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THE T CELL PHENOTYPE AND RECEPTOR REPERTOIRE IN DIFFERENT STAGES OF RHEUMATOID ARTHRITIS – an immunological timeline

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The T cell phenotype and receptor repertoire in different stages of rheumatoid arthritis – an immunological timeline

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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To my family,

POPULAR SCIENCE SUMMARY OF THE THESIS

Rheumatoid arthritis is a chronic disease characterized by swollen and tender joints in hands and feet. Whereas this might bring destroyed and functionally impaired joints to mind, such consequences are now rare due to modern therapy. However, we are eager to better understand the causes of rheumatoid arthritis (arthritis meaning inflamed joint), with the ultimate goal to cure or possibly prevent the disease.

The cause of rheumatoid arthritis is not fully understood, but the risk of getting the disease increases if one has certain variations of genes that are related to the immune system, especially if combined with environmental hazards such as smoking. This is thought to start an immune response towards one-self (autoimmunity) in the lung or other mucosal tissues, that will later lead to joint inflammation.

In my work I have focused on the T cell (a white blood cell) and its features (phenotype) in rheumatoid arthritis, to build a timeline of these cells in relation to the disease course. Whereas a lot is known about T cells and rheumatoid arthritis in general, less in understood regarding T cells that are mistakenly reacting to specific self-molecules (autoreactive T cells) in the disease.

I find the T cell particularly interesting as it acts as a conductor of an orchestra, directing the other immune cells on what to do. If we can uncover what causes the T cells to lose control, on what they react to, perhaps we can stop them before they start to do harm.

The normal function of a T cell requires it to interact with other cells, using its unique receptor. In most of our studies we have worked with molecules that can imitate this interaction to detect the extremely rare autoreactive T cells. In addition, we have also studied what type of receptors the T cell can have in rheumatoid arthritis patients (the T cell receptor repertoire).

We can now show that autoreactive T cells can be detected in the blood, even before the disease start, in individuals that were known to later develop rheumatoid arthritis. During later stages of disease, the autoreactive T cells are there in slightly higher numbers (still, just a few per million T cells). In addition, we have discovered that some of them react to parts of a large protein (tenascin-C) and that this was the most frequent reaction over time. In patients that were just diagnosed with rheumatoid arthritis there were already many different T cells in the joint, including cells that was reacting to common viruses. This can indicate that virus reactive T cells were attracted to the on-going inflammation in the joint, while it remains an open question if they are involved in the disease development.

I have now added a small piece to the puzzle of rheumatoid arthritis, where my findings can direct the work towards tailored therapies to restore the balance of the immune system or possibly prevent rheumatoid arthritis in the future.

ABSTRACT

Rheumatoid arthritis (RA) is a systemic autoimmune disease, characterized by symmetrical inflammation in hands and feet. Antibodies recognizing citrullinated protein (ACPA) and/or rheumatoid factor (RF) are found in most patients and associates with a worse prognosis. Genetic predisposition (i.e., specific *HLA-DRB1* alleles) combined with environmental threats (e.g., smoking) increase the risk to develop seropositive RA and is thought to evoke a break in tolerance toward citrullinated autoantigens. Whereas it is well known that autoantibodies are present in the circulation long before disease onset, this is an unexplored area for autoreactive T cells recognizing citrullinated antigens. Additionally, little is known about such autoreactive T cells as the disease progress. The aim of this thesis was to explore the occurrence, phenotype, and T cell receptor repertoire of autoreactive CD4+ T cells during different stages of disease.

We have studied several cohorts including individuals at-risk of developing disease, patients with recent onset RA and patients with longstanding disease. Samples from peripheral blood, lymph nodes, synovial fluid, and synovial tissue have been assessed at the single cell level primarily using HLA-class II tetramers (≤12 citrullinated peptides), surface phenotyping, paired αβ-T cell receptor (TCR) sequencing or FluoroSpot. In addition, we have re-expressed TCRs into artificial cell lines to test antigen specificity. With the exception of TCR-repertoire analysis of synovial tissue, all individuals were *HLA-DRB1*04:01* carriers.

Autoreactive CD4+ T cells recognizing citrullinated peptides were rare, but detectable already prior to disease onset in individuals at-risk of developing RA. Notably, the frequencies were significantly lower in those who later progress to arthritis. We found citrullinated peptides from tenascin-C (a large extracellular matrix protein) to be a novel T cell autoantigen and notably, when assessed, this was the most frequent specificity during the different stages of disease, both in blood and synovial fluid. Citrullinated α-enolase, cartilage intermediate layer protein (CILP), fibrinogen-β and to a less degree vimentin peptides was also recognized by autoreactive CD4+ T cells during the course of RA. Intriguingly, lymph node CD4+ T cells primarily recognized citrullinated vimentin both in the at-risk phase and early stage of RA. The T cell receptor repertoire in the synovial tissue was diverse already at time of diagnosis, whereas both CD4+ and CD8+ cells contributed to clonal expansions. Viral reactive T cells constituted a small fraction of the clonally expanded CD4+ T cells, whereas the majority were orphans in terms of antigen specificity. However, a bias in gene usage of CD4+ T cell (TRBV20-1) was seen amongst ACPA+ patients and interestingly such bias was also noted in CD4+ T cells specific to citrullinated tenascin-C peptides derived from both synovial fluid and blood in longstanding RA.

To conclude, autoreactive CD4+ T cells constitute a small, but detectable part of the T cell compartment during the different stages of rheumatoid arthritis. The T cell receptor repertoire is diverse early in disease – whereas genetic bias might imply recognition of a common antigen. The knowledge of such cells and their specificity, of which tenascin-C was most frequent in our studies can direct future efforts in tolerizing therapies to cure or ultimately prevent disease.

LIST OF SCIENTIFIC PAPERS

I. **The T cell phenotype and occurrence of antigen specific T cells in autoantibody positive individuals at high risk of developing rheumatoid arthritis**
Sara Turcinov, Charlotte de Vries, Ravi K. Sharma, Christina Gerstner, Bruno Raposo, Anatoly Dubnovitsky, William W. Kwok, Karine Chemin, Vivianne Malmström* and Aase Hensvold*
Manuscript

II. **Multi-HLA class II tetramer analyses of citrulline-reactive T cells and early treatment response in rheumatoid arthritis**
Christina Gerstner, **Sara Turcinov**, Aase H. Hensvold, Karine Chemin, Hannes Uchtenhagen, Tamara H. Ramwadhdoebe, Anatoly Dubnovitsky, Genadiy Kozhukh, Lars Rönnblom, William W. Kwok, Adnane Achour, Anca I. Catrina, Lisa G. M. van Baarsen and Vivianne Malmström
BMC Immunology, 2020, 21:27

III. **Diversity and Clonality of T Cell Receptor Repertoire and Antigen Specificities in Small Joints of Early Rheumatoid Arthritis**
Sara Turcinov, Erik af Klint, Bertrand Van Schoubroeck, Arlette Kouwenhoven, Sohel Mia, Karine Chemin, Hans Wils, Carl Van Hove, An De Bondt, Ken Keustermans, Jeroen Van Houdt, Joke Reumers, Nathan Felix, Navin L. Rao, Pieter Peeters, Frederik Stevenaert, Lars Klareskog, Murray McKinnon, Daniel Baker, Anish Suri and Vivianne Malmström
Arthritis & Rheumatology, 2022, e-publication ahead of print

IV. **Shared recognition of citrullinated tenascin-C peptides by T and B cells in rheumatoid arthritis**
Jing Song, Anja Schwenzer, Alicia Wong*, **Sara Turcinov***, Cliff Rims, Lorena Rodriguez Martinez, David Arribas-Layton, Christina Gerstner, Virginia S. Muir, Kim S. Midwood*, Vivianne Malmström*, Eddie A. James, and Jane H. Buckner
JCI Insight. 2021, 6(5):e145217

V. **Biased TCR gene usage in citrullinated Tenascin C specific T-cells in rheumatoid arthritis**
Ravi K. Sharma, Sanjay V. Boddul, Niyaz Yoosuf, **Sara Turcinov**, Anatoly Dubnovitsky, Genadiy Kozhukh, Fredrik Wermeling, William W. Kwok, Lars Klareskog and Vivianne Malmström
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*These authors contributed equally

SCIENTIFIC PAPERS NOT INCLUDED IN THE THESIS

i. **Arthritis in systemic lupus erythematosus is characterized by local IL-17A and IL-6 expression in synovial fluid**
Natalie Sippl, Francesca Faustini, Johan Rönnelid, **Sara Turcinov**, Karine Chemin, Iva Gunnarsson, Vivianne Malmström
Clinical and Experimental Immunology, 2021, 205:44–52

ii. **Peripheral and Site-Specific $CD4^{+}CD28^{null}$ T Cells from Rheumatoid Arthritis Patients Show Distinct Characteristics**
Jennifer Pieper**, Sara Johansson***, Omri Snir, Ludvig Linton, Mary Rieck, Jane H Buckner, Ola Winqvist, Ronald van Vollenhoven, and Vivianne Malmström
Scandinavian Journal of Immunology. 2014, 79(2):149–155

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LIST OF ABBREVIATIONS

ACPA	Anti-citrullinated protein antibody
ACR	American College of Rheumatology
AID	Activation induced cytidine deaminase
AMPA	Anti-modified protein antibody
APC	Antigen presenting cell
CCP	Cyclic citrullinated peptide
CD	Cluster of differentiation
CILP	Cartilage intermediate layer protein
CMV	Cytomegalovirus
CRP	C-reactive protein
DAS28	Disease activity score 28 joints
DMARD	Disease modifying anti-rheumatic drug
csDMARD	Conventional synthetic DMARD
bDMARD	Biological DMARD
tsDMARD	Targeted synthetic DMARD
EIRA	Epidemiological Investigation of Rheumatoid Arthritis
ELS	Ectopic lymphoid structures
ESR	Erythrocyte sedimentation rate
EULAR	European League Against Rheumatism
FLS	Fibroblast-like synoviocytes
GWAS	Genome wide association studies
HHV-2	Human herpes virus 2
HLA	Human leukocyte antigen
IFN	Interferon
IgG	Immunoglobulin G
IL	Interleukin
LURA	Lung Investigation in Newly Diagnosed RA
mRNA	Messenger RNA
NK	Natural killer
PAD	Peptidylarginine deiminase
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PD-1	Programmed cell death protein 1
PMT	Post-translational protein modifications
RA	Rheumatoid arthritis

RF	Rheumatoid factor
RNA	Ribonucleic acid
SE	Shared epitope (in HLA-SE)
SFMC	Synovial fluid mononuclear cells
TCR	T cell receptor
Tfh	T follicular helper
Th1	T helper type 1
Th2	T helper type 2
Th17	T helper type 17
TNC	Tenascin-C
TNF	Tumor necrosis factor
tolDC	Tolerogenic dendritic cell
Tph	T peripheral helper
TRAJ	T cell receptor alpha-chain joining
TRAV	T cell receptor alpha-chain variable
TRBD	T cell receptor beta-chain diversity
TRBJ	T cell receptor beta-chain joining
TRBV	T cell receptor beta-chain variable
Treg	T regulatory

1 INTRODUCTION

The immune system is an intricate arrangement of cells, signaling molecules and structures that permits protection from foreign and internal dangers. Central to this is the ability of the immune system to discern when to react, to avoid misdirected action toward oneself. Unfortunately, this is not a fail-safe system and hence there are autoimmune diseases as rheumatoid arthritis.

The general interest in understanding the underlaying mechanisms behind such an autoimmune disease is profound. Within my own field of research there are more than 10,000 articles on the subject in the major medical database (i.e., searching for "rheumatoid arthritis AND T cells", PubMed-database, February 2023). Nevertheless, there are still many pending questions, only partially answered. What causes rheumatoid arthritis? How should we best select treatment for the patients? Can we find a cure or possibly prevent the disease?

While the answer to these big questions require work at the most basic and molecular level, this is not an academical matter, but of greatest importance for the patients that I meet in my everyday (parallel) work as a resident in rheumatology.

I have already mentioned the intricacy of the immune system and the same goes for the pathophysiology of rheumatoid arthritis, where my main interest is T cells – the conductors of the immune system. During my doctoral studies I have thus sought to understand if there are T cells recognizing specific antigens (possibly triggering or worsening the disease) and if this can be understood also by exploring the T cell receptor repertoire, without prior knowledge of such antigens.

To put any findings in perspective I have been particularly interested in understanding the relation to different stages of disease and I have even had the opportunity to investigate a window of time prior to disease onset.

When writing the framework of this thesis, my aim has thus been to describe our current understanding and hypothesis relating to the question "What causes rheumatoid arthritis?", with an emphasis on T cell contribution, and how my work adds on to this.

2 LITERATURE REVIEW

2.1 RHEUMATOID ARTHRITIS

Rheumatoid arthritis is a chronic systemic inflammatory disease characterized by symmetrical arthritis in hands and feet. Large joints as knees, ankles, elbows, and shoulders can also be affected, whereas the distal phalangeal joints are typically spared [1]. Joint erosions, with subsequent deformities and disability, are feared consequences of disease. In addition, extra-articular manifestations and co-morbidities (e.g., rheumatic nodules, vasculitis, interstitial lung disease and cardiovascular disease) can contribute substantially to morbidity and mortality [2, 3]. Although erosions and lung involvement can be present already early in the disease course, today's therapies have drastically changed the expected outcome from a debilitating to chronic, but manageable disease [4-7]. The prevalence of rheumatoid arthritis differs between countries, where the global prevalence has been estimated to 0.46%, with slightly higher numbers in Sweden (e.g., 0.66%) with a female to male (2:1) dominance [8, 9].

Rheumatoid arthritis is a clinical diagnosis, but a set of criteria jointly agreed upon by the European League Against Rheumatism (EULAR) and American College of Rheumatology (ACR) are used for research classification and as aid in the everyday clinical work (Table 1) [1]. Prior to the release of the 2010 ACR/EULAR criteria, the ACR 1987 revised criteria were used to classify rheumatoid arthritis (Table 2) [10]. Whereas the new criteria are aimed at identifying early stage disease [1], the old criteria included features that are associated with more longstanding disease, e.g., widespread distribution of arthritis, rheumatoid nodules and joint erosions [10].

