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STUDY OF AUTOIMMUNE REACTIONS IN RHEUMATOID ARTHRITIS

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Study of Autoimmune Reactions in Rheumatoid Arthritis

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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ABSTRACT

Rheumatoid arthritis (RA) is a systemic autoimmune disease that affects approximately 1% of the population worldwide. Despite being rather common, the etiopathology of RA remains unclear. Approximately two-thirds of patients have antibodies to citrullinated proteins (ACPAs), generally detected using the 2nd generation cyclic citrullinated peptide (CCP2) ELISA. A wealth of evidence implicates ACPA in the etiology of RA: the major risk factors - a group of HLA-DRB1 alleles referred to as the shared epitope (SE), and smoking - have been described to associate primarily with the ACPA-positive subset of RA. Moreover, ACPAs can be detected years before clinical onset, and their presence are highly predictive of progression to RA. However, the mechanism by which ACPAs might contribute to disease, as well as the definitive *in vivo* target, is not understood. The aim of this thesis was therefore to better characterize the ACPA response in RA, in terms of antigen specificity, association with genetic and environmental risk factors, cross-reactivity with carbamylated antigens, presence in CCP2-negative RA, and finally the antigen specificity and functional characteristics of ACPA-positive B cells.

Through studies using affinity purified anti-CCP2 IgG, we could demonstrate that the CCP2 ELISA directly captures the ACPA response, and that ACPA purified in this manner bound a variety of citrullinated peptide epitopes and exhibited binding to RA synovial tissue and immune cells (**Study I**). We also discovered that purified ACPA could bind both carbamylated and citrullinated proteins, and peptide absorption experiments confirmed extensive cross-reactivity between ACPA and anti-carbamylated protein (CarP) antibodies in the context of the candidate autoantigen α-enolase, casting doubt on the specificity of the anti-CarP response, which we posit may be cross-reactive ACPA (**Study II**). By screening 2,836 serum samples from the population-based case-control cohort EIRA on an autoantigen multiplex array, we then showed that "seronegative" RA is not truly a seronegative disease subset. Autoantibodies - including ACPA and rheumatoid factor (RF) - were present in a substantial proportion, and this subset resembled seropositive RA in terms of associations with risk factors (**Study III**). This study highlights the need for new biomarkers, better classification of seronegative RA, and more sensitive clinical tests for seropositive RA. Finally, we utilized a method of B cell immortalization to derive ACPA-producing B cell clones from RA synovial fluid that retained surface immunoglobulin expression. We successfully generated a CEP-1-positive B cell clone from a SE-positive RA patient, and visualized surface binding to citrullinated (but not native) protein (**Study IV**). This pilot study lays the ground for in-depth investigation of the characteristics of the ACPA lymphocyte population, specifically in regards to HLA-DRB1 SE-mediated antigen presentation.

It is my hope that the data presented in this thesis can provide a basis for future studies into the putative specificity and mechanism of the ACPA response, in order to elucidate disease processes in RA and ultimately improve the diagnosis and treatment of the disease.

LIST OF SCIENTIFIC PAPERS

I. Ossipova E, Cerqueira CF, Reed E, Kharlamova N, Israelsson L, Holmdahl R, Nandakumar KS, Engström M, Harre U, Schett G, Catrina A, Malmström V, Sommarin Y, Klareskog L, Jakobsson PJ, Lundberg K. Affinity purified anti-citrullinated protein/peptide antibodies target antigens expressed in the rheumatoid joint. Arthritis Res Ther. 2014;16(4):R167.

II. Reed E, Jiang X, Kharlamova N, Ytterberg AJ, Catrina A, Israelsson L, Mathsson-Alm L, Hansson M, Alfredsson L, Rönnelid J, Lundberg K. Antibodies to carbamylated α-enolase epitopes in rheumatoid arthritis also bind citrullinated epitopes and are largely indistinct from anti-citrullinated protein antibodies. Arthritis Res Ther. 2016;18(1):96.

III. Reed E, Hedström AK, Hansson M, Mathsson-Alm L, Brynedal B, Saevarsdottir S, Jakobsson PJ, Holmdahl R, Skriner K, Serre G, Alfredsson L, Rönnelid J, Lundberg K. How seronegative is ”seronegative” RA – Characterization of autoantibody responses in anti-cyclic citrullinated peptide antibody positive and –negative rheumatoid arthritis. *Manuscript.*

IV. Reed E, Germar K, van Uden N, Hansson M, Israelsson L, Rizzi T, Kwakkenbos M, de Vries N, Spits H, Lundberg K, Baeten D, Malmström V. Isolation, immortalization and characterization of an anti-citrullinated protein antibody producing B cell clone from rheumatoid arthritis synovial fluid. *Manuscript.*

CONTENTS

LIST OF ABBREVIATIONS

Ab	Antibody
ACPA	Anti-citrullinated protein antibody
ACR	American College of Rheumatology
Anti-CarP	Anti-carbamylated protein
Anti-Jo1	Anti-histidyl tRNA synthetase
Anti-Ro/La	Anti-Sjögrens-syndrome-related antigen A/B
Bcl-6	B-cell lymphoma 6 protein
Bcl-xL	B-cell lymphoma-extra large
BCR	B-cell receptor
Carb-CEP-1	Homocitrullinated alpha-enolase peptide-1
CEP-1	Citrullinated alpha-enolase peptide-1
CCP	Cyclic citrullinated peptide
CCP2	Cyclic citrullinated peptide, second generation
CD19	B-lymphocyte antigen 19
CD20	B-lymphocyte antigen 20
CD27	CD27 molecule
CD40-L	Cluster of differentiation 40 ligand
CI	Confidence interval
CRP	C-reactive protein
DAPI	4',6-diamidino-2-phenylindole
DAS28	Disease Activity Score 28
DMARD	Disease-modifying antirheumatic drug
ELISA	Enzyme-linked immunosorbent assay
EULAR	European League Against Rheumatism
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FT	Flowthrough
HLA	Human leukocyte antigen
IgA	Immunoglobulin A
IgG	Immunoglobulin G

IgM	Immunoglobulin M
IL-21	Interleukin 21
MHC-II	Major histocompatibility complex class II
OR	Odds ratio
PBS	Phosphate-buffered saline
PTPN22	Protein tyrosine phosphatase, non-receptor type II (lymphoid)
RA	Rheumatoid arthritis
RF	Rheumatoid factor
SE	Shared epitope
SF	Synovial fluid
ssDNA	Single-stranded DNA
TBS	Tris-buffered saline
TNF-alpha	Tumor necrosis factor alpha

1 INTRODUCTION

1.1 RHEUMATOID ARTHRITIS

Rheumatoid arthritis (RA) is a severe autoimmune disease characterized by chronic inflammation of the joints. RA is endemic in almost all human populations, affecting approximately 0.5-1% of the population worldwide (1). RA affects primarily women, with a roughly 3:1 ratio of women to men (2), and typically occurs later in life (3). In addition to chronic pain, fatigue and possible disability, RA patients also exhibit increased morbidity and mortality, primarily from cardiovascular disease (CVD) (4).

The primary tissue affected by RA is the synovium of the joint. In healthy individuals, the synovial membrane is comprised of a thin cell layer of synovial fibroblasts and macrophages, which maintain homeostasis in the joint. In RA, synovial inflammation leads to the development of a thick cellular layer called the pannus, which begins to grow over the articular surface. Synoviocytes and infiltrating immune cells, most notably neutrophils, macrophages, and T- and B-lymphocytes, fill the inflamed joint with pro-inflammatory cytokines, chemokines, prostaglandins and cartilage-degrading enzymes such as metalloproteinases (5), destroying cartilage as well as driving an inappropriate activation of osteoclasts to resorb bone, ultimately leading to permanent destruction of the joint (6).

RA typically presents as polyarthritis, and is currently diagnosed according to the new unified ACR / EULAR criteria for RA, established in 2010 (7) (Table 1). The 2010 ACR / EULAR criteria additionally classify patients as seropositive or seronegative, based on the presence/absence of specific autoantibodies: rheumatoid factor (RF) directed against the constant region of immunoglobulin G, and anti-citrullinated protein antibodies (ACPA) directed against citrullinated proteins.

Upon diagnosis, treatment is initiated with anti-inflammatory therapies such as glucocorticoids to quickly reduce pain and inflammation, coupled with administration of disease-modifying antirheumatic drugs (DMARDs) and/or biologicals aimed at long-term disease control (8). Aggressive treatment early in diagnosis can delay or prevent many of the more serious manifestations of the disease (9).

