Synovial fibroblast activation in rheumatoid arthritis

Role of disease-associated risk genes and effects of methotrexate

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Petit à petit, l'oiseau fait son nid

ABSTRACT

The overall aim of this thesis was to increase the knowledge of molecular mechanisms shaping the pathogenic phenotype of activated fibroblast-like synoviocytes (FLS), which are drivers of chronic inflammation and destruction of the joints in rheumatoid arthritis (RA). As a main approach, we studied the role of previously identified multi-evidence, RA-associated risk genes in these cells. Moreover, we investigated effects of anti-rheumatic drugs, in particular methotrexate (MTX), on the expression of RA risk genes and pathogenic behaviors of activated RA-FLS. Cellular and molecular studies were primarily performed on joint tissue and cultured FLS from patients with RA and controls. For *in vitro* experiments, cytokines or growth factors known to activate FLS were added to simulate the inflammatory environment of the RA joint.

Paper I demonstrated that the RA risk gene *AIRE* (autoimmune regulator), mostly known as a transcriptional regulator in the thymus, is induced in RA-FLS by pro-inflammatory cytokines. Using *AIRE* silencing and RNA sequencing, we found that AIRE promotes the expression of a set of chemokines in activated RA-FLS. **Paper II** showed that MTX increases the transcription of the RA risk gene *LBH* (limb-bud and heart development) and other tumor suppressor genes in activated RA-FLS, possibly via epigenetic changes. Also, cell cycle analysis demonstrated that MTX inhibits proliferation of activated RA-FLS. **Paper III** applied transcriptomic analysis to further explore the influence of MTX and of a Janus kinase inhibitor on these cells. Therapeutic concentrations of MTX altered the expression of a vast number of genes in activated RA-FLS, and unexpectedly upregulated pro-inflammatory mediators like *IL1A* (interleukin-1α) and the RA risk gene *CSF2* (granulocyte-macrophage colony-stimulating factor, GM-CSF). We propose a mechanism whereby MTX increases the production of GM-CSF via autocrine IL-1α signaling, with potential to enhance inflammatory interactions between FLS and macrophages in the RA synovium.

In conclusion, our results support the biological relevance of multi-evidence RA risk genes in activated RA-FLS and add new knowledge on the pharmacodynamics of MTX in RA. The findings emphasize that RA-FLS are important targets in future treatment strategies to improve outcome in RA.

SAMMANFATTNING PÅ SVENSKA

Reumatoid artrit (RA), ledgångsreumatism, är en inflammatorisk sjukdom som främst angriper lederna men även kan drabba andra delar av kroppen. Risken att insjukna i RA påverkas av genetik, miljö och livsstilsfaktorer. Vid sjukdomen infiltreras leden av immunceller som i samspel med bindvävsceller i ledhinnan, s.k. fibroblast-lika synoviocyter (FLS), driver kronisk inflammation och förstörelse av brosk och ben. Aktiverade RA-FLS får tumörcellslika egenskaper och producerar inflammatoriska substanser (cytokiner och kemokiner), tillväxthormon och vävnadsnedbrytande enzymer. En stor andel av RA-patienter har otillräcklig effekt av dagens antireumatiska behandling, som framför allt riktar sig mot immunförsvaret. För att kunna förbättra behandlingsstrategier för RA är det viktigt med ökad kunskap om de molekylära mekanismerna som styr aktivering av aggressiva RA-FLS men även förståelse för hur dessa celler påverkas av befintliga läkemedel.

Ett hundratal gener har kopplats till RA och en fjärdedel av dessa ”riskgener” uttrycks av FLS. Vi har fokuserat på att undersöka funktionen av några av dessa gener i aktiverade RA-FLS, i första hand genom analys av ledvävnad från patienter med RA samt experimentella försök på odlade RA-FLS. I **delarbete I** visade vi att riskgenen *AIRE*, som främst är känd för sin roll i utvecklingen av immunologisk tolerans i tymus, uttrycks i aktiverade FLS i RA-leden. Genom att utsätta odlade RA-FLS för en inflammatorisk miljö kunde uttryck av AIRE framkallas. Vidare fann vi att AIRE ökar produktionen av pro-inflammatoriska ämnen i dessa celler. I **delarbete II och III** studerade vi hur läkemedlet metotrexat (MTX) inverkar på uttrycket av riskgener och sjukdomsalstrande beteenden hos aktiverade RA-FLS. MTX utgör första linjens behandling för RA sedan över 30 år tillbaka, men dess exakta verkningsmekanismer vid denna sjukdom är inte helt klarlagda. Vi visade att MTX ändrar uttrycket av tusentals gener i aktiverade RA-FLS. MTX ökade uttrycket av cellcykelreglerande gener, t.ex. RA-riskgenen *LBH*, och hämmade celldelningen av aktiverade RA-FLS, vilket kan ha fördelaktiga behandlingseffekter. Ett oväntat fynd var dock att MTX även förstärkte uttrycket av pro-inflammatoriska gener, såsom riskgenen *CSF2* kodande för proteinet GM-CSF som ökar aktiveringen av makrofager, en typ av immuncell som också är viktig i RA-leden. Sammanfattningsvis bidrar våra resultat med ny kunskap om effekter av riskgener och av MTX, vilka kan ha betydelse för det sjukdomsalstrande beteendet hos aktiverade RA-FLS.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. **Bergström B***, Lundqvist C*, Vasileiadis G K, Carlsten H, Ekwall O, Ekwall AK H.
The rheumatoid arthritis risk gene *AIRE* is induced by cytokines in fibroblast-like synoviocytes and augments the pro-inflammatory response.
Front Immunol. 2019; 10: 1384.
* These authors contributed equally to this work.

II. **Bergström B**, Carlsten H, Ekwall AK H.
Methotrexate inhibits effects of platelet-derived growth factor and interleukin-1beta on rheumatoid arthritis fibroblast-like synoviocytes.
Arthritis Res Ther. 2018; 20(1): 49.

III. **Bergström B**, Selldén T, Bollmann M, Svensson MND, Ekwall AK H.
Methotrexate promotes the release of granulocyte-macrophage colony-stimulating factor from rheumatoid arthritis fibroblast-like synoviocytes via autocrine interleukin-1 signaling.
Arthritis Res Ther. 2024; 26(1): 178.

ABBREVIATIONS

7-AAD	7-amino-actinomycin
ACPA	Anti-citrullinated protein antibody
ACR	American College of Rheumatology
AICAR	5-aminoimidazole-4-carboxamide ribonucleotide
AIRE	Autoimmune regulator
ANOVA	Analysis of variance
bDMARD	Biological disease-modifying anti-rheumatic drug
CCL	C-C motif chemokine ligand
CDK	Cyclin-dependent kinase
cDNA	Complementary DNA
csDMARD	Conventional synthetic disease-modifying anti-rheumatic drug
Ct	Cycle threshold
CXCL	C-X-C motif chemokine ligand
DAS28	Disease Activity Score using 28-joint counts
DHFR	Dihydrofolate reductase
DMARD	Disease-modifying anti-rheumatic drug
DMEM	Dulbecco's modified Eagle's medium
DNMT	DNA methyltransferase
ECM	Extracellular matrix
eTAC	Extrathymic AIRE-expressing cell
EULAR	European League Against Rheumatism
FAP	Fibroblast activation protein
FBS	Fetal bovine serum
FLS	Fibroblast-like synoviocytes
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GSEA	Gene Set Enrichment Analysis
GWAS	Genome-wide association studies
HLA	Human leukocyte antigen

IFN	Interferon
IL	Interleukin
IPA	Ingenuity Pathway Analysis
JAK	Janus kinase
JNK	Jun N-terminal kinase
LBH	Limb-bud and heart development
MAPK	Mitogen-activated protein kinase
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinase
mRNA	Messenger RNA
mTEC	Medullary thymic epithelial cell
MTX	Methotrexate
NF-κB	Nuclear factor-κB
OA	Osteoarthritis
PDGF	Platelet-derived growth factor
qPCR	Quantitative polymerase chain reaction
RA	Rheumatoid arthritis
RANKL	Receptor activator of NF-κB ligand
RF	Rheumatoid factor
RNA-seq	RNA sequencing
SEM	Standard error of the mean
siRNA	Small interfering RNA
SNP	Single nucleotide polymorphism
STAT	Signal transducer and activator of transcription
TGF	Transforming growth factor
TNF	Tumor necrosis factor
TOFA	Tofacitinib
TRA	Tissue-restricted antigen
tsDMARD	Targeted synthetic disease-modifying anti-rheumatic drug

CONTENTS

Introduction **1**
Rheumatoid arthritis 1
Etiology 1
Pathogenesis 2
Clinical manifestations 4
Treatment 5
Joint inflammation in RA 11
Healthy synovium 11
RA synovium 11
Fibroblast-like synoviocytes in RA 14
Pathogenic features of activated RA-FLS 14
RA-FLS subsets and markers 16
Epigenetic alterations of RA-FLS 17
Multi-evidence RA-FLS candidate genes 18
Effects of DMARDs on RA-FLS 20
Aims **23**
Methods **25**
Patient samples and ethical considerations 25
In vitro experiments 26
Cell culture 26
Stimulations and treatments 27
Gene silencing 28
Sample analysis 29
Quantitative polymerase chain reaction 29
RNA sequencing and bioinformatics workflow 30
Immunofluorescence staining and confocal microscopy 31
Flow cytometry 31
Statistical analysis 33

Results and discussion **35**

PAPER I:
Expression, regulation and function of AIRE in RA-FLS 35

PAPER II:
Effects of MTX on mitogen-induced proliferation of RA-FLS 39

PAPER III:
Effects of MTX on disease-associated risk genes and pro-inflammatory mediators in RA-FLS 41

Conclusions and future perspectives **45**

Acknowledgements **49**

References **51**

INTRODUCTION

Rheumatoid arthritis (RA) is one of the most common systemic autoimmune diseases and primarily affects joints. It is characterized by chronic inflammation of the synovium, the inner membrane of the joint capsule, leading to degradation of cartilage and bone. The pathogenesis involves both innate and adaptive immune responses, as well as dysregulated stromal cells such as fibroblast-like synoviocytes (FLS). Increased knowledge of how FLS are activated and contribute to persistence of inflammation is important in the development of novel treatment strategies for RA.

The following sections will first provide an overview on RA, before going deeper into the joint pathology and the role of activated FLS in the disease.

RHEUMATOID ARTHRITIS

RA has probably affected people since ancient times, but the first written clinical description is from the year 1800. The French doctor Landré-Beauvais wrote about a group of patients, predominantly women, with severe joint pain and swelling, exhibiting a chronic disease course and deterioration in general health (1). In 1869, the rheumatologist Garrod in England coined the term rheumatoid arthritis, which is based on the Greek words for watery and inflamed joints.

The prevalence of RA varies around the world, with estimates ranging from 0.25% to 1% (2). Higher prevalence rates have been recorded in Native American populations (3). The geographic differences in RA susceptibility may be attributed to distribution of risk genes as well as to environmental factors. RA usually strikes between the fifth and seventh decade of life but can affect individuals at any age (4). The disease is two to three times more common in women than in men (5).

Etiology

The exact cause of RA is unknown, but multiple risk factors contribute to the development of the disease, including female sex, genetic predisposition, and environmental factors.

First-degree relatives of individuals with RA are at around three-fold higher risk of developing the disease compared with the general population (6), which

supports the role of genetic susceptibility. Genome-wide association studies (GWAS) have identified over 100 genetic risk loci for RA, but the majority of these single nucleotide polymorphisms (SNPs) have only modest effect sizes (7). The most significant genetic associations for RA are within the human leukocyte antigen (HLA; also known as major histocompatibility complex (MHC)), mainly alleles of the *HLA-DRB1* gene encoding a specific five-amino acid sequence motif called the "shared epitope" (8). Examples of other RA risk genes are *PTPN22*, *TYK2*, and *STAT4*, which encode proteins involved in immune signaling, particularly in the Janus kinase (JAK)/Signal transducer and activator of transcription (STAT) pathway (9). SNPs associated with RA susceptibility are also found in the *PADI4* gene, which is implicated in protein citrullination capable of inducing a breach in immunological tolerance (10).

Cigarette smoking is the strongest environmental or lifestyle factor that has been associated with an increased risk for RA (11). Other risk factors include silica dust inhalation (12), obesity (13), vitamin D deficiency (14), microbiota alterations, infections, and chronic inflammation of mucosal sites (15-17). The pathways by which environmental exposures influence RA susceptibility may include epigenetic modifications, i.e. heritable alterations affecting gene expression without changing the DNA sequence (18).

Pathogenesis

RA probably evolves over many years before clinical onset, as a result of complex interactions between genetic and non-genetic factors in a multistep process (Figure 1). It is a highly heterogenous disease, where a diversity of pathways and cell types can contribute to the pathogenesis.

