T cells and chemokines in rheumatoid arthritis

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“If no mistake you have made, losing you are.
A different game you should play”
- Yoda

ABSTRACT

In this thesis, we investigated if circulating proportions of specific $CD4^+$ T cell subsets and blood chemokine levels were associated with disease activity and/or could predict remission in patients with early rheumatoid arthritis (eRA). We also compared the effect of different biological treatments on both T cell subset proportions and chemokine levels. Finally, we examined which T helper cell subsets are most abundant in the synovial fluid of inflamed joints, and which T cell associated cytokines induced the secretion of proinflammatory cytokines and chemokines by fibroblast-like synoviocytes (FLS).

To enable these studies, we analysed blood samples and assessed disease activity in patients with untreated eRA who participated in the NORD-STAR randomised treatment trial. Synovial biopsies and paired blood and synovial fluid were sampled from patients with established RA. FLS were propagated from synovial biopsies. The proportions of T cell subsets were analysed by flow cytometry and cytokine and chemokine levels were measured by bead-based immunoassays and ELISA.

In untreated eRA, circulating proportions of Th2, Th17 and CTLA-4^+ conventional $CD4^+$ T cells associated positively with disease activity in male, but not female patients. In patients treated with CTLA-4Ig, but not anti-TNF or anti-IL6R, baseline proportions of PD-1^+TFh and CTLA-4^+ conventional $CD4^+$ T cells predicted remission at week 24. Only treatment with CTLA-4Ig reduced the proportions of PD-1^+TFh. Plasma chemokine levels decreased in all treatment groups except in patients given anti-IL6R. Baseline chemokine levels did not predict remission in eRA. TPh, Th1 and $CXCR3^+$Th2 were the most abundant $CD4^+$ T cell subsets in RA synovial fluid, and the majority of B cell supporting TPh and PD-1^{high}TFh cells expressed a Th1 or $CXCR3^+$Th2 phenotype. IL-4, IL-13 and IL-17 induced FLS to secrete CXCL8, CCL2 and CXCL1, while IFNγ induced CXCL10.

In conclusion, we show that baseline proportions of circulating T cell subsets may be used as biomarkers of remission for CTLA-4Ig treatment in eRA. Our findings also indicate that both classical and non-classical $CXCR3^+$ T cell subsets mediate joint inflammation in RA and their associated cytokines induce secretion of proinflammatory chemokines by FLS.

Keywords: Rheumatoid arthritis, $CD4^+$ T cell, chemokines, disease activity, biomarker, remission, CTLA-4Ig, anti-IL6R, anti-TNF

POPULÄRVETENSKAPLIG SAMMANFATTNING

Ledgångsreumatism (reumatoid artrit, RA) är en kronisk autoimmun sjukdom som orsakar inflammation och skada i leder och andra organ. RA är en av de vanligaste autoimmuna sjukdomarna och drabbar ca 1 procent av befolkningen. RA mycket vanligare bland kvinnor vilka utgör ca 75% av alla patienter. En rad olika immundämpande läkemedel används idag för att behandla RA. Men det finns ingen säker metod som kan förutse vilken behandling som passar varje patient bäst. Genetiska studier har visat att T-hjälpar celler är starkt kopplade till denna sjukdom, men det är ännu inte helt klart vilka typer av T-hjälpar celler som driver sjukdomen.

T-hjälpar celler är en viktig del av vårt specifika immunförsvar. Vid en infektion aktiveras T-hjälpar cellerna när de stöter på ett proteinfragment från infektionen som passar cellens specifika receptor. T-hjälparcellen aktiverar sedan andra immunceller som B celler och makrofager genom att producera inflammatoriska signalmolekyler (cytokiner). Vid RA har T-hjälpar celler istället aktiverats av modifierade proteinfragment som finns i kroppens egna vävnader. Detta gör att immuncellerna börjar att attackera kroppens egen vävnad vilket leder till inflammation och att vävnaden förstörs. I den inflammerade vävnaden produceras också varningssignaler (kemokiner) som attraherar ännu fler immunceller och en ond cirkel startar. I min avhandling studerar jag rollen av T-hjälpar celler och kemokiner i RA.

Vi upptäckte att andelen av vissa typer av T-hjälpar celler i blodet samvarierade med sjukdomsaktivitet hos manliga men inte kvinnliga patienter med tidig obehandlad RA. Vi upptäckte också att förekomsten av två specifika typer av T-hjälpar celler kunde förutse hos vilka patienter behandling som blockerar aktivering av T-celler fungerar bäst. Dessa fynd kan tänkas leda till en metod för att optimera behandlingen av varje patient.

När vi mätte nivåer av kemokiner i blodet på patienter med tidig RA upptäckte vi att nivåer av CXCL10 och CCL2 korrelerade starkast med sjukdomsaktiviteten. Vi fann sedan att nivåerna av dessa kemokiner var mycket högre i ledvätskan från inflammerade leder jämfört med blodet från patienter med kronisk RA. Det visade sig också att andelen T-hjälpar celler som uttryckte receptorerna för dessa kemokiner var högre i ledvätskan än blodet.

När vi slutligen stimulerade fibroblaster från en inflammerad led med cytokiner som produceras av T-hjälpar celler så utsöndrade

fibroblasterna flera av de kemokiner som också fanns i ledvätskan från inflammerade leder. Dessa fynd ger oss nya ledtrådar till hur den onda cirkeln av inflammation och fortsatt rekrytering av immunceller fortskrider i lederna hos patienter med RA.

LIST OF PAPERS

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

I. **Aldridge J**, Pandya JM, Meurs L, Andersson K, Nordström I, Theander E, Lundell AC, and Rudin A. Sex-based differences in association between circulating T cell subsets and disease activity in untreated early rheumatoid arthritis patients. *Arthritis Research & Therapy 2018; 1:150.*

II. **Aldridge J**, Andersson K, Gjertsson I, Hultgård-Ekwall AK, Hallström M, van Vollenhoven R, Lundell AC, and Rudin A. Blood PD-1^{+}TFh and CTLA-4^{+}CD4^{+} T cells predict remission after CTLA-4Ig treatment in early rheumatoid arthritis. *Rheumatology (Oxford) 2021; e-published before print.*

III. **Aldridge J**, Lundell AC, Andersson K, Mark L, Lund Hetland M, Østergaard M, Uhlig T, Schrumpf Heiberg M, A. Haavardsholm E, Nurmohamed M, Lampa J, Nordström D, Hørslev-Petersen K, Gudbjornsson B, Gröndal G, van Vollenhoven R, and Rudin A. Blood chemokine levels are markers of disease activity but not predictors of remission in early rheumatoid arthritis. *Submitted manuscript.*

IV. **Aldridge J**, Hultgård-Ekwall AK, Mark L, Bergström B, Andersson K, Gjertsson I, Lundell AC, and Rudin A. T helper cells in synovial fluid of patients with rheumatoid arthritis primarily have a Th1 and a CXCR3^{+}Th2 phenotype. *Arthritis Research & Therapy 2020; 1:245.*

LIST OF PAPERS NOT INCLUDED IN THE THESIS

Zhang Y, **Aldridge J**, Vasileiadis G.K, Edebo H, Hultgård-Ekwall AK, Lundell AC, Rudin A, and Maglio C.
Recombinant adiponectin induces the production of pro-inflammatory chemokines and cytokines in circulating mononuclear cells and fibroblast-like synoviocytes from non-inflamed subjects.
Frontiers in immunology 2021; 11:569883.

Stockfelt M, Lundell AC, Lund Hetland M, Østergaard M, Uhlig T, Schrumpf Heiberg M, A. Haavardsholm E, T. Nurmohamed M, Lampa J, Nordström D, Hørslev Petersen K, Gudbjornsson B, Gröndal G, **Aldridge J**, Andersson K, Blennow K, Zetterberg H, van Vollenhoven R, and Rudin A.
Plasma interferon-alpha is associated with double-positivity for autoantibodies but is not a predictor of remission in early rheumatoid arthritis.
Arthritis Research & Therapy, 2021; Accepted.

ABBREVIATIONS

ACPA	Anti citrullinated peptide antibody
ACR	American College of Rheumatology
APC	Antigen-presenting cell
AUC	Area under the curve
BAFF	B cell activating factor
bDMARD	Biological disease-modifying anti-rheumatic drug
CCL	C-C motif chemokine ligand
CCR	C-C motif chemokine receptor
CD	Cluster of differentiation
CDAI	Clinical disease activity index
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
CRP	C-reactive protein
CXCL	C-X-C motif chemokine ligand
CXCR	C-X-C motif chemokine receptor
DAS28	Disease activity score 28
DMARD	Disease-modifying anti-rheumatic drug
ELISA	Enzyme-linked immunosorbent assay
ELS	Ectopic lymphoid-like structures
eRA	Early rheumatoid arthritis
ESR	Erythrocyte sedimentation rate
EULAR	European League Against Rheumatism
FLS	Fibroblast-like synoviocytes
GM-CSF	Granulocyte macrophage-colony stimulating factor
HC	Healthy control
HLA	Human leukocyte antigen
IFN	Interferon
IL	Interleukin
IL6R	Interleukin-6 receptor
MBDA	Multi-biomarker disease activity
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinase
MTX	Methotrexate

OA	Osteoarthritis
OPLS	Orthogonal projection to latent structures
OPLS-DA	OPLS discriminant analysis
PAD	Peptidyl-arginine deiminase
PAMP	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cells
PCA	Principal component analysis
PD-1	Programmed cell death-1
PDPN	Podoplanin
RF	Rheumatoid factor
RA	Rheumatoid arthritis
ROC	Receiver operating characteristic
SF	Synovial fluid
SJC	Swollen joint count
TFh	Follicular T helper cell
TFreg	Follicular regulatory T cell
TGFβ	Transforming growth factor β
Th	T helper cell
TJC	Tender joint count
TNF	Tumor necrosis factor
TPh	Peripheral T helper cell
Treg	Regulatory T cell

CONTENT

INTRODUCTION

The immune system

The human immune system consists of a complex network of tissues, cells and proteins that are divided into two main parts: the innate and the adaptive immune system. The innate system consists of both specialised cells and physical barriers within the body. The innate system is key to the removal of pathogens but lacks adaptability and cannot learn to combat recurring threats more effectively. In contrast, the adaptive immune system can adapt to new threats and has the ability to form an immunological memory. This memory allows the immune system to mount a faster and stronger response to previously encountered pathogens.

Innate immunity and acute inflammation

The innate immune system is the first line of defence against external attacks. The cells of the innate immune system including neutrophils, monocytes, macrophages and dendritic cells recognise threats via pattern recognition receptors [1]. These receptors are sensitive to molecular structures that are common across many types of pathogens. When innate immune cells encounter these pathogen-associated molecular patterns, or PAMPs, they are engulfed and degraded by phagocytosing cells such as neutrophils and macrophages. Recognition of PAMPs also triggers the release of proinflammatory cytokines that enhance the local immune response and cause inflammation.

Inflammation can be divided into acute and chronic inflammation. Acute inflammation is triggered by cells of the innate immune system such as tissue resident macrophages when they encounter PAMPs or products of tissue damage. This triggers the release of proinflammatory cytokines such as IL-1β, TNF and IL-8. IL-8 (CXCL8) is a chemotactic cytokine that generate an influx of neutrophils into the tissue that aid in clearance of pathogens and cellular debris. Acute inflammation abates once the trigger of the inflammatory response has been eliminated.

Adaptive immunity, chronic inflammation and autoimmunity

The primary cells of the adaptive immune system are T and B lymphocytes (T and B cells). While different cell types of the innate immune system can express the same pattern recognition receptor, each T cell or B cell clones express a unique receptor that recognise specific

antigens. During development T cells are educated in the thymus with the aim that only cells that recognise foreign (non-self) antigens join the circulating T cell pool [2]. This process is known as central tolerance. However, T cells that recognise self-antigens may escape this education process and join the circulating T cell pool.

To mount an adaptive immune response T cells must first be activated by an antigen-presenting cell (APC). As a result of their education in the thymus T cells only recognise antigens that are presented on the major histocompatibility complex (MHC) proteins. T cells can be divided into two main types based on their affinity for either MHC class I or II proteins. Cytotoxic ($CD8^+$) T cells recognise antigens presented on MHC class I and are able to selectively kill cells infected by an intracellular pathogen. $CD4^+$ T cells recognise antigens presented on MHC class II. These cells both activate and regulate other cells of the innate and adaptive immune system. $CD4^+$ T cells can activate B cells into antibody-producing plasma cells and enhance the ability of macrophages to phagocytose specific pathogens.

Chronic inflammation involves both the innate and the adaptive immune system. In chronically inflamed tissue, cellular infiltrates consist of macrophages and T and B cells. Neutrophil infiltration is often limited in chronically inflamed tissue, although there are exceptions. Chronic inflammation can be caused by bacteria that survive inside of macrophages, such as *Mycobacterium tuberculosis,* but can also be caused by autoimmunity. Autoimmunity occurs when an adaptive immune response is initiated against the body's own tissues. The cause for this is often not known. In some autoimmune disorders the specific antigens that trigger and perpetuate the immune reaction are also not known. Since the immune system is unable to clear the antigen or antigens autoimmunity often leads to chronic inflammation and degradation of the affected tissue.

In the autoimmune disease rheumatoid arthritis (RA) the synovial membrane is the main site of a chronic inflammatory reaction [3]. Synovial macrophages produce proinflammatory cytokines such as IL-1β, IL-6 and TNF. T cells and B cells infiltrate the tissue. T cells, B cells and follicular dendritic cells can form organised structures reminiscent of germinal centres, so called ectopic lymphoid-like structures (ELS) [4]. Similar structures are found in lung tissue infected with *Mycobacterium tuberculosis* that has transitioned from an initial acute inflammatory state dominated by neutrophils to a chronic inflammatory state dominated by lymphocytes [5].

Immunity in males and females

The type of immune response and distribution of immune cell subsets differ between the sexes particularly in the adaptive immune system. On average, female individuals have higher circulating proportions of $CD4^+$ T cells and B cells but lower proportions of $CD8^+$ T cells compared to male individuals [6]. In both humans and mice, females also have higher antibody titres compared to males in response to infections or vaccines [7-9]. Both genetic and hormonal differences between the sexes likely contribute to these differences.

Females have double X chromosomes (XX), while males have only a single copy (XY). In both humans and mice, it has been shown that incomplete silencing of the second X chromosome in females causes increased expression of several immune-related genes found on the X chromosome [10-12]. Thus, some but not all T and B cells express two different copies of these genes in females. Notable examples include *CD40LG*, an important co-receptor in T cell mediated activation of B cells, and the chemokine receptor *CXCR3*. In mice, T cells that express two different copies of *CXCR3* were shown to produce higher levels of the cytokines IFNγ and IL-2 after activation [12]. *FOXP3* is also located on the X chromosome, and is the master transcription factor for regulatory $CD4^+$ T cells [13]. Despite its location on the X chromosome male regulatory T cells have been shown to express higher levels of *FOXP3* [14], possibly due to regulation by sex hormones [14, 15].