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

A. Joint involvement ▪ 1 large joint ▪ 2−10 large joints ▪ 1−3 small joints (with or without involvement of large joints) ▪ 4−10 small joints (with or without involvement of large joints) ▪ >10 joints (at least 1 small joint)	*Score* 0 1 2 3 5
B. Serology ▪ Negative RF and negative ACPA ▪ Low-positive RF or low-positive ACPA ▪ High-positive RF or high-positive ACPA	 0 2 3
C. Acute-phase reactants ▪ Normal CRP and normal ESR ▪ Abnormal CRP or abnormal ESR	 0 1
D. Duration of symptoms ▪ <6 weeks ▪ ≥6 weeks	 0 1

Sum of A-D. Definite RA is classified as score ≥6/10. Only patients with at least one joint with clinical sign of synovitis, not better explained by any other disease, should be tested. Patients with typical joint erosions combined with a history of prior criteria fulfilment and patients with longstanding disease where retrospective data imply criteria fulfilment should also be classified as RA. Adapted from **[1]**. *RF = rheumatoid factor, ACPA = anti-citrulline protein antibody, CRP = C-reactive protein, ESR = erythrocyte sedimentation rate.*

Table 2. ACR 1987 revised criteria for the classification of rheumatoid arthritis	
1. Morning stiffness	Morning stiffness in and around the joints, lasting at least 1 hour before maximal improvement
2. Arthritis of 3 or more joint areas	At least 3 joint areas simultaneously have had soft tissue swelling or fluid (not bony overgrowth alone) observed by a physician. The 14 possible areas are right or left PIP, MCP, wrist, elbow, knee, ankle, and MTP joints
3. Arthritis of hand joints	At least 1 area swollen (as defined above) in a wrist, MCP, or PIP joint
4. Symmetric arthritis	Simultaneous involvement of the same joint areas (as defined in 2) on both sides fo the body (bilateral involvement of PIPs, MCPs, or MTPs is acceptable without absolute symmetry)
5. Rheumatoid nodules	Subcutaneous nodules, over bony prominences, or extensor surfaces, or in juxtaarticular regions, observed by a physician
6. Serum rheumatoid factor	Demonstration of abnormal amounts of serum rheumatoid factor by any method for which the result has been positive in <5% of normal control subjects
7. Radiographic changes	Radiographic changes typical of rheumatoid arthritis on posteroanterior hand and wrist radiographs, which must include erosions or unequivocal bony decalcification localized in or most marked adjacent to the involved joints (osteoarthritis changes alone do not qualify)
"For classification purposes, a patient shall be said to have rheuatord arthritis if he/she has satisfied at least 4 of these 7 cerieria. Criteria 1 trough 4 must have been present for at least 6 weeks. Patients with 2 clinical diagnosis are not excluded." ***[10]***	

2.1.1 Autoantibodies mark a disease entity of rheumatoid arthritis

In addition to joint inflammation, one of the parameters assessed upon diagnosis is the presence of antibodies directed towards self-antigens [1]. These autoantibodies, recognizing either citrullinated proteins (anti-citrullinated protein antibody, ACPA) or immunoglobulins (i.e., the Fc-portion of IgG) namely rheumatoid factor (RF) are found in up to 70 % of the patients [11, 12]. The presence of ACPA has a higher positive predictive value for rheumatoid arthritis than RF, but more so the combination of the two [12, 13]. In addition, patients with autoantibodies diverge from those that are seronegative regarding genetic and environmental risk factors, as well as prognosis – where seropositivity is associated with a more severe disease and joint erosion [4, 14-17]. Although not a part of the classification criteria, additional autoantibodies including anti-carbamylated protein and anti-acetylated protein antibodies are also associated with rheumatoid arthritis, referred to as AMPA (anti-modified protein antibody) [18-20].

Intriguingly, the occurrence of both ACPA and RF can precede disease onset by years, a phase referred to as systemic autoimmunity associated with rheumatoid arthritis [12, 13, 21, 22]. However, the frequency of patients having autoantibodies as well as ACPA-titers increase in closer proximity to symptom onset [12, 13, 21].

Similar to the serological findings, symptoms as arthralgia and morning stiffness can occur prior to clinical evident arthritis and patients with autoantibodies and such symptoms are considered to be in the at-risk phase of developing disease [22, 23].

Although ACPAs are considered specific for RA, they can be found in a small portion of (yet) healthy individuals and also be a transient finding [11-13, 24]. Furthermore, amongst individuals in the at-risk phase not all will develop rheumatoid arthritis [25-27]. Hence, indicating that the presence of ACPA *per se* is not sufficient to develop disease.

2.1.2 Treatment options in rheumatoid arthritis – a brief overview

Rheumatoid arthritis is an autoimmune disease, hence the treatment principle is to target the immune response using different disease modifying anti-rheumatic drugs (DMARDs), with the aim to reach clinical remission. The most recent treatment recommendation by EULAR were up-dated 2022 and the treatment algorithm starts with methotrexate followed by an active approach to evaluate and change/add on treatment if the goal (remission) is not achieved, i.e., treat-to-target [28]. Additionally, there are national and local guidelines in the management of rheumatoid arthritis.

Methotrexate, belonging to the group of conventional synthetic DMARDs (csDMARDs), is a well-known drug, that has been used in treatment of rheumatoid arthritis since the 1980s. Whereas first developed in the 1940s as a chemotherapeutic agent interfering with purine synthesis, the pharmacological mode of action in rheumatoid arthritis is not fully understood. Methotrexate is probably acting on a wide array of inflammatory cells (e.g., lymphocytes, macrophages and neutrophiles) and interaction with adenosine signaling has been proposed as one important mechanism [29, 30].

Infliximab, inhibiting the major pro-inflammatory cytokine tumor necrosis factor (TNF), was the first biological DMARD (bDMARD) to be used in rheumatoid arthritis, thus marking the start of a new era in disease management [31]. There are now additional bDMARDs inhibiting IL-6 (e.g., tozilizumab), depleting B-cells (e.g., rituximab), inhibiting co-stimulation of T cells (e.g., abatacept) and additionally targeted synthetic DMARDs (tsDMARDs) inhibiting janus kinases (JAK; e.g. tofacitinib). However, the treatment algorithm cannot predict who will respond to which treatment, and there are additionally patients considered having a difficult-to-treat disease [28, 32]. Notably, today's therapies are influencing major aspects of the immune system and there is (yet) no cure for the disease.

2.1.3 HLA is the major genetic risk loci in ACPA+ RA

The human leukocyte antigen (HLA) genes are coding for molecules that are essential to the adaptive immune system, i.e., HLA class I and HLA class II. HLA class I molecules are present on nucleated cells, thereby mainly presenting intracellular peptides to scouting CD8+ (cytotoxic T cells) allowing recognition and elimination of cells that express foreign or transformed antigens. HLA class II molecules are instead found on antigen presenting cells (e.g., dendritic cells, macrophages, and B-cells) and forms the basis for recognition of CD4+ T cells.

It was early noticed that an overrepresentation of certain HLA class II alleles were seen amongst rheumatoid arthritis patients, later confirmed in large genome wide association studies

(GWAS) showing that *HLA* is the major genetic risk factor for ACPA+ rheumatoid arthritis [33-35]. Other well defined risk loci include *PTPN22* and *TRAF-1-C5*, whereas there are over 150 single nucleotide polymorphisms associated to the disease [36-38]. Furthermore, there are evident gene-gene interactions between *HLA-DRB1* and other non-HLA risk variants which by them self would only have a modest effect [39].

The genetic risk conferred by HLA was initially attributed to a common amino acid motif (position 70-74 of the *HLA-DRB1* allele) shared amongst different HLA-class II alleles – forming the shared epitope (SE) hypothesis [40]. *HLA-DRB1*04:01*, *HLA-DRB1*04:04, HLA-DRB1*01* and *HLA-DRB1*10:01* all represent such shared epitope alleles [41]. A more recent data imputation of six genome wide association studies has though refined the genetic association by pin-pointing positions 71, 74 and 11 in the *HLA-DRB1* locus as well as single polymorphism in *HLA-B* and *HLA-DPβ1* (all affecting the peptide binding groove of the HLA-molecules), to account for almost all HLA-associated risk in RA. Still, all the above-mentioned alleles remain as high-risk alleles for RA-development with *HLA-DRB1*04:01* having the highest odds ratio (OR 4.44, CI 4.02-4.91) [42].

The frequency of different HLA-alleles varies geographically and in Sweden close to 25 % of the population (control subjects) carry the *HLA-DRB1*04:01* allele, in comparison to less than 5 % in Spain [43]. In the Swedish EIRA (Epidemiological Investigation of Rheumatoid Arthritis) cohort 41% of ACPA+ patients have either an *HLA-DRB1*04:01* or *HLA-DRB1*04:04* allele, whereas the frequency is 24% of ACPA- patients (personal communication, Dr Padyukov, Karolinska Institutet). If looking at any HLA-SE-allele the frequencies are 59 % amongst ACPA+ patients compared to 30 % of the controls [39].

Consequently, even with HLA being the major genetic risk factor, this only confer a risk – not the sole reason for developing rheumatoid arthritis.

2.1.4 Additive effect of smoking upon genetic predisposition

Smoking has long been known as a risk factor for rheumatoid arthritis [44-46], however linkage of genetic risk factors to environmental exposure provides additional clues on disease etiology. This interaction was first shown in RF+ and later in ACPA+ RA, where carrying *HLA*-shared epitope alleles (even more prominent if double), in combination with a smoking history drastically increase the risk to develop ACPA+ rheumatoid arthritis, whereas smoking alone only confers a slight effect [47-49]. Additional combinations of smoking, *HLA-SE*, *PTPN22*, specific autoantibodies and being ACPA+ increase this risk even further [50, 51]. Exposure for other inhaled air way irritants as silica, textile-dust and asbestos has also been associated with an increased risk of rheumatoid arthritis [52-54].

2.2 CITRULLINATED PROTEINS – THE TARGET OF ACPA

Protein citrullination (also denoted deimination) is a physiological post-translational protein modification where the positively charged amino acid arginine (i.e., peptidylarginine) is converted to neutrally charged citrulline (i.e., peptidylcitrulline) [55]. This reaction is calcium

dependent and catalyzed by peptidylarginine deiminase (PAD) (Fig. 1) [56]. The finding of autoantibodies directed toward citrullinated proteins in rheumatoid arthritis patients have put much focus on citrullination as a source of autoantigens that can trigger this response.

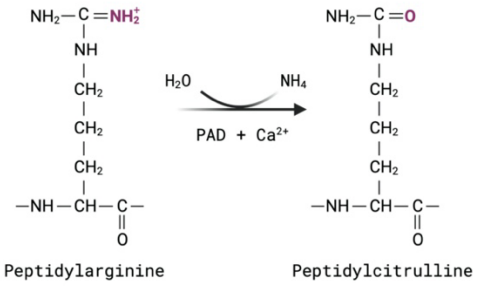

Figure 1. Peptidylarginine deiminase (PAD) catalyzes the conversion of peptide bound arginine residues to citrulline in a Ca2+ dependent reaction.

2.2.1 Extracellular citrullination in rheumatoid arthritis

There are five known isoforms of the PAD-enzyme, with different tissue and cellular niches influencing features ranging from keratinocyte differentiation and cytoskeleton structure to gene and immune regulation [57]. PAD2, a cytosolic enzyme, has been shown to be upregulated during monocyte differentiation into macrophages [58]. Notably, PAD2 is significantly higher in bronchoalveolar lavage fluid (BAL), BAL-cells (constituted predominantly by macrophages) and lung tissue in smokers compared to non-smokers [59, 60]. Moreover, citrullinated proteins are found in lung tissue specimens from both smokers and non-smokers [60]. The lung has thus been postulated as a potential site for breach of tolerance to citrullinated antigens [17].

PAD4 is predominately found in the nuclei of granulocytes and monocytes [56]. In addition to intracellularly active PAD-enzymes, neutrophils have been shown to express PAD4 on the cell surface and to secrete PAD2 [61]. NETosis, where neutrophils expels their interior and form extracellular traps for pathogens, also serves as a source of citrullinated peptides recognized by ACPA [62, 63]. In the advent of inflammation and cell death, PAD-enzymes and calcium may additionally leak from the intracellular compartment potentiating extracellular citrullination [56]. Both PAD2 and PAD4 are found in the synovial fluid and tissue of arthritis patients and corresponding to this, citrullinated antigens are found in the same compartments e.g. α-enolase, vimentin and tenascin-C in synovial fluid and citrullinated fibrinogen in synovial tissue [64-68]. Notably, citrullination is not a feature unique to rheumatoid arthritis, but rather represent a general finding of inflammation [67, 68].

2.2.2 P. gingivalis – a bacteria with citrullinating capacity

In addition to endogenous citrullination, the oral bacteria *Porphyromonas Gingivalis (P. gingivalis)* have the unique feature of producing a PAD-enzyme with subsequent citrullination [69, 70]. This bacterium is one of the pathogens implicated in periodontitis, chronic inflammation of the gingival tissue, the latter associated with rheumatoid arthritis [71-73]. *P. gingivalis* share sequence homology between its bacterial enolase and human α-enolase, and antibodies from RA-patients have been shown to cross-react with both of these citrullinated

antigens [74]. The occurrence of antibodies towards *P. gingivalis* (i.e., anti-RgpB IgG), as a sign of an invasive, immunogenic infection in combination with having a *HLA-SE* -allele has also been shown to be highly associated with ACPA+ RA, whereas smoking was an additive factor in this setting. *P. Gingivalis* infection could thus explain the association between periodontitis and ACPA+ rheumatoid arthritis [75].

2.2.3 The target of anti-citrullinated antibodies

A lot of effort has been made in defining the fine specificities and immune dominant epitopes for ACPA and a selection of the described B cell reactivities include citrullinated α-enolase, vimentin, fibrinogen, collagen type II and tenascin-C [76-80]. However, recent studies shows that monoclonal antibodies originating from RA-patients recognize specific citrulline containing motifs (e.g. citrulline adjacent to glycine) in a multi-reactive fashion, rather than specific proteins. Notably, this is not a function of an unspecific polyreactivity, rather the antibodies have preferential binding patterns towards the citrullinated epitopes [20, 81]. The finding of ACPAs derived from synovial fluid being highly mutated does however indicate a prior T cell interaction [81].

2.3 FROM GENETIC PREDISPOSITION TO ESTABLISHED DISEASE – A HYPOTHESIS

Our current hypothesis of ACPA+ rheumatoid arthritis development thus implies that genetically susceptible individuals (e.g. *HLA-DRB1* carriers) who are challenged by environmental factors leading to citrullination at mucosal sites (e.g., the lung and/or gingiva) loses tolerance to self and develop autoantibodies towards these citrullinated antigens – leading to a phase of systemic autoimmunity (e.g. the occurrence of autoantibodies prior to symptoms). A second hit might direct the immune response in these at-risk individuals to the joint, where arthralgia can proceed the development of arthritis and subsequent the chronic inflammatory condition that feature rheumatoid arthritis (Fig. 2) [17].

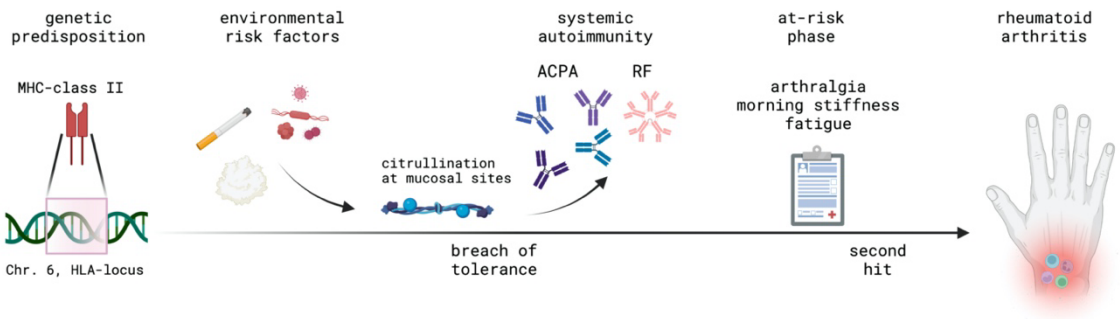

Figure 2. Proposed transition from having a genetic predisposition to the development of rheumatoid arthritis.