Table 1 The 2010 ACR/EULAR classification criteria for RA

	Score*
Symptom Duration	
< 6 weeks	0
≥ 6 weeks	1
Joint Distribution	
1 large joint	0
2-10 large joints	1
1-3 small joints (*with or without involvement of large joints*)	2
4-10 small joints (*with or without involvement of large joints*)	3
> 10 joints (*at least 1 small joint*)	5
Serology	
RF- *and* ACPA-	0
Low RF+ *or* low ACPA+	1
High RF+ *or* high ACPA+	2
Acute Phase Reactants	
Normal CRP *and* normal ESR	0
Abnormal CRP *or* ESR	1

***** Scores of 6 or greater are classified as having RA. CRP = C-reactive protein; ESR = erythrocyte sedimentation rate. Adapted from Aletaha et al (10).

Methotrexate (MTX) remains the standard first-line DMARD in RA (11); however, during the past few decades, a variety of biological DMARDs have entered the clinic, primarily targeting inflammatory cytokines. The most common of these have been biologicals inhibiting anti-tumor necrosis factor (TNF), including adalimumab, certolizumab, etanercept, golimumab and infliximab. In patients refractory to anti-TNF, B-cell depletion via the anti-CD20 antibody rituximab can be effective (12). Other biologicals include anakinra, the IL-1 receptor antagonist, the CTLA4-Ig fusion protein abatacept, which targets T cells, and the anti-IL-6 receptor antibody tocilizumab. The high cost of biologicals precludes their use as first-line DMARDs in many countries; they can also become immunogenic, leading to a loss of efficacy over time (13). Furthermore, many patients do not respond satisfactorily to biologicals due to side effects or for unknown reasons. In addition, even if inflammation subsides, many patients still experience pain and fatigue.

The development of novel therapies requires a greater understanding of the pathological mechanisms underlying RA, which yet remain unclear. Elucidating these mechanisms could provide better tools for earlier diagnosis, individual tailoring of therapies, and potentially make possible the development of antigen-specific tolerizing therapies (14) to treat or even prevent the disease in pre-disposed individuals.

1.2 RISK FACTORS FOR RA: CLUES TO ETIOPATHOLOGY?

Risk for developing RA has a definitive genetic component, with a concordance rate of 7-15% in monozygotic twins (15,16). The major genetic risk factors for RA are associated with adaptive immunity. Chief among these are certain alleles of the gene HLA-DRB1, coding for the beta chain of major immunohistocompability complex type II (MHC II).

1.2.1 Shared epitope

A group of HLA-DRB1 RA risk alleles were found to share the same 5-amino acid sequence motif in the third hyper variable region in the peptide-binding groove, and are therefore collectively referred to as shared epitope (SE) (17). These alleles were later found to share a common arrangement of specific amino acids in certain positions in the peptide binding groove of MHC-II (18). The SE hypothesis proposes that these specific MHC II molecules may efficiently present specific peptides to autoreactive T cells. Conversely, other DRB1 alleles have been found to have a protective effect in RA (19). Importantly, SE is only a risk factor for ACPA-positive disease (20).

1.2.2 PTPN22 polymorphism

In addition to SE, polymorphism in protein tyrosine phosphatase nonreceptor 22 (PTPN22), a protein tyrosine phosphatase expressed in lymphoid cells, have been associated with increased risk for autoimmunity, including RA (21). PTPN22 acts as a negative regulator of lymphocyte activity, and the common C1858T (rs2476601) allelic variant, resulting from a non-synonymous Arg620Trp single nucleotide mutation, is thought to contribute to autoimmunity by increasing survival in autoreactive lymphocytes (22). PTPN22 C1858T is understood to be a shared risk factor for autoimmunity (23), and healthy carriers of the risk allele have been shown to have more autoreactive B cells in the periphery

compared to non-carriers. Interestingly, a gene-gene interaction between SE and PTPN22 has been described to increase RA risk progressively, and while PTPN22 associates with both seropositive and seronegative RA, the gene-gene interaction is only present in the seropositive subset (24).

1.2.3 Other genetic risk factors

In light of the ACPA response in RA, it is interesting that polymorphisms in the gene encoding the citrullinating enzyme peptidyl-arginine deiminase 4 (PAD4) have been associated with RA, primarily in Asian populations (25,26). A wide range of other risk genes have also been identified in RA, a large number of which govern immune function (27). Most of these genetic risk factors seem to associate mainly with ACPA-positive RA.

1.3 ENVIRONMENTAL RISK FACTORS

In addition to genetic risk factors, environmental exposures, hormones, and lifestyle factors contribute the development of RA, with smoking being the most established non-genetic risk factor for RA (28). Interestingly, a gene-environment interaction between SE and smoking exists in seropositive RA (29).

1.3.1 Smoking

An epidemiological association between smoking and RA was first described in 1987 (28) and ten years later explicitly demonstrated in a twin cohort where participants were discordant for RA (30). The association of smoking with RA has since been confirmed in a large number of studies (31). Similarly, silica exposure (32) and textile dust exposure (33) have also been identified as risk factors for RA, collectively implicating inflammation in the lung in the etiology of the disease. Interestingly, it has been shown that chronic inflammation in the lungs of smokers as well as chronic obstructive pulmonary disease (COPD) cause increased protein citrullination (34,35), thereby increasing the exposure of ACPA targets to the immune system; RA-related autoantibodies have also been found (albeit at low levels) in several inflammatory lung diseases (36), and in bronchoalveolar lavage of RA patients (37).

These studies pinpoint the lung as a possible site for initiating the ACPA response. Of note, the lungs are one of the most commons sites of extra-articular manifestations in RA (38).

1.3.2 Microbial exposure

A microbial basis for RA has long been hypothesized, with Epstein-Barr virus, parvovirus and cytomegalovirus infection historically implicated (39), though there is still no consensus regarding these viruses due to contradictory data (40). An association between periodontitis and RA has also long been discussed, with the epidemiological association between these two diseases being recently confirmed in a systemic meta analysis (41). It has been proposed that the link between RA and periodontitis could be accounted for by the oral bacteria *Porphyromonas gingivalis* (*P.g*), a major causative agent in the development of chronic periodontitis, that intriguingly expresses an enzyme capable of protein citrullination (42,43). A number of studies have shown increased levels of antibodies against *P.g.* in RA compared to healthy controls, especially in ACPA-positive RA (44). Moreover, an interaction between elevated anti-*P.g.* antibody levels and HLA-DRB1 SE, as well as cigarette smoking, has been described in the ACPA-positive subset of RA (45). More recently, *Aggregatibacter actinomycetemcomitans (Aa)*, a Gram-negative bacterium linked to the development of aggressive periodontitis, has been demonstrated to induce hypercitrullination in neutrophils via a membranolytic pathway (46). Furthermore, association between SE and the presence of ACPAs and RF was found only in RA patients possessing anti-*Aa* antibodies, suggesting a causal link between exposure to AA and the development of ACPA-positive RA. Notably, citrullinated proteins are present in inflamed gingival tissue (43,47), and ACPA have been reported in non-RA patients with chronic periodontitis (48,49).

1.4 ROLE OF AUTOANTIBODIES IN RA: BIOMARKERS AND POTENTIAL PATHOLOGICAL AGENTS

Originally described in the 1930s, rheumatoid factor (RF), autoantibodies to the Fc portion of IgG, is one of the earliest known biomarkers for RA (50). While IgM RF is the most common isotype and most often measured clinically, IgG and IgA forms of RF also exist. IgA RF in particular is associated with smoking in RA patients (36,51), implicating mucosal immunity in the pathogenesis of the disease. Around two-thirds of RA patients are RF-positive, and RF-positive disease has been associated with a more severe disease course (52). While RF positivity has long been used to diagnose RA, its utility as a biomarker is

handicapped by its low sensitivity: among other instances, RF is found in other autoimmune diseases (53), B-cell lymphomas, as well as in systemically healthy individuals during infections (54). RF would therefore seem to be more associated with states of immune activation than RA *per se.*

In addition to RF, a variety of other autoantibodies have been historically associated with RA. Of these, the most important were antibodies binding to keratin (anti-keratin antibodies, or AKA), and the so called anti-perinuclear factor (APF) (55), later identified as antibodies to filaggrin, a keratin-binding structural protein (56). As filaggrin is not expressed in the joint, further research into the true antigenic target of these autoantibodies eventually led to the historic discovery that their real target was in fact citrullinated proteins (57,58). These autoantibodies are now collectively referred to as anti-citrullinated protein antibodies, or ACPAs. The cyclic citrullinated peptide (CCP) test, which was the first commercially available assay for ACPA positivity, was based on cyclic citrullinated filaggrin-derived peptides (59); however, the second generation CCP test (CCP2) currently used in many clinics uses a combination of synthetic citrullinated peptide epitopes derived from a phage display library to provide an optimal combination of sensitivity and specificity for diagnosing RA (60). Importantly, the CCP2 peptide does not contain sequences derived from human proteins.