Male sex hormones (androgens) are largely considered immunosuppressive [16]. Androgens can reduce the proportion of $CD4^+$ T cells in circulation [17, 18], and delay B cell maturation [19, 20]. B cell maturation may be inhibited through in decreased expression of B cell activating factor (BAFF) in secondary lymphoid tissue. BAFF levels have been shown to be higher in males with low androgen levels compared to those with normal levels [20]. In mice, testosterone appear to limit the expression of BAFF by fibroblastic reticular cells in the spleen and reduce B cell numbers independently of $CD4^+$ T cell numbers [20].

Compared to androgens, the *in vivo* effect of female sex hormones (oestrogens and progesterone) on immune regulation may be more complex and can have both proinflammatory and anti-inflammatory effects [16]. In mice, the oestrogen estradiol (E2) has been shown to inhibit T cell and B cell maturation [21], although it may also promote T cell activation and proliferation [22]. E2 also increase antibody production by bone marrow resident B cells in mice [23], and *in vitro*

activated human B cells [24]. In humans, progesterone dampen the activity of $CD4^+$ T cells activated *in vitro* and suppress their production of cytokines such as IL-4, IL-13, IL-17A and TNF [25]. In females, blood levels of oestrogens and progesterone vary throughout life as a consequence of the menstrual cycle, pregnancy and menopause, although the proportions of $CD4^+$ T cells appear to be consistently higher in females throughout life [26].

Ultimately the differences in immune response between the sexes manifest in a skewed prevalence of several diseases between male and female individuals. Males have a lower resilience against viral infections compared to females. For example, COVID-19 related mortality is on average 1.7 times higher in males compared to females [27]. For the majority of cancers, the incidence is also higher among males compared to females [28]. In contrast, most autoimmune diseases are more prevalent in females. RA for example has a female to male bias of approximately 3:1, while the bias for systemic lupus erythematosus is as high as 10:1 [29].

$CD4^+$ T cells

Development and elimination of self-reactive T cells

T cells originate in the bone marrow where they differentiate from bone marrow stem cells. Immature T cell precursors then migrate to the thymus where they fully commit to become T cells. In the thymus the immature T cell produces its unique T cell receptor (TCR). The TCR is a heterodimer of a α- and β-chain. The α and β chains are formed via the semi-random assembly of gene segments known as V(D)J-recombination [30].

At this stage the immature T cell must commit to either a CD4 or CD8 lineage. Thymus epithelial cells present parts of the body's own proteins (self-antigens) to the T cells via the MHC class I and II proteins [2]. T cells with a TCR that binds weakly to MHC class II protein becomes $CD4^+$ T cells. This is known as positive selection and cells that pass this stage join the circulation as mature $CD4^+$ T cells. In contrast, T cells with a TCR that binds to a MHC-self-antigen complex with a high affinity go into apoptosis, become inert or can become regulatory T cells [31]. This is known as negative selection.

The purpose of this process is to generate a large pool of T cells each with a TCR that has a unique binding specificity but is not reactive to the body's own proteins. This is known as central tolerance.

$CD4^+$ T cell subsets

$CD4^+$ T cells can be divided into different subsets that have different functions. The classical T helper 1 (Th1), Th2 and Th17 subsets direct the innate and adaptive immune system through the production of cytokines. The follicular T helper (TFh) subset promotes B cell activation and differentiation into antibody secreting plasma cells. Regulatory T cells (Tregs) suppress excessive T cell mediated immune responses and promote tolerance.

Traditionally each subset has been identified by the expression of a specific transcription factor. The transcription factors T-bet (encoded by the gene *TBX21*), GATA3 (*GATA3*) and RORγt (*RORC*) are key to the differentiation of Th1, Th2 and Th17 cells, respectively [32-34]. Differentiation of Tregs is dependent of the transcription factor FOXP3 (*FoxP3*) [13], while BCL-6 (*bcl-6*) is associated with the formation of TFh cells [35]. Which transcription factor that is expressed is determined by the cytokine environment during the activation of the T cell by an antigen-presenting cell (APC) (Figure 1). Each subset is also associated with the secretion of particular effector cytokines, although these cytokines are not uniquely produced by that subset and are not the only cytokines they produce (Figure 1).

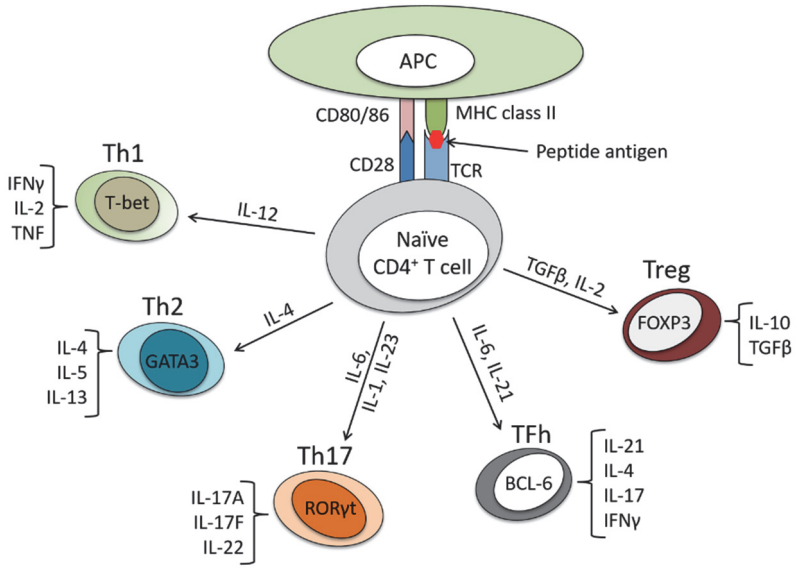


Figure 1. Schematic view of $CD4^+$ T cell activation and selected cytokines that promote differentiation of T helper 1 (Th1), Th2, Th17, follicular T helper (TFh) and regulatory T cells (Treg), respectively, and the characteristic transcription factor expressed and selected cytokines produced by each T cell subset. Antigen-presenting cell (APC), T cell receptor (TCR), major histocompatibility complex (MHC).

There are also a range of surface protein markers used to define each of the different subsets and subpopulations of $CD4^+$ T cells. Naïve $CD4^+$ T cells express the CD45RA isoform of the CD45 protein. CD45 is a tyrosine phosphatase that facilitates TCR signal transduction and T cell activation [36]. The antigen experienced (memory) T cell then lose the expression of CD45RA and instead express the CD45RO isoform. Classical circulating Th1, Th2 and Th17 are part of the memory pool and can each be delineated by a combination of the chemokine receptors CXCR3, CCR4 and CCR6 [37-39]. There are also non-classical subsets that express the features of several classical subsets. The Th1Th17 (also referred to as Th17.1) subset express CXCR3 and CCR6 and can secrete both IFNγ and IL-17, features of the Th1 and Th17 subsets, respectively [38]. Similarly, non-classical $CXCR3^+$Th2 and $CXCR3^+$Th17 cells also express markers and cytokines characteristic of the Th2 and Th17 subsets, respectively, but also the Th1 subset [38]. Thus, T helper phenotypes are a continuous spectrum rather than distinct subsets.

Studies in mice have shown that BCL-6 expression is required to generate $CXCR5^+$ TFh cells [40]. In humans, circulating TFh cells

express the chemokine receptor CXCR5 but the expression of BCL-6 is lower compared to bona fide TFh found in the secondary lymphoid organs [40]. TFh can also express programmed cell death protein 1 (PD-1), although not all TFh found in circulation do. In TFh, PD-1 expression indicates recent antigen exposure and PD-1^{+}TFh represent the activated TFh pool [40]. It is worth noting that the TFh cell subset is not completely distinct from the classical Th1, Th2 and Th17 subsets. Circulating TFh can be subdivided into cells with a Th1, Th2 and Th17 phenotype based on their expression of chemokine receptors CXCR3 and CCR6 [41]. Each of these TFh phenotypes also produce cytokines associated with each of the classical subsets i.e. IFNγ (Th1), IL-4, IL-5 and IL-13 (Th2), and IL-17A (Th17) and express the respective transcription factors.

A subset of peripheral T helper cells (TPh) that share B cell helping characteristics with classical TFh have been identified. TPh express high levels of PD-1 but lack the expression of the CXCR5 characteristic of TFh [42]. TPh instead express chemokine receptors such as CCR2, CX3CR1 and CCR5. The circulating proportions of these cells are higher in individuals with autoimmune disease compared to healthy controls (HC), although TPh comprise only a small proportion of circulating CD4^{+} T cells (often <1 percent) [42-44]. It is not known whether TPh can be subdivided into cells with a Th1, Th2 and Th17 phenotype similarly to classical TFh.

Tregs express high levels of the IL-2 receptor α-chain (CD25) but only low levels of the IL-7 receptor (CD127) [45]. Indeed, studies in mice have shown that Tregs require IL-2 in order for long term survival and to maintain effective suppressor function [46]. Tregs also constitutively express cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), both on the surface and intracellularly. CTLA-4 shares affinity for the CD80/86 co-receptors with the stimulatory receptor CD28, although CTLA-4 binds CD80/86 with a higher affinity compared to CD28 [47]. CTLA-4 is a key part of Treg suppressor function. During Treg-APC interactions CTLA-4 captures CD80/86 from the APC and internalise and degrade it, known as trans-endocytosis [48]. CTLA-4 is also expressed by activated T helper cells as a mechanism of self-regulation [49].

Migration and homing of T cells

In homeostatic conditions naïve $CD4^+$ T cells and central memory T cells circulate between the blood and secondary lymphoid organs. Both naïve T cells and central memory T cells express the chemokine receptor CCR7 [50], and they are attracted to the lymph nodes by the chemokines CCL19 and CCL21 produced by fibroblastic reticular cells. In contrast, $CCR7^{neg}$ effector T cells circulate in the blood and home to sites of inflammation via several different chemokine receptors.

The migration of T cells into inflamed tissue is a complex multi-step process shown in Figure 2. In inflamed tissues, blood endothelial cells lining the blood vessel express E- and P-selectins as well as integrin ligands VCAM-1 and ICAM-1 in response to inflammatory cytokines such as TNF [51]. Effector T cells but not naïve T cells express functional selectin ligands and the low affinity interaction between the T cells and selectins result in a rolling motion along the vascular wall [52]. In this rolling motion chemokines presented on the surface of the endothelium come into contact with the chemokine receptors on the rolling T cell. Chemokine receptor activation and integrin binding to VCAM-1 or ICAM-1 then allows the T cell to attach firmly and migrate through the endothelial wall into the target tissue, known as transendothelial migration [53].

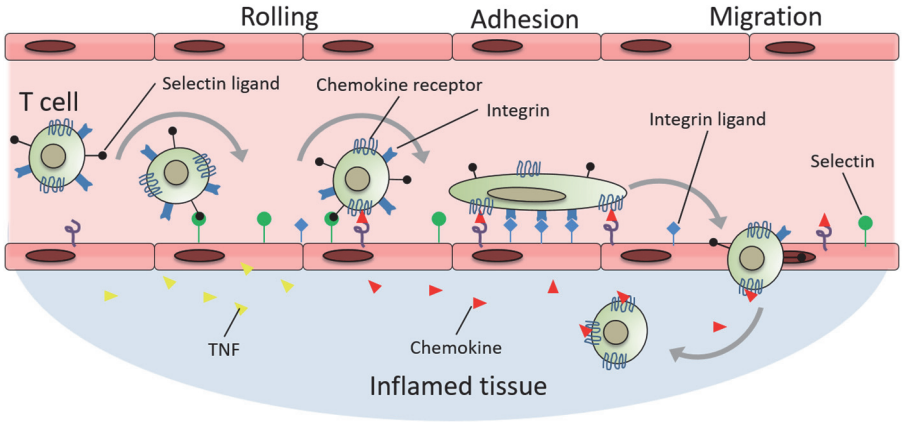


Figure 2. Schematic description of T cell migration into an inflamed tissue.

Chemokines and chemokine receptors

The recruitment of immune cells to sites of infection or tissue damage is mediated by a group of chemotactic cytokines known as chemokines

[54]. Chemokines are a diverse subgroup of cytokines and approximately 50 different chemokines have been identified and more than 20 different chemokine receptors. Chemokines are divided into four primary subfamilies based on the location of cysteine residues at the terminus of their amino acid sequence: XC, CC, CXC and CX_3C (C=cysteine, X=other amino acid). The corresponding chemokine receptors are grouped accordingly as XCR, CCR, CXCR and CX_3CR. A list of selected chemokine receptors and which $CD4^+$ T cell subsets that express them is shown in Table 1.

Table 1. Selected chemokine receptors expressed by T helper subsets

Chemokine receptor	$CD4^+$ T cell subsets that express the receptor	Receptor ligands
CCR2	TPh	CCL2
CCR4	Th2, Th17, TFh & TPh	CCL17, CCL22
CCR6	Th17, Th1Th17, TFh & TPh	CCL20
CCR7	Naïve & central memory T cells	CCL19, CCL21
CXCR3	Th1, Th1Th17, $CXCR3^+$Th2, $CXCR3^+$Th17, TFh & TPh	CXCL9, CXCL10, CXCL11
CXCR5	TFh	CXCL13

Peripheral T helper cell (TPh); T helper (Th); follicular T helper cell (TFh).

In autoimmune diseases such as RA excessive chemokine production and immune cell recruitment is a key aspect of disease pathology [55]. However, clinical trials of agents that block specific chemokines have only shown limited efficacy in RA. RA patients treated with a CXCL10 blocking antibody in a phase II trial showed a moderate reduction in disease activity after 3 months [56], while CCL2 blockade did not significantly reduce disease activity after 6 weeks of treatment [57]. This may in part be due to redundancy in the chemokine receptor signalling system [58].

Rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterised by inflammation of the synovial membrane, although extra-articular inflammation can be present in other tissues, e.g. the lungs [59, 60]. Initially, the proximal joints of the hands and feet are affected but larger joints can also become affected. The prevalence of RA is approximately 0.5-1 percent [61], except in specific indigenous populations who appear more susceptible [62]. RA is a highly heterogeneous disease, with differences in clinical presentation, severity

and pathological mechanisms between patients and at different stages of disease. The disease also afflicts female individuals 2-3 times more often than male individuals.

Multiple risk factors have been associated with the development of RA. Female sex is a risk factor but variants of the human leukocyte antigen (HLA), specifically alleles of the HLA-DRB1 subdomain that contain the "shared epitope", carry the largest genetic risk [63, 64]. The "shared epitope" is a conserved positively charged region in position 70-74 of the amino acid sequence of DRB that is thought to determine which peptides can be accommodated in the pocket of the HLA protein [65]. Cigarette smoking has been shown to be a major environmental risk factor, particularly in individuals who also carry the genetic susceptibility associated with the HLA-DRB1 subdomain [66, 67]. Chronic respiratory disease may also increase risk of RA [68].

Pathogenesis of RA synovial inflammation

RA is a systemic autoimmune disease. It is not known how the chronic inflammatory reaction in the RA synovium is initiated but signs of autoimmunity such as rheumatoid factor (RF) and/or anti-citrullinated protein antibodies (ACPA) in the blood can appear months or years prior to arthritic symptoms [69]. It has been proposed that autoimmunity to citrullinated peptides (and generation of ACPAs) may be initiated in the lungs, particularly in smokers [70]. Smoke particles increase the expression of peptidyl-arginine deiminase (PAD) [71], which convert arginine to citrulline (citrullination) (Figure 3).

