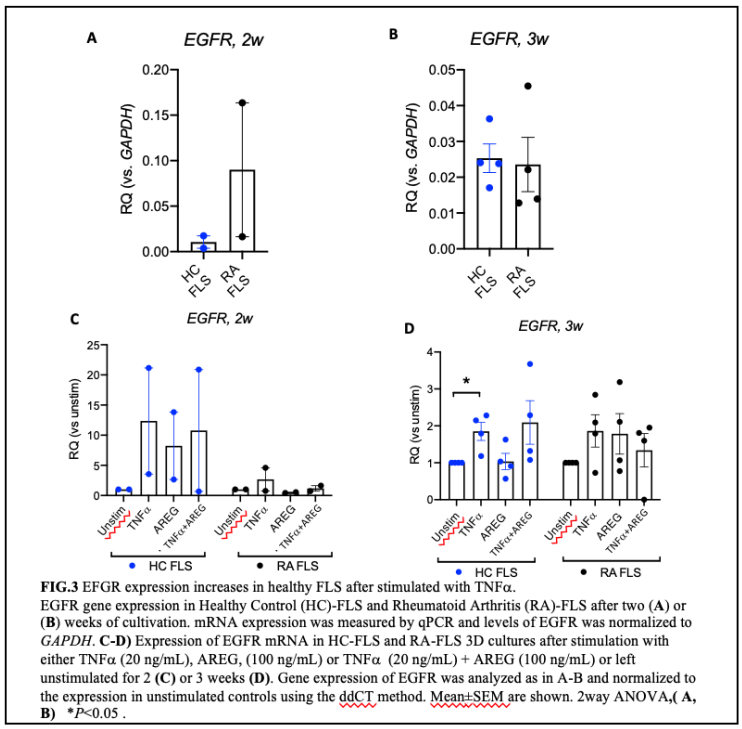

FIG.3 EFGR expression increases in healthy FLS after stimulated with TNFα.
EGFR gene expression in Healthy Control (HC)-FLS and Rheumatoid Arthritis (RA)-FLS after two (**A**) or (**B**) weeks of cultivation. mRNA expression was measured by qPCR and levels of EGFR was normalized to *GAPDH*. **C-D)** Expression of EGFR mRNA in HC-FLS and RA-FLS 3D cultures after stimulation with either TNFα (20 ng/mL), AREG, (100 ng/mL) or TNFα (20 ng/mL) + AREG (100 ng/mL) or left unstimulated for 2 **(C)** or 3 weeks **(D)**. Gene expression of EGFR was analyzed as in A-B and normalized to the expression in unstimulated controls using the ddCT method. Mean±SEM are shown. 2way ANOVA,**(A, B)** **P*<0.05 .

The epidermal growth factor receptor, (EGFR) is known to play an important role for the growth of hyperplastic tissue as it is the receptor for AREG (*36*). In RA, the hyperplastic synovial tissue contributes to the pathogenesis for the disease and the involvement of EGFR has been implicit. ERBB2 (codes for HER-2 receptor, another receptor in the EGFR-family that have been showed to bind AREG) and AREG are important members of the EGF-family (*36*).

Fig 3, A, B, shows indifferent expression of EGFR in HC-FLS and RA-FLS compared to expression level of GAPDH but increased expression of EGFR after stimulated with TNFα (20 ng/mL) and cultivated for three weeks (fig 3, D). Worth mentioning is also what seems to be an increase in EGFR expression in RA-FLS (fig 3, D) after stimulated and incubation for three weeks, however this did not reach significance.

Table 1. Gene expression of ERBB2 show tendencies to decrease after stimulation with TNFα + AREG in healthy FLS, marked by *
Gene expression of ERBB2 and AREG after three weeks of cultivation. Stimulated with TNFα (20 ng/mL), AREG (100 ng/mL), TNFα (20 ng/mL) +AREG (100 ng/mL) or left unstimulated. Mean ±SEM is shown.

	HC-FLS			RA-FLS		
ERBB2	Mean	SEM	N	Mean	SEM	N
UNSTIM	1.000	0.000	3	1.000	0.000	3
TNFα	0.467	0.073	3	0.876	0.054	3
AREG	0.765	0.111	3	1.192	0.260	3
TNFα+AREG	0.475*	0.043	3	0.775	0.081	3
AREG						
UNSTIM	1.000	0.000	3	1.000	0.000	3
TNFα	3.409	4.045	3	4.428	2.538	3
AREG	1.974	1.372	3	4.367	2.941	3
TNFα+AREG	82.795	82.408	3	15.737	10.263	3

Gene expression of ERBB2 (table 1) decreases in HC-FLS after stimulated with TNFα (20 ng/mL) and TNFα (20 ng/mL) + AREG (100 ng/mL), marked with *.

The gene expression of AREG show no tendencies to change after stimulation.

Stimulation of TNFα promotes increased gene expression of EGFR in RA FLS, decreased gene expression of ERBB2 in HC FLS and indifferent gene expression of AREG on both HC-FLS and RA-FLS

5.2 Stimulation with TNFα and AREG show tendencies to increase gene expression of IL6.

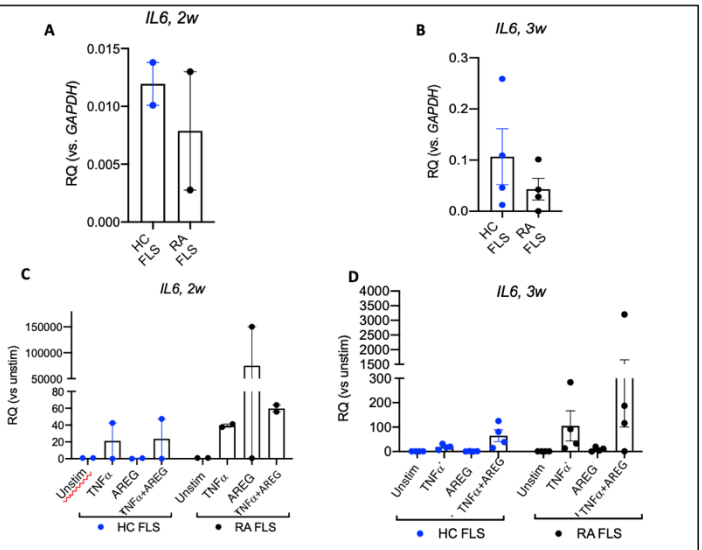

Fig 4. IL6 shows tendencies to increase expression in FLS after stimulation with TNFα and AREG.
A. *IL6* gene expression in Healthy Control (HC)-FLS and Rheumatoid Arthritis (RA)-FLS after two **(A)** or three **(B)** weeks of cultivation. mRNA expression was measured by qPCR and levels of IL6 was normalized to *GAPDH*. **C-D)** Expression of IL6 mRNA in HC-FLS and RA-FLS 3D cultures after stimulation with either TNFα (20 ng/mL), AREG, (100 ng/mL) or TNFα (20 ng/mL) + AREG (100 ng/mL) or left unstimulated for 2 **(C)** or weeks **(D)**. Gene expression of IL6 was analyzed as in A-B and normalized to the expression in unstmulated controls using the ddCT method. **D.** No significant difference were detected of IL-6 after three weeks. Mean±SEM are shown. 2way ANOVA**,(A, B)**

IL6 is an important cytokine in the pathogenesis of RA. It is involved in activation of osteoclast and leukocytes and is a crucial factor for the differentiation of B-cells. Further function is regulation of acute-phase response, anemia of chronic disease and lipid-metabolism (*11*).