2.4 LOOKING INTO THE ARTHRITIC JOINT

In the healthy synovial joint, the articulating bones are separated by cartilage and synovial fluid while kept together as a functional unit by a fibrous capsule, tendons, ligaments, and muscles [82]. The synovium, composed of thin fibrofatty tissue, an intimal layer of fibroblast-like synoviocytes (FLS) and to a lesser extent macrophages [83], covers the inside of the capsule and the small portion of bones not articulating and thus are not covered by cartilage [82]. Other inflammatory cells and mediators are very scarce if any [83].

The hallmark of rheumatoid arthritis is on the other hand marked inflammation with thickening of the synovial tissue, infiltration of lymphocytes, neutrophils, macrophages and other inflammatory cells as well as increased levels of cytokines. Angiogenesis with immature vessels is evident and pannus-formation, i.e., invasive synovial growth, can develop followed by bone and cartilage damage [82]. On the contrary, bone loss can also be seen prior to clinical synovitis [84, 85]. ACPA derived from RA patients have been suggested to target citrullinated antigens on osteoclasts and their precursors, with enhanced osteoclastogenesis and bone resorption *in vitro* and in mouse models *in vivo* [86-89] – posing a pathological function of such autoantibodies.

The pathological findings in the synovium can be divided into three groups, depending on the type and degree of cell infiltration; 1) lymphoid 2) myeloid and 3) fibroid, where the first represents a rich lymphocyte infiltration and the formation of ectopic lymphoid structures and the last a very scarce or absent cell infiltrate [90]. These histological subsets also sub-divides in relation to genetic expression measured by RNA-sequencing of synovial tissue although authors suggest a continuum in-between these groups. When investigating the response to therapy in priorly treatment naïve patients by this subdivision, a shift to a less inflammatory state correlated with decreased disease activity and similarly the most inflammatory profile (e.g. involving toll like receptor-signaling and type 1 IFN signatures) at baseline was associated with treatment response [91].

2.4.1 Ectopic lymphoid structures – where T and B cells meet

The ectopic lymphoid structures (ELS) forms a basis for antigen driven T and B cell interaction at site of inflammation resulting in affinity maturated B cell clones [90]. In secondary lymphoid structures (i.e., lymph nodes) antigen experienced T cells expressing CXCR5 (T follicular helper cells – Tfh) provides the essential help for B cells to survive, mature and transform into plasma cells [92]. It has been assumed that the same would occur also in ectopic lymphoid structures, whereas these cells have been difficult to find. Instead, a new T cell subset, the T peripheral helper cell (Tph), has been described both in synovium and synovial fluid, being virtually absent in peripheral blood, of rheumatoid arthritis patients. The Tph are PD-1hiCXCR5- and similar to the Tfh they are potent secretors of CXCL13 (ligand for CXCR5) and IL-21 [93-95]. The Tph cells also show co-localization with B cells not only in the ectopic tissue, but also more widespread in the synovium [94]. In support of a functional tertiary structure in the synovium, expression of activation induced cytidine deaminase (AID),

necessary for initiation of class switch and somatic hypermutation of the B cell immunoglobulin genes, has been shown in areas with follicular dendritic cells adjacent to ACPA+ plasma cells in synovial tissue from RA patients [96]. Interestingly, rheumatoid fibroblast-like synoviocytes has also been shown to promote B cell AID expression and class switching in B cell-activating factor/a proliferation-inducing ligand (BAFF/APRIL) dependent manner [97].

One suggested mechanism for the local immune response in the joint would thus be deposition of immune complex (e.g., ACPA and RF) subsequent to a minor insult to the joint (e.g. trauma or infection), leukocyte extravasation and complement activation, followed by a perpetuated inflammatory response involving release of citrullinated antigens during NETosis and the establishment of ELS in the synovium [90].

2.5 T CELLS IN HEALTH AND DISEASE

2.5.1 T cell development and the concept of tolerance

The T cell progenitor originates in the bone marrow but migrate and mature in the thymus (hence the name), where it acquires the T cell receptor (TCR) – a unique identifier of the cell defining its antigen specificity. Importantly, cells that are overly activated in this process are deleted to ensure tolerance to self.

2.5.1.1 The T cell receptor locus enables a diverse repertoire

The loci coding for T and B cell receptors are the most complex in the genome, providing the basis for a broad immune response to yet unknown antigens [98]. The T cell receptor, consisting of an α- and β-chain (i.e., for the αβ-T cell), is formed in a stepwise fashion to ensure a diverse T cell repertoire. The α-chain is randomly re-arranged from variable (V) and joining (J) gene segments, and the β-chain from V, diversity (D) and J gene segments – with nucleotide (N) insertions and deletions further increasing junctional diversity. Additionally, there are also genes coding for the constant (C) domain [99]. Each gene segment is composed of several different functional genes (Table 3), resulting in a T cell receptor diversity from 25 x 10^6 possible receptor combinations to theoretically 10^{15} [99-101]. The most variable part of the TCR is represented by complementary determining region 3 (CDR3; covering the V(D)J-junctions of the α- and β-chain respectively), which is centered over the peptide when interacting with the HLA-peptide complex on an antigen presenting cell [102]. Whereas there was long a consensus on the major docking properties of the TCR over the HLA-peptide (i.e., TCR-α over HLAβ), it has been shown that interaction with both HLA-class I and class II can form in reverse position [101, 103, 104].

2.5.1.2 Positive and negative selection

In the process of TCR-rearrangement, the T cell receptor is tested to ensure recognition of a HLA:peptide complex (positive selection), and subsequently hinder autoreactivity (negative selection) where T cells with increased reactivity to self-antigens are either deleted or passed

Table 3. Number of functional T cell receptor gene segments			
	Variable (V)	Diversity (D)	Joining (J)
α-chain	TRAV 42–45 [105, 106]		TRAJ 51 [107, 108]
β-chain	TRBV 40-42 [109, 110]	TRD 2-3 [111, 112]	TRBJ 13 [111, 113]

onto a regulatory path as regulatory T cells [114, 115]. A specific feature of medullary thymic epithelial cells allows them to express and present antigens, that would normally be restricted to other tissues, to the T cells which thus promote tolerance to self-peptides [116]. However, it has been hypothesized that not all post-translational protein modifications (PMTs) are represented in the thymus – why citrullination could be a source of neoepitopes for autoreactive lymphocytes that passed the test in the thymus. In line with this, it has been shown in mouse that post-translational modifications (N.B. not citrullination) might not be presented to T cells in the thymus, thus not being subjected to central tolerance induction [117].

During these developmental steps the β-chain of the TCR is re-arranged first, followed by proliferation of the thymocytes and subsequent expression of both CD4 and CD8. The α-chain is then re-arranged separately in the progeny cells, and not until the αβ-TCR has been positively selected (or the cell has undergone apoptosis) on basis of recognizing an HLA:self-peptide this rearrangement stops. Hence, one cell can produce >1α chain to pair with the β-chain. The recognition of a peptide on either the HLA class I or HLA class II molecule further decide if the cell will commit to the CD8+ or CD4+ lineage [118-120]. Initial theories regarding the T cell and its receptor postulated "one cell, one receptor" and that a second α-chain did not render two distinct TCRs [121, 122]. However, it was later shown that one cell can carry two functional receptors consisting of one β-chain and two different α-chains, posing a possible mechanism for evading tolerance if the cell gets activated by one receptor and then recognize another antigen by the second one [123, 124].

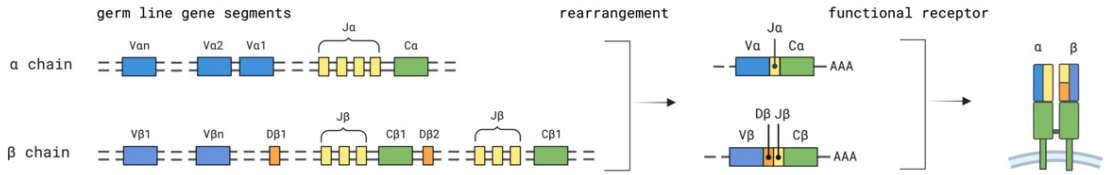

Figure 3. Schematic representation of how rearrangement of the germ line encoded genes results in a functional T cell receptor constituted by an α- and β-chain.

2.5.2 Naïve T cells give rise to different memory subsets

T cells can broadly be divided upon expressing CD4 (T helper cells) or CD8 (cytotoxic cells), but with emerging knowledge on specific surface markers, cytokine secretion- and

transcriptional profiles as well as tissue preferences the picture painted is much more complex, exemplified by the newly described T peripheral helper cells mentioned previously.

The majority of T cells are found in the lymphoid tissues, followed by mucosal sites, but are present in more or less every organ [125]. Naïve T cells, that have not yet encountered their cognate antigen, can be recognized by the combined expression of the surface receptor CD45RA and the lymphoid homing molecule CCR7 [126]. Upon activation by an antigen presenting cell, they proliferate and exert effector functions as cytokine production, whereas only a small proportion of cells persists as memory cells [125].

Memory T cells (CD45RA-) can be divided into central memory T cells (Tcm) expressing CCR7+, thus homing to secondary lymphoid structures, and the CCR7- effector memory T cells (Tem) that efficiently secrets effector function cytokines (e.g. IFN-γ) upon re-encounter with its antigen [126, 127]. In addition to this, there is also a terminally differentiated effector memory subset re-expressing CD45RA (Temra; CD45RA+CCR7-), predominantly found in the CD8+ subset, but to a less degree also amongst CD4+ T cells [125, 126]. Memory T cells that reside in tissues (e.g. mucosal tissues and skin) express CD69 and are denoted tissue resident memory T cells (Trm). Additionally, they can also express CD103 as well as other homing receptors [125, 128].

It has been postulated that an additional memory cell subset with stem cell-like properties also exists (CD45RA+CCR7+CD62L+CD95+), where all markers apart from CD95 phenotypically overlap with naïve cells. In the CD8 compartment, these memory stem cells (Tscm), have been shown to respond with rapid cytokine secretion upon antigen stimulation after polyclonal TCR stimulation, and while giving rise to progeny of memory phenotype a proportion of cells retain their naïve like phenotype [129]. Further, the proportion of CD4+Tscm has been shown to be significantly increased peripheral blood of RA-patients with active disease, compared to healthy controls and following treatment [130].

2.5.3 Major T cell subsets in rheumatoid arthritis

The immune response in rheumatoid arthritis is very complex, involving many cells in both the adaptive and innate immune system where a broad overview of important T cell subsets in rheumatoid arthritis will be given below.

2.5.3.1 Th1 cells as major drivers of disease?

Rheumatoid arthritis has long been considered primarily a T helper type 1 (Th1, controlled by T-bet) driven disease, with CD4+ T cells secreting IFN-γ and TNF – exerting its main functions by activating macrophages (e.g. potentiating the respiratory burst and upregulating HLA class II expression) and endothelial cells (e.g. upregulating expression of adhesion molecules) [131-134]. In addition, activated macrophages are also potent secretors of TNF [135]. Notably, when studying the TNF expression in the synovium of rheumatoid arthritis patients prior to treatment with anti-TNF, this level could predict clinical response with the source of the cytokine including both macrophage subsets and T cells [136].

2.5.3.2 The Th17 subset – an efficient neutrophil recruiter

T helper type 17 (Th17) cells represent a separate subset – characterized by RORγt transcription factor and secretion of IL-17 (IL-17A and IL-17F). The function of IL-17A includes inducing chemokine production (e.g. CXCL1 and IL-8) with subsequent recruitment of neutrophils and myeloid cells, as well as IL-6 and G-CSF further influencing mainly neutrophils [137-140]. Th17 cells and IL-17 are found both in the synovial fluid and synovium of rheumatoid arthritis patients [141-143]. The effect of IL-17 on rheumatoid synoviocytes (e.g. in producing IL-6 and IL-8) has also been shown to be greatly enhanced by simultaneous TNF stimuli [144]. Additionally, IL17 has been shown to increase matrix metalloprotease secretion and induce osteoclast activity *in vitro* – thus posing a link to tissue damage in the rheumatic joint [141, 145-147]. Albeit, whereas Th17 emerged as the most important T cell type in the pathology of rheumatoid arthritis, specific treatment blocking IL-17 have not had the expected effect. Instead, it has been shown to be a potent treatment strategy for psoriasis and psoriatic arthritis [148].

2.5.3.3 Cytotoxic CD4+ T cells in rheumatoid arthritis

Activation of the naïve T cell is dependent on co-stimulation via CD28 and the absence of such signal render the cell anergic [149, 150]. However, a CD4+ memory cell subset lacking this molecule (the CD28null T cell) is found in patients with autoimmune disease as rheumatoid arthritis and multiple sclerosis [151-154]. In contrast to being anergic, the CD28null T cells are potent secretors of IFN-γ and also have cytotoxic features (e.g. produces perforin and granzyme b) and expresses natural killer (NK)-cell surface markers as NKG2D and KIR [151, 152, 155-158]. Recently, it was also shown that the synovial fluid of ACPA+ patients harbor significantly more cytotoxic CD4+ T cells than that of ACPA- patients [95].

2.5.3.4 Regulatory T cells – keepers of peripheral tolerance

Opposed to the proinflammatory functions of the previously described cell subsets in disease pathology of rheumatoid arthritis, the intricate balance with regulatory cells is also an important factor for keeping immune tolerance. In 1995, *Sakaguchi et al.* described a CD25+T cell subset able to maintain peripheral tolerance in mice [159], which correspond to cells with the FOXP3 transcription factor [160, 161]. In humans FOXP3 and CD25 expression is not equal to suppressive functions, but instead confined to subsets of these cells (i.e., resting/naive and activated regulatory T cells; Tregs) [162]. Natural Tregs are derived from the thymus, presumably by having a self-reactive TCR, albeit not efficient enough to drive clonal deletion [118, 163]. Proposed mode of action is cell-to-cell suppression via CTLA-4 blocking co-stimulatory molecules on antigen presentation cells, and deprivation of IL-2 needed for replication of the conventional T cells [164]. In rheumatoid arthritis the findings have been inconclusive regarding changes in circulating Tregs, however an increased frequency of this cell subset has been shown in the joint compartment [165-168].

2.5.4 A molecular link between HLA and citrulline autoimmunity

The specific amino acid positions coded by the *HLA-DRB1*-alleles that convey the risk of ACPA+ rheumatoid arthritis all affect the peptide binding cleft of the HLA class II molecule [42]. A link between these variants and citrulline autoimmunity has been proposed by the finding that the positively charged P4 pocket of these HLA class II molecules (e.g. *HLA-DRB1*04:01* and **04:04*) favors neutrally charged citrulline over positively charged arginine in this position [169-171]. However, it has also been shown that citrullinated peptides can bind HLA with different bindings registers, where the citrulline residue is not engaging with the P4 pocket – but nonetheless constitute a neoantigen (Fig. 4) [172-174]. This is exemplified by α-enolase$_{26\text{-}40}$ where the native and citrullinated version bind to HLA class II in the same conformation, with the arginine and citrulline residue respectively pointing toward the T cell receptor. *In vitro* stimulation of peripheral blood monocytes from *HLA-DRB1*04:01* rheumatoid arthritis patients however show a significantly higher IFN-γ response to the citrullinated α-enolase$_{26\text{-}40}$ peptide in comparison to the native (arginine) version [172].

The common denominator in the immune response that connect genetic alterations of HLA to B cells producing autoantibodies is consequently an CD4+ T cell – recognizing its cognate antigen on the HLA-molecule, which enables providing help to the B cell to finetune the humoral response.