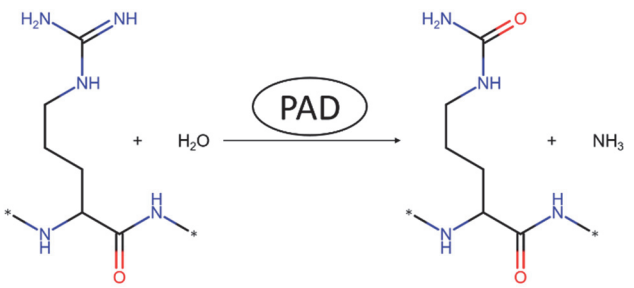


Figure 3. Schematic drawing of the conversion of arginine into citrulline, known as citrullination, by peptidyl-arginine deiminase (PAD).

Citrullinated peptides have a higher affinity for HLA molecules that contain the "shared epitope" [65], which may facilitate antigen presentation and T cell activation [72]. In line with this, untreated eRA patients that are ACPA-positive more often present with T cell and B

cell infiltration in lung tissue compared to ACPA-negative individuals [60]. ACPAs are also highly promiscuous and bind to several different citrullinated antigens [73], which may facilitate the spread of the autoimmune reaction to other tissues. This theory, however, does not account for the development of seronegative RA.

In RA, the synovial inflammation is most often characterised by an infiltration of macrophages and T cells, as well as activation of fibroblast-like synoviocytes (FLS). T cells, B cells and follicular dendritic cells can eventually form ectopic-lymphoid structures (ELS) capable of autoantibody production within the tissue [4]. Different cell types in the inflamed synovium produce proinflammatory cytokines and catabolic enzymes. Single-cell transcriptional analyses of synovial tissue have shown that macrophages express high levels of IL-1β, while TNF expression is abundant in macrophages, T cells and B cells [74]. IL-6 is most highly expressed by B cells and FLS, while matrix-metalloproteases (MMPs) are secreted by FLS.

Histological and bulk transcriptional analysis of the synovium in eRA have also shown that there exist three distinct synovial pathotypes which differ in the level of immune cell infiltration [75, 76]. A lymphoid-myeloid pathotype characterised by abundant macrophage, T cell and B cell infiltration and organised ELS formation. Gene pathway analysis shows enrichment for the Th1 and Th2 activation pathways along with IFNγ, IL-21 and CD40L [75]. TFh express both IL-21 and CD40L to mediate B cell activation. Pathways linked to dendritic cell maturation, B cell development and expression of type 1 interferons (IFNα and IFNβ) are also enriched in the lymphoid-myeloid pathotype [75], and are consistent with the formation of functional ELS in the synovium [4]. The diffuse myeloid pathotype has significant macrophage infiltration but few T cells or B cells and no ELS formation. Macrophages show a proinflammatory phenotype, and upstream regulator analysis found genes regulated by IL-1β, TNF, IL-6, IL-4, IFNγ and IL-15 to be upregulated in this pathotype [75]. Lastly, a pauci-immune phenotype with few immune cells but rich in FLS. In established RA, this pathotype show a cell infiltration and transcriptional signature similar to that observed in osteoarthritis (OA) [74]. It is not yet known if these pathotypes represent distinct subsets of disease or are evolving states of the pathogenic inflammatory process in the synovium.

$CD4^+$ T cells in RA

Genome-wide association studies strongly support $CD4^+$ T cells as key promotors of RA pathology. In addition to specific HLA alleles, several of the non-HLA genes with the strongest genetic association to RA are linked to $CD4^+$ T cell activation and function. These include but are not limited to*: PTPN22, CD28, CTLA-4, CCR6, CXCR5 and CD40* [77].

Among classical $CD4^+$ T cell subsets Th1 and Th17 cells have been the main subjects in RA research [78, 79]. In established RA, the proportions of Th1 cells in circulation have been shown to be lower than in HC [80]. In the same study proportions of Th17 cells in circulation did not differ between patients with established RA and HC, and Th2 proportions were not measured. In eRA, we have shown that Th1 proportions in blood do not differ between eRA patients and HC, but that proportions of Th2 and Th17 cells were higher in eRA patients [38]. Plasma levels of the Th1 associated IFNγ, Th2 associated IL-4 and several other cytokines have also been shown to be higher in eRA patients compared to matched controls [81]. In that study, plasma levels of Th17 associated IL-17 did not differ significantly from matched controls. With regard to TFh cells, results concerning the proportions in eRA vs HC are conflicting [82, 83].

In the inflamed joint, the levels of T cell infiltration varies between patients in both early and established RA [74, 75]. Transcriptional analysis of synovial tissue from eRA patients with high levels of T cell infiltration showed an enrichment in the Th1 and Th2 activation pathways [75]. Indeed, elevated levels of IL-13, IL-2, IL-15 and IL-4 in the SF most strongly distinguished eRA patients from other early arthritides [84]. In established RA, patients with high levels of T cell infiltration also show an expansion of the TPh subset in the synovium [42, 74]. The TPh subset shares functionality with conventional TFh and can induce plasma cell differentiation *in vitro*. However, it lacks the expression of chemokine receptor CXCR5 characteristic of TFh and instead express CCR2 [42]. In RA patients, TPh only comprise approximately 0.5-1% of memory T cells in circulation [42, 85, 86], but up to 25-30% in synovial fluid and tissue [42]. It is not known if the TPh subset originates in the blood or if it differentiates locally in the inflamed synovium.

Fibroblast-like synoviocytes in RA

In the model of RA pathogenesis, fibroblast-like synoviocytes (FLS) have been promoted from bystanders to active mediators of

inflammation and cartilage destruction [87]. In a healthy joint the thin lining layer of the synovial membrane is comprised of FLS [88]. These cells primarily secrete proteins that lubricate the joint. In RA, the chronically inflamed synovium becomes hypertrophic and FLS take on distinct pathogenic phenotypes and are found both in the lining and sub-lining layers of the synovium [74, 89]. Studies in both mouse models and on human cells have shown that lining and sub-lining FLS confer distinct aspects of RA pathogenesis [89, 90]. Sub-lining FLS secrete proinflammatory cytokines and chemokines, e.g. IL-6 and CCL2. Lining FLS promote cartilage destruction through secretion of matrix metalloproteinases (MMPs).

In vivo, lining and sub-lining FLS can be delineated by their expression of surface proteins. Lining layer FLS express higher levels of CD55 and cadherin-11, while sub-lining FLS are recognised by the expression of CD90 (THY1) [89, 91]. It was recently shown that the phenotypic distinction between lining and sub-lining FLS is regulated by NOTCH-dependant signalling between FLS and perivascular endothelial cells [92]. NOTCH3 expression in particular was upregulated in FLS from patients with active RA and blockade of NOTCH3 attenuated inflammation in a murine arthritis model.

Clinical presentation and classification of RA

The first clinical symptoms of RA are often pain and swelling of the proximal joints of the hands and feet accompanied by morning stiffness lasting from 30 minutes to several hours [61]. Although typically centred on the small joints and the wrists, the larger shoulder, elbow, hip, knee and ankle joints can also be affected. Elevated serum markers of inflammation such as erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) can also be present at this stage. Autoantibodies RF and/or ACPA can be detected in the blood in approximately two thirds of patients [93]. If the disease is not treated, extra-articular manifestations can arise. These include but are not limited to rheumatoid nodules (hard subcutaneous lumps) and rheumatoid vasculitis (inflammation of arteries that cause necrosis of the blood vessels). Cardiovascular disease (CVD) is a common comorbidity and disease activity is a significant contributor to CVD risk in RA patients [94].

A number of classification criteria can be used as a basis for an RA diagnosis, although no formal diagnostic criteria exist. The most recent are the American College of Rheumatology (ACR)/European League Against Rheumatism (EULAR) 2010 criteria [95]. According to these

criteria a classification of RA requires that the patient has at least one swollen joint and a score ≥ 6 from the scoring system shown in Table 2.

Table 2. Rheumatoid arthritis classification [A]

Classification	Points
Swollen or Tender joint [B]	
1 large joint	0
2-10 large joints	1
1-3 small joints	2
4-10 small joints	3
>10 joints (≥1 small joint)	5
Serology [C]	
Negative RF and ACPA	0
Low positive RA or ACPA	2
High positive RF or ACPA	3
Acute phase reactants [D]	
Normal CRP and ESR	0
Abnormal CRP or ESR	1
Symptom duration [E]	
<6 weeks	0
≥6 weeks	1

[A] Adapted from American College of Rheumatology/European League Against Rheumatism classification criteria [95].
[B] Large: shoulder, elbow, hip, knee or ankle. Small: metacarpophalangeal joints, proximal interphalangeal joints, second through fifth metatarsophalangeal joints, thumb interphalangeal joints and wrists.
[C] NEGATIVE < below upper normal limit (UNL). UNL < LOW ≤ 3xUNL. HIGH > 3xUNL. UNL as determined by local laboratory standards.
[D] Normal/abnormal as determined by local laboratory standard.
[E] Self-reported duration of pain, swelling or tenderness of joints included at the time of assessment.
Rheumatoid factor (RF); anti-citrullinated protein antibody (ACPA); c-reactive protein (CRP); erythrocyte sedimentation rate (ESR).

For each individual patient the severity of the disease (disease activity) is quantified by a disease activity score. Frequently used scores include the disease activity score in 28 joints (DAS28) and clinical disease activity index (CDAI). Each score has cut-points that define specific disease activity states and are calculated based on a combination of clinical assessments as described in Table 3. According to EULAR recommendations treatment should aim to achieve a state of remission or at least low disease activity to minimise joint damage [96].

Table 3. Disease activity measures for patients with RA

Scoring method	Formula	Disease activity status		
		Remission	Low	Moderate to high
CDAI	SJC28 + TJC28 + PGA + EGA	≤ 2.6	>2.8-10	>10
DAS28-ESR	0.28√(SJC28) + 0.56√(TJC28) + 0.7ln(ESR) + 0.014(GH)	≤ 2.6	>2.6-3.2	>3.2

Clinical disease activity index (CDAI); disease activity score in 28 joints (DAS28); erythrocyte sedimentation rate (ESR); swollen/tender joint count in 28 joints (SJC28/TJC28); patient/evaluator global assessment 0-10 cm scale (PGA/EGA); patients global assessment of disease activity 0-100 mm (GH).

Treatment

Treatment of RA often involves the use of several different medications. Non-steroidal anti-inflammatory drugs and corticosteroids are used to reduce pain and inflammation, while disease-modifying anti-rheumatic drugs (DMARDs) offer both symptomatic relief and reduce disease activity.

DMARDs can be divided into conventional synthetic, targeted synthetic and biological DMARDs. The most used conventional DMARD is methotrexate (MTX). The increase in extracellular adenosine caused by MTX is thought to be one of its main mechanism of action in RA [97]. MTX trigger an increase in the secretion of adenine nucleotides which are converted into adenosine in the extracellular space [98]. Adenosine in turn inhibits the proinflammatory action of several cell types such as neutrophils, macrophages and T cells via adenine receptors, particularly A2a, and inhibits NFκB-signalling, cytokine secretion and T cell activation. Targeted synthetic DMARDs include Janus kinase (JAK)-inhibitors such as tofacitinib. These drugs block the intracellular signalling pathway of cytokines and inhibit immune cell activation.

The mechanism of action of biological DMARDs (bDMARDs) is dependent on their target. Anti-TNF and anti-IL6 receptor drugs each block the proinflammatory effect of the cytokine TNF and IL-6, respectively. The CTLA-4Ig fusion protein abatacept consists of the extracellular part of the CTLA-4 receptor and the Fc-portion of an IgG1 antibody. The CTLA-4Ig bind to the CD80/86 co-receptors expressed by APCs and thereby inhibit their ability to activate T cells. The anti-CD20 antibody rituximab binds to CD20 expressed by B cells and leads to depletion of these cells.

Recommendations for first line treatment of RA in Sweden are described in detail in guidelines by EULAR and the Swedish

rheumatology society [96, 99]. In brief, first line treatment consists of MTX escalated to a maximal dosage of 20-30 mg/week during the first 4-8 weeks combined with oral corticosteroids (5-7.5 mg/day). Corticosteroid dosage is often higher initially and reduced during the first month of treatment. Intra-articular injections of corticosteroids are used to complement the primary treatment. First line treatment can also be supplemented with a bDMARD if the patient shows signs of an aggressive disease, i.e. large number of swollen joints, ACPA or RF positive, or erosions.

An evaluation of the treatment effect should be performed 3 months after initiation. If the response to first line MTX therapy is inadequate treatment can be supplemented with a bDMARD or a targeted synthetic DMARD. There are no quantitative biomarkers that can predict the optimal treatment for each patient [96]. Thus, bDMARD treatment is often a trial and error strategy. Owing to long-time experience and lower cost the first preferred bDMARD option is anti-TNF. If contraindications for anti-TNF treatment are present CTLA-4Ig, anti-IL6 receptor or JAK-inhibitor therapy can be used. If the first bDMARD is not effective anti-CD20 can be used.

Despite the large number of different available treatments a significant proportion of eRA patients do not achieve strict disease remission (CDAI≤2.8) in response to first line treatment (MTX + corticosteroids) [100, 101]. A recent study showed that approximately 50 percent of DMARD-naïve eRA patients achieve strict remission after 6 months of treatment with active conventional therapy (MTX + corticosteroids or MTX + sulfasalazine + hydroxychloroquine) or MTX combined with anti-TNF, CTLA-4Ig or anti-IL6R [102]. Only MTX + CTLA-4Ig treatment resulted in a higher rate of remission compared to active conventional therapy (52.0% vs 42.7%, respectively).

AIMS

The specific aims of each paper in the thesis were to investigate:

Paper I

- If circulating proportions of $CD4^+$ T cell subsets and their association with disease activity differ between male and female patients with untreated early rheumatoid arthritis (eRA).

Paper II

- If circulating proportions of $CD4^+$ T cell subsets specifically predict disease remission after treatment with CTLA-4Ig in eRA.
- If biological treatments with different immunological targets affect $CD4^+$ T cell subset proportions differentially.

Paper III

- If baseline plasma chemokine levels predict remission after 24 weeks of treatment in eRA.
- If treatments with different modes of action affect chemokine levels differentially.

Paper IV

- Which $CD4^+$ T cell subsets that are most abundant in the synovial fluid of inflamed joints in RA.
- Which T cell associated cytokines can induce the secretion of proinflammatory chemokines by fibroblast-like synoviocytes.

METHODS

In this section I present the patient cohorts that were studied and selected methods that were used. A more detailed description of each method can be found in the individual papers.

Patient cohorts and biological samples

The clinical data and biological samples analysed in **Paper I-III** were collected from three overlapping patient cohorts that were part of the NORD-STAR treatment trial. This trial only includes patients with newly diagnosed untreated eRA. The biological samples examined in **Paper IV** were collected from patients with early or established RA. The demographic and clinical features of each patient cohort are summarised in Table 4.