Fig 4, C, D, shows tendencies to increased gene expression in both HC-FLS and RA-FLS after stimulated with TNFα (20 ng/mL) and AREG (100 ng/mL) after incubated for both two and three weeks. Stimulation with only TNFα seems to increase the gene expression of IL6, but stimulation with both cytokines, TNFα+AREG show a greater increase (fig 4, D). Since stimulation with only AREG show no increase in gene expression, it is to be believed that AREG potentiate the effect of TNFα.

Further, we also tested cytokines involved in AREG-expression but results of IL7, IL18, IL33, CSF2RB, and TSLP were undetermined. These are cytokines which can influence expression of AREG in joints as they stimulate immune cells that produces AREG and therefor involvement in pathogenesis of Rheumatoid Arthritis can be investigated.

5.3 TNFα increases gene expression of MMP3

Matrix metalloproteinases, MMPs is involved in the pathogenesis of RA and answers for the alterations in ECM with decreased expression of lubricin and collagenases, it also makes alteration on the cartilage-surfaces, permitting invasion and adhesion of FLS (*11*).

In fig 5, A, gene expression of MMP3 show tendencies to be increased in RA-FLS compared to HC-FLS after being incubated unstimulated for three weeks. It also shows increased gene expression in RA-FLS after stimulated with TNFα (20 ng/mL) and cultivated for three weeks (fig 5, B). Similar tendencies were showed in HC-FLS. Only stimulation with AREG do not seem to have any effect on gene expression and stimulation with AREG and TNFα did not show any synergistic effects, AREG do not potentiate TNFα in this case.

MMP9 show no tendencies to be increased after stimulation.

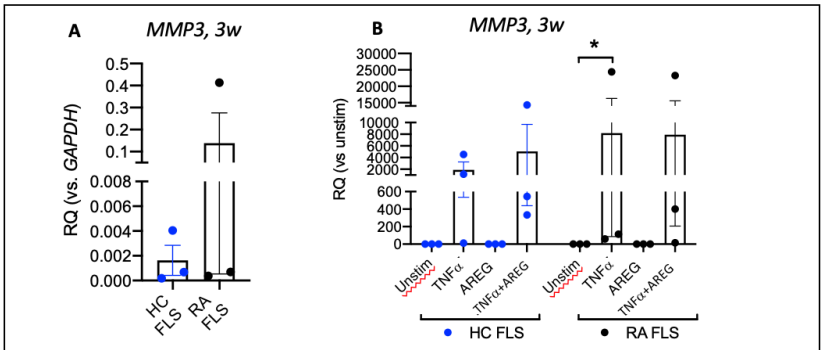

FIG. 5. MMP3 gene expression increase in RA-FLS after stimulation with TNFα.
MMP3 gene expression in Healthy Control (HC)-FLS and Rheumatoid Arthritis (RA)-FLS after 3 **(A)** weeks of cultivation. mRNA expression was measured by qPCR and levels of EGFR was normalized to *GAPDH*. **B)** Expression of MMP3 mRNA in HC-FLS and RA-FLS 3D cultures after stimulation with either TNFα (20 ng/mL), AREG, (100 ng/mL) or TNFα (20 ng/mL) + AREG (100 ng/mL) or left unstimulated for 3 weeks. Gene expression of MMP3 was analyzed as in (A) and normalized to the expression in unstimulated controls using the ddCT method. Mean±SEM are shown. 2way ANOVA, **(A).** *P<0.05 .

Table 2. Stimulation with TNFα and AREG show tendencies to decrease gene expression of MMP11 and MMP13, in HC-FLA and RA-FLS, marked with *.
Gene expression of MMP9, MMP11, MMP13 after three weeks of cultivation. Stimulated with TNFα (20 ng/mL), AREG (100 ng/mL), TNFα (20 ng/mL) +AREG (100 ng/mL) or left unstimulated. Mean ±SEM is shown.

	HC-FLS			RA-FLS		
MMP9	Mean	SEM	N	Mean	SEM	N
UNSTIM	1.000	0.000	3	1.000	0.000	3
TNFα	42.237	22.340	3	75.576	0.524	3
AREG	1.660	0.851	3	1.183	0.493	3
TNFα+AREG	72.626	37.141	3	29.741	11.227	3
MMP11						
UNSTIM	1.000	0.000	3	1.000	0.000	3
TNFα	0.114	0.127	3	0.659	0.681	3
AREG	0.734	0.428	3	3.512	4.804	3
TNFα+AREG	0.133*	0.102	3	0.101	0.034	3
MMP13						
UNSTIM	1.000	0.000	3	1.000	0.000	3
TNFα	0.916	0.492	3	3.085*	5.208	3
AREG	0.306*	0.412	3	4.619*	6.516	3
TNFα+AREG	2.150*	3.092	3	1.076	1.647	3

Gene expression of MMP11 decreases in HC-FLS after stimulated with TNFα (20 ng/mL) + AREG (100ng/mL) for three weeks, visible in table 2, marked with *.
Also, visible in table 2, MMP13 show tendencies to increase in RA-FLS after stimulation and decrease in HC-FLS after stimulated with TNFα and AREG separately. Stimulation with TNFα + AREG show increased gene expression.

We also studied the gene expression of MMP1, MMP2, MMP10, MMP14 and MMP17, which was undetermined. Normally, MMP1 and MMP2 is detected in FLS but since our results did not showed any detection of them, it might be due to too low gene expression not visible for detection or due to low function of primers being used.

Unstimulated, gene expression of MMP3 increases in RA-FLS. After stimulated with TNFα increased gene expression showed in both HC-FLS and RA-FLS. MMP9 show indifferent gene expression after stimulated, MMP11 show decreased gene expression in HC-FLS and MMP13 show increased gene expression in RA-FLS and HC-FLS, after stimulated with both TNFα and AREG. Decreased gene expression in HC-FLS after stimulated separately.