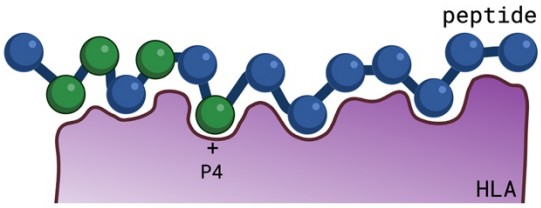

*Figure 4. Citrulline residues (green) can bind to the P4 pocket of e.g., HLA-DRB1*04:01 whereas positively charged arginine is unfavored in this position. Still, other binding registers with the citrulline facing towards the T cell receptor can also form neoepitopes.*

Adapted from Malmström et. al, Nature Reviews Immunology 2017

2.5.5 Autoreactive T cells recognizing citrullinated antigens

The aspiration to understand which type of cell that mediate the help to autoreactive B cells and which antigen that potentially start and promote the inflammatory response has led to search for antigen specific, autoreactive T cells.

The proposed T cell autoantigens has emanated predominantly from the B cell autoantigens and animal models of arthritis, e.g., citrullinated vimentin, α-enolase, aggrecan and collagen type II [74, 77, 79, 175]. Furthermore, native collagen type II, BiP (immunoglobulin binding protein) and the 65 kD M. tuberculosis antigen are examples of other potential antigens [176-178].

Aggrecan, collagen type II and cartilage intermediate layer protein are all constituents of cartilage, whereas vimentin is an important cytoskeletal protein [179-182]. A-enolase is a metabolic enzyme (phosphopyruvate hydratase) ubiquitously found in most human tissue. In addition, α-enolase also functions as a plasminogen activator present on the cell surface on

different cells including endothelial cells and lymphocytes [183]. Fibrinogen/fibrin is central to clot formation, but also in implied in e.g., wound healing and cell adhesion and deposits of fibrinogen is abundant in the synovial tissue in RA-patients [184, 185].

Each protein can have multiple arginine residues that are subjected to citrullination, but it does not necessarily mean that they will bind to HLA-class II molecules and elicit a T cell response. However, citrullinated versions of specific peptides emanating from these proteins have all been shown to bind HLA-class II SE molecules in competition assays thus providing the framework for CD4+ T cell recognition. Additionally, the crystal structure for the HLA:peptide interaction also has been solved for e.g. citrullinated α-enolase, vimentin and aggrecan [170, 172-174, 186-188].

Antigen specific T cells recognizing citrullinated autoantigens represent a very rare population in peripheral blood, with cell counts of 1-10 per million CD4+ T cells [173, 188]. Still, by using HLA-class II tetramer (further discussed in the methods section) our group and others have shown the occurrence of autoreactive CD4+ T cells recognizing citrullinated vimentin, α-enolase, aggrecan, collagen type II, fibrinogen and cartilage intermediate layer protein (CILP) in rheumatoid arthritis patients [172-174, 186-189]. If instead looking into the joint compartment a significant increase of autoreactive CD4+ T cells can be seen compared to peripheral blood [173].

The phenotype of the citrulline specific autoreactive T cells tends to be that of memory T cells, indicating a previous encounter with the antigen *in vivo* [188]. Stem cell like T cells has also been shown to constitute a part of citrulline specific CD4+ T cells in RA [130]. In contrast, autoreactive CD4+ T cells found in healthy donors are relatively less frequent and phenotypically more likely to be naïve [188].

Upon stimulation with citrullinated peptides *in vitro* CD4+ T cells from rheumatoid arthritis patients have been shown to secrete proinflammatory cytokines as IFN-γ, TNF-α and IL-17 [172, 174, 186, 189, 190]. Furthermore, *Law et al.* saw that the *in vitro* response to citrulline peptides, measured by IL-6 secretion differed depending on disease duration. Patients with short duration responded to citrullinated aggrecan or not at all, in comparison to patients with >5 years of disease duration who were more likely to respond to more than one antigen – indicating epitope spreading [190]. In contrast to this, antigen specific T cells recognizing several different citrullinated antigens (as measured by HLA-class II tetramers) has shown to be more abundant earlier in disease (<5 years of duration) and also significantly lower in patients on biological-, compared to non-biological treatment [188], suggesting targeting or indirect effects by these therapies.

2.5.6 T cell receptor repertoire studies in rheumatoid arthritis

The TCR repertoire have been studied in rheumatoid arthritis over the years, although the results have not been conclusive. This can be appointed to the heterogenous nature of the disease and that classification criteria have included different set of patients (i.e., regarding

ACPA status) in different stages of disease. Furthermore, downstream methodology has been varying over time and as a consequence the definition of a clonal expansion – making comparisons utterly difficult.

Methods priorly used include; flowcytometry to stain different Vβ-genes (old nomenclature), PCR on Vβ-genes, spectratyping and CDR3-lengthdistribution assessment of bulk RNA. Whereas the next-generation sequencing (NGS) has allowed high throughput analysis of RNA, these studies have primarily been done in bulk – focusing on the TCR β-chain.

Additionally, large scale analysis and comparisons between datasets and databases is sometimes hampered by lacking information of the HLA-type of the research subjects. *Glanville et al.* have though shown that for certain defined foreign (i.e., viral and bacterial) epitopes TCR specificity groups could be defined using an algorithm, without prior knowledge of epitope or HLA-restriction element [191].

2.5.6.1 T cell receptor repertoire in peripheral blood and joints of rheumatoid arthritis patients

In 2019, *Liu et al.* performed a large study of the β-chain repertoire of rheumatoid arthritis and systemic lupus erythematosus (SLE) patients showing a differential V gene usage in comparison to healthy controls. Public sharing of clonotypes within each diagnosis group were considered disease specific and some of these clonotypes were also shared between patient groups. However, the patients were not separated on ACPA status, nor were there any information on HLA-type. Additionally, the analysis was performed on genomic DNA of peripheral blood – while the cell type was not assessed [192].

A previous study found a higher frequency of clonally expanded CD4+ and CD8+ cells (as determined by dominant spectratype peak) in peripheral blood of RA patients compared to healthy controls, whereas no apparent skewing in variable β-gene usage was seen [193]. Similarly, next generation sequencing of single CD4+ cells from five rheumatoid arthritis patients showed more clonal expansions in comparison to healthy donors (both naïve and memory subsets) [194].

Jiang et al. performed bulk RNA-sequencing on sorted CD4+ T cells subsets from treatment naïve early RA patients, of whom 75 % were ACPA+ (HLA-type not known) and showed a TRBV3-1 overrepresentation in both naïve, effector memory and Th17-cell subsets compared to healthy controls [195]. *Goronzy et al*. studied Vβ-genes from CD4+ T cells from peripheral blood and synovial fluid and cloned them after CD3 polyclonal activation, showing a skewed TCR repertoire towards Vβ-3, Vβ-14 and Vβ17 with sharing between peripheral blood and synovial fluid for specific clonotypes assessed by PCR of the Vβ-chain amongst clones with skewed gene usage [196].

Early studies of synovial tissues and synovial fluid T cells showed skewed usage of either Vα or Vβ-genes although not conclusive between cohorts [197]. More recent studies have focused

on large joint, performing bulk analyses of the Vβ-repertoire of the T cells (i.e., not subdividing on CD4+ or CD8+ cells). Interestingly, with this approach a significant difference in Vβ-usage was seen between ACPA+ and ACPA- patients [198]. Still, the results are variable in terms of preferred gene usage in the different studies [198-200].

A major finding has instead been that clonotypes are shared between joints in the same individual, also when comparing small and large joints [200-202]. In contrast, this sharing was not evident when comparing synovial tissue repertoire to either synovial fluid or peripheral blood and instead enrichment of clones has been shown in the synovium [201, 202].

2.5.7 T cell targeting treatment approaches

There has been a revolution in regard to the treatment options and patient outcome over the last 15-20 years after introduction of biological treatment, but also new approaches on how to use well known medications and applying different treatment strategies (treat-to-target) [203].

As mentioned in the first section, methotrexate is the first in line treatment option for rheumatoid arthritis excerting its effect on a wide array of cells, including T cells where the presumed mode of action includes increasing the T cells sensitivity to apoptosis, as well as inhibiting the pro-inflammatory transcription factor NF-κB [28, 30].

Abatacept (CTLA4-Ig) is a biologic disease modifying drug (bDMARD) that have been approved for treatment of rheumatoid arthritis since 2007 and currently included in the EULAR-treatment algoritm [28, 204]. This soluble fusion protein is the first treatment aimed at targeting T cells by binding to CD80/86 on antigen presenting cells, thus blocking co-stimulation via CD28 on T cells. The effect might however be broader than targeting T cell activation, as this interaction also affect Tregs and the cells expressing CD80/86 including the T cell itself but also B cells [205]. Our group has previously shown a decrease in cytokine secretion (e.g. IFN-γ, TNF) amongst ACPA+, but not ACPA- patients, upon *in vitro* stimulation of PBMC following abatacept treatment. However, the frequency of Tregs also decreased [206]. Interestingly, the frequencies of e.g., Tfh cells, TfhPD1+ cells, memory Th17 cells and Tregs also decreased in a study of early, treatment naïve rheumatoid arthritis patients in Sweden. Further, TfhPD1+cells at baseline was proposed as one factor that might predict remission [207]. In a large registry-based study it was shown that being ACPA+ and/or RF+ was associated with lower risk of discontinuation with abatacept treatment, suggesting that treatment is more effective in this group of patients [208].

Given the proposed involvment of CD4+ T cells in rheumatoid arthritis pathology there are two trials the ARIAA (Abatacept Reversing subclinical Inflammation as measured by MRI in ACPA+ Arthralgia) and APPIPRA (Arthritis prevention in the pre-clinical phase of RA with abatacept) where abatacept has been used to treat individuals at risk of developing rheumatoid arthritis with the aim to delay or prevent disease [209, 210]. Additionally, in the ICoSRA (inhibition of co-stimulation in rheumatoid arthritis) open label trial abatacept has been used to

study changes in the repertoire of antigen specific CD4+ T cells recognising citrullinated antigens [211]. Whereas inclusion has ended, the final results are yet to be published.

Rituximab, an anti-CD20 antibody that target B cells with subsequent depletion, is one of the treatment options in rheumatoid arthritis [28]. The clinical effect of B cell depletion could propose a less importance of T cells in the disease. Interestingly, the plasma cell compartment, mainly responsible for antibody production, is not affected due to the lack of CD20-expression of these cells. Importantly, B cells are also acting as antigen presenting cells, why depletion of such cells could in fact act also on on T cells [212]. In the PRAIRI (Prevention of clinically manifest rheumatoid arthritis by B-cell directed therapy in the earliest phase of the disease) study rituximab was used in an at-risk cohort (ACPA+/RF+ with arthralgia) compared to placebo, where a single administration delayed arhtritis onset in the treatment group [213].

2.6 UNMET NEEDS AND FUTURE PERSPECTIVES

Although advances have been made, there are still many unmet needs in the RA-field. The European League Against Rheumatism (EULAR) pinpoints the following 1) new therapeutics/approaches that can improve outcome (ideally a cure), 2) repair of tissue damage caused by the disease 3) individually tailored treatment. Focus areas of research are for example finding prognostic markers of treatment response, understanding cellular and molecular markers of remission/flare and developing medicines, i.e. “resetting immune function” [214]. Ultimately, the goal apart from cure, would be to prevent rheumatoid arthritis.

2.6.1 Immune monitoring

The rationale behind immune monitoring is that immune cells have a specific phenotypic profile, that could be assessed and followed to allow a new set of biomarkers to define immune tolerance. Finding specific T cells involved in disease onset and or progression would provide such a setting [215].

2.6.2 Immune therapy as an approach to cure rheumatoid arthritis

‘Specific allergen immunotherapy’ is a well-established method to induce long-term immunomodulating effect in allergic patients. In allergy research the benefits are that the allergens (i.e., antigens) are known, whereas this is not as straight-forward in autoimmune diseases as rheumatoid arthritis. Knowing the antigen would provide a basis for peptide-based vaccines, with the goal to induce a tolerogenic state (i.e., induce T cell anergy or induce Tregs), without instead causing activation of the inflammatory pathways. Length of peptides, dose and rout of administration appears to be of great importance in this instance [216].

One approach for tolerance inducing therapy is using tolerogenic dendritic cells (tolDC). In contrast to the inflammatory antigen presenting dendritic cell, these cells have been shown to promote T cell hyporesponsivness and Tregulatory induction. Induction of such dendritic cells can be done *in vitro* in an antigen specific manner, further used in two clinical phase 1 trials in RA [217].

In the “Rheumavax” study, autologus tolDC loaded with four citrullinated peptides originating from collagen type II, fibrinogen-α, fibrinogen-β and vimentin were injected intradermally [218]. In “Autologous tolerogenic dendritic cells for rheumatoid and inflammatory arthritis (AuToDeCRA)” autologous tolDC primed with antigens from autologous joint effusion, were administered into the patients joint [219]. Both approaches were safe, whereas the study designs limited conclusions regarding efficacy [218, 219]. Liposomes loaded with calcitriol and antigenic peptide have been shown to induce tolerogenic features of dendritic cells *in vivo* in mice arthritis model and *in vitro* with human DCs as well as affecting the T cell subsets favoring a regulatory phenotype, which could provide yet another method for tolerance induction [220].

HLA-class II tetramers, targeting the autoreactive T cells has also been proposed as carrier molecules for toxins and immunomodulatory substances to delete or modulate the immune response [221].

3 RESEARCH AIMS

The overall goal of my doctoral studies has been to explore the existence and phenotype of autoreactive T cells in rheumatoid arthritis patients and how this relates to different stages of disease. Such information can increase our knowledge of disease development and form the basis for more precise therapeutic interference with the autoimmune component of rheumatoid arthritis.

Hypothesis: Autoreactive CD4+ T cells recognizing citrullinated antigens are important drivers of the immune response in rheumatoid arthritis and targeting of such cells could restore tolerance and cure or possibly prevent disease.

- Which are the common (and the private) CD4+ T cell specificities of rheumatoid arthritis patients with the same HLA-DR allele?
- Are there differences during the stages of rheumatoid arthritis development and progression?
- What is the αβ-T cell receptor repertoire in rheumatoid arthritis?
- Which T cell epitopes should be included into a future tolerizing vaccination trial?

4 MATERIALS AND METHODS

Our methodology to understand the immunopathology of rheumatoid arthritis is based on the possibility to study samples from individuals with, or being at-risk of developing, the disease. The advantages of this strategy can be easily apprehended as our findings will reflect a clinically relevant setting. We can assess which T cells that are to be found, their phenotype and changes that might occur over the disease course. An essential approach if wanting to examine what characterize the T cell features in rheumatoid arthritis. The challenges are nonetheless evident, as patients display interindividual differences (e.g., in terms of age, sex, treatment and co-morbidities) resulting in heterogenous populations to study. Working with human samples also implies a limitation in terms of number of samples, their origin and sample size, why we need to handle these precious samples with care and consideration.