In **Paper I-II**, blood samples were drawn before treatment (baseline, **Paper I-II**) and after 4, 12 and 24 weeks of treatment (**Paper II** only) from patients recruited to the NORD-STAR study in Gothenburg and Lund. Blood samples were also drawn from 31 healthy age- and sex-matched individuals who were included as controls in **Paper I**.

As a spin-off to the NORD-STAR study (**Paper III**), plasma samples were collected at baseline and after 24 weeks of treatment and frozen for later analysis from all per protocol Swedish participants.

In **Paper IV**, paired samples of blood and synovial fluid were collected from eight patients with active RA. Synovial tissue samples were collected from nine RA patients undergoing joint replacement surgery. FLS were expanded form synovial tissue. Synovial tissue from non-inflamed joints were also collected from individuals undergoing exploratory arthroscopy but were not included in the paper. Analysis of synovial fibroblasts from non-inflamed joints are included as additional results in this thesis.

All studies were approved by the regional ethics committee of Gothenburg, Lund and Stockholm. The studies were performed in accordance with the Declaration of Helsinki and all participants signed an informed consent form.

Table 4. Patient characteristics in each respective study.

I	**Total number**	**72**	
	Age, *yr** (range)	59 (21-80)	
	Female, *n* (%)	50 (69)	
	Smoker, *n* (%)	11 (15)	
	Symptom duration, months* (range)	5 (1-23)	
	ACPA$^+$, *n* (%)	59 (82)	
	RF$^+$, *n* (%)	52 (72)	
	CDAI* (range)	28.1 (10.1-68.7)	
	DAS28-ESR* (range)	5.3 (2.6-8.7)	
	DAS28-CRP* (range)	5.1 (2.7-8.3)	
II	**Total number**	**60**	
	Age, *yr** (range)	56 (21-77)	
	Female, *n* (%)	39 (65)	
	Smoker, *n* (%)	10 (17)	
	Symptom duration, months* (range)	5.5 (1-23)	
	ACPA$^+$, *n* (%)	50 (83)	
	RF$^+$, *n* (%)	43 (72)	
	CDAI* (range)	27.9 (10.1-68.7)	
	DAS28-ESR* (range)	5.3 (2.6-8.7)	
	DAS28-CRP* (range)	5.0 (2.7-8.3)	
III	**Total number**	**347**	
	Age, yr* (95% CI)	58 (55-61)	
	Female, *n* (%)	235 (68)	
	Smoker, *n* (%)	72 (21)	
	Symptom duration, months* (95% CI)	5.2 (4.5-5.7)	
	ACPA$^+$, *n* (%)	285 (82)	
	RF$^+$, *n* (%)	256 (74)	
	CDAI* (95% CI)	28.1 (27.1-33.6)	
	DAS28-ESR* (95% CI)	5.5 (5.4-5.7)	
	DAS28-CRP* (95% CI)	5.1 (5.0-5.2)	
IV	**Total number**	**17**	
	Biological samples	Blood & synovial fluid (n=8)	Synovial tissue (n=9)
	Age, yr* (range)	57 (30-72)	62 (27-70)
	Disease duration, years* (range)	6.5 (<1-13)	10.5 (1-22)
	Female, *n* (%)	6 (75)	8 (89)
	ACPA$^+$ and/or RF$^+$, *n* (%)	6 (75)	5 (56)
	SJC28	1.5 (1-5)	N/A
	TJC28	1 (1-4)	N/A

* Median, n – number; N/A - data not available

The NORD-STAR study

NORD-STAR is an international, phase four investigator-initiated, randomised, assessor-blinded clinical trial that included 812 patients with eRA from Denmark, Finland, Iceland, Norway, Sweden and the Netherlands. The study consisted of two parts. The aim for part one is to compare the proportion of patients who achieve disease remission with active conventional therapy versus three different biological treatments. The aim for part two is to compare two different dose reduction strategies for patients who respond to first-line therapy.

For the work included in this thesis, clinical data and biological samples collected during the first 24 weeks of part one were used. The analysis was also limited to Swedish patients only and the following study description is specific for these patients. A complete description of the larger trial is available in the NORD-STAR study protocol [103].

Patients

All patients were diagnosed with RA according to the American College of Rheumatology (ACR)/European League Against Rheumatism (EULAR) 2010 criteria. Key inclusion criteria include: ≥ 18 years of age, ≥ 2 swollen joints and 2 tender joints (66 and 68 joint counts, respectively), disease activity score in 28 joints (DAS28-CRP) of ≥ 3.2 and a symptom duration < 24 months (retrospective patient-reported symptoms). All patients also had to be ACPA-positive, RF-positive or have a CRP level ≥ 10 mg/L. Key exclusion criteria were: previous DMARD treatment or a current active inflammatory joint disease other than RA.

Treatment and clinical evaluation

Patients were first randomised to one of four treatment arms (Figure 4). The treatments were methotrexate (MTX) + oral prednisolone (arm 1), MTX + anti-TNF (arm 2), MTX + CTLA-4Ig (arm 3) or MTX + anti-IL6R (arm 4). All patients received MTX escalated to 25 mg/week within the first 4 weeks. Oral steroids were not allowed in arm 2-4. In arm 1, intra-articular (i.a.) corticosteroid injections were allowed when clinically indicated. In arm 2-4, i.a. injections were allowed when needed during the first 12 weeks. Corticosteroid injections were not allowed during week 20-24 from baseline in either treatment arm. Disease activity was assessed and blood samples were collected at baseline and after 4, 12 and 24 weeks of treatment. The primary clinical

outcome was remission according to the clinical disease activity index (CDAI≤2.8) at week 24.

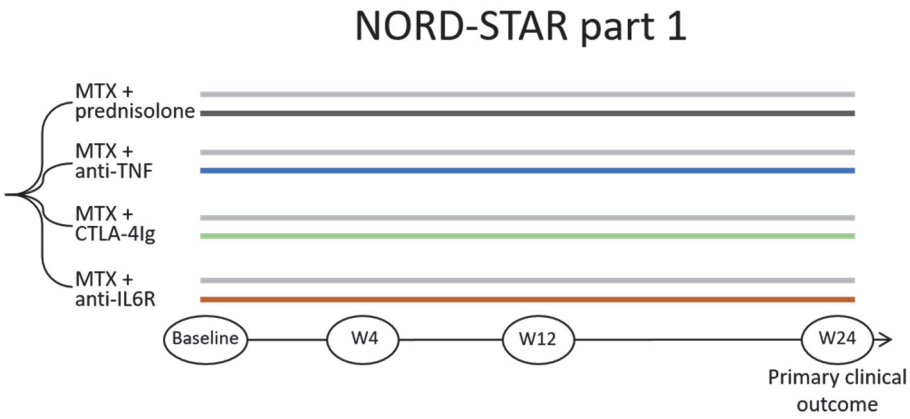


Figure 4. Schematic description of part one of the NORD-STAR randomized treatment trial and time points for clinical evaluation and blood sampling. Methotrexate (MTX), certolizumab-pegol (anti-TNF), abatacept (CTLA-4Ig) and tocilizumab (anti-IL6R).

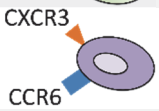
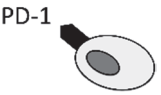
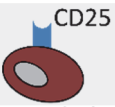
Flow cytometry

In order to measure the circulating proportions of specific T cell subsets flow cytometry was used to determine protein expression on lymphocytes. Lymphocytes were isolated from fresh whole blood or synovial fluid by density centrifugation and live cells were stained with fluorophore-conjugated antibodies that targeted surface proteins CD4, CD45RA, CCR4, CCR6, CXCR3, CD25, CD127 and CXCR5. To stain the intracellular proteins CTLA-4 and FOXP3, isolated lymphocytes were first fixed and permeabilised prior to incubation with antibodies. The analysed T cell subsets are presented in Table 5.

The classical Th1, Th2 and Th17 subsets were identified by combinations of chemokine receptors, including CCR4, CCR6 and CXCR3 (see gating strategy below). The gating strategy and phenotypes of these T cell subsets have previously been validated by subset specific transcription factor expression and cytokine secretion [38]. The advantage of using chemokine receptors is that live cells can be analysed directly without *ex vivo* stimulation. Identification of each of the classical subsets by their intracellular cytokine expression or transcription factor expression would require *ex vivo* stimulation to

induce cytokine production and fixation before permeabilisation to label intracellular components.

Table 5. $CD4^+$ T cell memory subsets

T cell subset	Main cytokines produced	Receptors used for identification	
Th1*	IFNγ, IL-2, TNF		($CCR6^{neg}$ & $CCR4^{neg}$)
Th1Th17**	IL-17A, IFNγ		($CCR4^{neg}$)
Th17*	IL-17A, IL-17F, IL-22		($CXCR3^{neg}$)
$CXCR3^+$Th17**	IFNγ, IL-17A		
Th2*	IL-4, IL-5, IL-13		($CCR6^{neg}$)
$CXCR3^+$Th2**	IFNγ, IL-4		($CCR6^{neg}$)
TFh	IL-21, IL-4, IL-17A, IFNγ		(can be $PD\text{-}1^{+\text{ or neg}}$)
TPh (Paper IV only)	IL-21		($CXCR5^{neg}$)
Treg	IL-10, TGFβ		($CD127^{neg}$)
TFreg	IL-10, TGFβ		($CD127^{neg}$)

*Classical T helper (Th) subsets; **non-classical Th subsets; follicular T helper (TFh); regulartoy T cell (Treg); follicular regulatory T cell (TFreg); peripheral T helper (TPh); interferon (IFN); Interleukin (IL);

Gating strategy for CD4$^+$ T cell subsets

Flow data was analysed using FlowJo software (Tree Star, Ashland, OR, USA) and the minimum number of cells allowed within a gate was 50 cells. Forward and side scatter were used to identify single lymphocytes and naïve/memory CD4$^+$ T cells were identified by the expression of CD45RA (Figure 5).

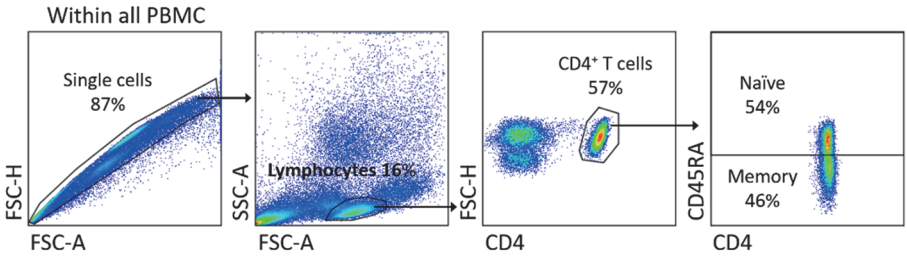


Figure 5. Gating strategy for CD45RA$^+$ (naive) and CD45RA^{neg} (memory) CD4$^+$ T cells. Each gate specifies the indicated cell subset as a percentage of the previous population. Peripheral blood mononuclear cells (PBMC).

Classical Th1, Th2 and Th17 and non-classical Th1Th17, CXCR3$^+$Th2 and CXCR3$^+$Th17 subsets were gated within memory cells and identified based on the expression of a combination of the chemokine receptors CCR4, CCR6 and CXCR3 (Figure 6).

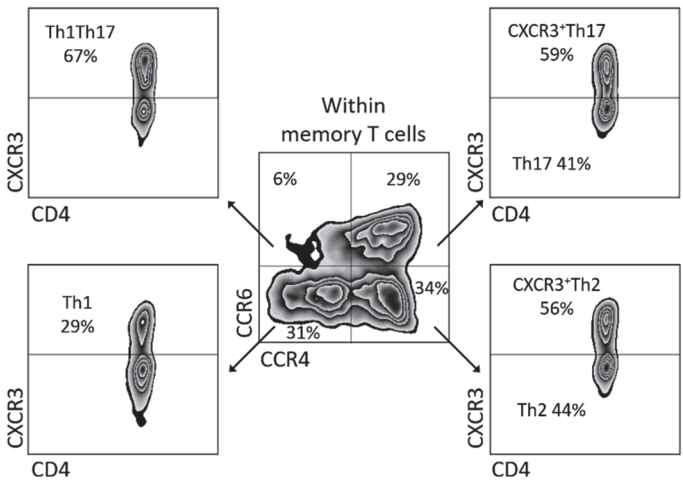


Figure 6. Gating strategy for the Th1, Th1Th17, Th2, CXCR3$^+$Th2, Th17 and CXCR3$^+$Th17 subsets from CD4$^+$ memory T cells. Each gate specifies the indicated cell subset as a percentage of the previous population.

Regulatory T cells (Tregs) were gated among $CD4^+$ cells and the remaining cells were termed NonTregs (Figure 7). Follicular Tregs (TFregs) were gated within Tregs, while follicular T helper cells (TFh) were gated within NonTregs. TFh cells were also subdivided based on PD-1 expression. Cells that expressed CTLA-4 intracellularly and/or on the surface were gated from within Tregs and NonTregs. The CTLA-4 gates were set as described in Figure 8.

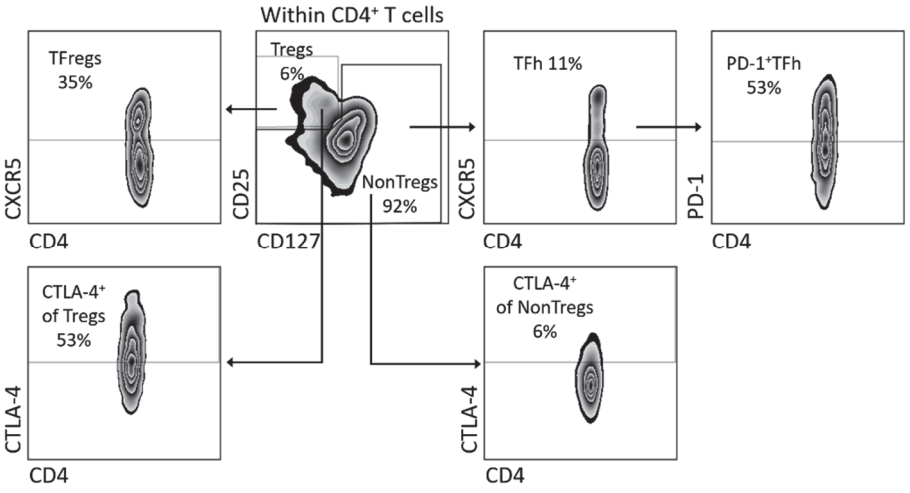


Figure 7. Gating strategy for regulatory T cells (Tregs), NonTregs, follicular regulatory T cells (TFregs), Follicular T helper cells (TFh), PD-1^+ fraction of TFh, and the fraction of CTLA-4^+ Tregs and NonTregs, respectively. CTLA-4 positivity was determined by comparing a stained sample with a control that only contained the streptavidin-PE fluorophore but not the biotinylated CTLA-4 antibody. Each gate specifies the indicated cell subset as a percentage of the previous population.

The fraction of $CD4^+$ cells that expressed CTLA-4 or FOXP3, respectively, were gated as shown in Figure 8. CTLA-4 positivity was determined by the use of a negative control sample. FOXP3 positivity was determined by using the $CD4^+CD25^{neg}$ cell population which are FOXP3 negative.