5.4 TNFα+AREG increases expression of monocyte attracting chemokines by FLS

Chemokines serves an important function in our immune response as it is responsible for attraction of immune cells to tissues (*39*). CC-chemokine ligand-2, CCL2 is responsible for the attraction of monocyte of FLS in RA. After recruiting the cells from the vasculature, monocytes often differentiation into macrophages, which are the main source of TNFα in the synovium. In turn, they activate FLS to produce pro-inflammatory chemokines, cytokines and matrix metalloproteinases. By that, CCL2 is a major contributor to the pathogenesis of RA (*14*).

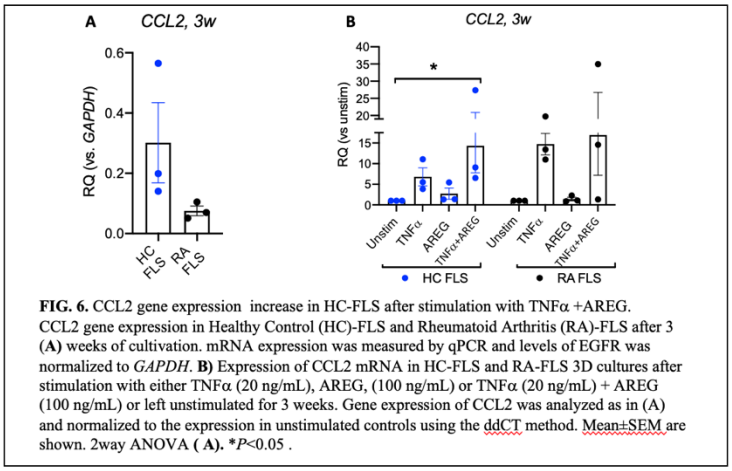

FIG. 6. CCL2 gene expression increase in HC-FLS after stimulation with TNFα +AREG. CCL2 gene expression in Healthy Control (HC)-FLS and Rheumatoid Arthritis (RA)-FLS after 3 **(A)** weeks of cultivation. mRNA expression was measured by qPCR and levels of EGFR was normalized to *GAPDH*. **B)** Expression of CCL2 mRNA in HC-FLS and RA-FLS 3D cultures after stimulation with either TNFα (20 ng/mL), AREG, (100 ng/mL) or TNFα (20 ng/mL) + AREG (100 ng/mL) or left unstimulated for 3 weeks. Gene expression of CCL2 was analyzed as in (A) and normalized to the expression in unstimulated controls using the ddCT method. Mean±SEM are shown. 2way ANOVA **(A).** *P<0.05 .

Table 3. Gene expression of CXCL1 show tendencies to increase after stimulation.
Gene expression of CXCL1 and CXCL8 after three weeks of cultivation. Stimulated with TNFα (20 ng/mL), AREG (100 ng/mL), TNFα (20 ng/mL) +AREG (100 ng/mL) or left unstimulated. Mean ± SEM is shown.

	HC-FLS			RA-FLS		
CXCL1	Mean	SEM	N	Mean	SEM	N
UNSTIM	1.000	0.000	3	1.000	0.000	3
TNFα	702.860	379.719	3	698.248	333.063	3
AREG	2.527	0.817	3	4.955	3.004	3
TNFα+AREG	2598.399	2323.191	3	798.125	551.748	3
CXCL8						
UNSTIM	1.000	0.000	3	1.000	0.000	3
TNFα	901.071	616.755	3	1960.962	1464.299	3
AREG	1.483	0.324	3	1.571	0.481	3
TNFα+AREG	5773.018	5414.658	3	572.405	516.538	3

Fig 6, A, shows results of increased gene expression of CCL2 in HC-FLS after stimulated with TNFα (20 ng/mL) + AREG (100 ng/mL) and cultivated for three weeks. It is to be believed that AREG potentiate the effect of TNFα as gene expression after stimulation with only TNFα also seems to be increased but not significantly.

RA-FLS show tendencies to increased gene expression after stimulated with TNFα. In table 3, CXCL1 show tendencies to increase equally in HC-FLS and RA-FLS after stimulation but no significant difference was found.

CXCL8, which is responsible for attraction of neutrophils (*4*), show no tendencies to be increased after stimulation.

Results of CXCL10 were undetermined.

CCL2 show increased gene expression in HC-FLS after stimulated with TNFα+AREG. No tendencies are shown for increased gene expression of CXCL1 or CXCL8 after stimulation.

5.5 TNFα reduce expression of COL1A1 in FLS

COL1A1 and COL1A2 are involved in the production of collagen, primarily type I collagen, which is found in most connective tissues and is involved in the production of bone and tendons (*40, 41*).

Figure 7, B, shows results of decreased expression of COL1A1 in RA-FLS after stimulated with TNFα (20ng/mL) + AREG (100ng/mL) and cultivated for three weeks.

In table 4, gene expression of COL1A2 show tendencies to decrease after stimulation but no significant result was found.

ACTA2, codes for Alpha Smooth Muscle Actin, which is usually used as a marker for whether a fibroblast has differentiated into a collagen producing myofibroblast (*41*). Tendencies of decreased gene expression after stimulation with TNFα is shown in both HC-FLS and RA-FLS, visible in tab 4, marked with *.

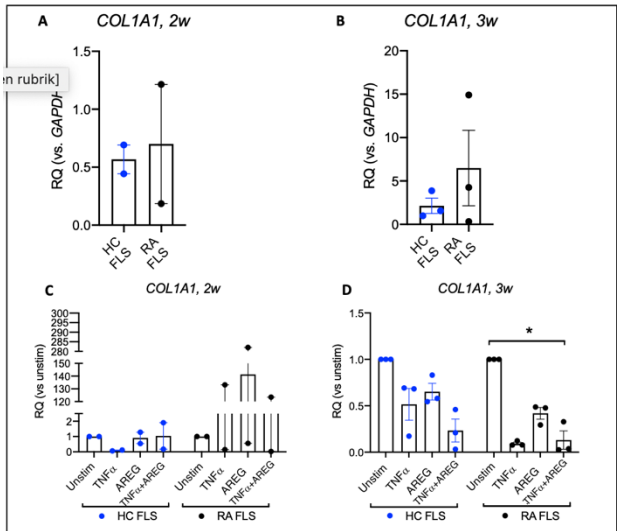

FIG 7. Gene expression of COL1A1 decrease in RA-FLS after stimulated with TNFα +AREG.
COL1A1 gene expression in Healthy Control (HC)-FLS and Rheumatoid Arthritis (RA)-FLS after two (**A**) or three (**B**) weeks of cultivation. mRNA expression was measured by qPCR and levels of COL1A1 was normalized to *GAPDH*. **C-D)** Expression of BIRC5 mRNA in HC-FLS and RA-FLS 3D cultures after stimulation with either TNFα (20 ng/mL), AREG, (100 ng/mL) or TNFα (20 ng/mL) + AREG (100 ng/mL) or left unstimulated for 2 **(C)** or 3 weeks **(D)**. Gene expression of COL1A1 was analyzed as in A-B and normalized to the expression in unstimulated controls using the ddCT method. Mean±SEM are shown. 2way ANOVA,**(A, B)** . **P*<0.05 .