The presence of ACPAs detected in this manner is highly specific for RA (i.e. 98%) (61), and around two-thirds of RA patients are positive for ACPAs. Together with RF, ACPA positivity is now a part of the current ACR EULAR criteria for RA (7), and the presence of ACPA and/or RF defines the seropositive subset of the disease. As with RF (62), ACPAs can be present for many years before joint symptoms develop, and are predictive of progression to RA (63,64), as well as a more destructive disease course, suggesting a direct pathogenic involvement. Crucially, it is now understood that SE and smoking are associated mainly with the development of ACPA-positive disease (65). However, given the extensive co-occurrence of ACPA and RF, and the strong gene-environment interaction between SE and smoking, it becomes difficult to elucidate the mechanistic relationship between ACPA and RF and SE/smoking in the etiopathogenesis of seropositive disease. Still, in recent years, focus has moved from RF to ACPA, when trying to understand the pathways underpinning RA.

1.5 CITRULLINATION IN HEALTH AND DISEASE

Whereas L-citrulline is an ubiquitous non-coded amino acid generated as an intermediate in the urea cycle, peptidyl citrulline is found exclusively as a post-translational modification (PTM) arising from deimination of arginine residues by a class of calcium-dependent enzymes, the peptidyl arginine deiminases, or PADs. Five different PAD isoforms have been described in humans, with differences in tissue expression, substrate specificity and subcellular localization (66). Of these, only PAD2 and PAD4 have been identified in the synovium; their increased expression has been correlated with the degree of inflammation in synovitis (67,68).

Physiological citrullination is widespread: for example, the production of functional myelin in the central nervous system requires citrullination, and citrullination takes place during the formation of cornified layer of the epidermis (69). While upregulation of citrullinated proteins was at first posited to be a specific feature of rheumatic synovitis, citrullination has also been observed to be increased in non-RA synovitides (70). It has since been observed that inflammation-related upregulation of citrullination is not specific to joints, but seems to be a general feature of inflammation (71). For instance, increased protein citrullination has been described in the lungs of smokers (34,35), in the gingival tissue of periodontitis patients (47), in inflamed tonsils (71), in the brain of patients with Alzheimer's disease (72) and multiple sclerosis (73), and in the muscles of myositis patients (29). Citrullinated proteins are also present in atherosclerotic plaques, providing a potential mechanistic link to cardiovascular disease in ACPA-positive RA (74).

The link between increased protein citrullination and inflammation is not fully understood, but could be related to increased necrotic cell death. PAD activity is dependent on high Ca^{2+} concentrations, which are normally not present intracellularly, but when the cell membrane integrity is lost - as a result of terminal differentiation of cells or necrosis - the intracellular Ca^{2+} concentration is increased, and PAD can become activated. During necrosis, PAD could also be released to the extracellular space, with the potential to citrullinate extracellular proteins (75).

1.6 CITRULLINATED CANDIDATE AUTOANTIGENS

While first described as binding to citrullinated fibrinogen present in the synovium (76), the "true" antigenic target of ACPAs is unknown: ACPAs have been demonstrated to bind to a variety of candidate citrullinated autoantigens also present in the synovium: vimentin (77), collagen type II (78), α-enolase (79) and more recently histones (80,81).

1.6.1 Fibrinogen

Fibrinogen is a hexameric plasma glycoprotein central to coagulation. As the inactive precursor of fibrin, fibrinogen is cleaved by the protease thrombin to fibrin monomers, which then polymerize to form a clot. Antibodies to citrullinated fibrinogen epitopes are found in approximately 50-60% of RA patients (82). Deposition of fibrin in the synovium is a feature of early RA synovitis, and has been posited to be a possible mechanism for the initiation of pannus formation (83).

1.6.2 Collagen type II

Collagen type II is a major constituent of hyaline cartilage, and is therefore a logical candidate antigen for RA. Immunization with collagen type II in rodents leads to the development of autoimmune arthritis resembling RA (collagen-induced arthritis, or CIA) (84), a widely-used animal model dependent on activation of both T (85) and B (86) lymphocytes. Antibodies to citrullinated collagen type II are found in up to 40% of RA patients (78).

1.6.3 Vimentin

Vimentin is a ubiquitously expressed component of intermediate filament, a major constituent of the cytoskeleton in eukaryotic cells. Antibodies to a mutated citrullinated isoform of vimentin (MCV) are found in approximately two-thirds of RA patients, and are used clinically as an alternative to the anti-CCP2 test, with comparable sensitivity and specificity (87), and potentially superior to CCP2 as a marker for joint destruction (88). While primarily an intracellular protein, one potential source of extracellular vimentin could be secretion by activated macrophages (89), which could then be citrullinated in an inflammatory microenvironment.

1.6.4 α-Enolase

α-enolase, or non-neuronal enolase, is an isoform of enolase, an ubiquitously expressed glycolytic enzyme. Antibodies to the immunodominant citrullinated peptide epitope CEP-1 are found in approximately 40% of RA patients (90). α-enolase is expressed in the cytoplasm and in the nucleus, and on the surface where it may function as a plasminogen receptor, which is upregulated during inflammation (91). Increased expression of α-enolase has for example been demonstrated in RA synovial fluid and -synovial tissue (79,92). Additionally, isoforms of enolase with a high sequence similarity to human α-enolase are expressed by several pathogenic bacteria, including *Porphyromonas gingivalis*. Anti-CEP-1 antibodies purified from RA sera bind citrullinated *P.g.* enolase, suggesting that molecular mimicry may play a role in the generation of the ACPA response in RA.

1.6.5 Histones

Large numbers of activated neutrophils infiltrate the RA synovial fluid and pannus (93), where they comprise a source of inflammatory signals and cause tissue damage through the generation of reactive oxygen species (ROS). They may also contribute specifically to the ACPA response through NETosis, a mechanism normally used by neutrophils to kill extracellular bacteria, in which activated neutrophils release their chromatin extracellularly, creating a sticky mesh of chromatin complexed with cytotoxic granules called neutrophil extracellular traps (NETs) to snare and kill pathogens (94). Hypercitrullination of histones by PAD4 is understood to be crucial to this process (95). NETosis is upregulated in RA and correlates with ACPA status (80), and ACPAs have been shown to specifically bind to citrullinated histones (81).

1.7 AN ETIOLOGICAL HYPOTHESIS FOR THE DEVELOPMENT OF ACPA-POSITIVE RA

Based on the epidemiological association between RA and smoking, and between RA and periodontal disease, and the observation of increased PAD activity/protein citrullination in inflamed lungs and gingival tissue, it has been suggested that ACPA-positive RA may be triggered at mucosal sites, and a potential etiological model has emerged for the development of ACPA-positive RA (Figure 1, reviewed in (96)).

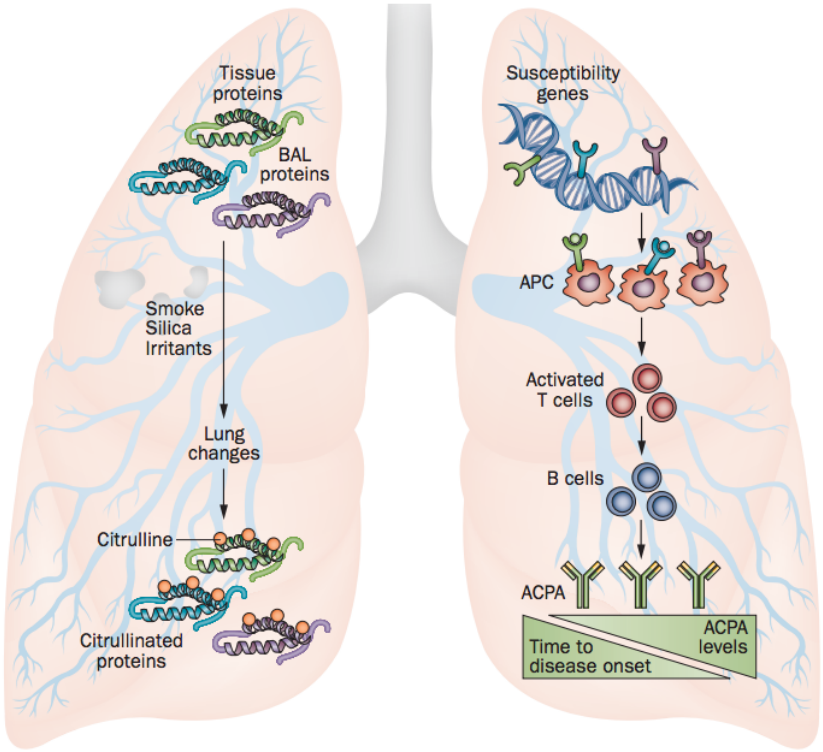

Figure 1: An etiological model for the etiology of ACPA-positive RA. Genetic risk factors (SE and *PTPN22*) and environmental insults (smoking, particulates, microbes) interact to cause a break in immunity towards citrullinated self-antigens in mucosal tissue, leading to autoimmunity and the production of ACPAs prior to joint pathology, which likely requires a second "hit" to initiate RA. Reproduced with permission from Nature Reviews Rheumatology (97).