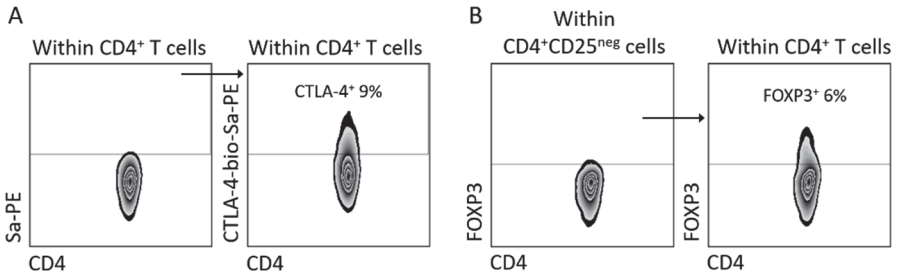


Figure 8. (A) CTLA-4 positivity was determined by comparing a stained sample with a control that only contained the streptavidin-PE fluorophore but not the biotinylated CTLA-4 antibody. (B) FOXP3 positivity was determined by comparing the whole $CD4^+$ cell population with the $CD4^+CD25^{neg}$ subpopulation.

In **Paper IV**, T cell subsets were analysed in paired samples of blood and synovial fluid. Classical and non-classical T helper subsets were gated as previously shown in Figure 6. PD-1^{high}TFh and peripheral T helper cells (TPh) cells in synovial fluid were gated within $CD4^+$ memory cells and identified by the expression of CXCR5 and PD-1 (Figure 9A). In RA, TPh cells have mainly been found in the arthritic joint and only a small fraction can be found in circulation [42]. Therefore, the gates set for TPh and PD-1^{high}TFh in synovial fluid were used as a reference for setting these gates in blood (Figure 9B).

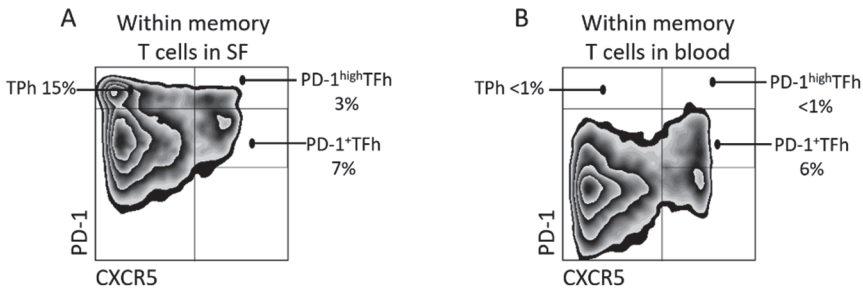


Figure 9. Gating example for peripheral T helper cells (TPh) and follicular T helper cells (TFh) in a paired sample of synovial fluid (SF) and blood. TFh are divided based on the levels of PD-1 expression into PD-1^+ and PD-1^{high}. Each gate specifies the indicated cell subset as a percentage of $CD4^+$ memory cells in SF and blood, respectively.

Bead-based immunoassay

Chemokine and cytokine levels in plasma, synovial fluid and cell culture supernatant were measured by bead-based immunoassay (Legendplex™, BioLegend, San Diego, CA). The technique is described in Figure 10.

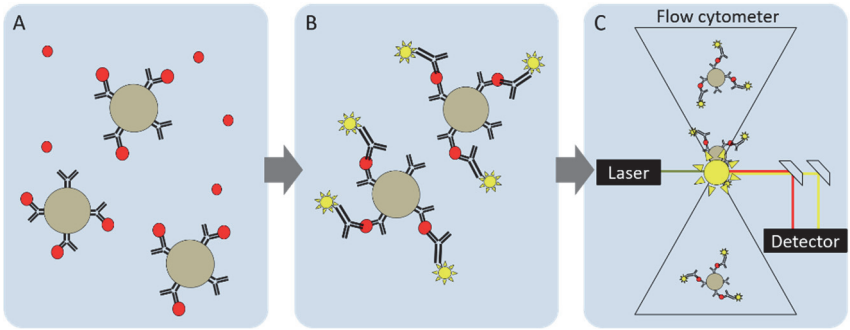


Figure 10. Schematic description of a bead-based immunoassay. (A) The target protein first binds to the antibody covered beads. The coverage is proportional to the concentration of the protein in the sample. (B) The secondary antibody binds to the target protein. (C) During flow analysis the fluorescent intensity of each bead is proportional to the amount of bound protein bound to the bead.

The bead-based method was chosen since it made it possible to analyse the concentration of up to 13 different cytokines or chemokines simultaneously in a sample volume of 25 µl. Nevertheless, we validated the results of the bead-based assay for selected chemokines by conventional enzyme-linked immunosorbent assay (ELISA). When we measured the concentration of CCL11 (eotaxin) in 26 plasma samples both methods produced comparable results (Figure 11). Cytokines and chemokines that were not available in bead-based assays were only analysed by ELISA.

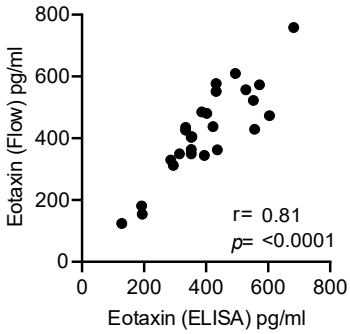


Figure 11. The plasma concentration of CCL11 (eotaxin) in 26 patients with early rheumatoid arthritis individuals as measured by bead-based immunoassay and ELISA Spearman's rank correlation test.

Isolation, expansion and stimulation of fibroblast-like synoviocytes

In **Paper IV**, fibroblast-like synoviocytes (FLS) were cultured from synovial biopsies from patients with established RA who underwent joint replacement surgery (n=9). Patient characteristics are shown in Table 4. Expanded FLS were used for transcriptomic, cytometric and cytokine/chemokine secretion analyses (Figure 12).

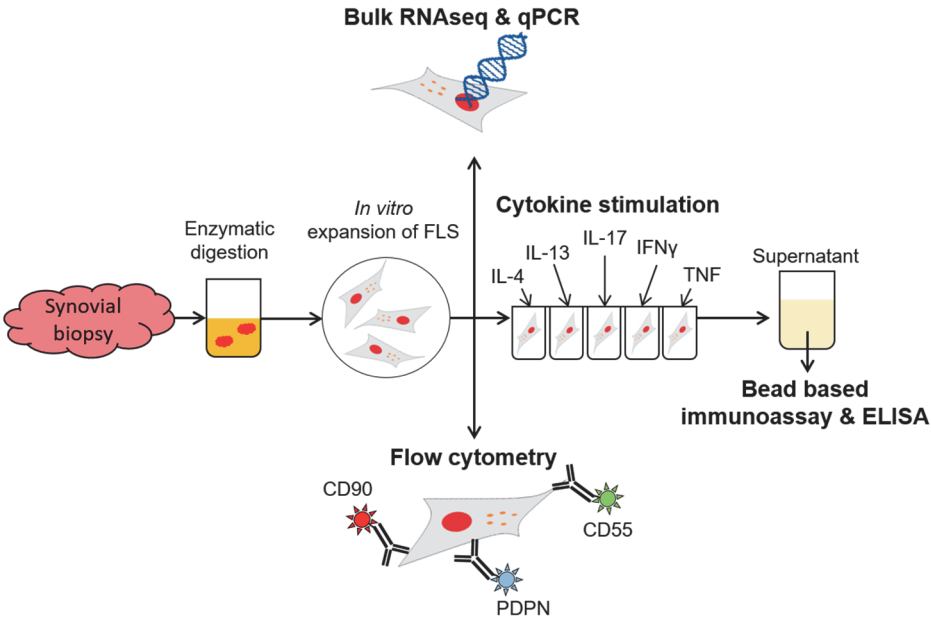


Figure 12. The transcriptional expression of IL-4, IL-5, IL-13, IL-17, IFNγ and TNF receptors in cultured fibroblast-like synoviocytes (FLS) was examined by RNA sequencing (RNAseq) and quantitative PCR (qPCR). The surface expression of CD90, podoplanin (PDPN) and CD55 was examined by flow cytometry. In the cytokine stimulation experiments FLS were cultured for 48 hours in culture media supplemented with 10 ng/ml of IL-4, IL-13, IL-17, IFNγ or TNF. The levels of 13 different chemokines and 15 cytokines were measured in the culture supernatant by bead-based immunoassay or enzyme-linked immunosorbent assay (ELISA). Detailed methods can be found in **Paper IV**.

Statistics

Univariate statistical analyses were performed using Graphpad Prism (GraphPad Prism Software, La Jolla, CA, USA) and SPSS (IBM SPSS Statistics, Armonk, NY, USA). Since most comparisons were made between small sample groups or data that were not normally distributed non-parametric statistical tests were used. In the analysis of paired data

Wilcoxon matched-pairs signed rank test was used to compare two groups. If the analysis included three or more groups Friedman test followed by Dunn's post test was used. For unpaired data the Mann-Whitney u-test was used to compare two groups and the Kruskall-Wallis test followed by Dunn's post test was used for three or more groups. For categorical variables Fisher's exact test was used when comparing groups. Correlation analysis was performed by Spearman's rank correlation test. For all univariate analyses a p-value ≤ 0.05 was considered statistically significant. In **Paper III**, Bonferroni correction was used to compensate for the increased likelihood of type 1 error after multiple-testing.

Univariate and multiple logistic regressions in **Paper II** were performed using Graphpad Prism. The results of logistic regression were presented as area under the curve (AUC) in receiver operating characteristic curves (ROC curves).

Multivariate factor analysis

Multivariate factor analyses were performed using SIMCA-P+ software (Umetrics, Umeå, Sweden) and included principal component analysis (PCA), orthogonal projection to latent structures (OPLS) and OPLS-discriminant analysis (OPLS-DA). Multivariate factor analysis is mainly used as tool for exploratory data analyses and is particularly useful for hypothesis generation and guiding selection of specific variables to study further.

In **Paper I-IV**, PCA, OPLS or OPLS-DA were used to find associations between specific variables within larger datasets. The variables that contributed most to each of the multivariate models were then examined further by univariate analysis. In this way multivariate modelling decreased the number of statistical comparisons that were needed and reduced the risk of type 1 error. PCA, OPLS and OPLS-DA are sensitive to differences in variance between variables. Variable X that ranges from 1-100 will have a larger variance than variable Y that ranges from 1-10. If all variables should be given equal weight in a model, the variance must be equalised (scaled) across all the included variables. Therefore, each variable included in the analyses was divided by one standard-deviation (known as unit variance scaling). All variables were also log transformed to normalise the data.

In SIMCA, the quality of the multivariate models is estimated by the variables R2 and Q2. R2 is a sum of the variance that is explained by all the principal components in your model (range 0-1). Thus, if the first

component in your model contain 35 percent of the data variance and the second component contains 15 percent the R2 for this model will be 0.5 (50 percent). As you add more principal components to your model R2 will increase until you have created one principal component for each observation in your dataset. The R2 will then be equal to 1 as your model contains 100 percent of the data variance. Q2 functions as an internal estimate of the predictive ability of the model and how reliable it is. It is estimated by dividing the dataset into seven parts. A new model is then created from $6/7^{th}$ of the data and this model tries to predict the $1/7^{th}$ that was left out. This is repeated for each of the seven parts until the whole dataset has been predicted. Q2 is then calculated from the difference between the predicted data and the original data (range 0-1). This means that a higher Q2 indicates that the model is more reliable and that the observed associations within the model are not dependent on the original data that was used to generate the model. A negative Q2 value is possible and a $Q2 \leq 0$ instead indicates that the model is not at all reliable.

RESULTS

In this section I present the key findings of each paper with an emphasis on results that address the specific aims of this thesis. A complete account of our findings and the supporting results can be found in each respective paper or manuscript. The results section also includes additional findings not included in the original papers or manuscripts. Brief comments are provided for the additional results.

Sex-specific relations between T cell subset proportions and disease activity in early RA (Paper I)

Genetic association studies indicate that $CD4^+$ T cells have an important role in initiating RA pathology [63]. We have previously found that circulating proportions of Th2 and Th17 cells are higher in patients with untreated eRA than in healthy individuals [38]. However, no significant relation between T cell subset proportions and disease activity was observed. Approximately 75% of RA patients are female and the circulating proportions of $CD4^+$ T cells also differ between males and females [26]. Therefore, we examined whether the relation between circulating $CD4^+$ T cell subset proportions and disease activity differ in male and female patients with untreated eRA.

Circulating proportions of $CD4^+$ T cell subsets were analysed by flow cytometry in a cohort of 72 DMARD-naïve eRA patients (see Methods). Patient characteristics are shown in Table 4. T helper subsets were identified based on their expression of chemokine receptors and included: classical Th1, Th2 and Th17, non-classical Th1Th17, $CXCR3^+$Th2 and $CXCR3^+$Th17. Additionally, Tregs, TFregs, TFh and PD-1^+TFh were also included in the analysis along with the fraction of $CD4^+$ T cells that express CTLA-4 or FOXP3. Disease activity was assessed by CDAI, DAS28, swollen and tender joint counts, ESR, and CRP.

In **Paper I**, we first examined the relation between $CD4^+$ T cell subset proportions and measures of disease activity in male and female eRA patients, respectively, by multivariate factor analysis (PCA and OPLS, see description in Methods). In male patients, the proportions of Th2 and Th17 cells, as well as the fraction of $CD4^+$ T cells that express CTLA-4, were positively associated with measures of disease activity. In contrast, proportions of Th1Th17 cells showed a negative association with disease activity measures. In female patients, OPLS analysis showed a

negative association between the proportion of FOXP3$^+$ T cells and several disease activity measures.

We next examined the relation between individual T cell subsets and measures of disease activity by univariate correlation analysis. In male patients, proportions of Th2 cells showed a significant positive correlation with CDAI, DAS28-ESR, SJC28, SJC66, TJC28 and TJC68. The proportion of Th17 cells and the fraction of CD4$^+$ cells that expressed CTLA-4 correlated only with DAS28-ESR. The proportion of Th1Th17 cells showed a negative correlation with DAS28-ESR and CDAI. In female patients, the proportions of FOXP3$^+$ T cells were negatively correlated with DAS28-ESR, TJC68, TJC28 and SJC66, and CXCR3$^+$Th17 cells were inversely related to SJC28 and SJC66.

In conclusion, these results show that there are sex-based differences in the association between T cell subsets and disease activity in eRA.

Additional results

In OPLS analysis, the proportions of follicular T helper (TFh) and follicular regulatory T cells (TFregs) showed a pattern of negative association with several measures of disease activity in male but not female patients (Figure 13).