Table 4. Gene expression of ACTA2 show tendencies to decrease after stimulation with TNFα, marked with *.
Gene expression of COL1A1 and ACTA2 after three weeks of cultivation. Stimulated with TNFα (20 ng/mL), AREG (100 ng/mL), TNFα (20 ng/mL) +AREG (100 ng/mL) or left unstimulated. Mean ± SEM is shown.

	HC-FLS			RA-FLS		
COL1A2	Mean	SEM	N	Mean	SEM	N
UNSTIM	1.000	0.000	3	1.000	0.000	3
TNFα	0.327	0.058	3	0.424	0.103	3
AREG	1.172	0.456	3	0.845	0.304	3
TNFα+AREG	0.237	0.047	3	0.697	0.525	3
ACTA2						
UNSTIM	1.000	0.000	3	1.000	0.000	3
TNFα	0.595*	0.178	3	0.757*	0.098	3
AREG	1.481	0.581	3	1.047	0.412	3
TNFα+AREG	0.702	0.255	3	0.644	0.348	3

Decreased gene expression is shown in COL1A1 after stimulation with both TNFα+AREG in RA-FLS. Gene expression of ACTA2 seems to be decreased in both HC-FLS and RA-FLS after stimulation.

5.6 TNFα show tendency to increase expression of BIRC5 in FLS

Baculoviral inhibitor of apoptosis repeat-containing 5, BIRC5, is a gene that promotes production of cytokines and growth factors as well as inhibits apoptosis. BIRC5 codes for the protein Survivin, which in synovial fluid is found in a higher level in RA patients (*42*).

BIRC5 is also known to promote a more aggressive phenotype of FLS due to the increase in quantity of cells which becomes a component of the hyperplastic synovial tissue in RA (*19*).

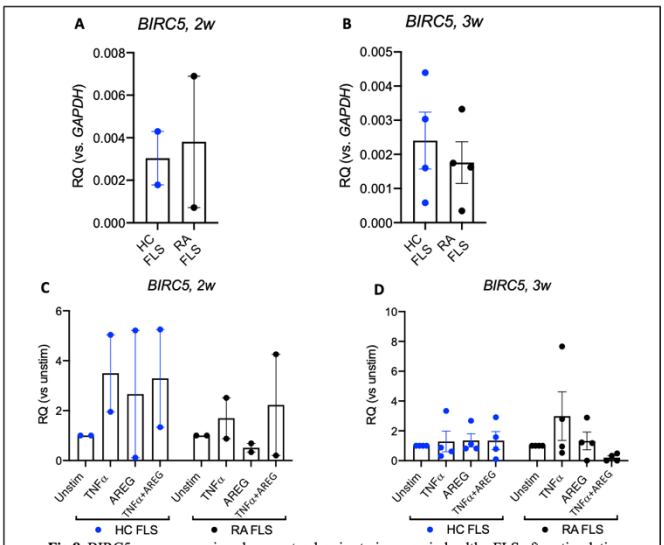

Fig 8. BIRC5 gene expression show no tendencies to increase in healthy FLS after stimulation. BIRC5 gene expression in Healthy Control (HC)-FLS and Rheumatoid Arthritis (RA)-FLS after two (**A**) or three (**B**) weeks of cultivation. mRNA expression was measured by qPCR and levels of ERBB2 was normalized to *GAPDH*. **C-D)** Expression of BIRC5 mRNA in HC-FLS and RA-FLS 3D cultures after stimulation with either TNFα (20 ng/mL), Amphiregulin (AREG, 100 ng/mL) or TNFα (20 ng/mL) + AREG (100 ng/mL) or left unstimulated for 2 **(C)** or 3 weeks **(D)**. Gene expression of BIRC5 was analyzed as in A-B and normalized to the expression in unstimulated controls using the ddCT method. Mean±SEM are shown. 2way ANOVA,**(A, B)**

In fig 8, B, results show tendencies to increased gene expression of BIRC5 in RA-FLS after stimulated with TNFα (20 ng/mL) and cultivated for two and further, three weeks.

MKI67 is a gene that codes for cell proliferation (*43*). Results show, in table5, tendencies to increase in RA-FLS after stimulation with TNFα (20 ng/mL), marked with *.

Table 5. Gene expression of MKI67 show tendencies to increase after stimulation with TNFα in RA-FLS marked with *.
Gene expression of MKI67 after three weeks of cultivation. Stimulated with TNFα (20 ng/mL), AREG (100 ng/mL), TNFα (20 ng/mL) +AREG (100 ng/mL) or left unstimulated. Mean ± SEM is shown.

	HC-FLS			RA-FLS		
MKI67	Mean	SEM	N	Mean	SEM	N
UNSTIM	1.000	0.000	3	1.000	0.000	3
TNFα	1.968	1.1317	3	5.053*	3.966	3
AREG	1.911	0.892	3	2.174	0.768	3
TNFα+AREG	1.972	1.212	3	0.323	0.176	3

As gene expression of Birc5 show tendencies to be increased after stimulation with TNFα in RA-FLS and as well for MKI67, this speaks for increased proliferation and increased production of cytokines which plays an important part in the pathogenesis of the disease.

5.7 Cross section of three dimensional FLS-cultures

Histology slides of HC-FLS and RA-FLS after stained with hematoxylin and eosin, which is the most commonly used staining technique. Hematoxylin stains acidic structure, for example the nucleus, purple and eosin stains basic structure, for example the cytoplasm, pink (*44*).

Fig 9 show cross section of HC-FLS. A, showing HC-FLS unstimulated with a round structure with FLS in an outer thin layer consisting of approximately 2-5 cells. B, showing HC-FLS stimulated with TNFα with FLS in a thicker layer on the border of the structure. White structures are presumable air bubbles from when making the 3D-culture, making it hard to determine if numbers of FLS has increased in same proportions compared to the other stimulations.

C, showing HC-FLS stimulated with AREG with a thicker outer layer of FLS, and a denser inner structure of what seems to be a mix of irregular ECM and FLS and lastly D, showing HC-FLS stimulated with TNFα+AREG with a visible denser outer layer of FLS.

Results show increased number of FLS in 3D-border after stimulated with AREG and TNFα+AREG (C, D).