Mechanisms involved in the disease initiation include post-translational modifications of proteins in the lung, oral or gut mucosa in response to environmental stressors. For example, smoking and infectious agents can induce peptidylarginine deiminases which mediate citrullination of a range of proteins (19). Citrullinated peptides may be recognized by the immune system as foreign antigens. Presence of the "shared epitope" in the MHC class II molecules of antigen-presenting cells facilitates the presentation of citrullinated peptides to T cells (20). Thus, these events may elicit an adaptive immune response with activation of T and B cells and production of autoantibodies, such as anti-citrullinated protein antibodies (ACPAs) which are highly specific for RA (21). Antibodies against other protein modifications (e.g. carbamylation and acetylation) have also been associated with RA (22). It is well-described that ACPAs and rheumatoid factor (RF), an autoantibody

targeting the Fc portion of immunoglobulin G, can be detected in blood several years before symptoms occur (23), manifesting early immune dysregulation. During the preclinical RA phase, the levels and specificities of ACPAs gradually increase along with an elevation of circulating cytokines, indicative of emerging systemic inflammation (24). The pathogenesis of seronegative RA, where ACPAs and RF are absent, is poorly understood.

The transition of the immune activation into clinically evident inflammation of the joints is hypothesized to require a "second hit", e.g. immune complex formation, vascular microtrauma, or infection (25). Characteristic features of the RA synovium will be described in more detail later. Briefly, the synovium is infiltrated by numerous immune cells, including lymphocytes, monocytes, neutrophils, and dendritic cells. Additionally, there is an activation and expansion of tissue-resident macrophages and FLS, which further promote the recruitment of immune cells and contribute to an inflammatory build-up (26). Interactions between the different cell types of the RA synovium are mediated by multiple pro-inflammatory cytokines, such as interleukin (IL)-1, IL-6, and tumor necrosis factor (TNF)-α (27).

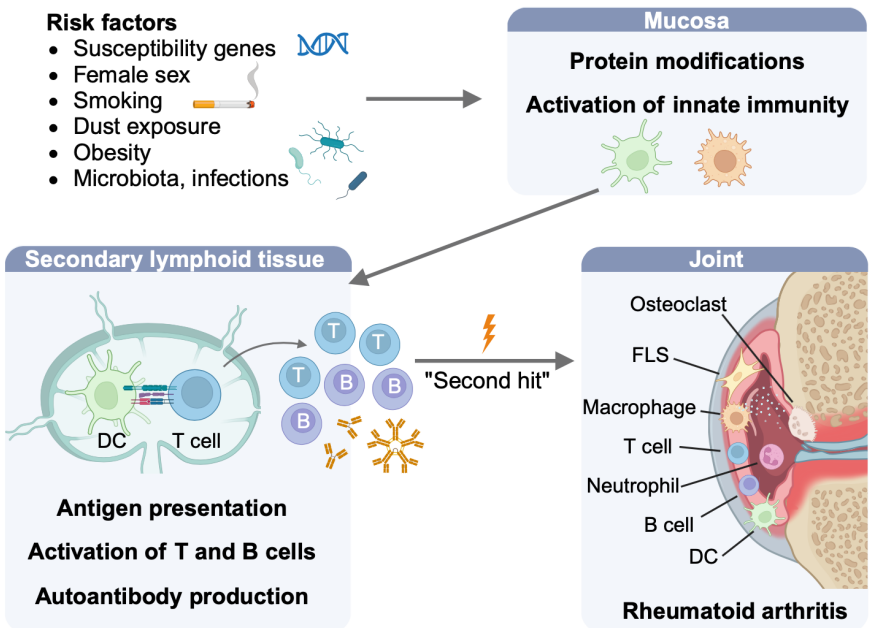

Figure 1. Pathogenesis of rheumatoid arthritis. In genetically susceptible individuals, environmental triggers may lead to a breach of tolerance, later transitioning to clinical inflammation of the joints. Abbreviations: DC, dendritic cell; FLS, fibroblast-like synoviocyte. Created with BioRender.com

The inflamed synovium transforms into a hyperplastic “pannus” tissue which, if left untreated, invades adjacent structures. Articular cartilage is destroyed by matrix-degrading enzymes produced mainly by FLS (28). The inflammatory milieu also increases the differentiation and activity of osteoclasts, which are responsible for RA-associated bone erosion (29).

Presence of ACPAs in established RA predicts a more severe disease course with joint destruction, supporting involvement of these antibodies in the pathophysiology (30). Suggested mechanisms include immune complex formation, macrophage activation, osteoclast activation and bone loss, and modulation of FLS mobility (31).

Ultimately, actions of the various pathogenic players of innate and adaptive immunity and the stromal compartment create self-sustaining inflammatory loops, leading to chronic synovitis and potential systemic complications (26).

Clinical manifestations

The onset of RA can be acute or insidious. In most patients, the disease presents gradually with morning stiffness, fatigue, pain and swelling of joints. Within a few months, it usually develops to a symmetrical polyarthritis. Typically, the small joints of the hands and feet are first affected. However, any synovial joint may be involved. Without proper treatment, RA can cause progressive joint damage, leading to deformities and functional disability (32).

Although the main pathology of RA is in the joints, manifestations in other organs and tissues are also common. Examples of such findings are rheumatoid nodules in the skin, secondary Sjögren’s syndrome, interstitial lung disease, anemia, neuropathy, and vasculitis (33). In addition, RA patients have a higher prevalence of comorbidities, including osteoporosis, lymphoma, and cardiovascular disease, contributing to increased mortality compared to the general population (34).

RA is a clinical diagnosis based primarily on the patient’s medical history and the physical examination, with help from blood tests (ACPA, RF, and inflammatory markers) and sometimes radiology (conventional radiography, magnetic resonance imaging, or ultrasonography). RA classification criteria have been developed by the American College of Rheumatology (ACR) and the European League Against Rheumatism (EULAR) for the purpose of patient stratification in clinical studies, but are often used as a guide to diagnosis in clinical praxis. While the 1987 ACR criteria distinguished RA from other types

of arthritis (35), the current criteria from 2010 (Table 1) emphasize features of early RA that predict an aggressive disease course, thereby identifying patients who may benefit most from intervention with effective treatment (36).

Table 1. The 2010 ACR/EULAR classification criteria for rheumatoid arthritis.

Joint involvement (tender/swollen)		**Acute-phase reactants**	
1 large joint	0	Normal CRP and normal ESR	0
2-10 large joints	1	Abnormal CRP or abnormal ESR	1
1-3 small joints	2	**Symptom duration**	
4-10 small joints	3	<6 weeks	0
>10 joints (at least 1 small joint)	5	≥6 weeks	1
Serology			
Negative RF and negative ACPA	0	**Total score ≥6 = definite RA**	
Low positive RF or low positive ACPA	2		
High positive RF or high positive ACPA	3		

Abbreviations: ACPA, anti-citrullinated protein antibody; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; RF, rheumatoid factor.

Treatment

Early diagnosis and prompt initiation of treatment with disease-modifying anti-rheumatic drugs (DMARDs) are critical factors to prevent progression to joint destruction in RA (37). The EULAR recommends a "treat-to-target" strategy, consisting of tight control with frequent monitoring and adjustment of treatment if the therapeutic goal has not been achieved (38). While the target is a state of remission in early RA, low disease activity could be an alternative goal for patients with longstanding RA.

Over the last decades, the therapeutic arsenal for RA has expanded markedly (Figure 2). Still, the conventional synthetic DMARD (csDMARD) methotrexate (MTX) remains the first-line treatment and anchor drug in RA (39). For 50-60% of patients, the response to MTX monotherapy is insufficient (40, 41) and another drug is added, usually a biological DMARD (bDMARD). Alternatively, a targeted synthetic DMARD (tsDMARD), such as the novel JAK inhibitors, can be considered. In patients without poor prognostic factors, another option could be combination of csDMARDs, such as triple therapy with sulfasalazine and hydroxychloroquine added to MTX. Short-term glucocorticoids are used as bridging therapy when starting csDMARDs, and as treatment for flares (38).

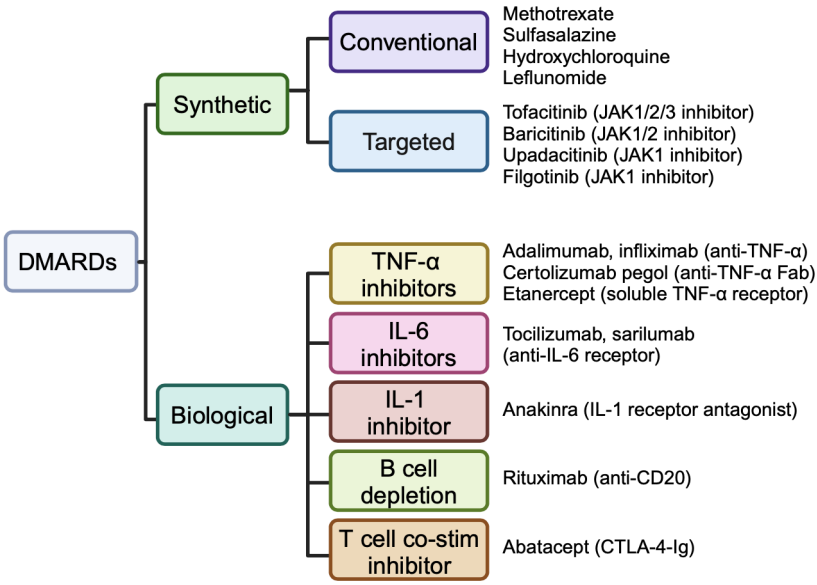

Figure 2. Overview of classes of disease-modifying anti-rheumatic drugs (DMARDs) for the treatment of rheumatoid arthritis, with examples of currently available medications. Abbreviations: Fab, fragment antigen-binding; IL, interleukin; JAK, Janus kinase; TNF, tumor necrosis factor. Created with BioRender.com

Methotrexate

The folic acid antagonist MTX was developed in the 1940's for the treatment of leukemia. Later, it was found that MTX at "low-dose" (compared to the high doses used in cancer therapy) was also effective in reducing disease activity in RA (42). MTX has now been the standard of care for RA treatment for more than thirty years. It is considered a low-cost drug with proven clinical efficacy, both as monotherapy and in combination therapies, as well as having an acceptable safety profile (39).

MTX is administered once weekly, either orally or parenterally. The starting dose is usually 15 mg/week, which is escalated to a maximum of 25 mg/week. MTX is a prodrug that is slowly polyglutamated intracellularly to its active form, which explains why it may take several weeks to demonstrate clinical effect (43, 44). Although MTX is generally well-tolerated, potential side effects include tiredness, headache, nausea, and gastrointestinal symptoms. Toxicities may also occur in the liver, bone marrow, and lung. Because of teratogenic effects, MTX is contraindicated in pregnancy. During MTX therapy, supplementation with folic acid can reduce the risk of adverse reactions and patient withdrawal from treatment (45).

Whereas the mode of action of MTX in cancer settings is inhibition of nucleotide synthesis required for cell proliferation, other mechanisms are thought to be important for the therapeutic effect of low-dose MTX in RA (Figure 3). Firstly, MTX potently inhibits the enzyme 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) transformylase, leading to increased extracellular levels of adenosine with anti-inflammatory effects on most immune cell types (46, 47). MTX also inhibits dihydrofolate reductase (DHFR), thereby diminishing production of polyamines and downstream toxic metabolites with joint damaging potential. Another effect of DHFR inhibition is nitric oxide synthase uncoupling (48). This process increases the production of reactive oxygen species, which activate Jun N-terminal kinase (JNK) and the transcription factor AP-1, ultimately leading to restored cell cycle checkpoint gene expression and sensitivity to apoptosis of activated T cells (49, 50). Moreover, the increased JNK activity as well as the adenosine release mediated by MTX suppress activation of the pro-inflammatory transcription factor nuclear factor-κB (NF-κB) in T cells and FLS (51). Lastly, it has been demonstrated that MTX can inhibit JAK/STAT signaling in a macrophage cell line (52).

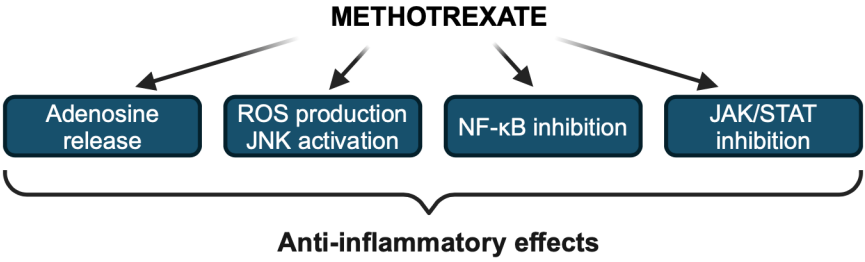

Figure 3. Mechanisms of action of low-dose methotrexate in the treatment of rheumatoid arthritis. Postulated pathways by which methotrexate exerts anti-inflammatory effects. Abbreviations: JAK/STAT, Janus kinase/Signal transducer and activator of transcription; JNK, Jun N-terminal kinase; NF-κB, nuclear factor-κB; ROS, reactive oxygen species. Created with BioRender.com

Biological drugs

The class of bDMARDs includes monoclonal antibodies, receptor antagonists and fusion proteins targeting the activity of specific components of the immune system, usually by binding to extracellular or membrane-bound molecules. These drugs are mainly administered by injection or infusion, due to degradation and absorption issues via the oral route. Currently available biological agents for the treatment of RA act by inhibiting pro-inflammatory cytokines (TNF-α, IL-6, IL-1), depleting B cells or blocking T cell activation (53). In case of inadequate response to initial therapy with a csDMARD, the EULAR recommends addition of any bDMARD for patients who have poor prognostic factors such as high disease activity, presence of RF/ACPA, or early erosions (38). Clinical trial data have demonstrated improved efficacy of combining a bDMARD with MTX compared to bDMARD monotherapy (54). Concomitant use of MTX may prevent the development of anti-drug antibodies, which are associated with impaired clinical response to bDMARDs (55). Biological therapies can increase the risk of chronic and opportunistic infections (56), motivating testing and vaccination for certain infections before initiation of treatment.