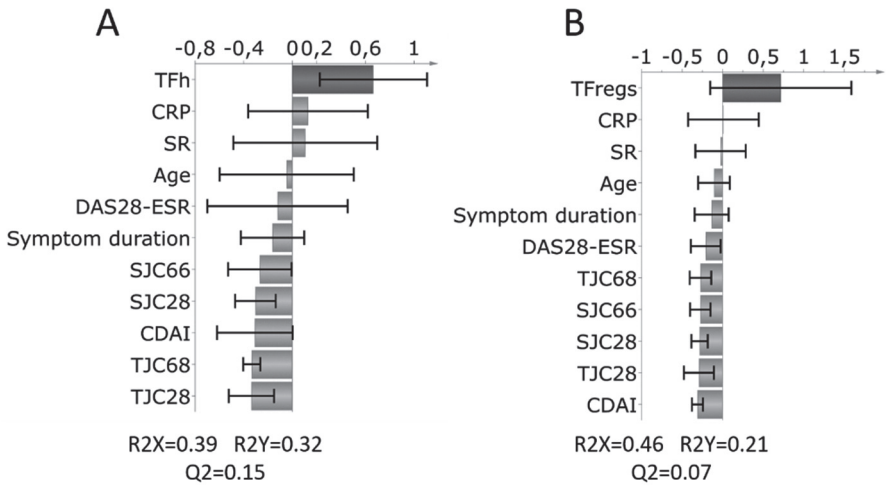


Figure 13. OPLS loading plots depicting the relationship between (A) follicular T helper cells (TFh) or (B) follicular regulatory T cells (TFregs) and disease activity measures in male patients with untreated early rheumatoid arthritis.

Univariate analyses showed that the proportions of TFh and TFregs were also negatively correlated with CDAI in male patients, although the relation was not statistically significant for TFh cells (Figure 14). The proportions of both subsets also showed a negative relation with swollen and tender joints, although the relation to swollen joints was not statistically significant (data not shown). Thus, lower proportions of the $CXCR5^+$ follicular subsets of T cells in the circulation indicated higher disease activity in male patients with eRA.

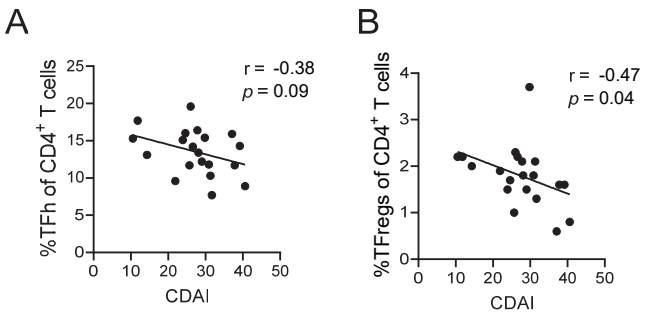


Figure 14. Correlation analysis between the proportions of (A) TFh or (B) TFreg cells and clinical disease activity index (CDAI) in male patients with eRA. Spearman's rank correlation test. Regression lines are presented in each plot.

Specific T cell subsets predict remission after treatment with CTLA-4Ig in early RA (Paper II)

The introduction of bDMARDs has improved the clinical outcome for many patients with RA. Still, about 50 percent of eRA patients do not achieve remission (CDAI≤2.8) after first line bDMARD treatment [102, 104, 105]. CTLA-4Ig (abatacept) is a bDMARD that inhibits T cell activation and, therefore, we examined whether baseline T cell proportions could predict disease remission after CTLA-4Ig treatment. For comparison, we also examined whether T cell proportions could predict remission after treatments that do not specifically target T cells. Furthermore, we investigated whether biological treatments with different mechanisms of action affect circulating proportions of $CD4^+$ T cell subsets differentially.

Circulating $CD4^+$ T cell subset proportions were analysed at baseline and after 24 weeks of treatment in 60 eRA patients as a part of a spin-off study to the NORD-STAR treatment trial. In statistical analyses samples

from baseline and week 24 were analysed as paired data. Patient characteristics are shown in Table 4. Since the aim of this study was to investigate the effect of targeted biological therapy only patients who were treated with bDMARDs were included. The patients were treated with MTX combined with either CTLA-4Ig, anti-IL6R or anti-TNF.

By OPLS-DA (see Methods), we first examined if baseline proportions of specific T cell subsets associated with remission (CDAI $\leq$ 2.8). We found that several T cell subsets associated with remission after treatment with CTLA-4Ig, and that the baseline proportions of PD-1^{+}TFh and NonTregs that express CTLA-4 were significantly higher in eRA patients who achieved remission by week 24 than those who did not.

Next we examined the diagnostic value of baseline proportions of PD-1^{+}TFh and NonTregs that express CTLA-4 by logistic regression. In ROC analysis (see Methods), the proportions of PD-1^{+}TFh and NonTregs that express CTLA-4 combined in a single regression model resulted in an AUC of 0.89 (Figure 15).

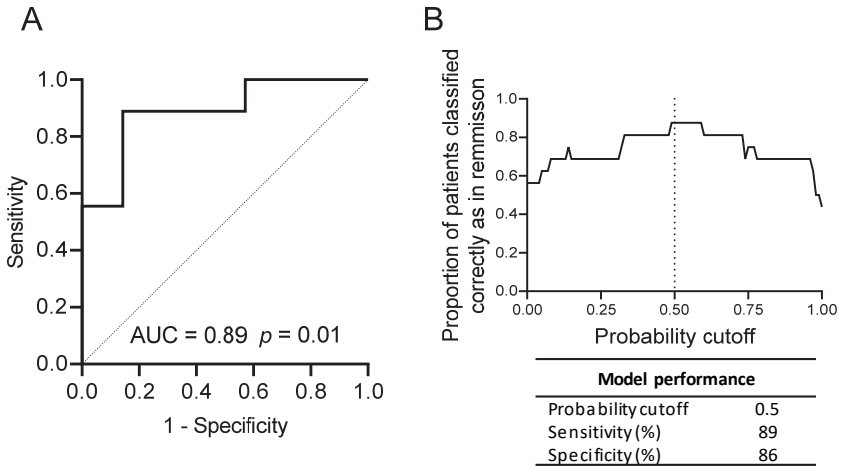


Figure 15. Multiple logistic regression model of baseline proportions of PD-1+TFh and NonTregs that express CTLA-4. (A) ROC curve and area under the curve (AUC). (B) Plot of the proportion of patients that are correctly classified as in remission by the model at the respective probability cutoff values.

Baseline T cell subset proportions did not predict remission after treatment with anti-IL6R or anti-TNF.

The effect of different biological treatments on circulating T cell subset proportions

The percentage of memory $CD4^+$ T cells decreased after treatment with CTLA-4Ig, while anti-IL6R had no effect and anti-TNF treatment increased the proportion of memory T cells in circulation (Figure 16). Similarly, the proportions of PD-1^+TFh cells decreased after CTLA-4Ig, were unaffected by anti-IL6R, and increased after anti-TNF treatment compared to baseline. Each respective treatment also showed distinct effects on the proportions of several other classical and non-classical T helper subsets. Two T cell subsets, the proportions of Tregs that expressed intracellular CTLA-4 and Th17 cells, decreased in all three treatment groups by week 24.

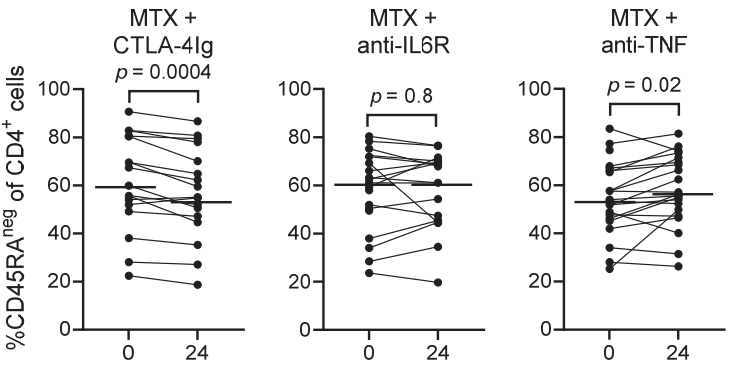


Figure 16. The difference in proportions of memory ($CD45RA^{neg}$) $CD4^+$ T cells between baseline and week 24 in each respective treatment group. Paired Wilcoxon matched-pairs signed rank test. Bars indicate median.

In summary, our results indicate that baseline proportions of PD-1^+TFh combined with NonTregs that express CTLA-4 might serve as a biomarker of remission in eRA patients treated with CTLA-4Ig. Furthermore, treatment with CTLA-4Ig, anti-IL6R or anti-TNF each resulted in distinct changes in circulating proportions of several subsets of T helper cells and regulatory T cells, as well as $CD4^+$ memory T cells in total.

The effect of targeted biological treatments on plasma chemokine levels in early RA (Paper III)

We have previously shown that plasma levels of CXCL10 and several other chemokines are higher in patients with eRA (n=43) than healthy individuals [106]. Plasma CXCL10 also correlated to measures of disease activity. Similar results have been found by another group for CXCL13 [107]. Blood chemokine levels have also been proposed as potential biomarkers of treatment response in RA, although the findings have not been conclusive [108, 109]. Therefore, we examined whether blood chemokine levels could be used as biomarkers for remission in eRA, and we investigated how chemokine levels are affected by biological treatments with different modes of action.

This study included all 347 per protocol Swedish patients who participated in the NORD-STAR treatment trial. Patient characteristics are shown in Table 4. Patients with eRA were randomised into one of four treatments arms, MTX combined with either prednisolone, anti-TNF, CTLA-4Ig or anti-IL6R. Plasma samples were collected at baseline and after 24 weeks of treatment and frozen for later analysis. The plasma concentrations of 14 different chemokines were measured at baseline and after 24 weeks by bead-based immunoassay and ELISA. In statistical analyses samples from baseline and week 24 were analysed as paired data.

Plasma chemokine levels and disease activity in early RA

We first examined the relation between baseline plasma chemokine levels and measures of disease activity by multivariate OPLS and univariate correlation analyses. We found that the plasma levels of CXCL10, CXCL9, CXCL11 and CCL2 were primarily associated with swollen joints and CDAI. In contrast, plasma levels of CXCL5 and CXCL8 were most strongly associated with ESR and CRP, respectively.

Plasma chemokine levels and prediction of remission in early RA

To examine whether chemokine levels could predict remission we first compared baseline chemokine levels between patients with different treatment outcome in the whole Swedish cohort. There were no significant differences in baseline chemokine levels between patients in remission (CDAI≤2.8), with low disease activity (2.8<CDAI≤10) or with moderate/high disease activity (CDAI>10) at week 24. By multivariate OPLS-DA we then examined if baseline chemokine levels

associated with remission at week 24 in each respective treatment arm. OPLS-DA models showed poor or no prediction (low or negative Q2 values) and could not distinguish between patients who achieved remission and those who did not based on baseline chemokine levels.

The effect of biological treatments of plasma chemokine levels in early RA

When we compared the plasma chemokine levels before and after 24 weeks of treatment we found that bDMARDs with different modes of action had distinct effects on plasma chemokine levels in eRA patients. For example, plasma levels of CXCL10, CXCL13, CXCL8, CXCL9 and CXCL11 decreased significantly after treatment with MTX combined with prednisolone, anti-TNF or CTLA-4Ig, but not anti-IL6R. The anti-IL6R treatment arm showed no reduction in plasma chemokine levels despite a significant decrease in CDAI.

In conclusion, baseline plasma chemokine levels could not predict remission in eRA patients treated with MTX combined with prednisolone, anti-TNF, CTLA-4Ig or anti-IL6R, but all treatments except anti-IL6R resulted in a significant reduction in several plasma chemokine levels.

T helper cells in synovial fluid from patients with RA (Paper IV)

The primary characteristic of RA is joint inflammation, and joint pain and swelling are key measures of disease activity. Previous findings indicate that the majority of T helper cells in inflamed joints express CXCR3 and were therefore denoted Th1 cells [110]. However, circulating subsets of Th2 and Th17 cells also express CXCR3 [38]. Therefore, we examined which T helper cell subsets are most common in the synovial fluid (SF) in inflamed joints. Additionally, since fibroblast-like synoviocytes (FLS) have been established as active mediators of inflammation in RA we also examined which chemokines FLS produce in response to Th1-, Th2- and Th17-associated cytokines, respectively.

The proportions of $CD4^+$ T helper subsets were analysed in paired samples of blood and SF from eight patients with active RA who had at least one swollen joint. Patient characteristics are shown in Table 4. Cultured FLS from synovial biopsies were stimulated with IL-4, IL-13, IL-17, IFNγ or TNF for 48 hours. The concentrations of 13 different chemokines were measured in FLS culture supernatant as well as plasma and SF.

T helper phenotypes in synovial fluid and peripheral blood

We first compared the proportions of $CD4^+$ T helper subsets in blood and SF. We found that the proportion of memory T cells was significantly higher in SF compared to blood and that the majority of the T cells in RA SF were $CXCR3^+$. The $CXCR3^+$ cells consisted mainly of Th1 and $CXCR3^+$Th2 cells, although $CXCR3^+$Th17 and Th1Th17 cells were also present. The proportions of TPh and PD-1^{high}TFh cells were also higher in SF than blood (Figure 17A-B). Proportions of TFh that express intermediate levels of PD-1 (PD-1^+TFh) did not differ between the two compartments. TPh share the B cell supporting characteristics of conventional TFh but lack the expression of CXCR5 associated with TFh cells, although both cell types express PD-1 [42]. In blood of healthy individuals, TFh can be subdivided into Th1, Th2 and Th17 phenotypes and each comprise approximately 25-30 percent of the TFh population [41]. When we subdivided TPh and PD-1^{high}TFh from RA SF we found that they primarily consisted of cells with a Th1 or $CXCR3^+$Th2 phenotype (Figure 17C-D).

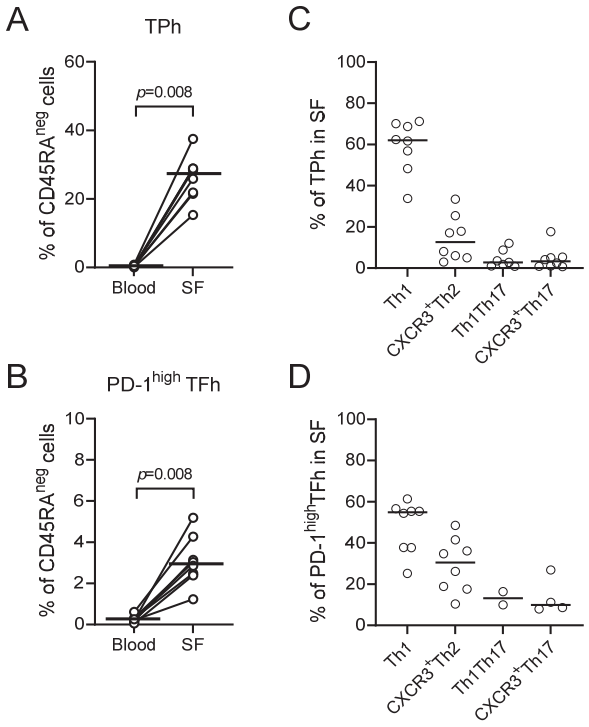


Figure 17. The difference in proportions of (A) peripheral T helper cells (TPh) and (B) follicular T helper cells with a high PD-1 expression (PD-1^{high}) between blood and synovial fluid (SF), and

the proportion of TPh and PD-1^{high}TFh with a Th1, CXCR3^{+}Th2, Th1Th17 or CXCR3^{+}Th17 phenotype. Paired Wilcoxon matched-pairs signed rank test.