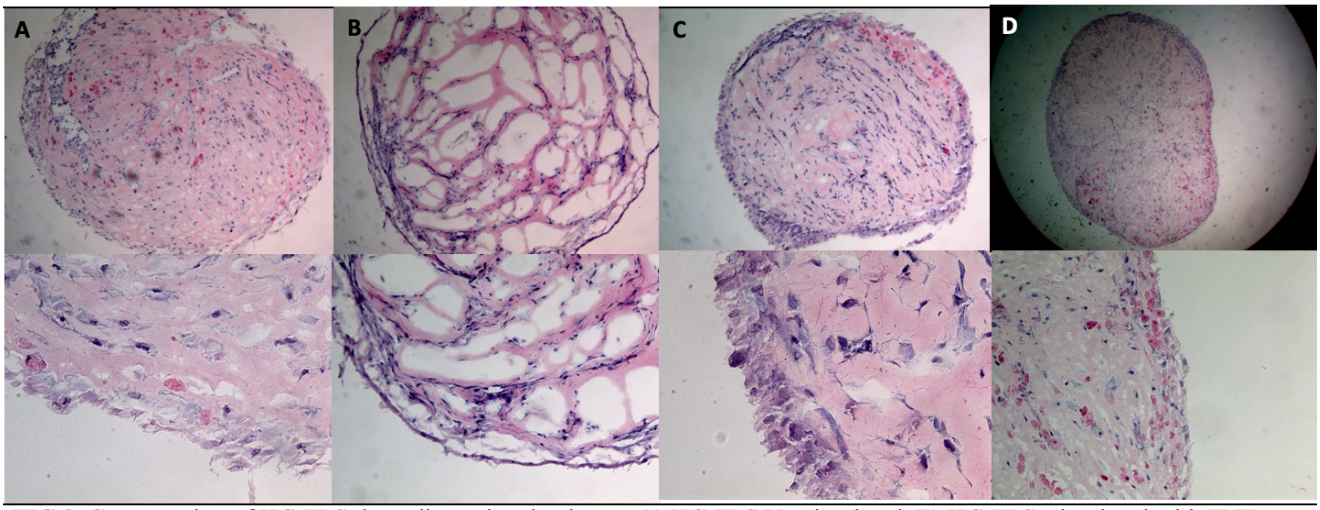

FIG.9. Cross section of HC-FLS three dimensional cultures. **A)** HC-FLS Unstimulated. **B)** HC-FLS stimulated with TNFα (20 ng/mL). **C)** HC-FLS stimulated with AREG, (100 ng/mL). **D)** HC-FLS stimulated with TNFα (20 ng/mL) + AREG (100 ng/mL).

Fig 10 showing cross section of RA-FLS in 3D-cultures. A, unstimulated RA-FLS, with a larger area of ECM with FLS organized in a loose irregular pattern. B, RA-FLS stimulated with TNFα showing a denser outer layer of FLS and a smaller area of ECM. C, RA-FLS stimulated with AREG. Similarly to that seen in HC-FLS, here is visible a thicker outer layer of FLS with a loose ECM in the middle. Lastly D, RA-FLS stimulated with TNFα + AREG. This slide show a denser area of FLS located in the middle and the ECM is moved to the outer part of the 3D-structure. Results show increased FLS in the sublining layer of synovium after stimulation with TNFα and AREG.

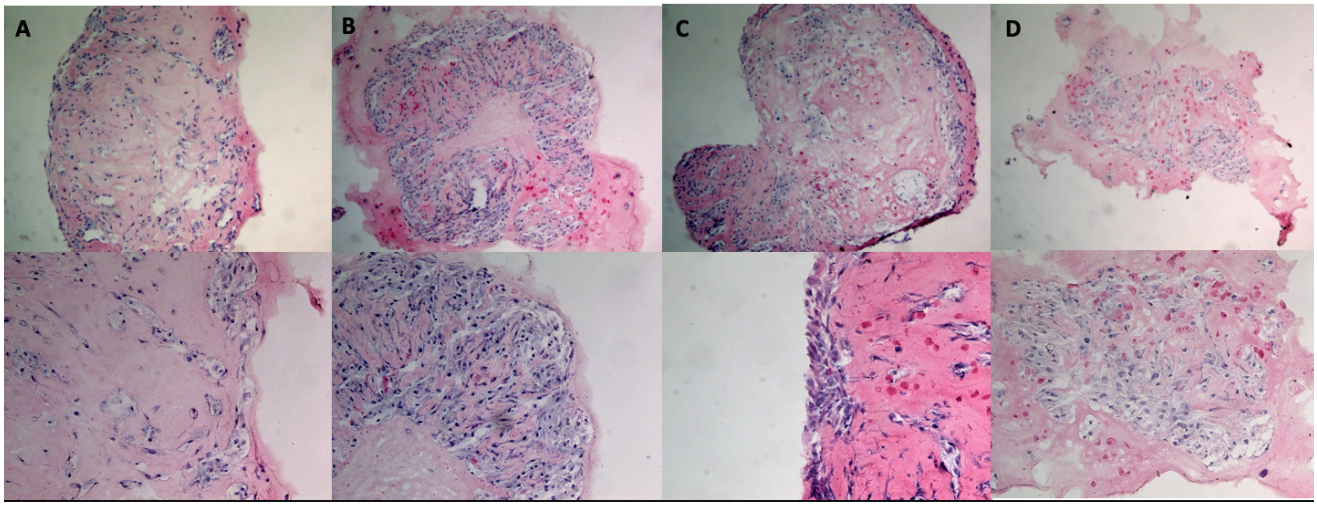

FIG.10. Cross section of RA-FLS three dimensional cultures. **A)** RA-FLS Unstimulated. **B)** RA-FLS stimulated with TNFα (20 ng/mL). **C)** RA-FLS stimulated with AREG, (100 ng/mL). **D)** RA-FLS stimulated with TNFα (20 ng/mL) + AREG (100 ng/mL).

6. Discussion

Rheumatoid arthritis, one of the most common autoimmune systemic disease, is very complex. The pathogenesis is multifactorial, involving environmental factors, susceptible genes, epigenetic modification and loss of tolerance, leading to a T-and B cells response and in turn, an inflammatory response. The immune system, both innate and adaptive, causes tissue response and degradation of cartilage and bones. Synovitis, caused by leukocyte infiltration and cell migration, which enables local fibroblast activation, increased expression of molecules and cytokines increases buildup of inflammatory tissue (*45*).

Fibroblast-like synoviocytes, FLS, located in the synovium of joints is found to be a more aggressive type when the disease is present. FLS produces lubricin, provide structure and maintain the ECM and is found in a higher number of cells in rheumatoid Arthritis, causing alterations and instability in the joints by destruction of cartilage, increased production of collagen degrading proteins and increased infiltration of cells (*19*).