First, environmental factors such as smoking or infections lead to increased protein citrullination as a result of inflammation and subsequent upregulation of PAD activity. In the presence of danger signals (such as toxic components of cigarette smoke and/or bacterial DNA/LPS etc.), a break in tolerance to citrullinated protein(s) may occur, especially in genetically predisposed individuals – most importantly through SE-restricted presentation of citrullinated peptides on MHC II (29) to pathogenic T cells, and through increased resistance of autoreactive lymphocytes to induction of tolerance in the presence of PTPN22 risk alleles. Activated T cells subsequently help B cells to produce pro-inflammatory cytokines and high affinity ACPA, through clonal selection and antigen-driven affinity maturation.

Since ACPAs can be present for many years prior to the development of synovitis, a "second hit" is likely required in these pre-disposed individuals, in which an initiating event gives rise to inflammation in the joint, causing an upregulation of citrullination and activation and broadening of the ACPA response (98). Ultimately this results in a vicious circle of cell death, PAD activation, citrullination, ACPA production, immune complex formation, Fc receptor and/or complement engagement, and the activation of osteoclasts leading to increased bone resorption (99), eventually causing chronic inflammation and RA.

The mechanism through which ACPAs exert these effects is not understood, but a wealth of experimental evidence implicates that they may act in a variety of ways to contribute to disease (100). For example, immune complexes containing citrullinated proteins and ACPA can active immune cells through Fc-receptor engagement (101), and are potentiated by RF (102). They might also function in an Fc-independent manner: the increased activation of osteoclasts by ACPAs has been demonstrated to occur through a chemokine-dependent mechanism (103), and a similar mechanism is thought to be responsible for pain behavior upon transfer of ACPAs to mice through activation of nocicecptors (104); low-avidity ACPA have also been demonstrated to be strong activators of complement (105).

1.8 ANTIBODIES TO CARBAMYLATED PROTEINS

Recently, antibodies to carbamylated proteins were identified in RA, denoted "anti-CarP antibodies" (106). This confirmed earlier studies which described similar autoimmune reactions in animal models of arthritis (107,108). Carbamylation is a non-enzyme-dependent process in which the primary amines in the side chains of lysine residues react with cyanate ions to form homocitrulline, which is chemically similar to citrulline but contains an additional methyl group (Figure 2). Carbamylation is upregulated in inflammation, primarily through the production of cyanate by neutrophil myeloperoxidase; notably, increased plasma cyanate is also found in smokers (109).

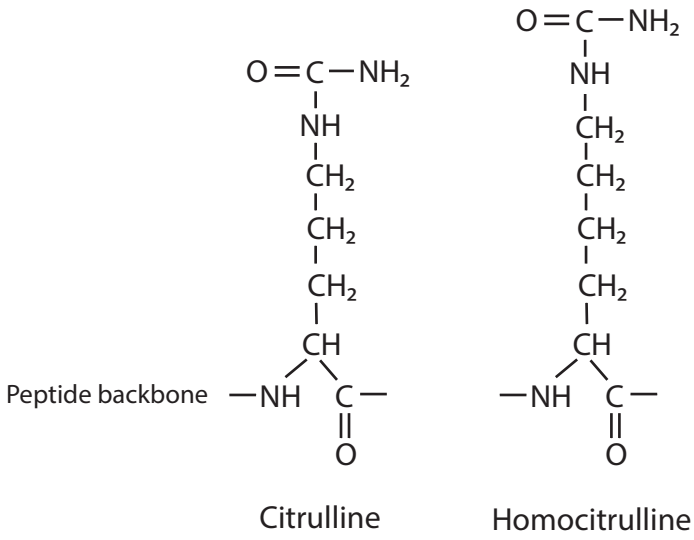

Figure 2: Peptidyl citrulline (left) and peptidyl homocitrulline (right). Citrulline is a noncharged amino acid resulting from deimination of arginine residues; homocitrulline is a noncharged amino acid resulting from carbamylation of lysine residues.

Anti-CarP antibodies share many similarities to ACPAs: they are specific for RA (though they have also been described at low frequencies in primary Sjögren's disease (110)), and they are present prior to diagnosis, in which case they are also predictive for the development of RA (111). It has since been shown that the most commonly used *in vitro* method for the detection of peptidyl citrulline, the Senshu method (112), does not discriminate between homocitrulline and citrulline (113). Additionally, work with carbamylated fibrinogen has revealed cross-reactivity with ACPAs (114); however, several animal models of RA have been described to develop anti-CarP antibodies without ACPA (115). Importantly, there is no association between the presence of anti-CarP antibodies and specific risk factors for RA, such as SE alleles or smoking (116). In addition to carbamylated fibrinogen, anti-CarP antibodies have been demonstrated to target carbamylated vimentin (117,118). Antibodies to acetylated vimentin have also been described in RA (119).

1.9 THE ROLE OF B CELLS IN RA

RA synovitis is typically characterized by infiltration of both innate and adaptive immune cells, and the formation of ectopic lymphoid structures, resembling germinal centers, has been described in the joints during chronic inflammation (120). While T cells have historically been implicated as major drivers of disease (121), the relative increase in efficacy of the B cell-depleting therapy rituximab in seropositive RA *versus* seronegative RA suggests an important role for B cells in ACPA-positive RA (122-124). Importantly, rituximab treatment does not fully deplete B cells in RA synovium, which is posited to be a consequence of the abundance of pro-survival factors in the inflamed synovial microenvironment (125), and as rituximab is an anti-CD20 antibody, it does not target plasma cells or plasma blasts, which do not express CD20. ACPA production is thought to also occur locally in inflamed joints: ACPA IgG is enriched in the synovium (126,127), and single-cell cloning experiments have revealed that a large proportion of synovial B cells are ACPA-positive (128).

In addition to their function as antibody secreting cells, B cells could also initiate or drive disease through cytokine secretion as well as their ability to serve as specific antigen-presenting cells (APCs). The latter is an intriguing possibility for initiation of disease in the absence of an obvious trigger, as even small amounts of citrullinated autoantigens could be efficiently captured and presented by ACPA-positive B cells, and could represent a direct mechanism for the contribution of SE alleles to RA pathogenesis.

2 AIMS

The general aim of the work presented in this thesis was to investigate specific autoimmune reactions in RA, with focus on the ACPA response, in order to learn more about disease processes in RA. This was to be accomplished in the following projects:

1. Purify and characterize the antigenic specificity of ACPAs (**Study I**)
2. Investigate the specificity of ACPAs in regards to the recently described autoantibodies to carbamylated proteins (**Study II**)
3. Analyze and characterize the CCP2-negative subset of RA in regards to ACPA fine-specificities, RF isotypes and potential reactivity to non-RA-specific autoantigens, in relation to RA risk factors (**Study III**)
4. Generate genetically reprogrammed B clones towards the investigation of the antigen specificity and functional charactersitics of ACPA-positive B cells (**Study IV**)

3 MATERIALS AND METHODS

3.1 RA PATIENT MATERIAL

In **Studies I** and **II**, anti-CCP2 IgG or anti-CEP-1 IgG were purified from non-paired synovial fluid (SF) and plasma samples collected with informed consent from RA patients attending the rheumatology clinic at Karolinska University Hospital, Stockholm, Sweden, from 2001 to 2011. SF was collected from patients requiring arthrocentesis. All patients fulfilled the ACR/EULAR criteria for RA and were selected on the basis of having high anti-CCP2 antibody levels (>300 AU/mL), or a strong anti-CEP-1 antibody response. Samples were stored at −20°C (short term) or −80°C (long term) until processed. In **Study I**, ACPA binding to *in vivo*-generated antigens was investigated using SF mononuclear cells isolated from RA patients requiring arthrocentesis, and synovial tissues obtained from RA patients undergoing hip or knee joint replacement surgery, at the Karolinska University Hospital. In **Study IV**, immortalized B cells were generated from SF mononuclear cells, obtained from two patients requiring arthrocentesis: an ACPA-positive / HLA-DRB1-SE-positive RA patient, attending the Karolinska University Hospital, and an ACPA-negative / HLA-DRB1-negative, non-RA control patient attending the Academic Medical Center (AMC), in Amsterdam, the Netherlands.