4.1 THE CLINICAL COHORTS AT A GLANCE

To assess T cells at different stages of disease individuals from several cohorts have been included in the subprojects of the thesis. Whereas these cohorts include many patients each, with a main scope reaching beyond T cell studies, I have had the privilege to also access samples and include patients on basis of HLA-type in the subprojects. Although the *HLA-DRB1*04:01* allele is enriched in rheumatoid arthritis, pre-selecting samples based on this HLA allele do affect sample size and hence the patient numbers mentioned below only refer to my subprojects.

4.1.1 At-risk phase to early rheumatoid arthritis

4.1.1.1 The Karolinska Risk-RA cohort

The Karolinska Risk-RA cohort comprise individuals with musculoskeletal symptoms (i.e., arthralgia) concomitant ACPA-positivity, without clinical or ultrasound signs of synovitis when included in the study. Follow-up time is three years (minimum), or until development of arthritis (end-point) [222]. T cells from peripheral blood have been studied in 20 individuals (all carrying ≥ one *HLA-DRB1*04:01* risk allele) in this setting and thus reflecting the at-risk phase, and in some individuals also arthritis onset. **Paper I**.

4.1.1.2 Lymph node biopsies from at-risk individuals and early RA patients

Our collaborators at Academic Medical Center, Amsterdam, Netherlands have recruited individuals with arthralgia being ACPA+ and/or RF+ (i.e., in at-risk phase), as well as early RA patients (disease duration ≤6 months, naïve to bDMARDS) to undergo inguinal lymph node biopsies in their cohorts [223, 224]. After performing HLA-typing of many individuals in these cohorts, we were able to identify 5 at-risk individuals, 5 early RA patients (of whom 4 were ACPA+RF+), 1 patient with undifferentiated arthritis (seronegative) and one healthy control all carrying an *HLA-DRB1*04:01* allele. Samples from these individuals were subsequently investigated by us for the occurrence of antigen specific CD4+ T cells. **Paper II**.

4.1.1.3 Lung Investigation in Newly Diagnosed RA – the LURA cohort

The LURA cohort has included patients at time of diagnosis (ACR 1987 criteria) at Karolinska University Hospital [6]. Of these, 10 patients with ≥ one *HLA-DRB1*04:01* risk allele were included in our study of antigen specific T cells in peripheral blood at time of diagnosis and at 6 months follow-up after initiating methotrexate treatment. **Paper II**.

4.1.1.4 Synovial biopsies in early rheumatoid arthritis cohort

This cohort include patients at time of RA diagnosis, independent of antibody or HLA-allele status, to undergo synovial biopsy. 16 patients (11 ACPA+ and 5 ACPA-) were included in our study and biopsies were taken from 13 small and 3 large joints, followed by paired αβ-TCR sequencing and flow cytometry phenotyping of the T cells. **Paper III**.

4.1.2 Established to longstanding disease

With the terminology established or longstanding disease we are referring to patients that are assessed during later stages of disease, often implying the concomitant (or prior) use of DMARDs and/or glucocorticoids.

4.1.2.1 The Benaroya Research Institute (BRI) Immune-Mediated Disease Registry and Repository

ACPA+ RA patients fulfilling the 2010 ACR/EULAR criteria, carrying an *HLA-DRB1*04:01* allele, were included from the Benaroya Research Institute (BRI) Immune-Mediated Disease Registry and Repository. Peripheral blood mononuclear cells (PBMC) were used for *in vitro* (n=14) and *ex vivo* (n=9) assessment of antigen specific cells recognizing citrullinated tenascin-C (mean disease duration 3.98 years, SD 2.23). **Paper IV**.

4.1.2.2 The Karolinska Synovial Fluid cohort

The Karolinska Synovial Fluid cohort includes patients with different rheumatic diseases that donate synovial fluid when being treated with arthrocentesis as part of the clinical routine. A paired sample of blood is also drawn if applicable. A majority, but not all patients, have longstanding disease. From this cohort, ACPA+ RA-patients carrying an *HLA-DRB1*04:01* allele have been included for studies of synovial fluid and/or peripheral blood for reactivity towards citrullinated antigens. Median disease duration for patients included in **paper IV** was 17.9 (0.2-43.7) years and 17 (8-43) years in **paper V**.

4.1.2.3 Longitudinal validation cohort

Fourteen patients with ACPA+ RA, being *HLA-DRB1*04:01* carriers, that were initially included in the Karolinska Institutet based EIRA (Epidemiological Investigation of Rheumatoid Arthritis [46]) study were asked to participate in this subproject. Peripheral blood samples were assessed for antigen specific T cells at repeated blood draws. Median symptoms duration was 6 (3-43) years. **Paper II**.

4.2 SAMPLING OF BLOOD VERSUS STUDYING SITE OF INFLAMMATION

In these projects the studies have been performed with T cells originating from different compartments. Peripheral blood samples have been assessed for the occurrence of antigen specific T cells and the overall CD4+ T cell phenotype (the latter in at-risk individuals). Additionally, T cells have also been studied in synovial fluid, synovial tissue of an inflamed joint (site of inflammation) and lymph nodes (i.e., an immunological organ).

An overview of the different type of samples in relation to the timeline of rheumatoid arthritis is given in Figure 5.

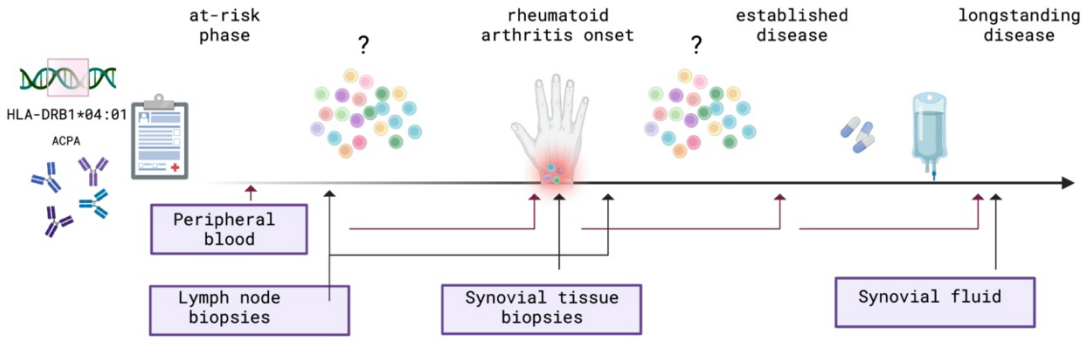

Figure 5. We have assessed T cells emanating from peripheral blood, lymph nodes, synovial tissue and synovial fluid at different stages of rheumatoid arthritis.

4.2.1 Peripheral blood – an accessible location

Sampling from the peripheral blood compartment has the obvious advantage of accessibility. This procedure confers minor discomfort for the patient and there are well established routines on sample handling in the general health care system. Analysis of blood samples are thus the method of choice for invasive sampling in the everyday clinical routine. From a scientific perspective the aforementioned advantages also apply, and a reasonable number of retrieved lymphocytes can be expected for immunological assays. However, the question arises if this compartment reflects what can be seen at site of inflammation. Comparisons are thus important to understand similarities and differences.

4.2.2 Synovial fluid

Studying the synovial fluid offers a possibility to assess cells from site of inflammation in a large joint. In our research we use synovial fluid samples that have been retrieved as a part of the clinical routine when treating local inflammation (predominantly knee arthritis) with arthrocentesis followed by injection of a corticosteroid. I.e., samples that otherwise would have been discarded. Although the sample is taken from the joint it cannot be concluded that the synovial fluid is equivalent to the synovium. Whereas it has been shown that overlap of T cell clones between different joints (synovial tissue) is high, this is not as evident between synovial tissue and synovial fluid in the same joint [201].

4.2.3 Synovial tissue biopsies

Ultrasound guided biopsies from small joints with ongoing inflammation has the advantages of sampling reflecting the disease and its distribution [82]. This is however an invasive technique rendering samples that are small in regard to tissue size and cell number, which limits the *ex vivo* analyses possible. We have processed samples in immediate proximity to sampling in order to ensure viability of the cells which also implies the need of meticulous planning limiting the potential clinical translation. This might however be overcome by the possibility of using cryopreserved tissue also for assessing live cells [225].

4.2.4 Lymph node biopsies

Lymph nodes are the secondary lymphoid organs where T cells are activated by professional antigen presenting cells and also confer help to B cells. Studies of lymph nodes are thus a common approach in animal studies of immunology and an intriguing locale also in humans. Ultrasound guided lymph node needle biopsy have been shown to be well tolerated by research subjects, although the cell numbers retrieved (median 1.2 million cells, IQR 0.4-2.4) [226] might be a limitation depending on research question.

To summarize, there are advantages and disadvantages with the different sampling sites, where the appropriate use of method makes them all valuable in understanding the immunology of rheumatoid arthritis.

4.3 EXPERIMENTAL PROCEDURES

4.3.1 Multiparameter flow cytometry, spectral flow cytometry and cell sorting

Flow cytometry is one of the most commonly used methods to assess immune cells, as it offers the possibility to study single cells in solution, using fluorescent antibodies towards surface markers as well as intracellular compartments and molecules (e.g., cytokines). Other applications include Ca^2-flux, mRNA and proliferation assays [227]. The fluorochromes used in flow cytometry are excited by lasers of different wavelength and band pass filters allows detection of the emitted light as separate markers [228]. It is however crucial to select appropriate fluorochromes to separate the emission, compensate the data for spill-over effect between different channels as well as having a stringent gating strategy when analyzing the data – including a viability marker to exclude dead cells [227]. Additionally, flow cytometry can also be used for cell sorting of bulk and single cells, where index data allow for downstream phenotypic analysis of specific cells in the latter.

The advent of spectral flow cytometry has however revolutionized the field, as this technology uses the full spectrum of each emitted fluorochrome as a unique fingerprint and as such multiple fluorochromes (>30) with close resemblance can be used to assess cells in the same sample [228]. Increasing possibilities does however also imply increasing complexity, where bioinformatic analysis will likely become more important as a complement to traditional flow cytometry analysis.

In my projects I have used both conventional flow cytometry for phenotyping studies (LSR Fortessa, BD) and flow cytometry sorting (Influx, BD) (e.g., **paper II, III, V**), whereas the last project (time wise) was carried out using spectral flow cytometry (CYTEK Aurora) (**paper I**).

4.3.2 HLA class II tetramers

HLA class II tetramers allow for identification and capturing of antigen specific CD4+ T cells by mimicking the HLA-TCR interaction, followed by flow cytometry analysis or sorting. The interaction between self-reactive T cells and the HLA peptide complex is of low affinity [229], where the use of tetramer increase the avidity by potentiating interaction with more than one TCR at the same cell (Fig. 6) [230, 231].

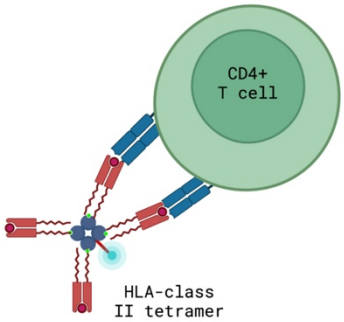

Figure 6. HLA-class II tetramers allow multiple interaction with the T cell receptor, enabling flow cytometry assessment.

4.3.2.1 Production an assembly of MCH class II tetramers

Soluble monomers of HLA class II molecules (i.e., DRB1*04:01) are produced *in vitro* in an S-2 insect cell line system, followed by biotinylation using Bir A enzyme. The monomers are loaded with the peptide of interest and tetramerized using a fluorochrome-streptavidin conjugate (e.g. phycoerythrin (PE)-streptavidin) [232].

4.3.2.2 Ex vivo and in vitro usage of HLA class II tetramers

Tetramers can be used on *in vitro* propagated cells as well as for *ex vivo* analysis. The use of magnetic bead enrichment, targeting the fluorescent dye (e.g., PE and its conjugates) coupled to the tetramer, enables capture of rare cells in the *ex vivo* setting. A starting number of at least 20 million cells is desirable for a reliable *ex vivo* assay, which however limit the use on smaller samples [233]. In order to capture even lower affinity interactions, the use of higher degree of multimerization e.g., dodecameres and affinity maturated class II molecules have also been proposed [234, 235].

Whereas *ex vivo* tetramer analysis allows for capturing rare cells, this is a low throughput system where only a limited number of samples (preferably ≤6) can be assessed on a single day to ensure proper conditions throughout the experiment. Additionally, a meticulous approach is needed in subsequent analysis.

4.3.3 FluoroSpot

This method is an evolution of the ELISpot, where the use of fluorophore labeled secondary antibodies makes it possible to analyze up to three cytokines at a time on the single cell level (Fig. 7).

Cells are stimulated *in vitro* with the preferred antigen and secreted cytokines are captured by plate bound cytokine specific antibodies. Soluble detection antibodies are added, followed by secondary antibodies. Each cell secreting cytokines will form a spot, which can be detected and automatically counted using a FluoroSpot reader [236-238]. In contrast to intracellular staining of cytokines using flow cytometry the cells are not treated to prohibit cytokine secretion prior to detection [238]. FluoroSpot assays can be performed with a smaller number of cells (300-500 thousand per well), compared to performing *ex vivo* MCH class II tetramer, making this a potentially more sensitive method. However, if using a mixed cell population, it is not possible to discern which cell type is secreting the cytokines (nor any additional phenotype). Adding HLA-blocking antibodies to study HLA-restriction in the response can also be used as a proxy. Additionally, cells can be harvested after the *in vitro* stimulation for additional experiments.

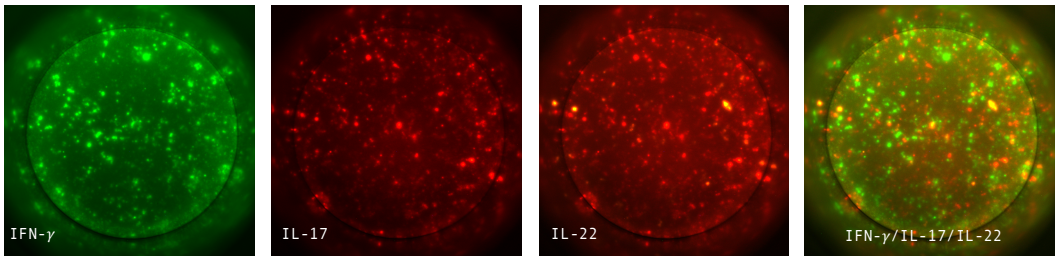

Figure 7. A three color FluoroSpot with simultaneous assessment of different cytokines at the single cell level. Each spot represents a cell. To the right an overlay of all colors.

4.3.4 Single cell TCR sequencing

Single cell T cell receptor sequencing allow for detailed analysis of the T cell receptor repertoire, also in samples where cell numbers are limited, e.g., sorted antigen specific cells and T cells from synovial biopsies. We have used a method described by *Han et al.* [239], where cDNA is synthesized from the RNA in the sorted cells, followed by a nested PCR reaction where the α- and β-chain of the TCR are amplified in parallel using multiple primers (in separate wells). Lastly, a PCR reaction is performed to add individual barcodes to each well, for downstream sequencing using an Illumina MiSeq instrument with subsequent deconvoluting, alignment and V(D)J-annotation. Information of the paired αβ-TCR allows for stringent analysis of the T cell receptor repertoire and re-expression of selected TCRs into cell line systems for functional analysis and evaluation, which is not feasible in bulk RNA sequencing. The latter however is more suitable for assessment of larger samples.