JAK inhibitors

The newest drugs in RA therapy are the JAK inhibitors, belonging to the category of tsDMARDs. These are orally bioavailable, small molecules that enter cells and inhibit the intracellular signaling of multiple cytokines and growth factors. Ligand binding to cytokine receptors trigger phosphorylation of receptor-associated JAKs, which in turn activate STAT proteins that enter the nucleus to induce transcription of effector genes (57) (Figure 4). The numerous types of cytokines signal via different combinations of four isoforms of JAKs and seven STAT family members, mediating different biological functions. Thus, the selectivity of JAK inhibitors may determine their effects on inflammatory responses but also influence their safety profile. Whereas tofacitinib, the first JAK inhibitor approved for RA, blocks the activity of multiple JAKs, more recently developed JAK inhibitors target only a single JAK isoform (58).

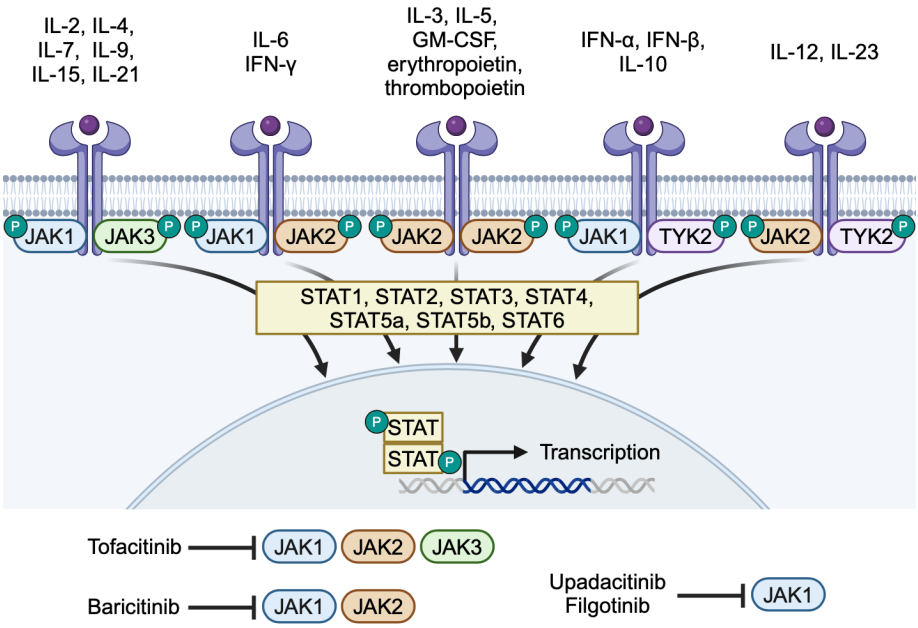

Figure 4. JAK/STAT signaling and inhibition. Examples of various cytokines and growth factors signaling through different combinations of JAKs (59), and an overview of the selectivity of JAK inhibitors approved for RA treatment. Abbreviations: GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN, interferon; IL, interleukin; JAK, Janus kinase; STAT, signal transducer and activator of transcription; TYK, tyrosine kinase. Created with BioRender.com

JAK inhibitors alone or in combination with MTX have demonstrated higher clinical efficacy compared to MTX monotherapy in active RA (60, 61). JAK inhibitors can also be more efficacious than the TNF blocker adalimumab, one of the most common bDMARDs used in RA treatment, when both are combined with MTX (62). Regarding safety, the ORAL Surveillance trial showed higher rates of infections, including herpes zoster, with tofacitinib versus TNF inhibitor treatment (63). Moreover, concerns have been raised regarding an increased risk of major adverse cardiovascular events and malignancy with tofacitinib (64). Until increased knowledge has been gathered, careful consideration of risk factors for such disorders is recommended when prescribing JAK inhibitors (38).

Glucocorticoids

Glucocorticoids were introduced in the treatment of RA in 1948 and are still widely used, owing to their capacity to rapidly suppress joint inflammation and potentially decrease radiographic progression in early disease (65). According to the current EULAR recommendations, short-term glucocorticoid therapy

should be considered when initiating or changing csDMARDs, which have a relatively slow onset of action compared to bDMARDs and tsDMARDs (38). Glucocorticoids can be administered orally or via injection (e.g. intramuscular, intraarticular) or by intravenous infusion, and exert their anti-inflammatory and immunosuppressive effects via modulation of a diversity of cell types and molecular pathways (66). However, prolonged use of glucocorticoids has been associated with an equally extensive list of adverse effects, including increased risk of infections, osteoporosis and fractures, hyperglycemia, and cardiovascular disease (67). The EULAR therefore strongly advocates tapering and discontinuation of glucocorticoids as quickly as clinically feasible, ideally within three months (38). Yet, the long-term safety and efficacy of glucocorticoids, influenced by dosage, treatment duration, and patient characteristics, are still subject to evaluation as well as debate (68).

Treatment response

The introduction of novel DMARDs and aggressive treatment strategies in early disease have improved outcomes for RA patients and made clinical remission a realistic goal (40). Disease activity and response to therapy can be monitored through a variety of composite measures. One of the most common measures used both in clinical practice and in studies is the Disease Activity Score using 28-joint counts (DAS28), which is calculated from clinical data (number of tender and swollen joints), inflammatory markers (C-reactive protein or erythrocyte sedimentation rate), and the patient's global assessment (69). Remission by DAS28 is defined as a score of less than 2.6.

In patients with sustained remission for at least six months, gradual tapering of DMARD therapy can be considered (38). However, complete DMARD withdrawal often leads to disease flares (70), which might be explained by the presence of joint inflammation that is insufficiently captured by current clinical measures and remission criteria (71).

Despite the therapeutic advances in RA, around 50% of patients fail to achieve clinical remission, as seen in randomized trials as well as in long-term real-world data (72-74). This illustrates gaps in the molecular understanding of the disease and a need for improved treatment approaches.

JOINT INFLAMMATION IN RA

Healthy synovium

Synovial joints, also called diarthrodial joints, are characterized by their mobility and the presence of a cavity filled with synovial fluid. This space is sealed within a fibrous joint capsule with an inner membrane termed synovium. The synovium in turn consists of a thin intimal lining layer, composed of resident macrophages and FLS, and a sublining layer of connective tissue also containing FLS and synovial macrophages in addition to adipocytes, blood and lymphatic vessels, and sparse immune cells (75). The lining layer lacks features of a classical epithelium, such as a continuous basement membrane and junctional complexes, and is rather considered a loose association of cells. However, a recent study described a specialized subset of locally renewing synovial macrophages, forming a protective barrier in the joint (76).

Apart from providing structural support, the synovium functions to lubricate the joint and supply nutrients to the articular cartilage. FLS in the lining layer secrete important constituents of synovial fluid, e.g. hyaluronan and lubricin. Moreover, FLS regulate and remodel the extracellular matrix (ECM) by producing matrix proteins as well as matrix-degrading enzymes (77).

RA synovium

The joint pathology of RA is characterized by transformation of the synovium into a hyperplastic, tumor-like, invasive tissue. The synovial lining layer expands from a thickness of 2-3 cells to a depth of up to 10-20 cells (Figure 5), owing to an increased number and activation of both FLS and macrophages in response to inflammation (26). The lining FLS area by histology has been demonstrated to correlate with clinical disease activity (78). Activated RA-FLS express a variety of pro-inflammatory mediators and joint-damaging enzymes, described in more detail later, contributing to destruction of cartilage and bone.

In the sublining layer, there is a marked influx of innate and adaptive immune cells, supported by significant neovascularization (26). Aggregates of lymphocytes, sometimes shaping ectopic lymphoid structures together with follicular dendritic cells, are capable of sustaining production of autoantibodies in the synovial tissue (79). Recent studies have revealed the expansion of a specific subset of peripheral T helper cells (PD-1^{hi}CXCR5^{-}), expressing factors

that support B cell activation and differentiation into plasma cells, in the synovium of RA patients (80).

Other immune cell types found in the sublining include macrophages and mast cells. The increase of macrophages in the synovium is partly due to differentiation of infiltrating blood monocytes, and partly resulting from activation of tissue-resident macrophages (81). Distinct populations of synovial tissue macrophages with different functions have been identified, e.g. anti-inflammatory MerTK^{+} macrophages (82), pro-inflammatory MerTK^{-} macrophages associated with cytokine production (83), and HBEGF^{+} macrophages inducing FLS invasiveness (84). Neutrophils are rarely found in the tissue but pass through into the synovial fluid. The release of neutrophil extracellular traps from activated neutrophils is a source of citrullinated antigens in the joint, possibly promoting ACPA production or immune complex formation (31).

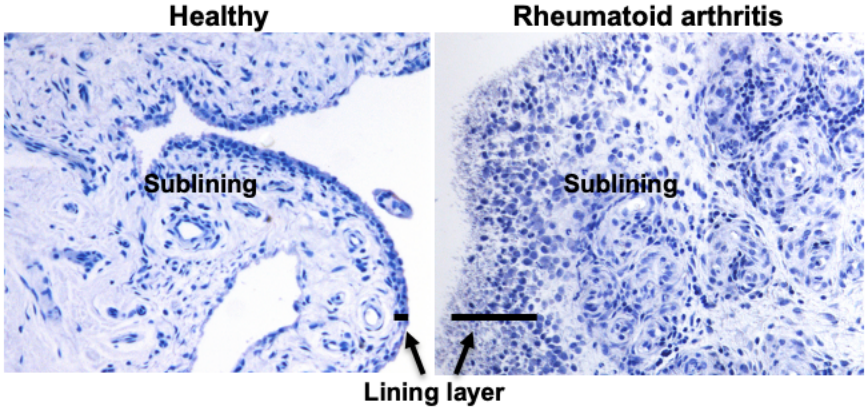

Figure 5. Histology of synovial tissue in rheumatoid arthritis compared to healthy state (hematoxylin staining of cell nuclei). In rheumatoid arthritis, there is a striking increase in cellularity of both the lining and the sublining layer of the synovium. Image courtesy of Anna-Karin H Ekwall.

The different synovial cell types produce a range of cytokines and other factors, creating complex networks that perpetuate inflammation (85). Furthermore, structural damage to cartilage in the affected joint is mediated by matrix metalloproteinases (MMPs) released primarily from activated FLS but also from macrophages and neutrophils (32). Bone degradation is caused by increased maturation and activation of osteoclasts. Receptor activator of NF-κB ligand (RANKL), produced by FLS and some T and B cells, promotes the differentiation of monocytes into osteoclasts. These destructive processes are

enhanced by pro-inflammatory cytokines like TNF-α, IL-1 and IL-6 from macrophages and FLS (29).

The activated and proliferative stromal and immune cells have high energy requirements, which in combination with dysregulated microvasculature lead to a hypoxic environment in the inflamed joint (86). During these conditions, the synovial cells undergo metabolic adaptions, e.g. enhanced glycolysis as displayed by FLS from patients with RA compared to osteoarthritis (OA), potentially supporting their pathogenic phenotype (87). Moreover, generation and accumulation of bioactive metabolites may contribute to further cell activation and inflammatory responses (88).

In recent years, large heterogeneity in RA synovial tissue has been described by both histology and transcriptomic analysis. Studies of synovial biopsies from patients with early untreated RA have suggested three distinct pathotypes: 1) lympho-myeloid, characterized by accumulation of T cells, B cells, plasma cells, and myeloid cells (monocytes or macrophages), 2) diffuse myeloid, rich in monocytes or macrophages but poor in B cells, and 3) pauci-immune/fibroid, lacking immune cells but containing stromal cells like FLS (89). Cellular and molecular profiling of synovium has the potential to refine patient stratification and to some extent predict response to therapy (90). For example, in RA patients with a synovial signature classified as B cell poor by RNA sequencing, the IL-6 receptor inhibitor tocilizumab was more effective than the B cell-depleting drug rituximab in the R4RA trial (91). In contrast, the fibroid synovial pathotype has been associated with resistance to multiple DMARDs (92).

FIBROBLAST-LIKE SYNOVIOCYTES IN RA

FLS have a key role in the RA joint pathology, by mediating synovial hyperplasia, chronic inflammation and tissue destruction. RA-FLS are activated locally by inflammatory mediators such as IL-1, TNF-α, and growth factors. Also, fragments released during cartilage damage and cell death can activate RA-FLS via Toll-like receptors (28).

Thus, RA-FLS act in concert with infiltrating immune cells, but they also display autonomous aggressive behaviors contributing to disease persistence and progression (93). Advances in the characterization of RA-FLS have highlighted their potential as novel therapeutic targets.