Chemokine production by fibroblast-like synoviocytes

Next, we examined the chemokine secretion profile of RA FLS that had been stimulated with cytokines associated with a specific T helper subset, i.e. IFNγ (Th1), IL-4 or IL-13 (Th2) and IL-17 (Th17), respectively. Culture media with no added cytokines was used as a negative control and stimulation with TNF was used as a positive control. IL-4, IL-13 and IL-17 all induced secretion of proinflammatory chemokines CCL2, CXCL1 and CXCL8 by RA FLS, while IFNγ induced secretion of CXCL10. When we subsequently measured chemokine levels in paired plasma and SF we found that the levels of CCL2, CXCL8 and CXCL10 as well as CXCL9 and CCL20 were significantly higher in SF than in plasma.

In summary, we show that the T helper cells found in the synovial fluid of inflamed joints are primarily CXCR3^{+} memory cells of a Th1 or CXCR3^{+}Th2 phenotype. Furthermore, we show that IL-4, IL-13 and IL-17 induce RA FLS to secrete proinflammatory CCL2, CXCL1 and CXCL8, whereas IFNγ induced CXCL10.

Additional results

The small volume of SF that could be collected from inflamed joints limited the number of subsets that could be examined. Thus, the proportions of Tregs and TFregs could only be analysed in two samples. The number of cells in SF was also insufficient to perform staining for CTLA-4 or FOXP3. We found that the proportions of Tregs were higher in SF than in blood of these two RA patients (Figure 18A), which is consistent with previous findings in larger groups of RA patients [111, 112]. In the two patients, only a small fraction of Tregs in SF expressed CXCR5 and the proportions of TFregs appeared lower in SF compared to blood. Thus, TFregs do not appear to concentrate in the SF of patients with RA, although this needs to be confirmed in a larger group of RA patients.

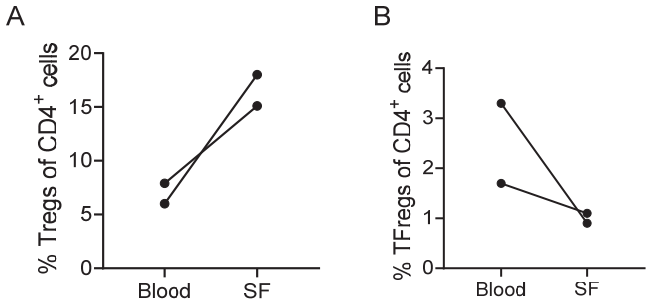


Figure 18. Proportions of (A) regulatory T cells (Tregs) and (B) follicular regulatory T cells (TFregs) of $CD4^+$ T cells in paired samples of blood and synovial fluid (SF) from two patients with established rheumatoid arthritis.

FLS have been shown to actively contribute to the inflammatory milieu of the RA joint [74]. FLS in the sub-lining layer of the synovium promote inflammation through the production of cytokines and chemokines, while FLS in the lining layer confer joint destruction through secretion of RANKL and matrix metalloproteases (MMPs) [90]. These two subsets of FLS can be distinguished by their expression of CD90 (sub-lining) and CD55 (lining), while podoplanin (PDPN) is expressed by both [91].

To investigate whether this phenotypic distinction was applicable to *in vitro* expanded FLS we analysed cultured FLS from RA patients and synovial fibroblasts from non-inflamed joints by flow cytometry. We found that after *in vitro* expansion RA FLS expressed all three markers, CD90, PDPN and CD55. Similarly, the majority of synovial fibroblasts from non-inflamed joints also expressed all thee markers, although one sample lacked CD55 expression (Figure 19). Thus, the distinct lining and sub-lining FLS phenotypes are not maintained during *in vitro* culture. Indeed, it was recently shown that the phenotypic difference between lining and sub-lining FLS is dependent on NOTCH mediated signalling between FLS and perivascular endothelial cells [92].

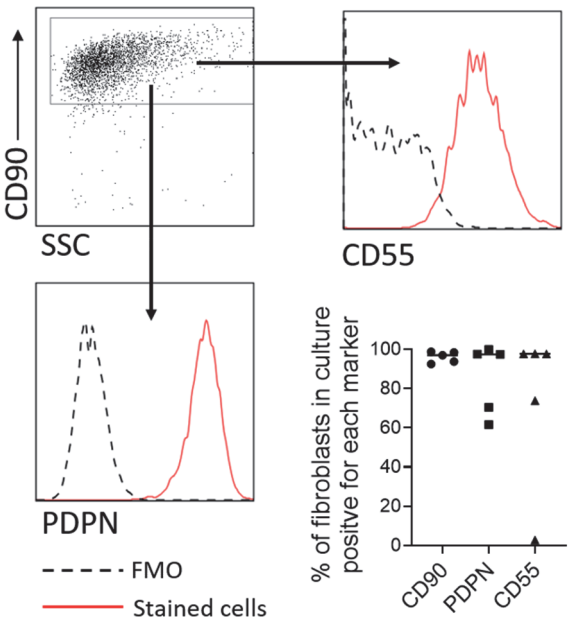


Figure 19. Representative dot plot and histograms CD90, podoplanin (PDPN) and CD55 expression on synovial fibroblasts from non-inflamed joints and the total percentage of fibroblasts in culture expressing CD90, PDPN and CD55.

Next, we compared the chemokine secretion of RA FLS and synovial fibroblasts from non-inflamed joints in response to stimulation with IL-4, IL-13, IL-17, IFNγ or TNF. The concentrations of CXCL1, CXCL5, CXCL8, CXCL9, CXCL10, CXCL11, CCL2, CCL3, CCL4, CCL5, CCL11, CCL17 and CCL20 were then measured in the culture supernatant. TNF triggered higher levels of CXCL1 from synovial fibroblasts from non-inflamed joints than RA FLS (p=0.01), while IL-13 triggered higher levels of CXCL8 from RA FLS (p=0.04) (Figure 20). No other significant differences in chemokine secretion were found. Thus, there is little disparity in chemokine secretion between RA FLS and synovial fibroblasts from non-inflamed joints. Potential larger variation in sensitivity to cytokine stimulation may have been lost as a result of repeated passages of *in vitro* culture.

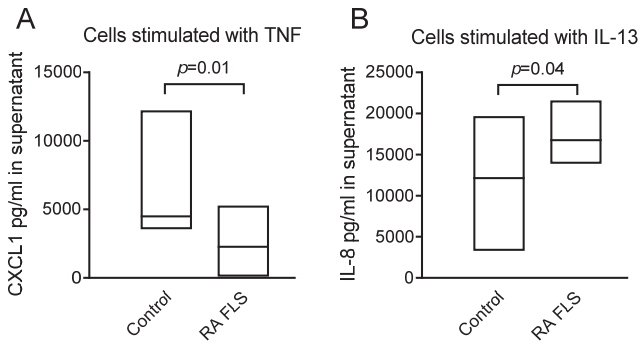


Figure 20. Levels of (A) CXCL1 or (B) CXCL8 in culture supernatant from synovial fibroblasts from non-inflamed joints (control) and fibroblast-like synoviocytes (FLS) from patients with rheumatoid arthritis (RA). Boxes indicate minimum and maximum value and the median. Mann-Whitney u-test.

DISCUSSION

In this thesis, we aimed to identify $CD4^+$ T cell subsets and chemokines that associate with disease activity and/or could predict remission in patients with eRA. Additionally, we investigated which T cell subsets and chemokines were present in the blood and synovial fluid of patients with established RA and what cytokines/chemokines were produced by FLS that were stimulated with T cell associates cytokines.

In the following sections I discuss the main findings of the included papers and how we addressed specific knowledge gaps in the field. I also expand on related topics that were beyond the scope of the individual papers.

$CD4^+$ T cells and chemokines in RA

At the onset of the work included in this thesis, RA had been considered a Th1 and Th17 mediated disease especially in established RA [79]. Indeed, proportions of citrulline-specific Th1 cells have been shown to be higher in RA patients compared to HC [113], although one study had shown that the most expanded circulating T cell clones in patients with RA were not Th1 or Th17 cells [114]. Early RA had not been extensively studied but one previous study had indicated a Th2 and Th17 bias in the cytokine profile of synovial fluid in patients with eRA [84]. Additionally, our research group had also reported that proportions of Th2 and Th17 cells in blood were higher in patients with eRA than HC [38]. The Th2 subset was of particular interest as the role of Th2 associated cytokines in RA had not been extensively studied and was thought to have only anti-inflammatory functions in RA. We had also found that CXCL10, the chemokine ligand to the CXCR3 receptor, correlated with measures of disease activity in patients with eRA [106]. Although CXCR3 was originally associated with Th1 cells, we and others had shown that non-classical subsets of Th2 and Th17 cells, as well as Th1Th17 cells also expressed CXCR3 [38, 39, 115]. It was not known whether these non-classical subsets were present in the synovial fluid of inflamed joints from RA patients. Recent findings had also characterised a novel pathogenic T cell subset with B cell helping capacity (termed TPh) in the synovium of RA patients [42]. It was not known if TPh cells also had a Th1 and/or non-classical T cell phenotype.

CXCR3$^+$ T cells and CXCR3 ligands in RA

Among CD4$^+$ T helper cells, CXCR3 expression was initially associated with the Th1 subset and IFNγ production [116]. The proportion of Th1 cells in circulation tend to be lower in patients with early or established RA compared to HC [38, 80]. Non-classical CXCR3$^+$Th2, CXCR3$^+$Th17 and Th1Th17 cells are also present in the blood of patients with early or established RA [38, 39, 115]. Akin to Th1 cells, non-classical CXCR3$^+$ T helper cells produce IFNγ when activated *in vitro* [38]. In **Paper IV**, we found that the CXCR3$^+$ T cell population in SF from patients with established RA included both Th1 cells and non-classical subsets. The majority of CXCR3$^+$ cells consisted of Th1 and CXCR3$^+$Th2 cells. Whether non-classical T helper subsets such as CXCR3$^+$Th2 are also present in the joint in eRA has not been investigated. However, single cell transcriptional analysis of synovial tissue with high lymphocyte infiltration show enrichment of both Th1 and Th2 activation pathways in eRA [75].

CD4$^+$ T helper cells migrate to the inflamed joint in response to local chemokine production. The dominance of CXCR3$^+$ T cells in the synovial fluid would indicate that their migration from the periphery into the inflamed joint is mediated at least in part by CXCR3 and its ligands CXCL9, CXCL10 and CXCL11. Plasma levels of CXCL9 and CXCL10 are higher in patients with RA compared to HC [106, 117], and deletion of CXCR3 have been shown to reduce CD4$^+$ T cell migration into the inflamed synovium in murine arthritis models [118]. However, in established RA, only the concentrations of CXCL9 and CXCL10 were significantly higher in the SF than in paired blood, while CXCL11 levels were higher in blood. Hence, CXCL11 likely does not mediate T cell recruitment to the RA joint. It is probable that the high levels of CXCL9 and CXCL10 in RA SF leak into the blood and result in the increase in systemic levels of these chemokines in patients with RA. Indeed, in **Paper II** we found that treatment with CTLA-4Ig or anti-TNF in eRA patients resulted in reduced blood levels of CXCL9 and CXCL10, while proportions of Th1 cells in circulation increased. This indicates a reduced migration of Th1 cells to the joint due to decreased levels of CXCL9 and CXCL10.

CXCL9 and CXCL10 secretion is mediated by interferons. Synovial tissue from RA patients display an IFN-signature characterised by the expression of IFN-stimulated genes [74]. Pathway enrichment analysis have shown that sub-lining FLS in particular show high expression of the IFNγ-mediated signalling pathway and express high levels of

CXCL9 [74]. In line with previous findings, we found that IFNγ stimulation increase the secretion of CXCL9 and CXCL10 by FLS [119], although the rise in CXCL9 secretion compared to media control was not statistically significant. Thus, FLS may be the primary source of CXCL9 and CXCL10 in the RA joint.

In **Paper III**, blood levels of CXCL9, CXCL10 and CXCL11 correlated with disease activity, particularly measures that included swollen joints. Indeed, treatment with anti-CXCL10 has been shown to reduce disease activity in patients with RA [56], and CXCR3 blockade or deletion abrogate arthritis in murine arthritis models [118, 120]. Thus, our findings indicate that infiltration of classical and non-classical $CXCR3^+$ T cell subsets and production of CXCR3 chemokine ligands in the RA joint likely mediate disease activity in both early and established RA.

Th2 and Th17 subsets and their associated cytokines in RA

The Th2 subset and its associated cytokines IL-4, IL-5 and IL-13 have traditionally been considered anti-inflammatory in RA [121]. Both classical Th2 and non-classical $CXCR3^+$Th2 cells secrete IL-4 when activated *in vitro* but $CXCR3^+$Th2 cells produce little to no IL-5 [38]. It is possible that IL-5 is responsible for the anti-arthritic effect associated with the Th2 subset in RA. IL-5 promotes eosinophil activation and eosinophils have been shown to promote resolution of arthritis in murine disease models [122, 123]. Treatment with anti-IL5 in patients with eosinophilic asthma and concomitant RA have also been shown to trigger a flare in RA disease activity [123]. Hence, IL-5 mediated eosinophil activation could in part be responsible for the anti-inflammatory effects that has been associated with the Th2 subset. We did not examine the effect of IL-5 stimulation on FLS since transcriptional analysis had shown that FLS do not express the IL-5 receptor.

Previous studies also indicated that IL-4/IL-13 could have anti-arthritic effects. In murine models and *in vitro* experiments it has been shown that these cytokines inhibit osteoclast differentiation and bone destruction [124, 125]. Contrary to this notion, in **Paper IV**, we found that IL-4 and IL-13 induced the secretion of proinflammatory cytokines and chemokines by FLS. Increased levels of IL-4 and IL-13 in SF has also been shown to be a distinguishing feature of early RA compared to other arthritides, which could indicate a pathogenic effect of these cytokines specific to early RA [84]. Specifically, IL-4 and IL-13 may be linked to the formation of ELS in the synovium. In Sjögren's syndrome,

IL-13 and TNF together promote a pathogenic phenotype in salivary gland fibroblasts characterised by increased VCAM-1 expression [126]. This fibroblast phenotype was shown to be key to the formation of ELS in Sjögren's syndrome. IL-4 and IL-13 along with TNF similarly induce a sustained expression of VCAM-1 in RA FLS [127].

In **Paper IV**, we found that the proportions of Th17 cells was higher in blood than SF, while proportions of $CXCR3^{+}$Th17 cells did not differ significantly in the two compartments. Nevertheless, the production of IL-17 by these cells might promote inflammation and cartilage degradation in RA through activation of FLS. Recent findings indicate that $CXCR3^{+}$ and $CXCR3^{neg}$ Th17 subsets activate FLS independently of IFNγ and promote the secretion of IL-6, CXCL8, MMP-1 and MMP-3 [115]. In line with these findings, we observed that IL-17 but not IFNγ significantly increased secretion of IL-6 and CXCL8 by FLS. Additionally, only IL-17 induced granulocyte-macrophage colony-stimulating factor (GM-CSF) in FLS. Treatment with anti-GM-CSF have been shown to reduce disease activity in RA in phase II trials [128, 129]. Thus, Th17 cell subsets may contribute to RA disease activity by promoting GM-CSF production in the joint. In the SKG arthritis model, Th17 and IL-17 induced GM-CSF secretion by FLS is essential to develop autoimmune arthritis [130].