3.2 EIRA

In **Studies II and III**, a serum biobank from the Swedish population-based case-control study EIRA (Epidemiological Investigation of RA) was used to investigate antibody reactivities. The EIRA cohort consists of newly diagnosed RA cases and controls matched for age, sex and residential area. Blood samples were collected at the time of recruitment, and information on cigarette smoking (as well as other environmental exposures) was obtained via self-reported questionnaire at baseline (129). Genotyping for the identification of SE alleles (i.e. HLA-DRB1*01 (except DRB1*0103), *04 and *10) and PTPN22 polymorphism (i.e. rs2476601) was performed on DNA from blood as previously described (24,65). Information on C-reactive protein (CRP) levels and disease activity score for 28 joints (DAS28) was obtained by linking EIRA with the Swedish rheumatology register, where clinical data is stored as part of standard care (130). Serum anti-CCP2 antibody levels were measured in house, as described below (20).

For the studies included in this thesis, information on genetics, smoking habits, CRP levels, DAS28 and anti-CCP2 antibodies were retrieved from the EIRA database.

3.3 ELISA

In all studies, CCP2 positivity was determined in house using the anti-CCP2 IgG ELISA assay Immunoscan CCPlus® from Euro-Diagnostica AB, Malmö, Sweden, according to manufacturer instructions. Cutoff for positivity was 25 AU/mL. For detection of ACPA fine-specificities, and anti-CarP antibodies, in house peptide and protein ELISAs were used as previously described (127,131). The use of a serum pool standard, made comparisons between different plates possible. Cutoff for positivity for each peptide ELISA was calculated on the basis of the 98th percentile in a group of 150 randomly selected EIRA controls.

3.4 ISAC MULTIPLEX

High-throughput investigation of antibody reactivities in the EIRA cohort was made possible through the use of the ImmunoCAP Immuno Solid-phase Allergen Chip (ISAC) peptide array system (Phadia AB, Uppsala, Sweden) containing antigens of interest, in **Studies II, III** and **IV**, as previously described (132,133). Antigens are spotted onto glass slides, which are then incubated with sample, washed to remove unbound antibody, and analyzed using a Cy3-conjugated goat anti-human IgG (Jackson ImmunoResearch Laborities, Newmarket, UK). Antibody binding is then recorded as fluorescence intensity using a laser scanner, which is converted to normalized arbitrary units (AU/ml) after normalization against an internal control spot on each slide. Cutoff for positivity for each antigen was calculated as the 98th percentile using a group of 370 EIRA controls.

3.5 ACPA PURIFICATION

In **Study I** and **Study II,** polyclonal ACPA IgG was purified from ACPA-positive RA patient plasma and SF samples for use in *in vitro* experiments through affinity chromatography using the CCP2 peptide(s), kindly donated by Euro-Diagnostica AB. In brief, SF samples were centrifuged and treated with hyaluronidase to reduce viscosity, then proteins were precipitated and diluted in PBS; plasma samples were centrifuged and diluted

in PBS. Bulk IgG was isolated on Protein G coupled columns before CCP2 IgG was purified on CCP2 affinity columns. The CCP2-depleted flow through (FT) fraction was also preserved. In **Study II**, anti-CEP-1 IgG was isolated using a similar method: CEP-1 peptides, and the arginine-containing version REP-1, were directly coupled to NHS-Sepharose columns at 1 mg/ml. Protein G column purified IgG fractions from five CEP-1-positive serum samples were subsequently purified on the CEP-1 affinity column following pre-absorption on the REP-1 column to eliminate any non-citrulline-specific antibodies. Also here, FT fractions were preserved. Purified anti-CCP2 IgG, anti-CEP-1 IgG and FT IgG were concentrated and buffer exchanged to PBS before aliquoted and stored at -20°C until further analyzed, or long-termed stored at -80°C. A total of three CCP2 pools were generated: one SF CCP2 pool from n=26 RA patients, and two plasma CCP2 pools from n=16 and n=38 RA patients, respectively. Purified anti-CEP-1 antibodies and FT fractions from the five CEP-1-positive RA patients were not pooled, but kept separate.

3.6 GENERATION OF POST-TRANSLATIONALLY MODIFIED PROTEINS

In **Studies I, II,** and **IV**, citrullinated and carbamylated protein antigens were generated in house for use in *in vitro* experiments. Citrullination was performed by incubating proteins at a concentration of 1 mg/ml in PAD buffer (100 mM Tris, 10 mM $CaCl_2$, 5 mM dithiothreitol (DTT), pH 7.6) with 2 U/mg protein of rabbit skeletal muscle PAD2 enzyme (Sigma, St. Louis, MO, USA) for 2 h at 37° C. The reaction was terminated by the addition of 20 mM ethylenediaminetetraacetic acid (EDTA), followed by thorough dialysis to calcium-free PBS. Carbamylation was performed by incubating proteins at a concentration of 1 mg/ml in PBS in the presence of 100 mM potassium cyanate overnight at 37°C, followed by thorough dialysis to calcium-free PBS. Successful citrullination and carbamylation were confirmed through mass spectrometry.

3.7 IMMUNOBLOTTING

In **Study I** and **Study II**, the purified ACPAs were analyzed for reactivity against modified proteins using Western Blot. Briefly, citrullinated, carbamylated or unmodified proteins were separated electrophoretically on NuPAGE® Bis-Tris 4-20 % gels and transferred to nitrocellulose membranes. Membranes were blocked (5% milk in TBS/0.05% Tween) before incubation overnight at 4°C with either purified anti-CCP2 IgG or FT IgG at a concentration

of 2 μg/ml in blocking buffer. Detection antibody (HRP-conjugated goat anti-human IgG) was added at a dilution of 1:10,000 in blocking buffer for 1 hour at RT, following detection using ECL chemiluminescence.

3.8 IMMUNOHISTO- / IMMUNOCYTOCHEMISTRY

In **Study I,** purified ACPAs were analyzed for binding to *in vivo* generated antigens using immunohisto- and immunocytochemistry. Briefly, synovial tissues from hip or knee biopsies were obtained from three RA patients undergoing joint replacement surgery. Snap frozen biopsies were cryostat sectioned, fixed with 2% formaldehyde, and stored at −80°C, before stained; SF mononuclear cells from three RA patients were isolated by ficoll separation, fixed in 2% formaldehyde, and stained. The SF ACPA IgG pool and corresponding FT IgG pool, described above, were biotinylated, before added at a concentration of 10μg/mL (on synovial tissues) or 5μg/mL (on SF mononuclear cells). Cells were permeabilized using PBS/saponine. Detection of bound antibodies was visualized using the vectastain detection system (on synovial tissues) or Streptavidin/HRP (on SF mononuclear cells).

3.9 PEPTIDE ABSORPTION ASSAY

In **Study II**, cross-reactivity between CEP-1 and carb-CEP-1 was determined through a peptide absorption assay. In brief, serum samples from CEP-1 single positive patients (n=4), carb-CEP-1 single positive patients (n=4) or CEP-1/carb-CEP-1 double positive patients were diluted 1:100 in RIA buffer and incubated with 100 μg/ml of either CEP-1 or carb-CEP-1 peptide for 2 h at RT, before assayed for reactivity to CEP-1 or carb-CEP-1 using ELISA.

3.10 B CELL CLONING

3.10.1 B cell immortalization and cell culture

In **Paper IV**, B cells were isolated from SF mononuclear cell samples (one RA patient and one non-RA disease control patient) via positive selection with CD22 Microbeads (Miltenyi Biotech). IgG-expressing memory B cells ($CD19^{+}CD27^{+}IgM^{-}IgA^{-}$) were subsequently isolated by flow cytometry on a FACSAria (Becton Dickinson); cells were then transduced with a retroviral vector expressing green fluorescent protein (GFP) marker, human Bcl-6 and

Bcl-xL, as previously described (134). GFP-positive clones were single-cell sorted by FACS into 96-well plates, expanded and maintained at a density of $2x10^5$ cells/ml in expansion medium (IMDM + 8% FBS, penicillin/streptomycin and 25 mg/ml recombinant mouse IL-21) in co-culture with γ-irradiated (50 Gy) mouse L-cell fibroblasts expressing human CD40L, plated at a density of $1x10^5$ cells/ml.