Pathogenic features of activated RA-FLS

The expansion of FLS in the RA synovium may result from a combination of increased proliferation and resistance to apoptosis (94). *In vitro* studies have demonstrated that RA-FLS proliferate when exposed to e.g. platelet-derived growth factor (PDGF) and IL-1β, which are abundant factors in the rheumatoid joint (95, 96). Dysregulation of apoptosis and cell cycle activity has been associated with abnormalities of the tumor suppressor p53 and its downstream mediator p21 in RA-FLS (97, 98). Other tumor-like properties of RA-FLS include increased invasive and migratory capacities, and possibly the ability to spread between joints as demonstrated in mice (99, 100). RA-FLS-mediated invasion and degradation of ECM and cartilage is promoted by high expression of adhesion molecules, e.g. cadherin-11, and production of proteolytic enzymes like MMPs (101). As mentioned, RA-FLS also produce RANKL that supports the differentiation of osteoclasts responsible for bone erosion.

Moreover, activated RA-FLS secrete multiple pro-inflammatory cytokines, chemokines, and growth factors (Figure 6). These mediators may further stimulate RA-FLS in an autocrine manner, or recruit and activate immune cells. For example, type I interferons (IFNs), e.g. IFN-β, synthesized by TNF-activated RA-FLS autocrinally drive the expression of IFN-stimulated genes, including several chemokines (102, 103). C-C motif chemokine ligand (CCL) 2, C-X-C motif chemokine ligand (CXCL) 8, and CXCL10 released from RA-FLS attract monocytes and macrophages (28). Macrophages are major sources of TNF-α and IL-1β that activate RA-FLS to produce e.g. IL-6 and granulocyte-macrophage colony-stimulating factor (GM-CSF). These in turn promote macrophage activation, creating a vicious cycle (104). Additionally, various cytokines and chemokines in conjunction with vascular endothelial

growth factor produced by RA-FLS stimulate angiogenesis, perpetuating the infiltration of immune cells into the inflamed synovium (105).

There is also a crosstalk between RA-FLS and lymphocytes, both via secreted mediators and direct cell-cell interactions, contributing to the activation and differentiation of T and B cells (106). In response to IFN-γ (produced by T cells), RA-FLS can upregulate MHC class II molecules and present antigens to $CD4^+$ T cells *in vitro* (107).

Interestingly, FLS derived from RA patients maintain an activated, cytokine-producing phenotype over several passages in cell culture, even in the absence of further external inflammatory stimuli. It has been proposed that RA-FLS are not simply "passive responders" to the inflammatory microenvironment (108). Rather, they are considered to be "imprinted" with pathogenic features that actively drive the disease (93). Compared with OA-FLS or healthy FLS, RA-FLS display molecular alterations that promote the activation of e.g. mitogen-activated protein kinase (MAPK) and NF-κB pathways, leading to increased production of cytokines and MMPs (28). Epigenetic modifications are believed to play an important role for the persistently activated state of RA-FLS (109).

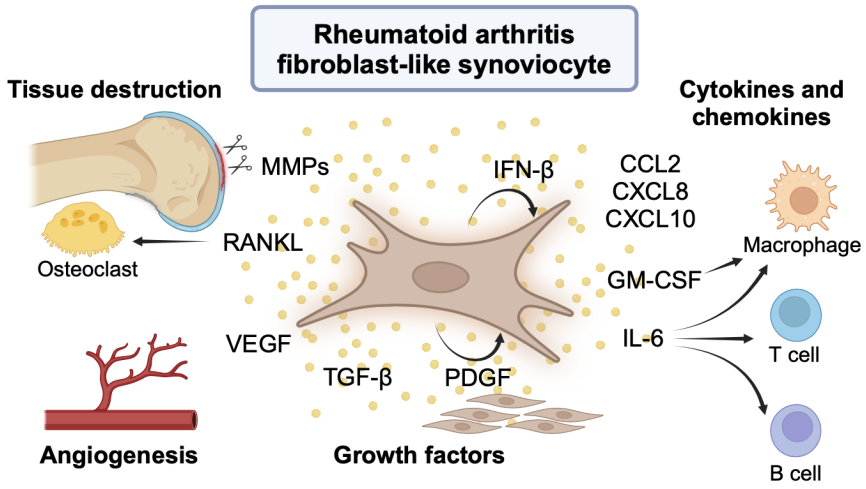

Figure 6. Major factors produced by rheumatoid arthritis fibroblast-like synoviocytes contributing to inflammation and joint destruction. Abbreviations: CCL, C-C motif chemokine ligand; CXCL, C-X-C motif chemokine ligand; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN, interferon; IL, interleukin; MMPs, matrix metalloproteinases; PDGF, platelet-derived growth factor; RANKL, receptor activator of NF-κB ligand; TGF-β, transforming growth factor-β; VEGF, vascular endothelial growth factor. Created with BioRender.com

RA-FLS subsets and markers

Recent work has demonstrated heterogeneity among RA-FLS in terms of surface marker expression, transcriptional profile, and pathogenic functions, leading to the characterization of several distinct RA-FLS subsets. For example, FLS in the synovial lining layer are positive for the surface protein CD55 but lack expression of CD90, the latter instead being a marker of sublining FLS (110). Further on, single-cell RNA sequencing studies have identified different RA-FLS subpopulations within these compartments, defined by expression of e.g. *DKK3*, *CD34* or *HLA-DRA* (111, 112).

The glycoprotein podoplanin is upregulated in human cancers but is also expressed by FLS mainly in the invasive lining layer of RA synovium (113). Moreover, it can be induced in RA-FLS *in vitro* by stimulation with pro-inflammatory cytokines (113, 114). Another marker associated with an activated RA-FLS phenotype is the fibroblast activation protein-α (FAPα), which is a membrane-bound protease found in both lining and sublining layers (115). In a mouse model of arthritis, deletion of cells expressing FAPα resulted in a reduction of joint inflammation and structural damage. Single-cell transcriptional analysis of inflamed synovium from murine arthritis and from RA patients, followed by cell transfer experiments in mouse joints, revealed two functionally different $FAP\alpha^+$ FLS subsets. $FAP\alpha^+CD90^+$ FLS localized in the sublining display high expression of chemokines and cytokines that drive inflammation, while $FAP\alpha^+CD90^-$ FLS restricted to the lining layer have a destructive profile, expressing mediators of cartilage and bone damage (116).

The molecular mechanisms shaping the differentiation of RA-FLS subsets may be influenced by the synovial microenvironment. NOTCH3 signaling from vascular endothelium has been reported to provide a positional cue for RA-FLS, creating a gradient of $CD90^+$ phenotypes (117). Another study suggested that distinct transcriptional states of RA-FLS are established as a result of exposure to cytokines derived from macrophages and T cells infiltrating the synovium (118).

Furthermore, recent data propose changes in FLS phenotypes during resolution of inflammation. Accumulation of FAP, imaged by positron emission tomography, was observed in inflamed, damaged joints of RA patients and decreased after anti-TNF treatment. The reduction in FAP signal could reflect a phenotypic shift from pro-inflammatory FLS towards $CD200^+$ FLS (119).

The latter subset was associated with a network of pro-resolving cells, highlighting that RA-FLS can also have immune regulatory functions.

Lastly, longitudinal studies on blood transcriptional profiles of RA patients have highlighted the occurrence of "pre-inflammatory mesenchymal" (PRIME) cells in the circulation. These cells share gene expression patterns of sublining FLS and are expanded in peripheral blood just before disease flares, preceded by B cell activation. Moreover, the levels of circulating PRIME cells were found to decrease during the duration of flares. The authors suggested a model whereby PRIME cells are activated by B cells and migrate from the blood into the synovium, where they contribute to or cause joint inflammation (120).

Epigenetic alterations of RA-FLS

Changes in the epigenetic landscape of RA-FLS contribute to the imprinted, pathogenic phenotype of these cells. Such alterations include DNA methylation, histone modifications, and expression of microRNAs or long non-coding RNAs (121). DNA methylation is mediated by DNA methyltransferases (DNMTs) acting mainly in promoter regions, and usually leads to silencing of gene expression. Several studies have demonstrated differences in DNA methylation between cultured RA-FLS and OA or healthy FLS, especially implicating genes involved in inflammation, ECM interactions, and cell movement (122, 123). Moreover, these patterns are stable over many cell passages (123).

When and how the epigenetic imprinting of RA-FLS occurs is incompletely understood. Differential DNA methylation of RA-FLS, compared to FLS from other types of inflammatory arthritis, can be detected early in the RA disease course but may also change over time (124, 125). These modifications may be induced by environmental triggers (e.g. smoking, infections, hormones) as well as factors in the local inflammatory milieu in the joint, or genetic RA risk variants (126). For example, exposure to pro-inflammatory cytokines such as IL-1 can suppress the expression and function of DNMTs in RA-FLS (127).

Furthermore, DNA methylation and transcriptional patterns have been found to differ between RA-FLS depending on the joint of origin (128, 129). The location-specific signatures might contribute to variations in treatment response observed across joints (130).

Multi-evidence RA-FLS candidate genes

The advent of high-throughput, genome-scale technologies (e.g. genomics, transcriptomics, epigenomics) has opened new frontiers for the understanding of disease processes and the identification of potential therapeutic targets. Although individual datasets provide large amounts of information, integration of results from multiple omics can provide broader biological insights (131).

As an approach to identify non-obvious candidate genes implicated in the pathogenicity of RA-FLS specifically, Whitaker *et al.* performed an analysis of three datasets: 1) RA-associated risk genes identified from GWAS, 2) differentially expressed genes in RA-FLS, and 3) differentially methylated genes in RA-FLS compared to OA and healthy FLS (132). In the overlap between these datasets, seven "multi-evidence" genes were found (Figure 7). This group included genes with known involvement in the RA pathogenesis, such as *CSF2* and *HLA-DQA1*, in addition to genes with previously less characterized functions in RA. A brief description of each of the seven genes is given below.

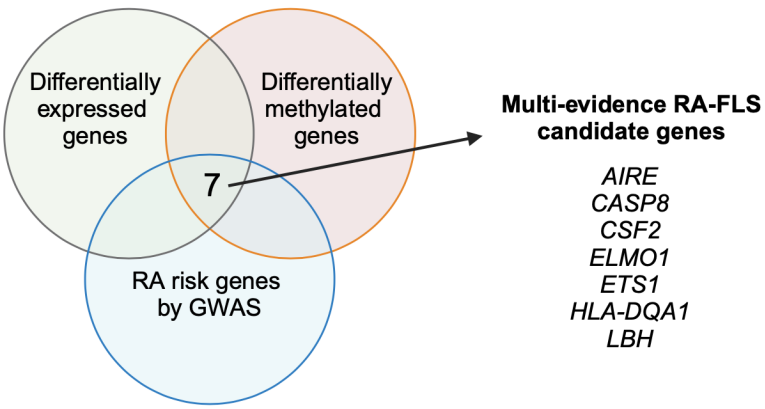

Figure 7. Multi-evidence candidate genes for the pathogenicity of fibroblast-like synoviocytes in rheumatoid arthritis as identified by integrative analysis of three omics datasets (Whitaker et al. (132)). Abbreviations: GWAS, genome-wide association studies; RA, rheumatoid arthritis; RA-FLS, rheumatoid arthritis fibroblast-like synoviocytes.

AIRE (autoimmune regulator) has a well-established role in the development of central tolerance in the thymus. AIRE is a master transcriptional regulator which induces the expression of thousands of tissue-restricted antigens (TRAs) in medullary thymic epithelial cells (mTECs). The TRAs are presented on MHC molecules to maturing T cells, leading to negative selection of self-reactive T cells or generation of regulatory T cells. Mutations in *AIRE* cause a

rare disorder called autoimmune polyendocrine syndrome type 1, typically manifested by hypoparathyroidism, adrenal insufficiency, and mucocutaneous candidiasis (133). Thymic AIRE expression is promoted by RANKL and other members of the TNF superfamily, and is also controlled by epigenetic mechanisms (134). Outside the thymus, AIRE expression has been described in secondary lymphoid tissues and in cancer tissues, but the regulation and function of peripheral AIRE, e.g. in the inflamed RA synovium, remain largely unknown (135). Interestingly, in skin tumor keratinocytes, AIRE was found to participate in the induction of pro-inflammatory genes (136).

CASP8 (caspase-8) is involved in various cellular processes, e.g. apoptosis and cell movement (137). A recent study demonstrated that caspase-8 regulates aggressive behaviors of RA-FLS. Silencing of *CASP8* in RA-FLS resulted in a decrease in cell adhesion, migration and invasion in response to PDGF (138).

CSF2 (GM-CSF) is, as mentioned, a critical regulator of macrophage activation. GM-CSF is abundant in RA synovial tissue and synovial fluid (139, 140) and is produced largely by RA-FLS. In an arthritis mouse model, GM-CSF blockade reduced inflammation and destruction of the joints (141). Early phase clinical trials have suggested efficacy of inhibitors of GM-CSF or its receptor in RA, and several studies are ongoing (142).

ELMO1 (engulfment and cell motility 1) is expressed constitutively by RA-FLS and has been demonstrated to promote migration and invasion of these cells (132). Knockout of *Elmo1* reduced arthritis severity in mice, but this effect was mainly attributed to altered migratory capacity of neutrophils (143).

ETS1 (ETS proto-oncogene 1) encodes a transcription factor that is significantly induced in RA-FLS upon stimulation with TNF and other pro-inflammatory cytokines. It promotes the expression of tissue-damaging factors such as RANKL and MMPs, and FLS-specific deletion of *Ets1* attenuated joint destruction in mouse models of arthritis (144).

HLA-DQA1 encodes the alpha chain of the class II HLA-DQ molecule and has been implicated in genetic susceptibility for RA, although the association has not been fully elucidated (145, 146).