6.1 Cultivation of FLS in three-dimensional structures

Growing cells in 3D cultures enhances the ability to study the cells functions. Results from previous study show that after being cultured in 3D, functional characteristics, such as production of lubricin and changes in ECM, simulate the same characteristics that FLS possesses in vivo (*30*). The impact of stimulation with AREG and TNFα both separately and together in cultivation of FLS is to be seen on various gene expression as well as in the growth of the 3D-cultures. Since the key events of the pathogenesis in RA is cell infiltration, chronic inflammation, destruction of cartilaginous structures and bones and inhibition of normal cell functions involved in autoimmunity, the genes chosen to study is major contributors to these events. Previous study

show that FLS cultivated in Matrigel have an intrinsic capacity to recreate synovial architecture (*32*), together with results of for examples, increased expression of genes involved in the alterations in ECM, cartilage production and cell proliferation, it is to be believed that this correlates whit what is visible in the slides. As the architecture of the culture changes in the slides with a thicker outer layer of FLS with stimulation of TNFα, AREG and combined stimulation of TNFα + AREG together with alterations in the ECM it correlates with previous studies.
The formation of the outer thicker layer of the 3D-structure with multiple cells after stimulation with for examples AREG is comparable to what is known in the pathophysiology of the disease and the formation of pannus. Further, this can indicate that cultivation of FLS in 3D more resembles the in vivo formation and characteristics of the cells which can develop further understanding for the disease.

6.2 TNFα induces a ECM degrading phenotype in FLS grown in 3D culture

TNFα might induce a severe phenotype of FLS due to changed gene expression but as similar results is seen in HC-FLS as well as RA-FLS, it is to be believed that TNFα not only matter for RA but more in general for FLS, especially since levels of TNFα is found already at early stages of the disease. Stimulation with TNFα, one of the major cytokines involved in Rheumatoid Arthritis cartilage metabolism, resulted in increased gene expression of EGFR in HC-FLS. As EGFR increases after stimulation with TNFα, thus causing increased sensitivity for ligands such as EGF, AREG and HB-EGF this might cause cell proliferation and growth (*36*). As well, results show increased gene expression of MMP3, BIRC5 and MIKI67 in RA-FLS. Previous studies have shown that stimulating FLS with TNFα induced condensation of cells and contributes to

increased MMP expression, leading to alterations of ECM but only a small number of cytokines and chemokines were expressed differently (*30*).

Since TNFα increases expression of EGFR, IL6 and MMP and decreases expression of COL1A1 and COL1A2 which codes for production of collagen it is to be believed that TNFα induces a joint degrading phenotype of FLS.

Since the increased gene expression due to stimulation with TNFα is responsible for growth, proliferation of cells and alterations in ECM it speaks for the more aggressive phenotype here as well. Correlations with previous results indicates that TNFα is one of the important key-players for the destruction of the disease.

The results from the gene expressions is consistent with what is shown in the slides from the 3D-structures. As a thicker layer of FLS is visible, and it is shown that with stimulation of TNFα, a lesser amount/ smaller area of ECM is visible and centrally located. Combining result of gene expression and slides it is to be believed that increased expression om MMP3, together with decreased expression ACTA2, coding for alpha smooth muscle actin which is a marker of myelofibroblast, and decreased expression of COL1A1 and COL1A2 is responsible for the alterations in ECM.

6.3 AREG might promote increased proliferation of FLS

Previous studies of AREGs impact in FLS show result of increased levels of AREG in RA, enhanced proliferation capacities, with an increased expression of ERBB2, increased levels of expression of inflammatory cytokines, such as IL6 and angiogenic factors (*36*). Compared to the

previous study, our results correlate even though it is not significant. In several genes, the expression increases but no significant results were detected after stimulation with AREG alone. Tendencies to increased gene expression might indicate that AREG is important but has a more potentiating effect together with TNFα than alone in this case. On the slides, a thicker layer of FLS is located on the border, which can be due to greater cell proliferation as of MKI67-expression induced by AREG. Between cells, a looser mesh of ECM is visible, and is likely to be the result of increased expression of matrix metalloproteins (MMP).

6.4 Effects of combined stimulation with TNFα and AREG

TNFα increases expression of EGFR, the receptor for AREG, in FLS and thus also increases sensitivity to AREG, which leads to cell growth and proliferation. Results showing a potentially potentiating effect of AREG for TNFα, and by that making combined stimulations important to study.

Stimulation with TNFα + AREG decreases gene expression of COL1A1 in RA-FLS, and therefore production of type I collagen. MMP11, which is involved in alterations of ECM and cartilage surfaces (*11*) is also decreased. CCL2, which contributes to a greater attraction of monocytes has an increased gene expression in HC-FLS after stimulation with TNFα and AREG. Since osteoclast derives from monocytes this might contribute to increased infiltration of cells as well as faster breakdown of bone structures. For IL6, results show a potentiating effect of AREG when combined stimulation, but no increased expression is visible when used separately. Previous study show results of increased production of IL-6 in the presence of TNFα (*46*) and the potentiating effect of AREG is visible as the gene expression increase only with stimulation with TNFα but even more with combined stimulation.

As fibroblast secrete IL6 it is to be believed that feedback loops are initiating, causing the FLS to stimulate itself to increase gene expression and proliferation further. This may lead to a more severe disease and it clarifies the benefits of make FLS a target for treatment. The results from combined stimulation of FLS indicates AREGs important role in the pathology in RA, as gene expression of several genes is increased after stimulation with TNFα alone but even more after combined stimulation.

The combining effect of increased expression due to stimulation with TNFα and AREG, together, potentially enhances damage in joints as it increases infiltration of monocytes and therefore osteoclast as its specific function is break down bone tissue.

The impact of TNFα + AREG is also visible on the slides from the 3D-structures, showing an even thicker layer of FLS on the border compared to stimulated separately, but as well, a denser area with cells is found in the middle of the slide. It indicates the benefits from growing FLS in 3D-structures as well as it become more visible on a microscopical point of view how and what FLS react to the stimulation.

6.5. Strengths, limitations and further studies

The strengths of this study are firstly that several different cell lines from different individuals are being used, which makes it possible to study individual variability. This method makes it possible to study the cells in its natural environment as 3D cultures were used, which also allows for studies of histological differences.

However, for further studies improvements can be made.

Firstly, corrections of number of cells being used for cultivations of 3D-cultures can be made, making it possible to improve and correct concentration of RNA used for generation of cDNA. Secondly, the quality of primers being used for PCR might be studied thus genes which is expected to be found in FLS showed undetermined results. With greater time interval, the opportunity to re-test and evaluate quality might be necessary to obtain potentially significant results.