4.3.5 TCR re-expression in cell lines

Re-expression of TCR sequences into artificial T cell lines enables testing of the receptor specificity *in vitro*. We have used three different cell line systems to assess antigen specificity;

1) transient re-expression in the human embryonic kidney cell line HEK293T [240], stable re-expression in 2) αβ-TCR negative 58 cells originating from a mouse T cell hybridoma [241, 242] and 3) αβ-TCR negative, CD4+ SKW-3 cells originating from a human chronic lymphoma cell line (although cross-contamination with a human acute lymphoblastic leukemia cell line has been reported) [243].

HEK-293T cells with re-expressed TCR, CD3 and CD4 can be used to confirm binding of sorted antigen specific cells after TCR sequencing to the same HLA class II tetramer by which it was selected. 58 cells with CD4 and a NFAT-GFP (nuclear factor of activated T-cells-green fluorescent protein) reporter can be used for *in vitro* antigen stimulation to test antigen specific using plate bound HLA class II monomer peptide complex for stimulation [242]. Similarly, the SKW-3 system allows for *in vitro* testing of antigen specificity using antigen presenting cells and the desired peptide for stimulation – by us used with CD69 upregulation as read-out. Since all cells in these systems carry the same TCR, an evident response in the read-out is expected. It is however technically impossible to re-express all TCRs and even if SKW-3 cells have been used to assess the antigen specificity of TCRs derived from autoreactive T cells [244, 245] it cannot be excluded that these low affinity TCRs would need additional co-stimulation to work properly.

4.3.6 HLA-typing

Data on HLA-type is instrumental in the majority of my projects and this is neither routinely assessed in the clinical care of rheumatoid arthritis patients, nor in all research projects. There are different strategies to perform HLA-typing, where two methods have been the primarily used by us. Most samples have been HLA-typed using the PCR-based Olerup method, which allows for low-resolution (two digit typing) analysis of the *HLA-DRB1*-alleles in one PCR-reaction. If having a *HLA-DRB1*04* allele, this is followed by a second round of PCR with another primer set to assess the four digit HLA-type (high resolution) [246]. In the EIRA and Karolinska Risk-RA cohort the samples have undergone genomic analysis, as part of genome wide association studies (GWAS) – from which the HLA-type can be imputed [37, 42].

4.4 ETHICAL CONSIDERATIONS

Research ethics can be approached from different angles where one is to question whether the research that we perform is ethically acceptable. I have been involved in writing ethical applications and patient information leaflets during my doctoral studies, which are apparent moments for such considerations.

We have a great responsibility towards the individuals in our research projects, to first of all communicate that all participation **is** voluntary. This might appear obvious, but even in a society as Sweden, where hierarchal structures are less prominent, a patient is in an inferior position to health care personnel. Thus, we as professionals must mean it when we say that the patient will be treated just as good independent on their decision whether to engage in research or not.

If we can be assured that everyone feels confident enough to decline participation, the subsequent reasoning is much easier. In many studies we are sampling blood one, or repeated times. Is that ethically acceptable? Most people would agree that this is only a minor intervention, routinely performed and hence so. However, if a patient has fragile and inaccessible blood vessels, drawing blood can be a painful and terrible experience. Again, we need to trust that we allow such an individual to make an unbiased decision (which might as well be to participate). In my opinion this reasoning is applicable for many situations where we want to sample different tissues, even if the intervention is more advanced (e.g., lymph node or synovial biopsy) – although not dangerous.

Another aspect regarding inclusion is when in the disease course (or maybe even prior to disease) we are asking the individual for involvement. It can be overwhelming to get a diagnosis as rheumatoid arthritis and at the same time be asked to participate in research. In my experience however, most patients are eager to contribute to drive research forward – for themselves and others. It can even be beneficial to be included in a project, exemplified by an individual without arthritis (with risk factors) that would otherwise be omitted from follow up, whereas in the project have a fast track to health care if symptoms exaggerate.

The personal integrity has rightfully gained increasing attention over the last years, and I completely agree that we need to be careful in handling of personal data, only accessing such information that we need, which is aided by pseudonymization. Central to research is also reproducibility, studying the group and identifying differences that lead us forward. I would however like to challenge the thought a little on this matter. When working as a physician the central aspect of my work is to see the individual. To know their history and make personalized decision based on this, of course in compliance to medical evidence. Nevertheless, I could not work if I did not have this specific individual information. In the era of striving for personalized medicine I have started to question if the ethical aspects of concealed personal data in combination with our group-based thinking is refraining us from making the major leaps forward. I am thus wondering if I, as a clinician scientist, could contribute even more if my approach to research would be more similar to my clinical work and thus the translational step back from bench to bedside.

Finally, I would like to address research ethics from yet another angle. Is it ethically correct to refrain from research? As I touched upon in the methods section, it is important that we are thoughtful in the use of human samples. They are precious and need to be used accordingly. However, we have been given a trust from the patient when they donate samples – and in return we are expected to use them to the best of our knowledge. Hence, not performing research can also be unethical. Keeping the ethical thinking alive is probable the most important for a sound and prosperous way forward.

5 RESULTS AND DISCUSSION

I have studied the occurrence of antigen specific T cells, the general T cell phenotype and the T cell receptor repertoire – with a focus of CD4+ T cells, during different stages of rheumatoid arthritis, including the pre-clinical at-risk phase. In this chapter the main findings and reflections of my thesis work will be presented. In depth data presentation is to be found in each paper.

In my work, I have worked with subsets of cohorts, where the individuals share a common genetic background – i.e., carrying at least one *HLA-DRB1*04:01*-allele, known to be the strongest genetic risk factor for developing rheumatoid arthritis [42]. However, this allele was also present in the healthy donors that were assessed in parallel. When investigating antigen specific T cells using HLA class II tetramers, selecting subjects on basis of HLA-type is a prerequisite to use the correct tetramer for the assay. In addition, the vast majority were also ACPA+, thus having a known B cell response to citrullinated antigens. Following this, we have focused our interest on T cell reactivity towards citrullinated peptides that can bind to and be presented by the HLA class II DRB1*04:01 molecule; e.g. citrullinated peptides derived from α-enolase, fibrinogen-β, cartilage intermediate layer protein (CILP) and vimentin [170, 172, 173, 186, 188]. Whereas these antigens have been previously described, we have also examined citrullinated tenascin-C (TNC), another target of ACPA [80] as a novel potential T cell autoantigen.

5.1 CAN CITRULLINE SPECIFIC CD4+ T CELLS BE IDENTIFIED IN THE AT-RISK PHASE OF RHEUMATOID ARTHRITIS?

We have studied the presence of citrulline specific CD4+ T cells in peripheral blood (**paper I**), as well as in lymph node biopsy samples (**paper II**), from ACPA+ and/or RF+ individuals with arthralgia (lacking clinical signs of arthritis), as a potential link between genetic predisposition (i.e., HLA-DRB1*04:01) and the occurrence of autoantibodies already in the at-risk phase of rheumatoid arthritis.

5.1.1 Assessment of T cells in the Karolinska Risk-RA cohort

Within this cohort where we have assessed peripheral blood mononuclear cells (PBMC) in 20 ACPA+ at-risk individuals (≤3 sample timepoints per individual). Ten individuals were selected upon being known to later progress to arthritis and matched with ten non-progressors (**paper I**).

5.1.1.1 Ex vivo analysis of citrulline specific CD4+ T cells

To explore if we could detect circulating autoreactive CD4+ T cells in individuals in the at-risk phase we used a setup with three HLA-class II tetramer pools recognizing 12 different citrullinated autoantigens (i.e., four antigens per tetramer channel, paper I, table 2), with an influenza as a positive control (Fig. 8). Additionally, we combined this staining with an extensive panel for surface marker phenotyping. Interestingly, autoreactive CD4+ T cells could

be found at low frequencies already at this phase and the vast majority of individuals had cells recognizing all antigens – i.e., citrullinated α-enolase/CILP, fibrinogen-β/vimentin and tenascin-C peptides.

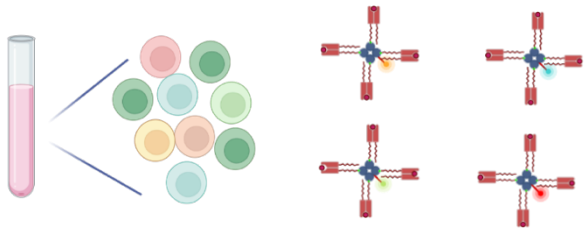

Fig. 8. Individual HLA-class II tetramers with the same fluorochrome were combined into a tetramer pool – allowing assessment of four different subsets of antigen specific CD4+ T cells, including influenza specific cells as a positive control.

However, the specificities did not distribute evenly and whereas cit-tenascin-C specific cells were the most frequent at baseline amongst future progressors (n=10), the cit-fibrinogen-β/vimentin specific cells were rare in this group. Intriguingly, the autoreactive cells were found at significantly lower frequency in the progressors compared to the non-progressors, whereas no difference was seen for influenza reactive T cells (**paper I, fig. 3A-D**). When further looking at the cit-tenascin-C specific CD4+ T cells in the progressor group we also saw a decline in frequency from baseline to arthritis onset (**paper I, fig 4A**). Together this observation could suggest that the citrulline specific CD4+ T cells already started homing to the joint, lymph-node or possibly the lung, posing a potential explanation to the somewhat counter intuitive finding of lower cell frequencies amongst progressors.

5.1.1.2 Phenotype of tenascin-C specific CD4+ T cells

Phenotypic analysis of the CD4+ T cells recognizing citrullinated tenascin-C showed that these cells were equally distributed amongst naïve and memory phenotype, in contrast to influenza specific cells that skewed towards a memory phenotype (**paper I, Fig. 6A-B**). The finding of memory cell subsets suggests a prior encounter with the antigen *in vivo*. The cit-tenascin-C CD4+ memory T cells were primarily of effector memory phenotype and the overall memory compartment showed CD95 and, to a slightly less degree, PD-1 expression (**paper I, fig. 6C-D, fig. 7A-C**). Cit-tenascin-C specific Tfh (CXCR5+) were rare and Tph (CXCR5-PD1hi) cells were virtually absent (**paper I, fig. 5A**). Neither could we find any Th1 or Th17 dominance. Instead, some of the cit-tenasin-C specific cells were of Treg phenotype (**paper I, fig. 5B-D**). The low count (absolute number) of cells that we could detect per specificity (**paper I, fig. 4E**) implies that caution has to be made when analyzing the cell phenotype, why our efforts were focused on the cit-tenascin-c specific cells. However, with this in mind, further analysis could be made to indicate potential difference in terms of phenotype between the specificities.

5.1.1.3 The global CD4+ phenotype do not define progressors from non-progressors

The global CD4+ phenotype was assessed in all individuals and when comparing specific T-helper subsets the frequencies were similar (**paper I, fig. 2A, B**). Likewise, the Treg frequencies and memory subsets (**paper I, fig. 2C, supplementary fig. 2B**) did not differ between the groups. CD4+CD28null T cells – previously shown in rheumatoid arthritis, could

also be identified (**paper I, fig. 7A**) [154]. If instead looking within each group a dominance of Th1 to Th17 phenotype was seen, notably with interindividual differences (data not shown).

5.1.2 Citrulline specific CD4+ T cells in the lymph node of at-risk individuals

The lymph node biopsies were also assessed with a multi tetramer approach (Table 3), although not including citrullinated tenascin-C, which was not known to be a T cell antigen at the time. Since the biopsy specimen contained few cells, these had been *in vitro* expanded with phytohemagglutinin (PHA) and IL-2 prior to cryopreservation. Our analysis revealed citrulline reactive CD4+ T cells in the five arthralgia individuals – including cells recognizing citrullinated vimentin (four individuals), citrullinated α-enolase (two individuals) and citrullinated CILP/fibrinogen-β (two individuals) (**paper II, fig. 3**). The procedure of *in vitro* propagating cells with a mitogen as PHA [247], will skew the phenotype of the cells, why no such analysis was performed. During the 20-28 months of follow up, no progression to arthritis was seen in these individuals.

Table 3. Overview of the peptides used for tetramer assessment in at-risk and early phase

Paper	Cohort (sample)	At-risk	Early RA	A-enolase (α-eno)	Cartilage intermediate layer protein (CILP)	Fibrinogen-β (fib-β)	Vimentin (vim)	Tenascin-C (TNC)
I	At-risk PBMC	+	(+)	α-eno$_{11-25}$ α-eno$_{26-40}$ α-eno$_{326-240}$	CILP$_{297–311}$	fib-β$_{70-81,}$ fib-β$_{69-81}$	vim$_{59-78}$ vim$_{418-431}$	TNC17$_{871-885}$ TNC22$_{1012-1026}$ TNC45$_{1633-1647}$ TNC56$_{2067-2081}$
II	Lymph nodes	+	+	α-eno$_{11-25}$ α-eno$_{26-40}$ α-eno$_{326-240}$	CILP$_{297–311}$ CILP$_{982–996}$	fib-β$_{69–80}$	vim$_{59-78}$ vim$_{418-431}$	
II	LURA PBMC		+	α-eno$_{11-25}$ α-eno$_{26-40}$ α-eno$_{326-240}$	CILP$_{297–311}$ CILP$_{982–996}$	fib-β$_{69–80}$	vim$_{59-78}$ vim$_{418-431}$	

The multi tetramer approached allowed assessment of multiple citrullinated antigens in parallel. Numbers in subscript refers to peptide position in the protein. N.B. the combinations of peptides per channel differed in the projects.

5.2 AUTOREACTIVE T CELLS ARE FOUND IN EARLY DISEASE

It could be argued that a patient diagnosed with rheumatoid arthritis per definition has chronic, hence established, disease. However, from an immunological perspective the early phase is very interesting as this reflect the transition from systemic to local inflammation and additionally, a timeframe where we might have the possibility to examine the immune cells prior to the influence of disease modifying drugs.

5.2.1 Citrulline specific CD4+ T cells in the lymph node of early RA patients

We had the opportunity to also assess lymph node biopsies (as described in previous section) from five patients with recent onset rheumatoid arthritis (≤ 6 months disease duration) (**paper**

II). Similar to what could be seen in lymph biopsies from arthralgia individuals, citrulline specific CD4+ T cells were present also in this phase. All five patients had CD4+ T cells specific for citrullinated vimentin and three of these patients also recognized citrullinated α-enolase and CILP/fibrinogen-β. Additionally, vimentin specific T cells were also seen in the patient with undifferentiated arthritis (accompanied by α-enolase) and in the healthy donor (**paper II, fig. 3**).

5.2.2 Autoreactive T cells in early disease have a T follicular helper cell phenotype

When investigating the occurrence of antigen specific CD4+ T cells in peripheral blood at time of diagnosis in the LURA-cohort, eight out of ten patients had CD4+ T cells recognizing citrullinated antigens (citrullinated α-enolase, CILP/fibrinogen-β and/or vimentin) (**paper II, fig. 4B)**. The cit-α-enolase and cit-CILP/fibrinogen-β specific CD4+ T cell were primarily memory cells, whereas the cit-vimentin specific cells had a more naïve phenotype (**paper II, fig. 4C and supplementary fig. 2C**). Intriguingly, CXCR5 expression, as a marker for T follicular helper cells, was a prominent feature of the citrulline specific cells and could indicate their function in promoting the ACPA-response. Th1 and Th17, as determined by CXCR3 or CCR6 expression, were however not a predominant phenotype (**paper II, supplementary fig. 3b**). In parallel to the baseline sample, we also had the opportunity to examine the 6 months follow-up sample, when the patients had been treated with methotrexate (6 patients also received low-moderate dose of glucocorticoids). Interestingly, we saw that the patients reaching remission based on a Disease Activity Score 28 (DAS28) score <2.6 showed a decrease in numbers of circulating citrulline specific CD4+ T cells (similar for all specificities), whereas this was only seen for one patient with DAS28 > 2.6 (**paper II, fig. 4B**).