LBH (limb-bud and heart development) variants have been associated with several autoimmune diseases (147, 148). It is a transcription co-factor involved in cell proliferation and differentiation during embryonic development. In RA,

LBH expression has been detected in the synovial lining layer. *In vitro* experiments showed that LBH controls cell cycle progression and thereby proliferation of RA-FLS (149). Deficiency of *Lbh* in mice with serum-transfer arthritis led to increased disease severity (150). The expression of *LBH* in RA-FLS is modulated by growth factors, e.g. reduced by PDGF and IL-1β (149). Moreover, it is regulated by an RA-associated SNP in combination with the DNA methylation state of an enhancer region (151).

Effects of DMARDs on RA-FLS

Despite their central contribution to the RA pathogenesis, RA-FLS are not specifically targeted by any of the currently available RA therapies. However, some DMARDs can affect the aggressive behaviors of RA-FLS, as exemplified below.

Methotrexate is a mainstay of RA treatment and acts via multiple mechanisms as mentioned earlier. FLS isolated from RA patients treated with MTX have a slower growth rate *in vitro* compared to FLS from non-MTX-treated RA patients (152). Moreover, MTX has the ability to inhibit NF-κB activation in RA-FLS, with potential consequences for pathways involved in cell proliferation and inflammation (51). A microarray study of cultured RA-FLS showed a decrease in gene expression of some pro-inflammatory cytokines (e.g. IL-6) and MMPs in response to MTX (153). Also, MTX has recently been shown to alter expression of microRNAs which could modulate migration and cytokine and chemokine production by RA-FLS (154).

Biological drugs targeting pro-inflammatory cytokines or immune cells may inhibit the activation of RA-FLS. For example, blockade of TNF or IL-6 can reduce the RA-FLS expression of chemokines and RANKL (155). TNF inhibitors could also induce apoptosis of RA-FLS in co-culture with peripheral blood mononuclear cells (156).

Effects of JAK inhibitors on RA-FLS include suppression of IL-6, RANKL and MMP production (157). Furthermore, tofacitinib has been demonstrated to interfere with TNF-induced, type I IFN-mediated chemokine expression in RA-FLS (102, 103). Tofacitinib could also decrease the RA-FLS expression of *CCL2* induced by IL-6 stimulation, and knee FLS were found to be less sensitive to this inhibition compared to FLS derived from hip (158). This suggests joint-specific differences in JAK/STAT signaling that could influence the response to JAK inhibitors (130).

Still, there is a need for enhanced understanding of the effects of DMARDs on pathogenic RA-FLS functions, in order to guide future treatment strategies. Synovial biopsy-based studies have shown that fibroblast signatures and increases of specific RA-FLS subsets (e.g. $DKK3^{+}$) are associated with poor treatment response to multiple DMARDs (92). Targeting RA-FLS could be a promising approach to improve RA remission rates without increasing immunosuppression.

AIMS

The overall aim of this thesis was to increase the knowledge of molecular mechanisms shaping the activated RA-FLS phenotype. Our main approach was to study the role of multi-evidence, RA-associated risk genes in these cells. Moreover, we wanted to investigate the effects of DMARDs, in particular MTX, on the expression of RA risk genes and pathogenic behaviors of activated RA-FLS in order to identify untargeted disease pathways.

The following research questions were addressed in the three papers:

Paper I

- Can the RA risk gene *AIRE* be detected in synovial tissue from RA patients?
- What regulates *AIRE* expression in primary FLS?
- What is the function of *AIRE* in activated RA-FLS?

Paper II

- Does MTX affect the proliferation of activated RA-FLS?
- Does MTX modulate the expression of the RA risk gene and cell cycle regulator *LBH* in activated RA-FLS and what is the potential mechanism?

Paper III

- How does MTX and tofacitinib change the transcriptome of activated RA-FLS?
- Which disease-associated risk genes are targeted by these DMARDs in activated RA-FLS?
- Which pathogenic responses of activated RA-FLS are not resolved by these DMARDs and what are the potential consequences for persistence of inflammation?

METHODS

This chapter will summarize and comment on the methodology used within the thesis. Further details are provided in the individual papers.

PATIENT SAMPLES AND ETHICAL CONSIDERATIONS

Synovial tissue specimens were collected at the time of joint replacement surgery from patients with established RA or OA. All RA patients fulfilled the 1987 ACR criteria for the disease. Anti-rheumatic drugs had been stopped at least two weeks before surgery. The obtained samples were remnant tissues that would otherwise have been discarded during the surgical procedure. Thus, no additional risk of physical harm could be expected from study participation. In **Paper III**, synovial biopsies, blood samples, and clinical data were collected from a cohort of patients with early RA (symptom duration ≤24 months) diagnosed according to the 2010 ACR/EULAR criteria. Ultrasound-guided synovial biopsy is a minimally invasive procedure demonstrated to be both safe and well-tolerated by patients (159). Potential adverse events associated with venipuncture are also rare but include discomfort, bruising, and fainting.

Human thymic tissue (used as positive control for AIRE expression in **Paper I**) was obtained from children undergoing corrective cardiac surgery for congenital heart defects. Removal of the thymus is routinely performed in this setting to facilitate surgical access and the tissue is normally discarded. However, early thymectomy has been associated with long-term immunological changes and an increased risk of infections, autoimmune disease, and cancers (160). These data have reinforced a more modern operative approach aiming to preserve thymic tissue when possible.

Research involving human subjects, especially in the era of genomics, may reveal information relevant to the participants' health, e.g. risk of disease other than the one directly under study. The management of such incidental findings should be considered, as they may potentially cause anxiety to the participants. All studies included in this thesis were approved by the Regional Ethics Committee of Gothenburg or the Swedish Ethical Review Authority (after 2019) and conformed to the Declaration of Helsinki. All samples were collected, used and biobanked according to the ethical approvals. All study participants (or their legal guardians in case of children) signed an informed consent form. The samples were coded to protect the participants' confidentiality. Results are presented without identifiable information.

IN VITRO EXPERIMENTS

Cell culture

Synovial tissue samples were enzymatically digested, and the dissociated cells were placed in culture. Not only FLS but also synovial macrophages adhere to the bottom of the culture vessel, but after around three to four passages the FLS are dominant (28) (Figure 8). At this point they form a relatively homogenous population and are used for experiments. FLS were not used after passage 9, due to senescence and reduced growth rate (161).

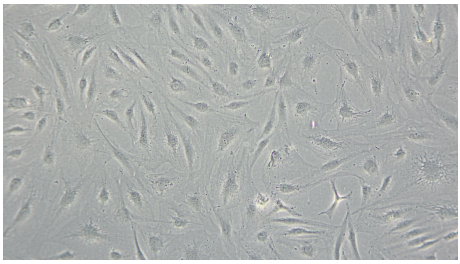

Figure 8. Monolayer culture of primary fibroblast-like synoviocytes.

The use of cell cultures can reduce the need of animal experiments and related ethical concerns. Primary FLS have the advantage over immortalized cell lines of more closely representing the biology of cells in the human joint. Thus, they also reflect the inherent variability existing between donors. As mentioned, primary cells have a limited lifespan in culture and may undergo phenotypic changes following passaging. Cell lines, on the other hand, are easier to expand, often provide more consistent results and may facilitate standardization.

FLS monolayer cultures are convenient to maintain, but a weakness of this structure is the loss of the physical influence of tissue architecture. Diverse methods for more complex three-dimensional cultures have been developed but were not assayed in our studies. Another limitation of conventional FLS cultures is the lack of interactions with immune cells which co-exist with FLS in the RA synovium. In **Paper III**, we performed an "*ex vivo* bioassay" as described by Kuo *et al.* (84), aiming to establish a direct co-culture of FLS and other synovial cells disaggregated from RA synovial tissue. However, one difficulty was monitoring the number of macrophages present in this system. We also set up a transwell FLS-macrophage indirect co-culture, which enabled better control and assessment of the paracrine communication between the two cell types.

Stimulations and treatments

During the first passages in cell culture, RA-FLS constitutively express some cytokines, MMPs and growth factors, but this production gradually decreases. The activated RA-FLS phenotype can be restored by exposure to an inflammatory milieu, e.g. by stimulation with cytokines like IL-1β and TNF-α (93). Another mediator that is abundant in the RA joint is the mitogen PDGF, which potently increases the proliferation of FLS (95). Previous studies have demonstrated that growth factors synergistically potentiate the effects of some cytokines on FLS (162).

The selected concentrations of the human recombinant proteins used to activate FLS in our studies (IL-1β, TNF-α, PDGF, as indicated in the papers) were based on earlier data of biologically active ranges. Serum-starvation was performed prior to stimulation, in order to synchronize cells to the same cell cycle phase and to reduce interference from other factors present in serum.

Regarding the doses of MTX (**Papers II** and **III**) and tofacitinib (**Paper III**) used in the experiments, we aimed to achieve physiologically relevant concentrations. However, data on the concentration of drugs in the joint of RA patients are scarce. Rather, we were guided by reports of plasma concentrations reached by these medications at dosages given for RA (163-165). Also, one study showed that the synovial concentration of MTX was around 10-fold higher than the simultaneous concentration in plasma (166).

A challenge posed to the experimental setup was the fact that MTX is gradually converted to its active form by intracellular polyglutamation, while the effect of mitogens (PDGF, IL-1β) come relatively fast. To capture the effects of MTX on FLS activation, we therefore developed a protocol where FLS were pre-treated with MTX for 24 hours before stimulation (Figure 9). The mitogens were then added in continued presence of MTX.

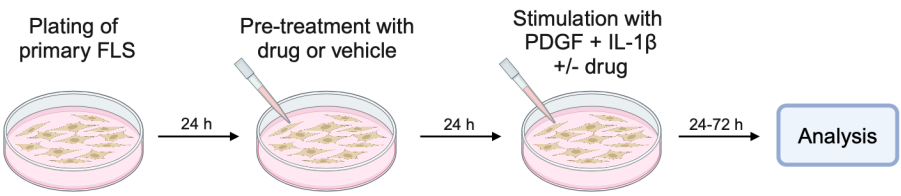

Figure 9. Design and timeline of treatment experiments with methotrexate or tofacitinib. Abbreviations: FLS, fibroblast-like synoviocytes; IL, interleukin; PDGF, platelet-derived growth factor. Created with BioRender.com

Gene silencing

In **Paper I**, the function of the RA risk gene *AIRE* in activated RA-FLS was studied by RNA sequencing of TNF + IL-1β-stimulated cells where *AIRE* was either expressed or had been silenced by small interfering RNA (siRNA). Synthetic siRNA is a commonly used tool to suppress gene expression through RNA interference, targeting a specific mRNA for degradation (Figure 10). Methods for delivery of siRNA into cells include viral vectors, transfection using cationic lipid-based reagents or, as in our experiments, electroporation of the cell membrane by means of an electrical pulse. The choice of transfection approach is largely governed by cell type.

The siRNA, consisting of an antisense strand and a sense strand forming a duplex of 21-23 base pairs length, is unzipped intracellularly. The antisense strand is incorporated with proteins into the RNA-induced silencing complex, which mediates binding and cleavage of the complementary target mRNA sequence (167). In this way, translation of the encoded protein is hindered. However, siRNA can also have non-specific effects resulting in unintended silencing of other genes, why a non-targeting siRNA should be included as negative control in the experiment. The gene knockdown induced by siRNA is transient, lasting up to around a week after transfection.

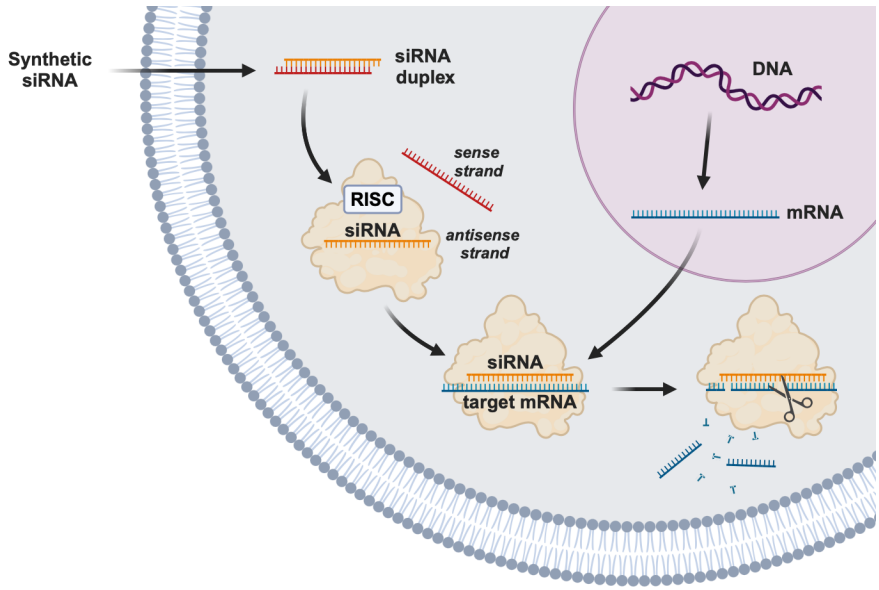

Figure 10. Mechanism of RNA interference by siRNA. Abbreviations: RISC, RNA-induced silencing complex; siRNA, small interfering RNA. Created with BioRender.com

SAMPLE ANALYSIS

Quantitative polymerase chain reaction

Analysis of gene expression was performed using real-time quantitative polymerase chain reaction (qPCR). First, RNA was extracted from cells by the use of commercial kits including a step of DNase I treatment to eliminate contamination of genomic DNA. Complementary DNA (cDNA) was then synthesized by reverse transcription and used as a template for qPCR. TaqMan assays, consisting of a pair of target-specific primers and a fluorogenic probe, enable specific and sensitive detection of a target of interest (168). At the start of each PCR cycle, the double-stranded cDNA is denatured at a high temperature, followed by lowering of the temperature to allow the primers and probe to anneal to their target sequences. Next, the Taq DNA polymerase binds to the primers and begins the extension phase, synthesizing new amplicon strands. When reaching the TaqMan probe, the DNA polymerase cleaves the probe via 5' nuclease activity, resulting in release of a reporter dye molecule from its quencher (Figure 11A). The reporter thus produces a fluorescence signal that increases in proportion to the amplified product and is continuously recorded by the instrument. A qPCR experiment typically runs for 40 cycles. The cycle number at which the fluorescence intensity passes a certain threshold above background levels, is called the cycle threshold (Ct) (Figure 11B). The greater the initial quantity of target mRNA in the sample, the earlier the amplification curve will cross the threshold and the lower the Ct value will be. Given an amplification efficiency of 100%, the PCR product doubles in each cycle, meaning a difference of 1 Ct corresponds to a 2-fold difference in the original number of target copies. A reference gene (e.g. *GAPDH*) is used to normalize the results for the amount of starting material added to the reactions. The relative changes in gene expression in treated samples compared to untreated control samples can be calculated using the $2^{-\Delta\Delta Ct}$ method (169).