T helper phenotypes of TPh cells in RA

The classical B cell supporting TFh subset is characterised by expression of the chemokine receptor CXCR5 [131, 132]. However, the majority of $CD4^{+}$ T cells capable of supporting B cell activation within synovial tissue and SF of RA patients have previously been shown to lack CXCR5 expression [42, 133]. These cells have been termed TPh and instead express chemokine receptors that attract them to sites of inflammation, e.g. CCR2. The TPh subset is highly specific to the joint in RA and often comprise <1 percent of memory $CD4^{+}$ T cells in circulation [42, 85, 86]. In **Paper IV**, we found that both TPh and PD-1^{high}TFh in the SF of patients with RA mainly consisted of cells with a Th1 or $CXCR3^{+}$Th2 phenotype. In line with our findings, transcriptional analysis has shown that both TPh and PD-1^{high}TFh from patients with RA express high levels of CXCR3 [42].

The combined expression of CXCR3 and CCR2 of TPh in RA SF would indicate that the higher levels of CCL2, CXCL9 and CXCL10 found in RA SF compared to blood contribute to the accumulation of these cells in the SF (Figure 21). It has recently been shown that TPh cells may also

expand locally in the joint rather than migrate from the blood [134]. CCL2 in particular may contribute to retention of TPh in the SF since these cells comprised a larger fraction of $CD4^+$ cells in RA SF compared to PD-1^{high}TFh, which do not express CCR2 [42].

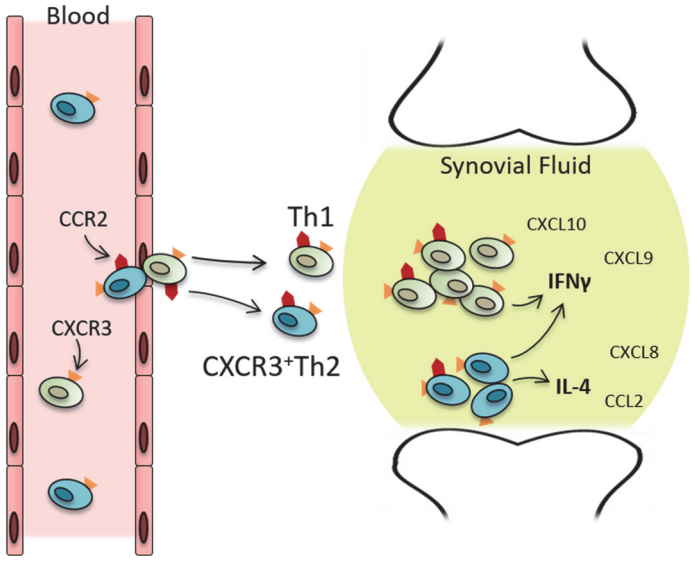


Figure 21. Simplified model for the migration of peripheral T helper (TPh) cells with a Th1 or $CXCR3^+$Th2 phenotype and chemokines in the synovial fluid of an inflamed joint.

In RNA analysis of synovial tissue, genes associated with TPh are highly expressed in both early and established RA patients that present with a lympho-myeloid synovial pathotype and distinct ELS formation [74, 75]. It has been shown that ELS support autoantibody production in the joint [4]. However, TPh and conventional TFh may differ in their ability to promote antibody production in B cells. TPh isolated from peripheral blood of patients with RA were not able to induce class switching in naïve B cells *in vitro* but did promote IgM, IgG and IgA secretion in memory B cells [85]. In contrast, PD-1^{high}TFh induced IgM, IgG and IgA in both naïve and memory B cells. Additionally, TFh cells with a Th1, Th2 or Th17 phenotype differ in their ability to induce antibody secretion by B cells *in vitro* [41]. Specifically, TFh with a Th1 phenotype were shown to have limited ability to induce antibody secretion compared to cells with a Th2 or Th17 phenotype. It is not known if these differences are also present among TPh cells with a Th1 or $CXCR3^+$Th2 phenotype found in the joints of RA patients.

Follicular regulatory T cells in RA

It is well established that proportions of Tregs are higher in RA SF than blood [111, 112]. In contrast, TFregs are less well studied in RA. In **Paper I**, we found that proportions of TFregs were negatively correlated to CDAI in male patients with eRA. A similar negative relation has been observed between proportions of TFregs and DAS28-ESR in patients with established RA [135]. It has been reported that TFregs infiltrate the salivary glands of patients with primary Sjögren's syndrome [136]. It is conceivable that the negative relation between blood proportions of TFregs and disease activity in RA is due to migration of TFregs into inflamed joints. In **Paper IV**, we could only compare the proportions of TFregs in blood and SF in two patients due to the limited number of cells we recovered from RA SF. In these two RA patients proportions of TFregs were lower in SF compared to blood. Thus, our results indicate that TFregs are not actively recruited to the inflamed joint in patients with RA, although this would need to be examined in a larger cohort.

Biomarkers of remission in RA

There are a wide selection of therapies available for the treatment of RA but the effectiveness of each drug varies considerably between individual patients. The heterogeneous pathogenesis observed in RA is likely a contributing factor. A biomarker that could predict the most appropriate drug for each individual patient could improve remission rates by optimising the use of each drug. Several studies have attempted to predict the efficacy of specific treatments based on baseline serum/plasma metabolites, cytokines, chemokines, soluble receptors or clinical characteristics [108, 109, 137-140]. However, the results have not been conclusive. Thus far, no quantitative biomarker has been identified and adopted into clinical practice [96]. Prediction is currently limited to prognostic indicators. Higher levels of disease activity, presence of autoantibodies and joint erosions early in the disease course advocate the use of MTX and bDMARD combination therapy as first line treatment [96]. The multi-biomarker disease activity (MBDA) score, an index of 12 different serum proteins, has also been shown to be an independent predictor of radiographic progression in eRA [141].

RA may have to be divided into different sub-classes or phases of disease in order to determine which treatment is optimal. Seropositive vs seronegative RA is one such distinction. The differences in pathogenesis between seropositive and seronegative RA is not well

understood. Seronegative RA patients lack the autoreactive antibodies ACPA and RF and comprise approximately one third of patients [93], although the fraction of patients that truly lack autoantibodies might be smaller. Up to 10 percent of patients considered ACPA negative by the widely used ACCP2-assays were shown to express ACPAs when a wider range of citrullinated peptides were included [142]. Additionally, other auto-antibodies such as anti-carbamylated peptides are also found in RA [143]. There is no clear distinction in synovial pathotypes between seropositive and seronegative individuals. Both disease subsets include patients with a lymphocyte rich pathotype and a leukocyte poor pauci-immune pathotype, although the lymphocyte rich pathotype might be more frequent in seropositive patients [76]. The genetic association to some $CD4^+$ T cells linked genes, for example HLA-DRB1 and PTPN22, appears weaker in seronegative RA compared to seropositive RA [67, 144, 145]. Seronegative patients also have lower proportions of pathogenic TPh cells in circulation [42, 86]. Thus, seronegative disease might be less T cell dependent compared to seropositive RA.

Treatment with CTLA-4Ig, anti-IL6R or anti-TNF

Studies have shown that CTLA-4Ig treatment is more effective in seropositive RA patients [146, 147], and that CTLA-4Ig treatment reduces ACPA and RF levels [148]. CTLA-4Ig acts as a suppressor of the adaptive immune response and inhibits T cell activation [149]. In **Paper II**, we found for the first time that baseline proportions of $PD\text{-}1^+$TFh cells and $CTLA\text{-}4^+$ fraction of NonTregs could predict remission in a group of eRA patients who were treated with CTLA-4Ig (94 percent of the patients were seropositive). PD-1 and CTLA-4 are expressed by T helper cells after antigen exposure and activation [40, 49]. Thus, CTLA-4Ig treatment appears more suitable for patients with overt signs of an active adaptive immune response.

bDMARDs have distinct molecular targets and different markers may be needed for each drug. Treatment with anti-IL6R has not displayed particularly superior efficacy in seropositive versus seronegative patients [150, 151]. This may in part be due to the unspecific and systemic role of IL-6 in inflammation, which might also make it more difficult to identify biomarkers for anti-IL6R treatment. Indeed, we found that neither circulating T cell subsets nor blood chemokine levels could predict remission in eRA patients treated with anti-IL6R. Transcriptional analysis have shown that the primary source of IL-6 in the inflamed RA synovium are $CD90^+$ sub-lining FLS [74]. Thus, the

$CD90^+$ sub-lining FLS population may contain prognostic value for anti-IL6R treatment in RA patients.

Earlier studies have indicated that anti-TNF is more effective in seronegative patients compared to seropositive patients [152, 153]. However, larger meta-analyses and registry studies showed no difference in the efficacy of anti-TNF between seropositive and seronegative patients [154, 155]. Greater synovial infiltration of B cell supporting TPh cells have been associated with poor response to anti-TNF treatment [156]. TPh cells have also been shown to be enriched in the synovium of seropositive RA patients [42]. Thus, anti-TNF therapy might not be suitable for patients with higher levels of TPh synovial infiltration. TNF secretion in the inflamed synovium contribute to immune cell recruitment as it triggers expression of selectins and integrin ligands by endothelial cells [51]. Reduced immune cell infiltration is likely an important mechanism of action for anti-TNF treatment. In line with this, low levels of immune cell infiltration in the synovial tissue (known as a pauci-immune synovial phenotype) have been shown to predict poor response to anti-TNF therapy [157]. Instead, patients who respond to anti-TNF display a marked reduction in $CD68^+$ synovial macrophages [157]. Thus, the monocyte/macrophage cell lineage might hold prognostic value for anti-TNF treatment in RA patients and could be of interest for future studies.

Baseline plasma chemokine levels have been examined as potential biomarkers of treatment efficacy [108, 109]. In eRA patients, we examined if baseline chemokine levels could predict remission after 24 weeks of treatment with MTX + corticosteroids, CTLA-4Ig, anti-IL6R or anti-TNF. In **Paper III**, we found that none of the analysed chemokines could predict remission in any of the treatment arms. Similarly, there was no difference in baseline chemokine levels between patients with different outcome (remission, low or moderate/high CDAI at week 24). Therefore, we conclude that plasma chemokines are most likely not suitable as biomarkers for treatment stratification in RA.

Sex differences in RA

In studies of RA male and female patients are most often examined together as a single patient cohort. Therefore, our understanding of immunological mechanisms that might contribute to potential sex differences in disease activity in RA are limited. The rate of remission after DMARD and/or bDMARD treatment have been shown to be lower in females compared to males [158-160]. However, sex differences in

treatment efficacy as measured by disease activity may in part be due to bias in the DAS28 disease activity measure caused by higher ESR values in females [161]. Additionally, females have been shown to report higher pain scores [162], and worse self-reported health [163], which might also contribute to higher reported disease activity in females.

In **paper I,** we investigated the relation between circulating $CD4^+$ T cell subset proportions and disease activity in eRA. In this cohort there was no significant difference in disease activity between male and female patients. However, the relation between T cell subset proportions and disease activity differed markedly between males and females. Most T cell subsets were related to disease activity in males only. One could speculate that the pronounced relation in males might be due to that the distribution of $CD4^+$ T cell subsets is more homogeneous in male patients compared to females. Both genetic and hormonal effects may promote greater heterogeneity in the T cell profile in female individuals. The XX phenotype and incomplete silencing of X-linked T cell related genes such as *CXCR3*, *CD40LG* and *FOXP3* cause variation in gene dosage in females [10-12]. Furthermore, blood concentration of sex hormones vary significantly in females in accordance with the menstruation cycle and the effect of sex hormones on T cell activity may differ depending on hormone concentrations. Estradiol can act to both promote and inhibit T cell activation and proliferation [21, 22], while progesterone seem to dampen the activity of $CD4^+$ T cells [25]. In contrast, androgens appear more consistently as immunosuppressive [17-20]. These factor could make it more difficult to identify statistical correlations between immune cell subsets and disease activity measures in female patients compared to males.

Greater homogeneity in male RA patients have also been observed in genetic association studies. Several genes that have been associated with the development of RA have shown a stronger association in male patients. These include but are not limited to *PTPN22* [164, 165], *PADI4* [166], and *FCGR3A* [167]. Thus, male RA patients might have a more homogenous risk gene profile compared to female patients.

In conclusion, our findings indicate a more homogeneous T cell profile in male patients with eRA compared to females. Therefore, future use of immune cell subsets as biomarkers of disease activity or treatment efficacy might have to be sex-specific.

CONCLUSIONS AND FUTURE PERSPECTIVES

The results presented in this thesis provide new insights on the role of specific $CD4^+$ T cell subsets and chemokines in early and established RA. Our findings indicate that recruitment of classical and non-classical T helper subsets to inflamed joints, in particular Th1 and $CXCR3^+$Th2 cells, is mediated by high levels of CXCL9 and CXCL10 in RA SF. In eRA, treatment with CTLA-4Ig or anti-TNF resulted in reduced plasma levels of CXCL9 and CXCL10 and increased proportions of Th1 cells in circulation. The increased Th1 proportions was likely caused by reduced migration of Th1 cells to the joint. Thus, blockade of the CXCR3 receptor might be an effective method to inhibit $CD4^+$ T cell infiltration of inflamed joints and could represent a potential therapeutic target in RA.

The TPh subset was first identified in the inflamed joint of patients with RA and is considered pathogenic in RA as it promotes B cell activation, and possibly ELS formation. We found that TPh in the SF of RA patients consisted of cells with different T helper phenotypes (mainly Th1 and $CXCR3^+$Th2). It would be of interest to examine if the TPh cells with a Th1, $CXCR3^+$Th2 or $CXCR3^+$Th17 phenotype from the SF differ in B cell helping capacity, as have been shown for circulating TFh cells with a Th1, Th2 or Th17 phenotype.

Our findings also indicate that cytokines produced by the classical Th2 and non-classical $CXCR3^+$Th2 subsets are proinflammatory in RA. Specifically, the cytokines IL-4 and IL-13 induced FLS to secrete proinflammatory cytokines and chemokines that were also abundant in RA SF. Still, the specific function of IL-4 and IL-13 in RA is not fully known. IL-13 has been shown to be essential for the formation of ELS in Sjögren's syndrome and it would be of interest to examine whether IL-4 and/or IL-13 mediate the formation of ELS in the synovium of RA patients.

A variety of drugs are available for the treatment of RA, although none of the treatments are fully effective in all patients. Current treatment strategies for RA rely on a trial and error approach. Biomarkers that could predict treatment effect of specific drugs in individual patients could reduce treatment costs and more rapidly improve the quality of life for the patients. Among 14 investigated chemokines, we found that blood levels of neither chemokine was suitable as a biomarker of

treatment outcome in eRA. This may in part be due to the large inter-individual variation in chemokine levels and/or redundancy in chemokine function. In contrast, higher baseline proportions of PD-1^{+}TFh cells and the fraction of CTLA-4^{+} of NonTregs in circulation predicted remission after treatment with CTLA-4Ig combined with MTX. If these findings can be replicated in a larger cohort of eRA patients and in established RA these T cell subsets could serve as viable biomarkers for CTLA-4Ig treatment.

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