5.3 HOMING MIGHT EXPLAIN DIFFERENCES IN FREQUENCY OF CIRCULATING AUTOREACTIVE T CELLS

We have adopted a similar method to assess the spectrum of circulating autoreactive T cells recognizing citrullinated peptides in the at-risk phase and early stages of disease, with the difference of including citrullinated tenascin-c in the most recent study of the at-risk phase (Table 3). Although comparisons must be made with care between *in vitro* propagated T cells and *ex vivo* analysis, it is an interesting finding that vimentin reactive T cells were the most frequent reactivity in the lymph nodes (notably also in control individuals), whereas the opposite was seen in at-risk (especially in future progressors) and early phase of disease. Similar to our hypothesis that an overall lower number of citrulline specific CD4+ T cells in at-risk individuals that are known to progress to rheumatoid arthritis represent homing, this could also be possible explanation for this discrepancy between lymph node and circulation.

When looking at the frequencies of autoreactive CD4+ T cells, these were seemingly lower per specificity in the at-risk phase (including sampling at arthritis onset) in comparison to the numbers from the LURA cohort. This difference could reflect that we are in fact studying individuals already before disease onset and that arthritis onset in this cohort represent an even

earlier time-point than time of diagnosis, as the individuals are followed within a research program. Another explanation is the probable effect of the very stringent gating in terms dump-channel (excluding monocytes, B cells and additionally NK cells), having a viability marker (excluding dead cells instead of apoptotic cells), CD3 and CD8 to get a clean CD4-population that the spectral flow cytometry allows without having to leave out other surface markers (**paper I, supplementary figure 1A**).

5.4 THE T CELL RECEPTOR REPERTOIRE IN THE SYNOVIUM AT TIME OF DIAGNOSIS

Whereas the use of HLA-class II tetramers enables the search for antigen specific CD4+ T cells, this also implies a hypothesis driven method, where one must decide which antigen to study and that only individuals with a specific HLA-type can be included in the studies. A different approach is to study the T cell receptor repertoire – i.e., an unbiased mapping of T cells, which we have done in patients with recently diagnosed rheumatoid arthritis. We had the possibility to take ultrasound guided biopsies from small joints of thirteen patients and additionally from large joints of three patients (**paper III**) for single cell sorting, phenotyping and T cell receptor sequencing of the paired αβ-TCR.

5.4.1 CD4+ T cells show a diverse repertoire in the synovium

The overall T cell phenotype in the biopsies showed a dominance of CD4+ T cells (**paper III fig. 1C**) in both ACPA+ and ACPA- patients and as expected also amongst the single cells sorted (**paper III, fig. 2A**). By analyzing the paired αβ sequence of the T cell receptor we could apply a stringent definition of clonality where cells were considered clonally expanded if the same αβ-sequence (CDR3) was found ≥2 times. We observed a broad CD4+ T cell repertoire, with most cells carrying a unique TCR – although clonal expansions were a constant finding in this compartment. It is known that T cells can have double α-chains that pair with the β-chain, although the occurrence of this has not been addressed in the rheumatoid joint with prior methods [123]. However, in this cohort double alpha chains was a frequent finding (**paper III, fig. 2E**) and the fact that we could re-express functional TCR with both such combinations in the SKW-3 cell line is even more intriguing. As previously hypothesized, this could imply that the T cell has been able to survive negative selection in the thymus with one TCR-combination ($\alpha_1\beta_1$), whereas the second ($\alpha_2\beta_1$) could have a different, potentially autoreactive, specificity [124].

5.4.2 CD8+ contribution to the T cell clonal expansions is substantial

In the patients where single CD8+ T cells were assessed, the trend was however towards a more restricted CD8+ repertoire. Notably, the numbers and size of the CD8+ T cell clones influenced the diversity of the overall CD3+ T cell compartment (**paper III, fig. 2 A-D**). This finding is interesting, as previous studies have assessed the T cell compartment identifying highly expanded T cell clones suggestive of a restricted repertoire in the synovium [200, 201]. However, those studies were performed without separating CD4+ from CD8+ T cells, thus not considering the CD8+ T cell effect on this. In our study we HLA-typed the patients for the

HLA-DRB1 allele, whereas we don't know if they would be carrier of the HLA-B-allelic variant also associated with rheumatoid arthritis and if that would have any influence on the CD8+ T cell repertoire [42]. Interestingly, a recent publication has shown that CD8+ T cells in peripheral blood of ACPA+ RA patients respond with proliferation to citrullinated (not native) antigens and that the clonally expanded cells had a cytotoxic profile and increased scores for synovial trafficking. In addition, when studying the synovial CD8+ T cell receptor repertoire, the large clones shared this cytotoxic phenotype [248]. We only had a limited phenotyping panel, with a focus on CD4+ T cells, although we could see that the CD8+ cells in the synovium were neither PD-1hi, HLA-DRhi nor CD25hi which were our surface markers (**paper III, fig. 3 b-c, supplementary fig 3**).

5.4.3 T peripheral helpers in the synovium, present also in early disease

The phenotype of clonally expanded CD4+ T cells showed a trend towards being CD4+PD-1hi (proxy for Tph-cells) and the CD4+PD-1hi cells constituted a well-defined population in the synovium (**paper III, fig. 3A**). This observation suggest that these cells could provide B cell help in the synovium and promote the local humoral response. Indeed, we have also studied patients from the same cohort using spatial transcriptomics and investigating the B cell repertoire showing the presence of T-B cell interaction, as well as plasma cell differentiation in synovial tissue specimen supporting this hypothesis [249]. In parallel to our work, *Murray-Brown et al.* recently published their findings of Tph cells (CD4+PD1hiCXCR5-) in large joint synovial biopsies of early RA patients, being significantly more frequent than Tfh cells, although co-localizing with B cells [250].

5.4.4 The antigen specificity of synovial CD4+ T cells

We have studied both ACPA+ and ACPA- patients in this study (**paper III**) and hence sought to understand if the TCR-repertoire differed between the groups. Here we could show that TRBV20-1 gene usage in non-expanded cells was more common amongst the ACPA+ patients and further a bias in the clonally expanded cells which was confined to shared epitope *HLA-DRB1*04* carriers (**paper III, fig. 4. A, D**). Although the TRBV-gene usage can be influenced by the HLA-type, this bias might represent the possibility to recognize a common antigen [251, 252]. When we have studied gene usage in antigen specific CD4+ T cells in established disease (**paper V**) we have found such bias in cells recognizing specific peptides derived from citrullinated Tenascin-C (**paper V, fig. 3**), whereas not in viral reactive cells. The concept of biased gene usage secondary to HLA-type could thus imply that the genetic risk associated to rheumatoid arthritis do not only affect the HLA, but also skew the T cell repertoire.

Additionally, when we re-expressed the TCR of clonally expanded cells from four patients with *HLA-DRB1*04:01* into SKW-3 cells we could identify ten viral reactive CD4+ T clones (including Epstein-Barr virus (EBV), cytomegalovirus (CMV) and Human Herpes virus (HHV-2)) – none of which had a TRBV20-1 gene usage. Although we also tested these 81 cell lines for reactivity against a wide array of native and citrullinated antigens previously described in the literature (e.g. α-enolase, CILP, fibrinogen-β, vimentin and others, **paper III,**

supplementary table 1) the remaining cells were orphans in terms of antigen specificity (**paper III, fig. 5**). Given the rarity of citrulline specific CD4+ T cells in circulation and the small biopsy specimen, the absence of such reactivity can only tell us that we could not identify them – not rule out that citrulline specific cells still reside within the joint. As discussed previously in the methods section, one cannot assume that low affinity autoreactive TCRs will have the same requirements for binding as high affinity TCRs.

The finding of virus reactive CD4+ T cells do trigger the question whether the inflammation in the rheumatic joint is driven by viral reactivity in a similar way that has been proposed for EBV in multiple sclerosis [253]. In favor of such hypothesis is the idea of a "second hit" needed to direct the immune response from a systemic phase (i.e., ACPA) to a clinical phase, where both infections and trauma has been pointed out as possible "hits". There are previous studies that have found EBV in the synovium of rheumatoid arthritis patients, although the results have been conflicting [254-258]. A complicating factor is the modus operandi of the virus to reside within lymphocyte. EBV at site of inflammation hence might just reflect migration of such cells [256]. Additionally, the subset of CD4+CD28null T cells found in rheumatoid arthritis patients have been shown to relate to CMV infection [154, 259, 260]. However, such infections are common to the general population, why causality might be difficult to assess. Nonetheless, when present at site of inflammation (even as bystanders), the virus reactive cells might well be contributing to inflammation.

5.5 TENASCIN-C A NOVEL T CELL AUTOANTIGEN?

Tenascin-C was mentioned as a potential T cell autoantigen in the beginning of this chapter. Whereas this large extra cellular matrix protein is scarcely found in the healthy adult, it is transiently up regulated during an insult as trauma or inflammation, being important for tissue repair, cell adhesion and migration [261, 262]. Serum levels of native tenascin-C has been shown to be increased in rheumatoid arthritis, as well as in other inflammatory conditions as psoriatic arthritis, SLE and inflammatory bowel disease [263, 264]. Tenascin-C has also been shown in synovium and synovial fluid of the rheumatic joint and when citrullinated also as a target for ACPA [80, 265-267].

In collaboration with our colleagues at Benaroya Research Institute (BRI, Seattle) and Kennedy Institute (University of Oxford) we thus sought to study the propensity of tenascin-C being a T cell autoantigen, as well as the existence of additional tenascin-C reactive autoantibodies in patients with established disease (**paper IV**).

5.5.1 Immunogenic properties of citrullinated tenascin-C peptides

An algorithm previously developed at BRI [188, 268] was used to identify and predict possible binding (in native and/or citrullinated form) to HLA class II DRB1*04:01 of arginine containing peptides derived from tenascin-C. This resulted in 64 such peptides, of which five citrullinated peptides had the capacity both to bind HLA-DRB1*04:01 in a competition assay, as well as to elicit prominent expansion of CD4+ T cells *in vitro* using PBMC from fourteen *HLA-DRB1*04:01* positive RA patients (**paper IV, table 1 and supplementary fig. 1**).

Using HLA-class II tetramers loaded with the five different immunogenic peptides (TNC17, TNC22, TNC45, TNC50 and TNC56) we could identify antigen CD4+ T cells recognizing citrullinated tenascin-C peptides in peripheral blood of all rheumatoid arthritis patients (n=9, **paper IV, fig. 2**). In this study the different peptides were assessed by separate tetramers, in comparison to the at-risk cohort (**paper I**), where they were combined in one channel. When combining all the tenascin-C positive cells the numbers were however >10 times what was found in the at-risk cohort and significantly higher than in the healthy donors (**paper IV, fig. 2A**). The cells showed a predominant Th2 phenotype in circulation and expressed CD38 in the memory compartment as a sign of activation (**paper IV, fig. 2 D-F**).

5.5.2 Synovial fluid mononuclear cells show profound response to citrullinated tenascin-C peptides

I also studied the reactivity to the five citrullinated tenascin-C peptides and their native version in synovial fluid mononuclear cells from ACPA+ rheumatoid arthritis patients (*HLA-DRB1*04:01* carriers). The cells were stimulated *in vitro* for 48 hours with the different peptides and a FluoroSpot assay was used to determine interferon (IFN)-γ, IL-17 and IL-10 secretion per cell (i.e., a spot) – normalized to spots per million synovial fluid monocytes (SFMC). As a comparison, tetramer positive cells are calculated per CD4+ T cells or CD4+memory T cells. IFN-γ response to the citrullinated version of the tenascin-C peptides (e.g., TNC17, TNC22, TNC45 and TNC56) was robust, whereas virtually no response was seen for the native version (**paper IV, fig. 3a**). To our surprise, the response to citrullinated tenascin-C was reaching that of influenza for some patients and as a comparison we also studied reactivity to α-enolase, fibrinogen-β, vimentin and CILP peptides. Reactivity was seen also for these previously known antigens, but the response towards tenascin-C was almost ten times higher (Fig. 9; **paper IV, fig. 3b**).

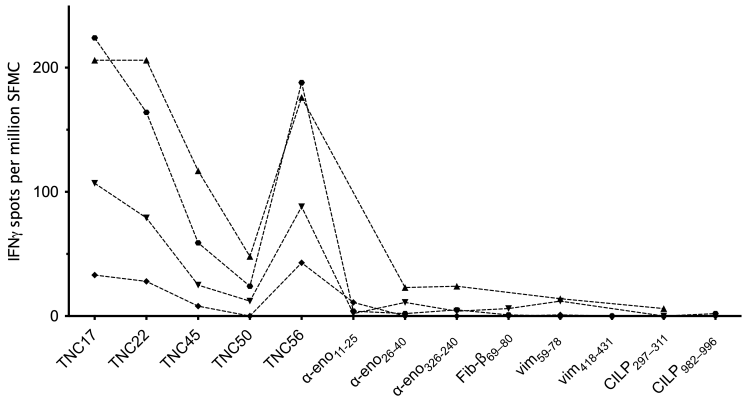

Figure 9. IFN-γ response to different citrullinated antigens. Each line represents a patient. SFMC = synovial fluid mononuclear cells

Blocking of HLA-DR (but not DQ) inhibited IFN-γ response to tenascin-C indicating an HLA-DR mediated (i.e., CD4+ Th1) response (**paper IV, fig 3C**). IL-17 secretion was limited, whereas 5 of 7 patients also displayed IL-10 secretion in response to citrullinated TNC22 although not significantly different to the arginine version (**paper III, supplementary fig. 2**). Whereas most patients in the synovial fluid cohort had long standing disease, two of the patients with marked IFN-γ secretion in the blocking experiment had a disease duration of median 3 months at sampling – which could further corroborate our previous hypothesis of migration of tenascin-C specific cells to the joint at disease onset.

5.5.3 ACPA recognize citrullinated tenascin-C – a feature of multireactivity

Furthermore, the occurrence of autoantibodies reactive to the same citrullinated tenascin-C peptides were also studied, where cit-TNC17 was the most prominent reactivity in the patients with concurrent CCP-positivity (**paper III, fig. 4A, 6A**). This peptide do however have a glycine (G) in position +1 to the citrulline residue (**paper III, table 1**) which previously has been shown to represent a specific binding motif (citrulline-glycine) of citrulline reactive antibodies – i.e., a multireactivity of this type of antibodies [81], why this reactivity could be anticipated amongst CCP+ individuals.

5.5.4 Reflections of tenascin-C in relation to rheumatoid arthritis

What makes tenascin-C an especially interesting antigen in relation to immunopathology of rheumatoid arthritis is the temporal distribution of the protein, being upregulated during inflammation and trauma [262]. Tenascin-C has also been shown to be deposited in the lungs (large airways – subepithelial, small airways – inner layer) in smokers with chronic obstructive disease, and when comparing smokers to never smokers [269, 270]. It can be anticipated that such protein deposits might also be citrullinated as citrullination is a known phenomenon in the different inflammatory tissues as well as in the lung of both smokers and non-smokers [60, 67] – posing a possible link between smoking, citrullination and the joint.