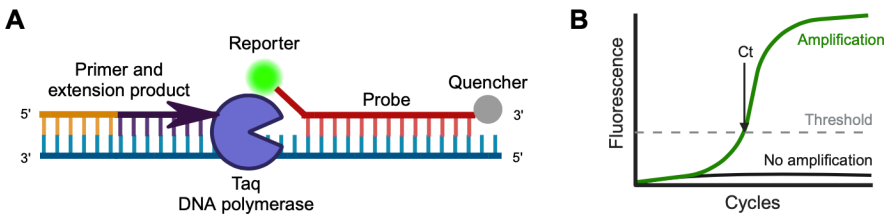

Figure 11. Quantitative polymerase chain reaction methodology. A) Overview of TaqMan assay. B) Principle of amplification curve and identification of cycle threshold (Ct) value. Created with BioRender.com

RNA sequencing and bioinformatics workflow

RNA sequencing (RNA-seq) was applied in **Papers I** and **III** for gene expression profiling at the whole-transcriptome level. This method is based on next-generation sequencing technologies and provides an unbiased view of coding and non-coding RNA transcripts in a sample, in contrast to qPCR or microarrays which use primers or probes to selected targets of interest. Library preparation and RNA-seq were conducted on an Illumina platform at the Genomics Core Facility, and bioinformatics analysis was performed with support from the Bioinformatics Core Facility at the University of Gothenburg.

Briefly, the TruSeq Stranded Total RNA Ribo-Zero protocol implies that samples are depleted of cytoplasmic and mitochondrial ribosomal RNA, followed by fragmentation and construction of a first-strand cDNA library. The cDNA molecules are ligated with index adapters binding to complementary oligonucleotides on the surface of the flow cell in the sequencing instrument. Through bridge amplification, a cluster is generated from each cDNA fragment. Sequencing-by-synthesis is then performed with paired-end strategy, meaning that cDNA fragments are read from both ends, which increases the precision of the alignment.

For bioinformatics analysis, a range of open-source tools can be utilized. The output sequencing data is subjected to quality assessment and filtering, e.g. based on read quality, read length, or adapter traces. The filtered reads are then mapped to the human reference genome and quantified. To analyze differential gene expression, we used the DESeq2 software package (170). The DESeq2 function normalizes the raw count data for differences in sequencing depth between samples. It performs statistical testing (Wald test followed by Benjamini-Hochberg correction for multiple testing) and outputs a log2 fold change for each gene, indicating how much the gene's expression has changed in one group in comparison to a control group.

To derive greater biological meaning from the list of differentially expressed genes, various functional analyses can be conducted. For example, the QIAGEN Ingenuity Pathway Analysis (IPA) application, which builds on a comprehensive repository of biological interactions, can identify pathways and functions relevant to a dataset. We also used Gene Set Enrichment Analysis (GSEA), a method that evaluates whether a gene set (a group of genes involved in a specific biological process according to previous knowledge) is overrepresented at the top or bottom of a ranked list of genes from the RNA-seq expression data (171).

Immunofluorescence staining and confocal microscopy

In **Paper I**, cultured FLS and sections of human synovial tissue and thymus were subjected to indirect immunofluorescence staining and confocal microscopy, enabling detailed visualization of proteins of interest. Tissues were fixed with paraformaldehyde and embedded in paraffin to preserve morphology. Since the fixation process can mask antigens by cross-linking proteins, it is necessary to perform an antigen retrieval step after sectioning the specimens, e.g. by heating the slides. For staining, samples are incubated with a blocking buffer to prevent non-specific binding, then a primary antibody which recognizes the target antigen, followed by washes and the addition of a fluorochrome-coupled secondary antibody that binds to the primary antibody. Advantages of using this approach (instead of direct immunofluorescence where the primary antibody is directly conjugated to a fluorochrome) include higher flexibility when choosing antibodies, and signal amplification as more than one secondary antibody can bind to each primary antibody. However, the indirect method requires longer time and carries the potential for cross-reactivity between secondary antibodies when samples are stained for several targets, and non-specific binding to endogenous immunoglobulins resulting in higher background fluorescence. Selecting the right primary antibody can be a challenge, given the growing number of commercial research antibodies and the lack of quality control standards for these products. Important aspects to consider are whether the antibody specificity has been properly validated and whether the antibody has been tested for the particular application.

The immunofluorescence preparations can be analyzed by confocal laser scanning microscopy, a specialized imaging technique for localization of the targets within the cells or tissues. It has the ability to provide high-resolution images by the use of point-by-point illumination and scanning of the specimen with a laser beam, and a confocal pinhole eliminating out-of-focus light, followed by electronic processing of the signal.

Flow cytometry

Flow cytometry works by detection of light scattering and fluorescence of cells and particles in a fluid stream as they pass one by one through a laser beam. Samples are usually stained with fluorescently labeled antibodies or ligands that bind to specific markers on the cell surface or intracellularly. The technique allows measurement of multiple parameters simultaneously and can collect data from a large number of cells. It has many different applications, including analysis of cell phenotypes as well as functional changes.

Intracellular protein staining

Apart from immunofluorescence, protein expression of AIRE in RA-FLS (**Paper I**) was also assessed by flow cytometry. An isotype control, indicating the level of non-specific background staining, was used to help define the gate for the positive population. To visualize the localization of AIRE staining within the cells, samples were also acquired on an ImageStream system. This instrument combines flow cytometry with fluorescence microscopy and produces high-resolution images of every cell in the sample.

Cell cycle analysis

In **Paper II**, effects of mitogens and of methotrexate on FLS proliferation were studied by cell cycle analysis using the fluorescent DNA dye 7-amino-actinomycin (7-AAD). The protocol involves fixation of samples with ethanol, which permeabilizes the plasma membrane to allow 7-AAD to enter the cells and access the DNA. The fluorescence intensity of 7-AAD measured by flow cytometry correlates to the amount of DNA in each cell. Cells in G2/M phase of the cell cycle have doubled their DNA content and will therefore fluoresce twice as brightly as cells in G1 phase (Figure 12A). We applied a gating strategy (Figure 12B) first identifying cells and excluding debris. Next, doublets were removed, as these could otherwise overestimate the proportion of G2/M cells. A DNA histogram of the single cells was then used to estimate the percentages of cells in G1 and G2/M phase, respectively.

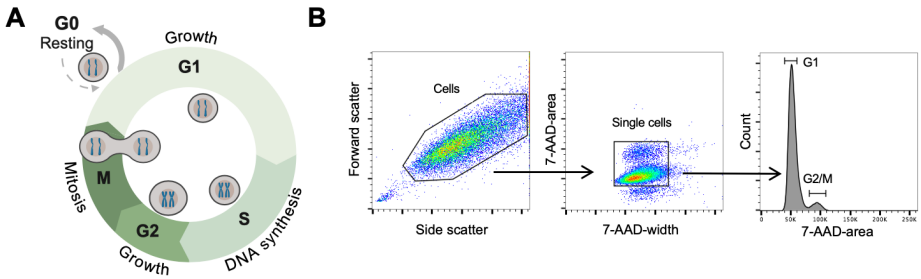

Figure 12. Cell cycle analysis by flow cytometry. A) Schematic overview of the cell cycle phases. Created with BioRender.com B) Gating strategy for experiments conducted in Paper II using the DNA dye 7-amino-actinomycin (7-AAD).

Bead-based immunoassay

Levels of secreted chemokines and cytokines were analyzed in cell culture supernatants (**Papers I** and **III**) and in human serum samples (**Paper III**) using flow cytometric, bead-based immunoassays. This method offers sensitive and specific measurement of analytes of interest and has greater multiplexing capabilities than conventional enzyme-linked immunosorbent assay. The

technology involves capture beads with distinct fluorescence patterns, each coated with antibodies specific to a single analyte. Target protein present in the sample or in the standard will thus be bound by a specific bead. Next, a biotinylated detection antibody is allowed to bind to its target, which is already bound by a capture bead, creating a "sandwich" with the analyte in between (Figure 13). A reporter dye (streptavidin-conjugated phycoerythrin) is then added and binds to the biotinylated antibodies. During flow cytometry, the beads are identified by fluorescence signature, and the signal from the reporter dye, which is proportional to the amount of bound analyte, is quantified. Concentrations are calculated using a standard curve.

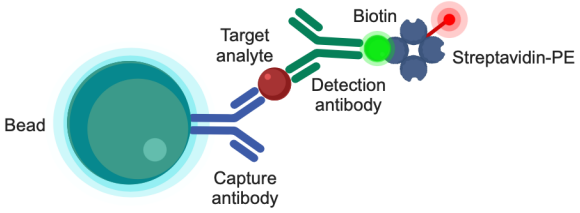

Figure 13. Basic principle of bead-based immunoassay. Created with BioRender.com

STATISTICAL ANALYSIS

The number of samples included in each cell culture experiment was most often three, due to practical reasons. These samples were biological replicates, i.e. independent primary FLS lines derived from different patients, capturing the variability in the population under study. Data from separate experiments that were performed in the same way but using different biological replicates, were subject to pooling. Results are presented as mean ± standard error of the mean (SEM) or median as indicated. Normal distribution of data was confirmed by Shapiro-Wilk test, followed by parametric analysis: Student's *t* test for comparison of means between two groups, or one-way analysis of variance (ANOVA) with post hoc tests for comparison of means among more than two groups. The non-parametric Wilcoxon signed rank test was applied on non-normal data. Paired/repeated-measures test was used when each biological sample was measured under different conditions/treatments or at multiple time points. Statistical analysis of differences in gene expression by qPCR was performed on ΔCt values. Correlations between fold changes in gene expression were tested with a non-parametric method, Spearman's rank correlation coefficient. Data were analyzed using the GraphPad Prism software. Bioinformatics analysis of RNA-seq data was conducted as stated above.

RESULTS AND DISCUSSION

This chapter will highlight and briefly discuss the main results of the three studies constituting this thesis. A complete account of results is provided in each paper.

PAPER I

Expression, regulation and function of AIRE in RA-FLS

The transcriptional regulator AIRE is primarily known for its critical function in the induction of central immune tolerance in the thymus. Genetic polymorphisms of *AIRE* have been associated with RA, although it is unclear how these variants contribute to disease pathology. The integrative analysis conducted by Whitaker *et al.* combining GWAS with multi-omics data of FLS from RA patients and controls, suggested a role of *AIRE* in RA-FLS (132). **Paper I** aimed to investigate the expression, regulation and function of *AIRE* in these cells.

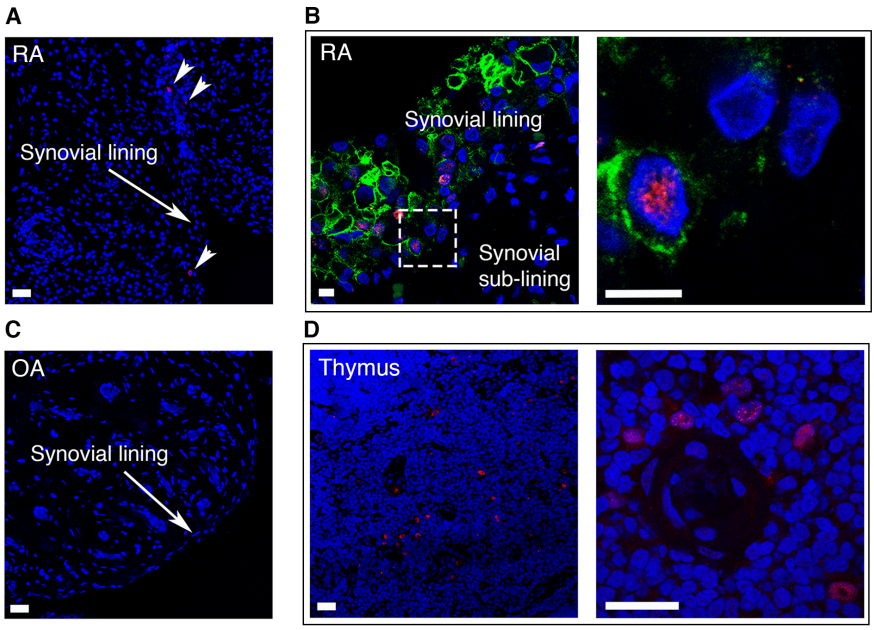

Figure 14. Immunofluorescence images of AIRE expression. A) RA synovium stained for AIRE (red) and cell nuclei (blue). Arrowheads point at AIRE positive cells in the hyperplastic synovial lining layer. B) RA synovium double-stained for AIRE (red) and the FLS activation marker podoplanin (green). C) OA synovium displaying a thin synovial lining and lacking AIRE positive cells. D) Thymus tissue as positive control for AIRE staining (red).