3.10.2 Antigen uptake assay

In **Paper IV**, the ability of B cell clones to bind and internalize citrullinated fibrinogen was assessed using an antigen uptake assay. Human fibrinogen conjugated to Alexa-594 (Fib-594) was citrullinated according to the protocol described above. GFP-expressing B cell clones at a density of $1x10^5$ cells/ml were then incubated with either unmodified (Fib-594) or citrullinated (cit-Fib-594) protein at a concentration of 1 μg/ml for 30 minutes at 4°C in PBS + 10% FBS. Cells were then washed with PBS at RT, resuspended in VECTASHIELD mounting medium (Vector Labs) containing 4',6-diamidino-2-phenylindole (DAPI) for nuclear visualization, and transferred to glass slides for fluorescence microscopy using a Zeiss LSM710 Laser Scanning Microscope (Zeiss).

3.11 STATISTICAL METHODS

Differences in antibody levels (**Study I, II** and **III**), number of ACPA fine-specificities (**Study II**), DAS28 and CRP levels (**Study III**), between groups, were analyzed using Mann-Whitney U test for independent groups. In **Study II** and **III**, odds ratios (OR) with 95% confidence intervals (CI) were calculated for associations between risk factors (i.e. smoking, HLA DRB1 SE and PTPN22 rs2476601) and different RA subsets. These analyses were performed in SAS version 9.3 (SAS Institute, Cary, NC, USA), and adjusted for age, gender and residential area. In **Study III**, co-occurrence of ACPA fine-specificities was calculated using Pearson correlation (R v. 3.3.3). For all statistical calculations, *P*-values <0.05 were considered significant.

3.12 ETHICAL CONSIDERATIONS

The purpose of this PhD project - to gain a better understanding of the autoimmune reactions in RA - is aimed at benefiting patients. The project has involved patient material, and ethical considerations included protection of privacy and handling of personal data and biological material. All biological samples were collected with informed consent and ethical approval granted by the regional ethics review board at Karolinska Institutet, Stockholm, Sweden, or in the case for the patient from AMC in **Study IV**, from the medical ethical committee of AMC, Amsterdam, the Netherlands. Personal information has been handled under PUL, and kept behind the Karolinska University Hospital's network firewall. Stored data does not include personal identifiers. All scientists and clinicians involved in the project have adhered to Good Clinical Practice / Good Laboratory Practice Guidelines. All studies were conducted in compliance with the Declaration of Helsinki: Ethical Principles for Medical Research Involving Human Subjects (135).

4 RESULTS AND DISCUSSION

4.1 STUDY I

While the CCP2 ELISA is commonly used for diagnosing ACPA-positive RA in the clinic, the CCP2 peptide (or peptides) itself is an artificial antigen whose sequence does not match any human proteins, and is therefore considered a surrogate marker for the true *in vivo* antigenic targets of ACPAs. Furthermore, whether or not the antibodies detected by the CCP2 ELISA are the same that react with the numerous candidate citrullinated autoantigens identified in RA had never been explicitly demonstrated, with the exception of citrullinated fibrinogen (136). Therefore, in our first study, we set out to characterize the specificity of ACPAs detected by the CCP2 ELISA by isolating them from ACPA-positive RA patient samples through CCP2 affinity chromatography. Synovial fluid (n=26) and plasma (n=16) samples were collected from ACPA-positive RA patients with higher anti-CCP2 antibody levels (>300 AU/mL). Bulk IgG was first purified using Protein G columns, followed by isolation of anti-CCP2 IgG using an affinity column containing the CCP2 peptide, kindly donated by EuroDiagnostica AB. Flowthrough IgG (FT) from the CCP2 column was also saved and analyzed.

We found that polyclonal ACPA IgG could be efficiently isolated using CCP2 peptide affinity chromatography, and that these antibodies constitute approximately 2% of the total IgG pool, in both plasma and SF. The resulting CCP2 column eluates were found to bind a range of citrullinated peptide autoantigens *in vitro* using ELISA, as well as specifically binding to citrullinated (but not native) versions of human fibrinogen, vimentin and α-enolase proteins in Western blot. Conversely, the flowthrough fraction showed no binding in either ELISA or WB to either citrullinated or native peptides or proteins. Importantly, purified anti-CCP2 IgG also bound *in vivo* targets in RA synovial tissue and on immune cells isolated from RA synovial fluid, whereas FT IgG did not (Figure 3). This could indicate a direct involvement of ACPAs in RA joint pathology.

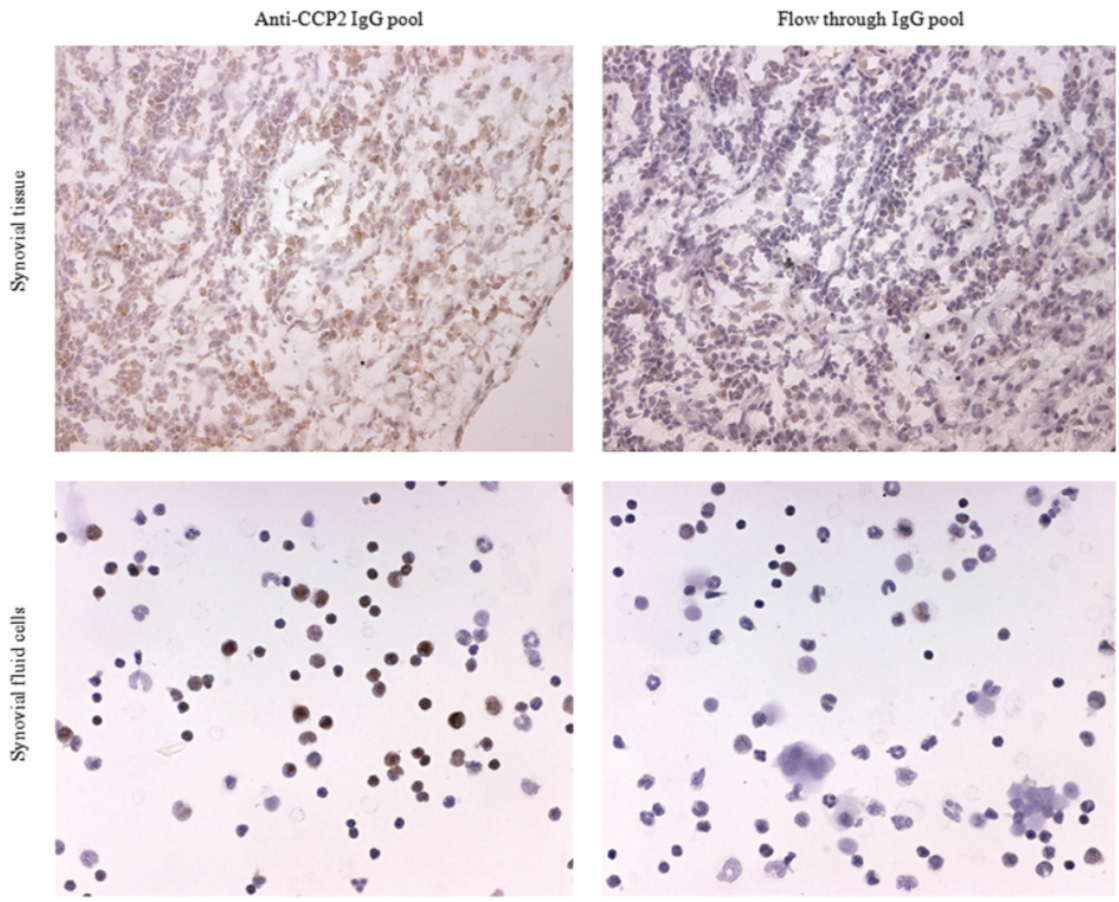

Figure 3: Immunohisto-/immunochytochemistry demonstrating binding of purified and biotinylated ACPA IgG to antigens expressed in RA synovial tissue and SF mononuclear cells. There is no binding of FT IgG. Figure from **Study I**.