Thirdly, a greater number of 3D-cultures could be made as the cells in several cultures did not grow properly or 3D-cultures were not being formed.

Lastly, future studies could be inclusive of immune cells in the culture, such as AREG producing type-2 innate lymphoid cells for developing greater understanding.

7. Conclusion

This study show that TNFα and AREGs impact gene expression and growth of FLS in 3D-structures.

TNFα induces FLS expansion and expression of inflammatory and cartilage degrading genes. AREG promotes cell proliferation and potentiates TNFα induced expression in FLS. We confirm that TNFα promotes an aggressive FLS phenotype which can be potentiated by AREG. Lastly, we conclude that HC-and RA-FLS respond similar to stimulation with TNFα and AREG. Cultivation of FLS in 3D-structures highlight mechanism in RA needed for further understanding and therapy targeting, making it possible to improve current treatment regime and prevent further sickness.

Thus, the study show no differences between FLS from RA compared to FLS form healthy individuals regarding gene-expression, response to stimulation with TNFα and AREG. This suggests that these cytokines might act early in the disease before FLS have acquired their unique epigenetic profile.

Populärvetenskaplig sammanfattning

3D-odlade-ledfibroblasters roll i Ledgångsreumatism

Jennie Lindau, Examensarbete, Läkarprogrammet på Göteborgs Universitet, Avdelning för reumatologi och inflammationsforskning. Göteborg, Sverige, 2021.

Ledgångsreumatism är en sjukdom som drabbar ca 0.5-1% av befolkningen och är vanligast hos kvinnor. De klassiska symtomen består inledningsvis av allmän sjukdomskänsla, feber, stelhet och smärta för att i senare skede leda till felställda och deformerade fingrar och leder.
Idag finns en god behandling av ledgångsreumatism bestående av framförallt DMARDS, en läkemedelsgrupp som förändrar sjukdomen och immunförsvaret med syfte att lindra symtom, men en stor del av patienterna blir inte hjälpta av detta och har fortsatta besvär av sin sjukdom.

Bakgrunden till sjukdomen innefattar ett komplext samband av vårt immunförsvar och kroppens förmåga att attackera sig själv, s.k. autoimmunitet. Efter att immunförsvaret aktiverats fortlöper sjukdomen dels genom inflammation och dels genom nedbrytning av brosk och ben.
De celler som har visat sig spela en stor roll i sjukdomen är ledfibroblaster. Det är en typ av celler, s.k. strukturceller som finns i våra leder och som i tidigare studier har visat sig vara mer aggressiva och förstörande hos patienter med ledgångsreumatism jämfört med friska individer. Amphiregulin är ett protein som kan aktivera ledfibroblaster och öka utsöndring av nedbrytande enzym. Det har hittats i ökade nivåer hos patienter med sjukdomen men dess specifika roll i är fortfarande oklar.

Tumor necrosis factor α, är en signalsubstans, med funktion att inleda celldelning samt produktion av enzym som bidrar till nedbrytning av leder, vilken också har visat sig ha en central roll i sjukdomen.

Odling av ledfibroblaster i 3D-kultur efterliknar cellernas naturliga miljö och kan därför främja studier av cellernas funktion. Det gör det även lättare att studera cellerna mikroskopiskt för att se skillnader i cellernas uppbyggnad. Efter att ha odlat cellerna studerades uttryck av olika gener vars funktioner kan påverka sjukdomsförloppet.

Syftet med denna studien är att undersöka hur ledfibroblaster från patienter med ledgångsreumatism skiljer sig avseende växt, svar på stimulering samt genuttryck, jämfört med ledfibroblaster från friska individer, efter att ha odlats i 3D-kultur.
Resultatet visar bland annat ökat uttryck av gener som orsakar cellförökning, bibehållen inflammation och förändringar i substansen som finns mellan våra celler samt tendenser till ökat uttryck av en gen som kodar för ökad nedbrytning av ben.

Konklusionen av studien är att stimulering med Tumor necrosis factor α, och Amphiregulin orsakar tillväxt, infiltration av inflammations-och brosknedbrytande gener samt främjar delning av ledfibroblaster. Resultaten tyder på, liksom i tidigare studier, en mer aggressivare typ av ledfibroblaster hos patienter med ledgångsreumatism, vilket utgör ett potentiellt framtida behandlingsmål.

Acknowledgements

I would like to express my deepest appreciation to my supervisor, Mattias Svensson, for making this thesis possible. Thank you for all advice and outstanding supervision, for always being available and answering my questions.

I would also like to thank my examiner Tao Jin for listening to my oral presentation and giving good feedback.