AIRE is expressed in activated FLS of the RA synovial lining layer

Immunofluorescence staining of AIRE protein was performed on synovial tissue samples from patients with established RA and patients with OA as controls. To determine if AIRE is expressed in areas of FLS activation, the samples were simultaneously stained for podoplanin. We demonstrated that AIRE is indeed expressed in activated FLS in RA synovial tissue, predominantly in the lining layer (Figure 14A-B). The nuclear, speckled staining of AIRE was similar to the pattern observed in mTECs of human thymic sections (Figure 14D), which also confirms the specificity of the antibody used. However, AIRE could not be detected in OA synovia (Figure 14C).

AIRE is induced in primary FLS by pro-inflammatory cytokines

TNF and IL-1β are abundant cytokines in the RA joint and known "activators" of RA-FLS. Using RNA-seq, we demonstrated that stimulation of cultured, primary RA-FLS with these pro-inflammatory factors increases the gene expression of a range of cytokines, chemokines, MMPs, and adhesion molecules, compared to unstimulated cells. Also, there was a differential expression of multiple known RA risk genes, including an upregulation of *AIRE*, following TNF + IL-1β stimulation. This supports the relevance of these genes in activated RA-FLS.

As confirmed by qPCR and by immunofluorescence, AIRE was not expressed in unstimulated FLS, but was strikingly induced by cytokine stimulation. This effect was observed in both RA-FLS and OA-FLS, although the induction of *AIRE* was considerably higher as well as more variable in RA-FLS. The differences may be influenced by "priming" of the RA-FLS as a result of previous repeated inflammatory exposure in the joint. It has been demonstrated that FLS *in vitro* can possess an inflammatory memory, mounting augmented pathogenic responses upon re-stimulation (172). Moreover, genetic and epigenetic factors may contribute to the differences in *AIRE* expression levels between RA-FLS and OA-FLS.

AIRE does not induce TRAs in RA-FLS

In the thymus, AIRE enables the expression of TRAs in mTECs, driving the negative selection of self-reactive T cells. Based on the knowledge that RA-FLS can act as antigen-presenting cells (107), we first hypothesized that AIRE induces the expression of TRAs also in RA-FLS. To study which genes are regulated by AIRE in RA-FLS, *AIRE* silencing and RNA-seq was performed

as outlined in Figure 15. Since AIRE is not expressed without stimulation, both the "high AIRE" and "low AIRE" samples were stimulated with TNF + IL-1β.

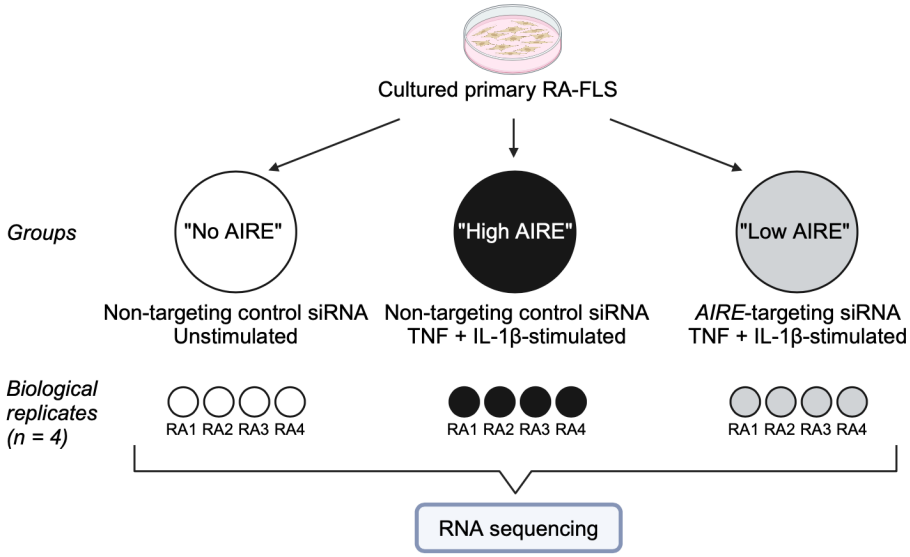

Figure 15. Experiment performed in Paper I to study the functional role of AIRE in RA-FLS, using RNA interference and RNA sequencing. Created with BioRender.com

A total of 217 genes were found to be differentially expressed in "high AIRE" compared to "low AIRE" RA-FLS. There was no enrichment of TRAs (defined as genes with an expression restricted to up to five tissues) among these AIRE-regulated genes, nor did AIRE induce MHC class II genes or MHC class II transactivator gene in RA-FLS. These data indicate that AIRE has another role in RA-FLS than in mTECs. The functions of AIRE in other described extrathymic AIRE-expressing cells (eTACs) remain elusive, but may include regulation of peripheral tolerance (135). For example, in secondary lymphoid tissues, eTACs with phenotypical similarities to dendritic cells expressing TRAs have been discovered (173). Moreover, studies in mice have found lymph node stromal cells expressing Aire and TRAs (174). In contrast, there are also reports of eTACs that lack TRA enrichment (175), and studies suggesting that AIRE promotes the expression of pro-inflammatory and cancer-related genes in certain tumor cells (136, 176). It seems that AIRE can play different roles in different cell types and contexts.

AIRE promotes expression of IFN-regulated genes in RA-FLS

Interestingly, the RNA-seq dataset revealed that AIRE increases the expression of several chemokines in RA-FLS (Figure 16A), e.g. CXCL10 which has been associated with disease activity in RA (177). Furthermore, we found that 93% (201/217) of the AIRE-regulated genes were classified as IFN-regulated genes, predominantly type II but also type I IFN-regulated genes (Figure 16B), according to the INTERFEROME database (178).

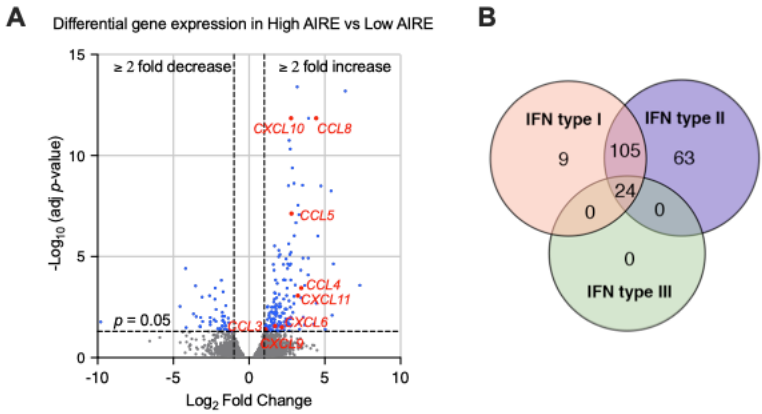

Figure 16. AIRE augments expression of an interferon gene signature in activated RA-FLS. A) Volcano plot showing differentially expressed genes (blue dots, adjusted p <0.05) by RNA-seq in "high AIRE" versus "low AIRE" samples. Chemokines highlighted in red. B) Classification of the 201 AIRE-regulated genes annotated as IFN-regulated genes by INTERFEROME.

The most significant biological functions identified among the AIRE-regulated genes by IPA were also related to processes where type I and type II IFN are involved, e.g. "antimicrobial response" and "inflammatory response". IFN-γ was not detectable in the cell culture supernatants nor upregulated in the RNA-seq data, suggesting that the IFN signature induced by AIRE in RA-FLS is independent of type II IFN production. Instead, the transcription of IFN-regulated genes might be more directly modified by AIRE. The significance of an increased interferon gene signature (mainly generated by type I IFN) has been demonstrated in patients with early untreated RA, where it predicts poorer six-month clinical outcomes (179). Also, there are associations between response to anti-TNF therapy in RA and changes in interferon signatures during treatment (180, 181). In summary, our results propose a pro-inflammatory role of AIRE in RA-FLS. Hypothetically, in genetically pre-disposed individuals, the induction of AIRE in FLS in an inflammatory environment may contribute to enhanced immune activation and chronic inflammation of the joint.

PAPER II

Effects of MTX on mitogen-induced proliferation of RA-FLS

Prominent features of activated RA-FLS include anomalies in pathways related to proliferation and apoptosis (93). MTX, the first-line treatment for RA, has been reported to restore defects in the expression of cell cycle checkpoint genes in T cells (49), but its effects on RA-FLS are incompletely characterized. **Paper II** aimed to study how MTX affects the proliferation and the expression of cell cycle regulators in RA-FLS in response to mitogens.

MTX inhibits effects of PDGF + IL-1β on RA-FLS cell cycle progression

PDGF is a known potent mitogen for FLS (95), and we found that the combination of PDGF with the pro-inflammatory cytokine IL-1β also has potential to promote cell cycle progression of RA-FLS. It has previously been shown that PDGF augments IL-1β-induced secretion of IL-6 from RA-FLS (162). Our results demonstrate that the cells are also capable of a proliferative response to this exposure. In the presence of MTX, however, the effect of PDGF + IL-1β on RA-FLS cell cycle progression was abolished, meaning cells were halted in G1 phase (Figure 17). This finding suggests a novel mode of action contributing to the efficacy of MTX in RA.

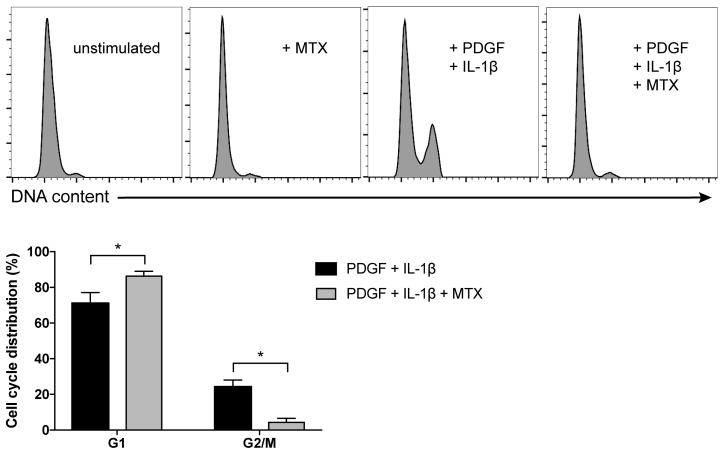

Figure 17. MTX inhibits mitogen-induced proliferation of RA-FLS. Cells were treated with MTX alone or stimulated with PDGF + IL-1β in the presence or absence of MTX for 24 h, followed by flow cytometric cell cycle analysis. Representative DNA content histograms and cell cycle distribution percentages at indicated treatments are shown.

The potential benefits of interfering with the cell cycle regulation of activated FLS have been demonstrated in animal models of arthritis. Cell cycling is

tightly controlled by protein complexes formed by cyclins and cyclin-dependent kinases (CDKs). In mice with collagen-induced arthritis, administration of CDK inhibitors suppressed FLS proliferation, by inducing cell cycle arrest at the G1 phase, and ameliorated disease (182, 183). In the ongoing TRAFIC clinical trial (phase Ib/IIa), the CDK inhibitor seliciclib is tested on active RA patients refractory to anti-TNF therapy, with the hypothesis that the combination of biological DMARDs with a drug targeting FLS proliferation may have additive anti-rheumatic effects (184).

MTX increases the expression of tumor suppressor genes in activated RA-FLS, possibly via epigenetic mechanisms

The multi-evidence RA-FLS candidate gene *LBH* has previously been demonstrated to regulate cell cycle progression of primary FLS by acting as a tumor suppressor (149). We confirmed that the gene expression of *LBH* by qPCR decreased in response to mitogen stimulation with PDGF + IL-1β. However, in the presence of MTX, the *LBH* expression was significantly higher upon mitogen stimulation compared to without MTX. Likewise, MTX was found to inhibit the effects of PDGF + IL-1β on the gene expression of several other known cell cycle regulators, e.g. the endogenous CDK inhibitor p21 (*CDKN1A*) which may play an important role in the joint pathogenesis of RA. In addition to inducing G1 cell cycle arrest, p21 has been implicated in suppressing pro-inflammatory responses of RA-FLS. Moreover, the expression of p21 is downregulated in the synovial lining of RA compared to OA (98). One approach to validate our data and assess to which extent MTX may restore synovial p21 expression, could be via synovial biopsies collected before and after treatment.