Taken together, these results provide direct evidence that the autoantibodies measured by the CCP2 ELISA are the same ACPAs identified as binding to citrullinated peptide and protein antigens *in vitro,* and provide further evidence for the direct binding of ACPAs to antigens expressed in the inflamed joint. In addition, this study provided a large pool of polyclonal ACPA IgG for use in-house and in collaborations to study the characteristics and potential pathogenic effects of ACPA in a variety of *in vitro* and *in vivo* systems. The purified ACPAs have for example been used to demonstrate that the Fc-glycan profile of ACPAs differs from non-ACPA IgG, suggesting differences in effector and immunoregulatory functions (137). Moreover, the ACPA pool was shown to induce pain in an experimental animal model (104), and to active osteoclasts *in vitro* (103). **Study I** additionally provided the technical basis for the efficient affinity purification of ACPA IgG, something that was implemented in **Study II**.

4.2 STUDY II

In addition to ACPAs and RF, antibodies to carbamylated proteins (anti-CarP antibodies) have been described in RA (106). While originally described as a distinct RA-specific autoantibody system, the biochemical similarity between citrulline and homocitrulline calls this into question, especially in light of the fact that purified ACPAs have been demonstrated to also bind carbamylated fibrinogen (114). Moreover, the presence of anti-CarP antibodies largely overlaps with ACPAs, which was demonstrated in both EIRA as well as the Dutch Early Arthritis Clinic (EAC) cohort (116). This led us to hypothesize that these antibodies were possibly no more than cross-reactive ACPAs. Prior to our study, the only protein that had been investigated in the context of anti-CarP reactivity was fibrinogen. Thus, we decided to focus on another candidate autoantigen in RA, α-enolase.

Using a pool of anti-CCP2 IgG purified from 38 RA patients, in the same manner as in **Study I,** we were first able to demonstrate that anti-CCP2 IgG could bind both citrullinated and carbamylated (but not native) fibrinogen and α-enolase in Western blot (WB) (Figure 4).

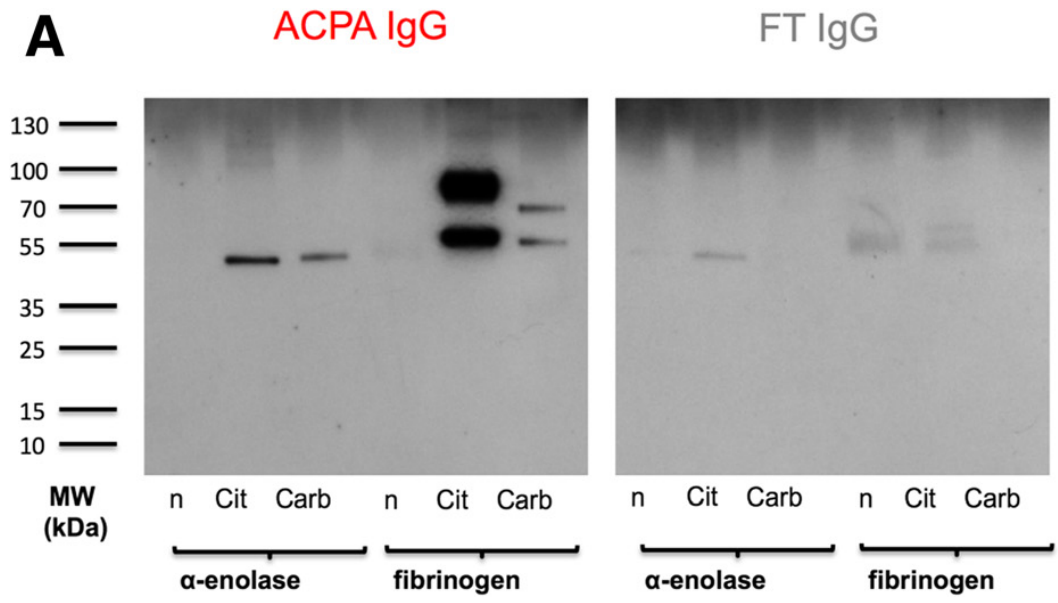

Figure 4: Western blot demonstrating binding of purified ACPA IgG to citrullinated and carbamylated α-enolase and fibrinogen. There is no binding to unmodified proteins, and no (or only weak/background) binding when blotting with FT IgG. Figure from **Study II**.

To investigate this crossreactivity in more detail, we then synthesized a "carbamylated" version of the immunodominant citrullinated α-enolase peptide epitope (CEP-1) - identical in sequence but with the citrullines replaced with homocitrullines - denoted carb-CEP-1. Sera from 2,836 EIRA patients and 373 EIRA controls were analysed for reactivity to CEP-1 and carb-CEP-1 in a high-throughput manner using the ISAC microarray. 41% of RA patients were found to be positive for anti-CEP-1 antibodies, in accordance with earlier data (131,138); the 21% of RA patients positive for anti-carb-CEP-1 antibodies were almost exclusively detected in the CEP-1-positive subset. Importantly, only 3% of patients were single positive for anti-carb-CEP-1 antibodies, and these patients had significantly lower anti-carb-CEP-1 IgG levels than the double positive subset. Moreover, this subset was nearly eliminated (<1%) when the assay specificity was increased from 98% to 100%.

In order to examine if the high co-occurrence of anti-CEP-1 and anti-carb-CEP-1 antibodies was due to crossreactivity, we then performed peptide absorption experiments. Using sera from patients positive for either anti-CEP-1 antibodies only (n=4), anti-carb-CEP-1 antibodies only (n=4), or both anti-CEP-1 and anti-carb-CEP-1 antibodies (n=4), we found crossreactivity to be consistently present, although at varying levels.

To examine this cross-reactivity further, we purified anti-CEP-1 IgG from five CEP-1-positive patients, using the same method as in **Study I** but with a CEP-1 column instead of CCP2. In ELISA, we found that purified anti-CEP-1 IgG showed strong and consistent binding to the CEP-1 peptide, but also varying degrees of binding to carb-CEP-1. FT IgG from the CEP-1 column did not bind to either peptide, indicating that antibodies binding to carb-CEP-1 were confined to the CEP-1 column eluate. These results clearly demonstrate that antibodies to citrullinated α-enolase also have the ability to bind to carbamylated epitopes.

Positing that this cross-reactivity might be explained by the genetic or environmental basis of the disease, we also examined association with RA risk factors. Smoking and PTPN22 polymorphism showed no specific association with carb-CEP-1 positivity, in line with previous reports (116), while SE was significantly associated with both CEP-1 and carb-CEP-1 positivity. The strength of the association was increased in double-positive patients compared to single-positive, indicating a SE-mediated effect on the development of cross-reactive antibodies. Notably, we also observed an overall stronger ACPA response in the carb-CEP-1/CEP-1 double positive subset, compared to the CEP-1 single positive subset.

Taken together, our data question the previous notion of anti-CarP antibodies as a separate autoantibody system in RA, and we propose that anti-CarP antibodies are cross-reactive ACPAs.

4.3 STUDY III

Most studies today are focused on trying to elucidate disease pathways in ACPA-positive RA, where the autoantigen is known (at least to a certain extent), and where most known genetic and environmental risk factors seem to cluster. Much less is understood about the ACPA-negative subset of the disease. Using the commercially available anti-CCP2 antibody assay frequently used in clinics, approximately 30% of RA patients will be considered ACPA-negative, and most of these patients will also be considered RF-negative. The lack of serological biomarkers presents an obstacle to prompt diagnosis for this group of patients. This subset comprises an intriguing complement to the "ACPA hypothesis" of RA pathogenesis: the relatively lower contribution of genetic and environmental risk factors important in ACPA-positive RA, combined with a relatively similar disease phenotype for both subsets suggests that disparate mechanisms in disease predisposition or initiation could lead to the development of a clinically similar disease.

The overarching aim of **Study III** was to investigate how "seronegative" seronegative RA really is. We desired to confirm data from previous studies demonstrating that a subgroup of CCP2-negative RA patients have specific ACPAs that are not picked up by the CCP2 test (138,139). We also examined the frequency of IgM, IgG and IgA RF, since most RF tests used in the clinic identify primarily IgM RF. In addition, we analyzed a bank of non-RA-related autoantibodies, including the anti-Ro/anti-La system, as the presence of non-RA-related autoantibodies could potentially indicate possible disease misclassification in some cases.