Lastly, I would like to thank Mathilda Nilsson for opponent and good feedback

References

1. M. N. D. Svensson *et al.*, Synoviocyte-targeted therapy synergizes with TNF inhibition in arthritis reversal. *Sci Adv* **6**, eaba4353 (2020).
2. B. Bartok, G. S. Firestein, Fibroblast-like synoviocytes: key effector cells in rheumatoid arthritis. *Immunol Rev* **233**, 233-255 (2010).
3. G. S. Firestein, Evolving concepts of rheumatoid arthritis. *Nature* **423**, 356-361 (2003).
4. J. A. Alberts B, Lewis J, Raff M, Roberts K, Walter P, in *The Molecular Biology of the Cell*. (Garlnad Science, New York, 2008).
5. L. Klareskog, A. I. Catrina, S. Paget, Rheumatoid arthritis. *Lancet* **373**, 659-672 (2009).
6. R. Medzhitov, Recognition of microorganisms and activation of the immune response. *Nature* **449**, 819-826 (2007).
7. H. K. Lee, A. Iwasaki, Innate control of adaptive immunity: dendritic cells and beyond. *Semin Immunol* **19**, 48-55 (2007).
8. G. S. B. R. C. Firestein. (Elsevier, 2017), vol. 1.
9. L. S. Klareskog, T, in *Reumatologi*. (Studentlitteratur AB).
10. G. S. B. R. C. Firestein. (Elsevier, 2017), vol. 2.
11. I. B. McInnes, G. Schett, The pathogenesis of rheumatoid arthritis. *N Engl J Med* **365**, 2205-2219 (2011).
12. G. D. Jay, K. A. Waller, The biology of lubricin: near frictionless joint motion. *Matrix Biol* **39**, 17-24 (2014).
13. D. A. Swann, F. H. Silver, H. S. Slayter, W. Stafford, E. Shore, The molecular structure and lubricating activity of lubricin isolated from bovine and human synovial fluids. *Biochem J* **225**, 195-201 (1985).
14. G. Nygaard, G. S. Firestein, Restoring synovial homeostasis in rheumatoid arthritis by targeting fibroblast-like synoviocytes. *Nat Rev Rheumatol* **16**, 316-333 (2020).
15. F. Sabeh, D. Fox, S. J. Weiss, Membrane-type I matrix metalloproteinase-dependent regulation of rheumatoid arthritis synoviocyte function. *J Immunol* **184**, 6396-6406 (2010).
16. E. H. Noss, M. B. Brenner, The role and therapeutic implications of fibroblast-like synoviocytes in inflammation and cartilage erosion in rheumatoid arthritis. *Immunol Rev* **223**, 252-270 (2008).
17. U. Muller-Ladner, T. Pap, R. E. Gay, M. Neidhart, S. Gay, Mechanisms of disease: the molecular and cellular basis of joint destruction in rheumatoid arthritis. *Nat Clin Pract Rheumatol* **1**, 102-110 (2005).
18. P. S. Burrage, K. S. Mix, C. E. Brinckerhoff, Matrix metalloproteinases: role in arthritis. *Front Biosci* **11**, 529-543 (2006).
19. N. Bottini, G. S. Firestein, Duality of fibroblast-like synoviocytes in RA: passive responders and imprinted aggressors. *Nat Rev Rheumatol* **9**, 24-33 (2013).
20. R. Lafyatis *et al.*, Anchorage-independent growth of synoviocytes from arthritic and normal joints. Stimulation by exogenous platelet-derived growth factor and inhibition by transforming growth factor-beta and retinoids. *J Clin Invest* **83**, 1267-1276 (1989).
21. T. C. Tolboom *et al.*, Invasive properties of fibroblast-like synoviocytes: correlation with growth characteristics and expression of MMP-1, MMP-3, and MMP-10. *Ann Rheum Dis* **61**, 975-980 (2002).
22. M. C. Miller *et al.*, Membrane type 1 matrix metalloproteinase is a crucial promoter of synovial invasion in human rheumatoid arthritis. *Arthritis Rheum* **60**, 686-697 (2009).
23. A. J. Naylor, A. Filer, C. D. Buckley, The role of stromal cells in the persistence of chronic inflammation. *Clin Exp Immunol* **171**, 30-35 (2013).
24. A. Lee *et al.*, Tumor necrosis factor alpha induces sustained signaling and a prolonged and unremitting inflammatory response in rheumatoid arthritis synovial fibroblasts. *Arthritis Rheum* **65**, 928-938 (2013).

25. K. Migita *et al.*, TNF-alpha-induced miR-155 regulates IL-6 signaling in rheumatoid synovial fibroblasts. *BMC Res Notes* **10**, 403 (2017).
26. Z. Jiao *et al.*, Notch signaling mediates TNF-alpha-induced IL-6 production in cultured fibroblast-like synoviocytes from rheumatoid arthritis. *Clin Dev Immunol* **2012**, 350209 (2012).
27. H. Perlman *et al.*, IL-6 and matrix metalloproteinase-1 are regulated by the cyclin-dependent kinase inhibitor p21 in synovial fibroblasts. *J Immunol* **170**, 838-845 (2003).
28. J. H. Humphreys *et al.*, Rheumatoid factor and anti-citrullinated protein antibody positivity, but not level, are associated with increased mortality in patients with rheumatoid arthritis: results from two large independent cohorts. *Arthritis Res Ther* **16**, 483 (2014).
29. X. Valencia *et al.*, Cadherin-11 provides specific cellular adhesion between fibroblast-like synoviocytes. *J Exp Med* **200**, 1673-1679 (2004).
30. H. P. Kiener *et al.*, Synovial fibroblasts self-direct multicellular lining architecture and synthetic function in three-dimensional organ culture. *Arthritis Rheum* **62**, 742-752 (2010).
31. C. Ritchlin, Fibroblast biology. Effector signals released by the synovial fibroblast in arthritis. *Arthritis Res* **2**, 356-360 (2000).
32. H. P. Kiener, D. M. Lee, S. K. Agarwal, M. B. Brenner, Cadherin-11 induces rheumatoid arthritis fibroblast-like synoviocytes to form lining layers in vitro. *Am J Pathol* **168**, 1486-1499 (2006).
33. S. Fillatreau, Cytokine-producing B cells as regulators of pathogenic and protective immune responses. *Ann Rheum Dis* **72 Suppl 2**, ii80-84 (2013).
34. T. W. LeBien, T. F. Tedder, B lymphocytes: how they develop and function. *Blood* **112**, 1570-1580 (2008).
35. D. R. Herbert, B. Douglas, K. Zullo, Group 2 Innate Lymphoid Cells (ILC2): Type 2 Immunity and Helminth Immunity. *Int J Mol Sci* **20**, (2019).
36. S. Yamane *et al.*, Proinflammatory role of amphiregulin, an epidermal growth factor family member whose expression is augmented in rheumatoid arthritis patients. *J Inflamm (Lond)* **5**, 5 (2008).
37. . (UniPro, https://www.uniprot.org/uniprot/P15514).
38. A. K. Ekwall *et al.*, The tumour-associated glycoprotein podoplanin is expressed in fibroblast-like synoviocytes of the hyperplastic synovial lining layer in rheumatoid arthritis. *Arthritis Res Ther* **13**, R40 (2011).
39. A. H. L. Abul K. Abbas, Shiv Pillai, in *Cellular and Molecular Immunology*. (Elsevier Saunders, 2015).
40. NCBI. (NCBI, https://ncbi.nlm.nih.gov/gene/1277.).
41. B. Hinz, Myofibroblasts. *Exp Eye Res* **142**, 56-70 (2016).
42. D. Chen *et al.*, Rheumatoid Arthritis Fibroblast-like Synoviocyte Suppression Mediated by PTEN Involves Survivin Gene Silencing. *Sci Rep* **7**, 367 (2017).
43. V. Badot *et al.*, Gene expression profiling in the synovium identifies a predictive signature of absence of response to adalimumab therapy in rheumatoid arthritis. *Arthritis Res Ther* **11**, R57 (2009).
44. K. L. Suvarna, C. Bancroft, B., in *Bancroft's Theory and Practice of Histological Techniques*. (Churchill Livingstone Elsevier, 2013), pp. 173.
45. R. Bucala, C. Ritchlin, R. Winchester, A. Cerami, Constitutive production of inflammatory and mitogenic cytokines by rheumatoid synovial fibroblasts. *J Exp Med* **173**, 569-574 (1991).
46. P. A. Guerne, B. L. Zuraw, J. H. Vaughan, D. A. Carson, M. Lotz, Synovium as a source of interleukin 6 in vitro. Contribution to local and systemic manifestations of arthritis. *J Clin Invest* **83**, 585-592 (1989).