To investigate if the effects of MTX on *LBH* expression in activated RA-FLS involve epigenetic mechanisms, we studied the gene expression of DNMTs. Stimulation with PDGF + IL-1β reduced *DNMT1* expression, consistent with earlier reports regarding IL-1β (127), but this effect was abrogated in the presence of MTX. Furthermore, there was a correlation between *LBH* and *DNMT1* expression in MTX-treated activated FLS. It has previously been demonstrated that MTX treatment reverts global hypomethylation and *DNMT1* expression of T cells and monocytes in peripheral blood of RA patients (185). Our data suggest that MTX might possibly also modulate cell cycle regulator gene expression and proliferation of RA-FLS via changes in DNA methylation. However, this would need to be confirmed e.g. by analysis of methylation status of the specific genes of interest.

PAPER III

Effects of MTX on disease-associated risk genes and pro-inflammatory mediators in RA-FLS

A better understanding of the molecular mechanisms contributing to the therapeutic efficacy of MTX may pave the way for the development of novel treatment strategies and markers to predict drug response. However, in light of the fact that around 50% of RA patients respond inadequately to MTX monotherapy (40, 41), it is equally important to identify pathogenic pathways that are *not* targeted by MTX and may perpetuate inflammation. In **Paper III**, we performed transcriptomic analysis to characterize the influence of MTX, and the JAK inhibitor tofacitinib (TOFA) as a comparator drug, on activated RA-FLS. We were especially interested in effects of these drugs on the expression of disease-associated risk genes and mediators involved in the inflammatory crosstalk between activated RA-FLS and immune cells.

MTX alters the expression of thousands of genes in activated RA-FLS

Primary RA-FLS were activated with PDGF + IL-1β in the presence or absence of MTX or TOFA for 48 hours prior to RNA-seq. We found that more than 6000 genes were differentially expressed by MTX, the majority being upregulated (Figure 18A), whereas around 1000 genes were differentially expressed by TOFA, compared to untreated activated control. MTX had strongest effects on pathways related to cancer and cell cycle regulation, supporting our findings from **Paper II**. For TOFA, the most significant pathway by IPA of differentially expressed genes was "interferon signaling", which is consistent with earlier reports of JAK inhibitors suppressing interferon responses in cytokine-activated RA-FLS (102, 103).

MTX targets disease-associated genes in RA-FLS but unexpectedly increases expression of pro-inflammatory mediators

Out of 103 RA risk genes previously identified by GWAS (7), 37 were differentially expressed by MTX in activated RA-FLS (Figure 18B). The expression of three multi-evidence RA-FLS candidate genes was altered by MTX, namely *LBH*, *ETS1*, and *CSF2*. Effects were also seen on other genes important to RA-FLS pathogenicity, e.g. upregulation of *CDKN1A* and downregulation of *PDPN* (podoplanin) and *MMP3* by MTX (Figure 18C). Surprisingly, MTX *increased* the expression of *CSF2* and also of other pro-inflammatory mediators like *IL1A*, *CCL20*, as well as of *TGFA* and *ETS1*. Similarly, a previous study reported the induction of pro-inflammatory

cytokines (IL-1, IL-6) by MTX in a monocytic cell line, with speculations that such effects could contribute to toxicities of MTX (186).

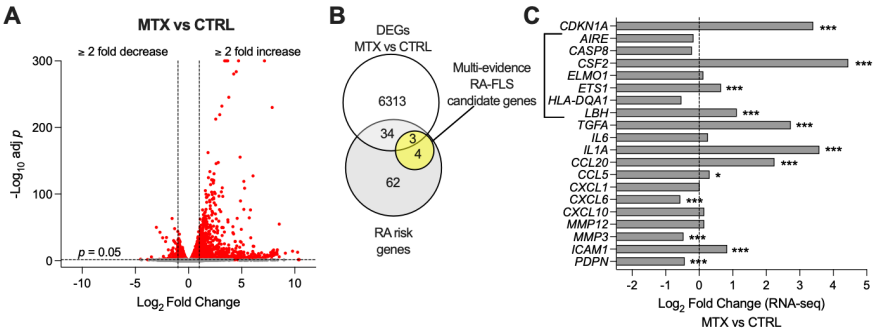

Figure 18. MTX alters the expression of a large number of genes in activated RA-FLS, including disease-associated risk genes. A) Volcano plot showing differentially expressed genes (DEGs; red dots) by RNA-seq of MTX-treated versus untreated activated RA-FLS. B) Venn diagram of DEGs by MTX, RA risk genes from GWAS, and multi-evidence RA-FLS candidate genes (132). C) RNA-seq-based fold changes in expression of pathogenic RA-FLS genes in response to MTX.

MTX augments the release of GM-CSF from RA-FLS, with implications for crosstalk with macrophages

CSF2/GM-CSF is a known mediator in the crosstalk between FLS and macrophages in the RA synovium, where TNF and IL-1β secreted by macrophages stimulate FLS to produce IL-6 and GM-CSF which in turn activate macrophages (104, 187). We demonstrated that PDGF + IL-1β induces *CSF2* expression in RA-FLS and that this effect is augmented in the presence of MTX. Also, increased levels of GM-CSF protein could be detected in the supernatants of MTX-treated compared to untreated activated RA-FLS. Interestingly, MTX promoted the expression of *CSF2* even in *unstimulated* RA-FLS.

In an indirect co-culture assay, MTX treatment of activated RA-FLS lead to increased expression of *IL1B* in macrophages. Thus, MTX may enhance the "vicious cycle" between these activated synovial cell types, potentially contributing to persistent joint inflammation. At the same time, MTX can act as a JAK/STAT inhibitor (52) and it is possible that it dampens the effects of GM-CSF on macrophages to some extent.

MTX promotes the production of GM-CSF in RA-FLS via increased IL-1 signaling

Through inhibition experiments, we demonstrated that the MTX-promoted expression of *CSF2* in activated RA-FLS could be mediated via either NF-κB or AP-1 transcription factors. Furthermore, the effect of MTX on *CSF2* expression was abrogated by IL-1 inhibitors (IL-1 receptor-associated kinase 4 inhibitor zimlovisertib and recombinant IL-1 receptor antagonist anakinra). We suggest a mechanism whereby MTX increases the release of IL-1α from activated RA-FLS, via increased transcription of *IL1A* and possibly also via secretion of intracellular preformed IL-1α, which in an autocrine fashion augments the expression of *CSF2*/GM-CSF.

Data from the recent PEAC study of patients with early treatment-naïve RA (89) show that transcript levels of *CSF2*, *IL1A* and *IL1B* in the joint positively correlate with ultrasound-detected synovial thickening, supporting the clinical relevance of these mediators. In order to translate our *in vitro* findings, it would be interesting to analyze how the synovial expression of GM-CSF and IL-1 change in response to MTX treatment and in relation to clinical outcome, but such data are currently not available. Blood samples are more often routinely collected, and we measured protein levels of the mentioned mediators in serum from early RA patients before and after two years of anti-rheumatic therapy. However, the sample size was rather small, the serum levels of GM-CSF and IL-1β were generally difficult to detect and it is uncertain how well blood samples reflect the processes taking place locally in the inflamed joints. Yet, an interesting finding was that a higher percentage of patients in the group that had received MTX had unchanged or increased serum levels of IL-1α at follow-up, compared to the group that had not been treated with MTX.

Biological drugs targeting GM-CSF are being evaluated for the treatment of RA (142). The IL-1 receptor antagonist anakinra is approved for DMARD-refractory, moderate to severe RA but is rarely used in clinical practice. Our results proposing that MTX promotes IL-1 signaling and thereby GM-CSF production of RA-FLS, may support the combination of MTX with inhibitors targeting the IL-1 pathway.

CONCLUSIONS AND FUTURE PERSPECTIVES

This thesis provides new knowledge on the role of disease-associated risk genes in activated RA-FLS and the effects of MTX on these cells.

In **Paper I**, we demonstrated that the RA risk gene *AIRE* is expressed in activated FLS in the RA synovium and can be induced *in vitro* by stimulation with pro-inflammatory cytokines. Unlike its role for tolerance induction in the thymus, AIRE does not seem to mediate expression of tissue-restricted antigens in RA-FLS. Instead, AIRE promotes the transcription of a set of interferon-regulated genes associated with pro-inflammatory responses.

In **Paper II**, we found that MTX increases the expression of the RA risk gene *LBH*, as well as other tumor suppressor genes, in RA-FLS activated with PDGF + IL-1β. Using cell cycle analysis, we demonstrated that MTX inhibits the effects of these mitogens on RA-FLS proliferation.

In **Paper III**, we described the broad transcriptional changes induced by MTX in activated RA-FLS, including an unexpected upregulation of known pro-inflammatory mediators like *IL1A* and the RA risk gene *CSF2*. Our data suggest that MTX promotes the production of GM-CSF in activated RA-FLS via autocrine IL-1 signaling, with potential implications for the FLS-macrophage crosstalk contributing to persistent synovitis.

The results support the biological relevance of multi-evidence RA-FLS candidate genes identified by integrative omics analysis, and that RA-FLS are important targets in the disease. Whereas most functional studies on RA risk genes by GWAS have been performed on immune cells, we demonstrate that some of these genes also influence the pathogenic responses of activated RA-FLS, i.e. tissue-resident stromal cells of the inflamed joints. A recent functional genomics study even provided evidence for a causal role of FLS in the development of RA, accounting for up to 24% of RA heritability (188).

A next step from **Paper I** could be to explore the mechanisms whereby AIRE promotes the expression of a limited number of interferon-regulated genes in activated RA-FLS independently IFN-γ production. Chromatin immunoprecipitation analysis could clarify if AIRE binds directly to the promotors of these genes in FLS. Furthermore, we are investigating if AIRE-

promoted chemokine production by RA-FLS depend on PDGF signaling, as the PDGF receptor-β gene (*PDGFRB*) was also identified as an AIRE-regulated gene in our RNA-seq dataset from **Paper I** (data not shown). Moreover, the "PDGF signaling pathway" was significant by IPA. PDGF is known to promote proliferation and migration of fibroblasts and can also potentiate the production of pro-inflammatory cytokines/chemokines in activated FLS (162). Interestingly, in vascular smooth muscle cells, activation of PDGFRβ has been reported to induce secretion of chemokines such as CXCL10 via JAK signaling independently of interferons (189). We have found that stimulation of RA-FLS with TNF + IL-1β decreases the transcription of *PDGFRB*. AIRE is induced in RA-FLS in this inflammatory setting and we hypothesize that in genetically susceptible individuals, the increase of AIRE is higher than in others and helps to maintain *PDGFRB* expression and PDGF-mediated responses of RA-FLS. We have genotyped primary RA-FLS with regard to three *AIRE* SNPs that have been associated with RA susceptibility in several populations (190), and are studying how these variants relate to functional responses *in vitro* (PDGF-induced proliferation, migration, and chemokine production, respectively). Although sample sizes are small, preliminary data indicate that *AIRE* genotype may influence the transcription of *AIRE* in RA-FLS in response to inflammatory stimuli and also the proliferation of these cells when subjected to PDGF + IL-1β. More samples and data on migration and chemokine production need to be collected. It would also be interesting to validate the findings *in vivo* by assessing *AIRE* expression in synovial fluid or synovial biopsies from patients with active arthritis. For example, we could investigate if *AIRE* genotype or the levels of *AIRE* expression correlate with expression of chemokines or *PDGFRB* in the joint or disease activity measurements.

As mentioned, clinical validation would also be relevant for the findings of **Papers II** and **III**, preferably by analysis of RA synovial biopsies collected before and after anti-rheumatic treatment (with MTX or TOFA). Our main interest would be to study the expression of cell cycle regulating genes, *CSF2*, *IL1A* and *IL1B*, as well as *AIRE* and AIRE-regulated genes identified in **Paper I.** The combination of such data with measurements of disease activity, could elucidate if these mediators have potential to act as therapeutic targets or markers for treatment response. Perhaps, it could be possible to identify specific subpopulations of RA patients with persistent synovitis where GM-CSF and IL-1 signaling is more prominent, that might benefit from treatment with anakinra or anti-GM-CSF. Moreover, we should investigate what the

levels of IL-1α in blood (**Paper III**) are reflecting. Importantly, local measurements of synovitis (e.g. ultrasound of joints) need to be included in such analyses, since clinical measures that only take into account the number of involved joints do not correlate with synovial *IL1A* nor blood IL-1α according to our analyses. Another interesting aspect to study is the association with pulmonary abnormalities, as there may be some common stromal-immune activation in joints and lungs in RA (191).

Other genes found to be differentially expressed by MTX in activated RA-FLS in **Paper III** also warrant further investigation. For example, *ETS1*, another multi-evidence RA-FLS candidate gene was *increased* by MTX. The consequences of this effect for the pathogenic, specifically tissue-destructive (144), behavior of RA-FLS should be examined.

Considering the established role of RA-FLS as drivers of chronic inflammation and joint degradation, it is critical to understand the mechanisms shaping the aggressive phenotype of these cells and how they are affected by treatment. Modulation of the pathogenic responses of RA-FLS may be a key to achieve molecular remission of the disease, at least in some patients. Multiple studies have highlighted the considerable cellular and molecular heterogeneity in RA synovitis, with associations to clinical outcomes. In the era of personalized medicine, future treatment strategies for RA will probably include integration of synovial tissue assessments into clinical management, so that the particular disease drivers in the individual patient can be identified and hopefully targeted.

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