5.6 PHENOTYPE OF AUTOREACTIVE T CELLS DIFFER OVER TIME AND WITH ANTIGEN

As a part of our development of the multi-tetramer approach used in paper II, we studied antigen specific CD4+ T cells in peripheral blood recognizing citrullinated α-enolase and/or citrullinated CILP/fibrinogen-β in consecutive samples from patients with >5 years disease duration. Here we could detect such cells in all fourteen patients (**paper II, fig. 2A**) with a dominance towards CILP/fibrinogen-β reactivity (found in 13 patients). Notably, in contrast to the patients with recent onset RA or tenascin-C reactivity these autoreactive cells showed a Th1 (CXCR3+) or Th17 (CCR6+) phenotype, although half of the CXCR3+ cells were double positive for CXCR5 (**paper II, fig 2C** – data on co-expression not shown). In a previous study from *James et al.* a predominant Th1 phenotype was seen amongst memory CD4+ T cells recognizing citrullinated peptides also included in our setup, although CCR6+ cells were rare [188].

5.7 A COMBINATORY APPROACH TO STUDY T CELL RECEPTOR REPERTOIRE IN AUTOREACTIVE T CELLS

In the work discussed until now, we have used HLA-class II tetramers to assess antigen specificity of CD4+ T cells (hypothesis driven) and TCR-sequencing as an unbiased approach to study the T cell receptor repertoire in rheumatoid arthritis patients.

In paper V we combined these two methods to describe an *in vitro* pipeline that allows for examination of the T cell receptor repertoire of rare antigen specific CD4+ T cells. The

rationale for such an attempt is the scarcity of antigen specific CD4+ T cells and the need of large cell number for *ex vivo* analysis limiting the use on smaller samples.

Tenascin-C derived citrullinated peptides were chosen for the assay due to the, in terms of autoreactivity, high frequencies of cit-tenascin-C specific CD4+ T cells in peripheral blood and the robust IFN-γ response upon *in vitro* stimulation of synovial fluid mononuclear cells in our previous work (**paper IV**).

In this setup we used cells derived from peripheral blood (n=4) or synovial fluid (n=4) of patients with longstanding rheumatoid arthritis disease. All but one patient had ongoing antirheumatic therapy, although sampling (including blood) was performed upon arthrocentesis implying ongoing inflammation.

The cells were *in vitro* stimulated with a cocktail of the citrullinated tenascin-C peptides for two weeks prior to sorting using HLA-class II tetramers. Cells were assessed after approximately 9 days to evaluate response (using a single tetramer for each peptide) and were further expanded *in vitro* until harvesting. Sequencing of the paired αβ-TCRs was then performed followed by analysis and re-expression of the selected TCR into either HEK293T or 58 cells (**paper V, fig. 1A**).

Prominent expansions were seen both in CD4+ T cells from peripheral blood and synovial fluid after *in vitro* stimulation. Following TCR-re-expression we could show that a TNC22 specific TCR recognized the cognate antigen both using tetramer staining and as measured by NFAT-upregulation. If instead using a mismatch in tetramer or stimuli (i.e., TNC17) this upregulation was not seen (**paper V, fig. 2B, C**).

We also studied similarities of the TCR-repertoire rendered by this method and whereas no shared αβ-TCR sequences were seen, we saw sharing of TCR β-sequences between three patients (both peripheral blood and synovial fluid). Interestingly, there was a skewed gene usage toward TRBV20-1 in cells sorted on being TNC22/TNC56 reactive – which was not reflected in the repertoire of TNC17/TNC45 or influenza specific cells (**paper V, fig. 3, supplementary fig. 3**). Gene usage was analyzed using the TCRdist algorithm, which also creates logo sequences of TCRs with biochemical features (e.g. similar charge, polarity etc.). This analysis also showed similarities between sequencies responding to the same antigen (**paper V, fig. 3C**).

To summarize, we were able to describe a pipeline which could be used to explore the T cell receptor repertoire of rare antigen specific CD4+ T cells. Furthermore, citrullinated tenascin-C provoked robust expansion of CD4+ T cells emanating from both peripheral blood and synovial fluid in patients with long standing rheumatoid arthritis.

6 CONCLUSIONS

In the introduction of this thesis, I referred to "what causes rheumatoid arthritis?" as a partially answered, but still central question in this field of research.

Our current understanding is that individuals with genetic predisposition, when exposed for certain environmental hazards such as smoking lose tolerance toward self-antigens – presenting as autoantibodies that can be detected in the circulation (i.e., ACPA and RF). However, not all these individuals will develop rheumatoid arthritis and neither do we know what event that direct the systemic immune response toward the joint – with subsequent arthritis development. The genetic association, i.e., HLA-class II-alleles and the occurrence of autoantibodies however indicates the importance of CD4+ T cells – possibly reactive to citrullinated autoantigens e.g., citrullinated α-enolase, vimentin and fibrinogen-β.

Whereas the serological aberrancy prior to disease onset is well described, I here show that autoreactive CD4+ T cells recognizing citrullinated antigens are also present in the circulation of individuals that will subsequently develop rheumatoid arthritis. The cells are very rare, but predominantly recognize citrullinated peptides derived from tenascin-C – that we also describe as a novel T cell autoantigen. Notably, there are high frequencies (from an autoreactive T cell perspective) of CD4+ T cells recognizing citrullinated tenascin-C peptides in later stages of disease, both in circulation and synovial fluid. An interesting feature about tenascin-C is its temporal distribution where the protein is normally absent in healthy tissue, but up-regulated during inflammation and injury [261]. Additionally, tenascin-c has been shown to be abundantly found in the lung tissue of smokers – a site known for citrullination [60, 269, 270]. With the lung being a proposed locations for loss of tolerance this finding might pose a link to autoreactive T cells.

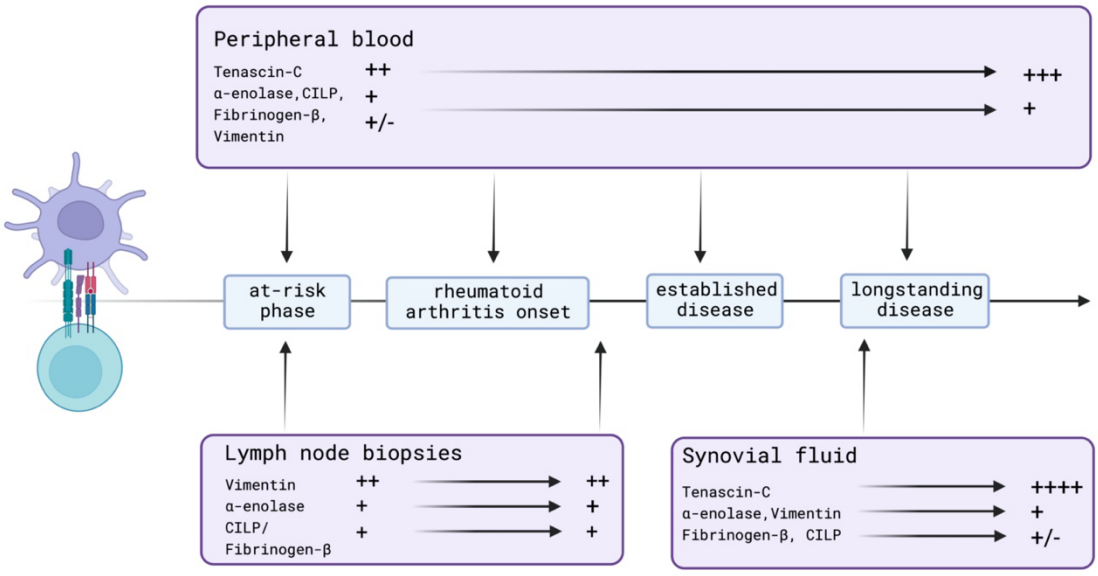

Figure 10. The timeline of autoreactive CD4+ T cells in different compartments.

During the time course from being at-risk of developing rheumatoid arthritis to having a longstanding disease we also identified cells recognizing citrullinated α-enolase, cartilage intermediate layer protein (CILP) vimentin and fibrinogen-β. Interestingly, such circulating cells had a T follicular helper phenotype in early rheumatoid arthritis, suggesting ability to provide B cell help. When instead looking into the secondary lymphoid tissue (i.e., a location for T and B cell interaction) we also detected citrulline specific CD4+ T cells (most often recognizing citrullinated vimentin) both in at-risk individuals and in early disease. The timeline of autoreactive CD4+ T cells in rheumatoid arthritis is summarized in figure 10.

Analysis of the T cell receptor provides yet another approach to understand the repertoire of T cells involved in rheumatoid arthritis and this work shows that the synovial tissue of rheumatoid patients (small joints at time of diagnosis) is infiltrated by a diverse set of T cells – of both CD4+ and CD8+ lineage. Additionally, the CD4+ T cell compartment comprised T peripheral helper cells, proposing capability to activate B cells at site of inflammation. There were also CD4+ T cells with reactivity to commonly acquired viruses as EBV and CMV, whereas we are left curious to whether there are also autoreactive T cells in place. A clue to this is however a biased gene usage in CD4+ T cells (TRBV20-1) in ACPA+ patients – also shared with autoreactive cells recognizing a set of citrullinated tenascin-C peptide in patients with longstanding disease. With continuing use of our in-vitro stimulation to TCR sequencing pipeline we will build upon our knowledge of autoreactive T cells and future experiments will give us more answers. Findings are summarized in figure 11.

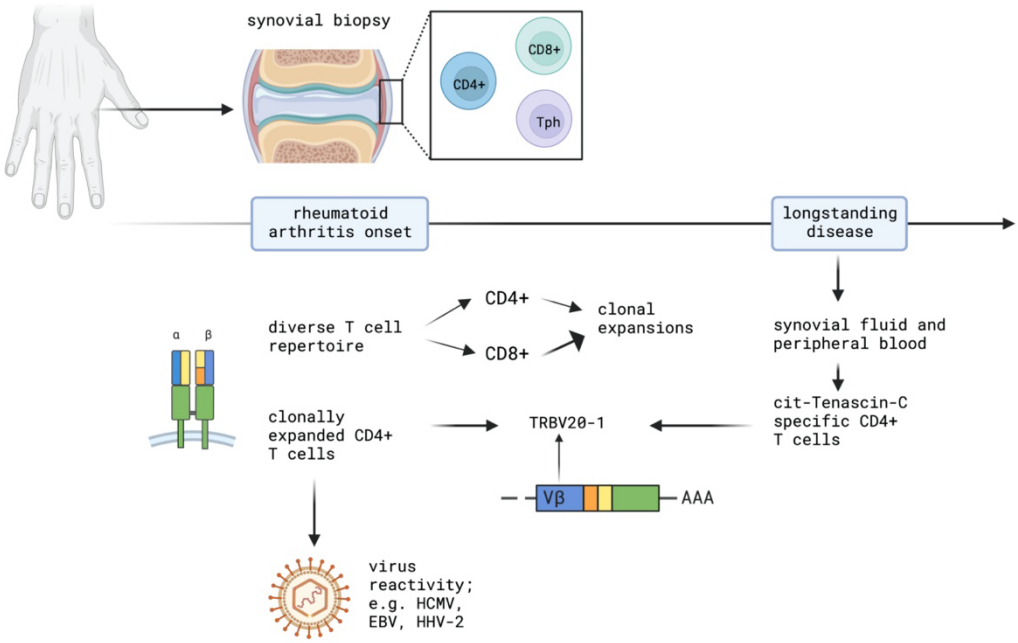

Figure 11. TCR-sequencing reveals a diverse repertoire in the synovium of ACPA+ patients at time of diagnosis. Still, a biased gene usage is seen, in suggesting recognition of a common antigen. Tenascin-C specific CD4+ T cells in longstanding disease interestingly shows the same bias. Additionally, there are Tph cells, indicating B cell helping potential as well as viral reactive CD4+ T cells in the synovium in this early phase of disease.

7 POINTS OF PERSPECTIVE

Performing research can be compared to putting an enormous puzzle together, where ones work hopefully build on to the bigger picture. When considering the future perspective in research, this relates both to which pieces to search for to complete the puzzle – but also to take a step back and look at the picture so far.

Working with *ex vivo* HLA-class II tetramers and deep phenotyping is a strenuous effort both in the lab as with subsequent data analysis. This is however balanced by the rewarding notion that it is possible to identify and characterize rare autoreactive T cells. Whereas there might be additional optimization steps or technical development to improve this technology, I believe that it is a way forward for further understanding of such cells. Additionally, we are just starting to grasp the complexity of our data sets. Hence, the first point of perspective is that we need to make use of the data that we already have, which include T cell receptor repertoire data, using advances in bioinformatics.

Second, the finding of autoreactive T cells is not equivalent to proof of action, why the next step involves examining if such T cell have the ability to cause or promote disease. In line with this our lab is currently establishing a TCR transgenic mouse to pursue this question. Another approach would be to study if autoreactive T cells in fact can help B cells *in vitro*, as it cannot be concluded that an autoreactive B cell is in fact activated by a citrulline specific T cell.

The third point of perspective reflects to the bigger picture. What do our findings mean, and how can we implement our knowledge in clinical practice? Whereas much of the research aim to understand the underlying mechanism of disease, perhaps the direct benefit to the patient is not so evident. However, to me, as a clinician scientist, it is fascinating that we are taking on such different approach in term of immunological assessment in the clinic and in the lab. Flow cytometry phenotyping is a core method in the lab, whereas rarely used in patient management in the clinic. Importantly, the flow cytometry analyses being made in the clinical immunology department need to be standardized and allow for automation which make the use of HLA-tetramers less likely in the clinical routine, although there are suggestions on immunological profiles (e.g., TfhPD1+ and abatacept) that could predict outcome. One way forward would be the person-centered approach that I mentioned in the section on ethical considerations – where one could immunophenotype patients and assess patterns in individual patients in close collaboration between clinic and lab – a step forward to personalized medicine. A different approach would be to perform a multicenter study (e.g., under the umbrella of European collaborations) where all newly admitted patients that are interested in participation are immunophenotyped with a core set of markers in a clinical immunology department (to ensure future feasibility). Additionally, follow-up sampling is made upon clinical follow-up during the first year. With enough samples we might be able to get enough granularity to draw conclusions that can be transferred back to the patient.

Future therapeutic approaches have already been touched upon, and here my work showing that several citrullinated antigens are recognized by T cells throughout the disease course could

form a guide on which antigens to include in tolerization trials. The use of antigen specific tetramers (or other molecules) to target autoreactive T cells for deletion is a tempting but also somewhat frightening thought. One could however imagine that such approach would be best applied already before disease onset, since the immune reaction once directed to the joint is profound and diverse.

Lastly, I am grateful to have been given the opportunity to make a small contribution to the puzzle and I am very much looking forward to seeing how the picture turn out over the years to come.

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FIGURE REFERENCES

All cartoons have been created using BioRender.com, where figure 3 was based on an available template by Jung-Hee Lee (Creator) and Akiko Iwasaki. When applicable additional references are found in the figure legend.

FluoroSpot pictures in figure 7 are taken from one of my experiments.