The basis for this study was a large serological dataset from the ISAC platform on the EIRA case-control cohort. Of the 2,836 RA sera examined, 989 were anti-CCP2 antibody negative, representing 35% of the whole EIRA RA population. Of these CCP2-negative RA patients, 67% were positive for at least one of the autoantibodies that we screened for; 34% were ACPA positive; 30% were RF positive; and 30% had antibodies to non-RA-related autoantigens. Cit-Fib$\beta_{60\text{-}74}$ was the most common ACPA fine specificity among CCP2-negative RA patients, with IgM RF as the most common RF isotype, and antibodies to the Ro/La antigens Ro60 and Ro52 as the most common non-RA-specific antibody.

More ACPA fine-specificities and higher ACPA levels were detected in CCP2-positive RA, compared to CCP2-negative RA. All RF isotypes were also more frequent in the CCP2-positive, compared to the CCP2-negative subset, while there was no such difference for other non-RA-specific autoantibodies. The co-occurrence of different ACPA, different RF isotypes,

and different antibody systems in general was more pronounced in CCP2-positive RA, compared to CCP2-negative RA (Figure 5). These findings suggest that there are at least two serologically distinct subsets of RA; and perhaps more importantly, that clinical screening for CCP2 and IgM RF clearly do not capture all "seropositive" RA patients.

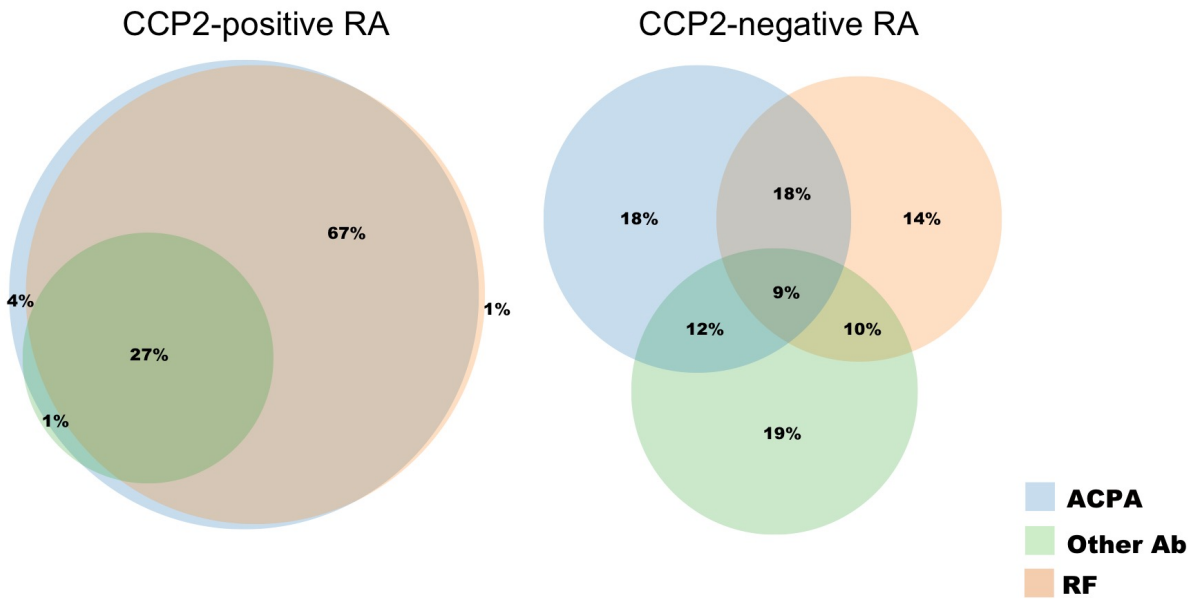

Figure 5: The distribution of ACPA, RF and other non-RA specific autoantibodies, among RA patients positive for at least one of these antibodies, in CCP2-positive and CCP2-negative subsets of EIRA. Figure from **Study III**.

The different autoantibodies were subsequently analyzed in relation to CRP, DAS28 and classical RA risk factors, in order to learn more about their potential role in RA etiology. These analyses showed only small differences in CRP levels and DAS28, indicating that presence/absence of these specific autoantibodies did not have a major impact on systemic inflammation or disease activity at the time of RA diagnosis, in line with what has been shown previously when comparing CCP2-positive and CCP2-negative early RA(140). Regarding HLA-DRB1 SE, we could confirm a previous report that SE associated with the presence of ACPA also in CCP2-negative RA, and in CCP2-positive RA we could demonstrate that the SE association was determined by six ACPA fine-specificities with high co-occurrence (or cross-reactivity). We could then show that presence of ACPA did not associate with smoking in CCP2-negative RA, but that presence of RF (and in particular IgA

RF) did, highlighting the importance of investigating ACPA in relation to RF when elucidating RA disease pathways. As shown previously, PTPN22 polymorphism, which has been linked to a number of autoimmune diseases as well as the presence of autoreactive B cells in systemically healthy individuals, seems to associate with RA, irrespective of ACPA and RF status (141,142). Interestingly, even when all autoantibodies were considered, the association with PTPN22 did not change significantly. The gene-environment and gene-gene interactions which are known to exist between SE and smoking (65) and between SE and PTPN22 (143) were not investigated in our study, but such analyses would add complexity to the interpretation of our findings.

In summary, **Study III** supports the idea of RA as comprising at least two disease entities, possibly with disparate etiopathogenic mechanisms leading up to a similar clinical picture. Moreover, our study confirms that "seronegative" RA is not entirely a seronegative disease subset, and that additional screening for ACPA fine-specificities and RF isotypes could potentially help diagnose a substantial proportion of patients with RA at an earlier time point than is currently possible. This is of great importance, as earlier treatment in RA is beneficial regardless of serological status (144-146).

4.4 STUDY IV

Research on the ACPA response has largely focused on the antibodies themselves. However, much less is understood about the characteristics and potential pathogenic contribution of ACPA-producing B cells, despite the clear implication of B cells themselves in RA by the efficacy of rituximab. The major barrier to this line of inquiry has been the technical difficulty of maintaining or expanding B lymphocytes *ex vivo* without differentiation into plasmablasts, which lose their ability to present antigen to T cells. However, a recently published method allows the immortalization of B cells through the ectopic expression of Bcl-6 and Bcl-xL - regulators of B cell differentiation and apoptosis - which can then be expanded in the presence of CD40L and IL-21 without differentiation (134). These cells have been described as possessing a germinal center-like phenotype, with preserved costimulatory and antigen-presenting capacity. We utilized this method in **Study IV** to generate ACPA-producing B cell clones, in order to establish a platform for investigating the antigen specificity and functional characteristics of the ACPA B cell repertoire.

We first selected a SE-positive, CCP2-positive RA patient (denoted RA1003) with a high CCP2 titer, from which we isolated IgG-positive memory SF B cells ($CD22^+/CD19^+/CD27^+/IgM^-/IgA^-$) using microbeads and flow cytometry. These memory B cells were then stimulated in the presence of CD40L and recombinant human IL-21 and subsequently immortalized by transduction with a retroviral vector expressing the transcription factors Bcl-6 and Bcl-xL, as well as GFP marker. Transduced GFP-expressing clones were then single-cell sorted into 96-well plates and expanded in the presence of CD40L and IL-21. We also derived B cell clones from SF of a non-RA, CCP2-negative disease control (denoted BB5327), in the same manner.

Transduced B cell supernatants were assayed for ACPA reactivity using the CCP2 ELISA. Of the 40 SF B-cell clones derived from RA1003, five tested positive for CCP2 (12.5%); as expected, none of the 50 clones derived from BB5327 were positive in the CCP2 ELISA. The proportion of ACPA-producing B cells derived from the RA patient is somewhat lower than previous reports for synovial B cells (25-40%) (128,147), but greatly increased when compared to the frequency in peripheral blood (approximately 1 in 12,500) (148), confirming the enrichment of SF B cells for ACPA positivity in seropositive RA.

Next, we determined the ACPA fine-specificity of the CCP2-positive clones using the ISAC array containing citrullinated peptides, as in **Study II and III.** Only two of the five CCP2-positive clones (RA1003.3 and RA1003.4) produced IgG that bound citrullinated peptides on ISAC, with each of these clones binding strongly to CEP-1 and weakly to a citrullinated fibrinogen peptide (Cit-Fib$\beta_{36\text{-}52}$). No binding was seen to the corresponding arginine-containing control peptides. Sequencing of the immunoglobuline sequence of the two CEP-1-positive clones revealed that they were in fact the same clone, expressing IgG1.

As we wished to use this method to investigate the ability of ACPA B cells to present peptides from citrullinated proteins, we next determined the specificity of RA1003.3 to *in vitro* citrullinated or carbamylated α-enolase and fibrinogen in ELISA. RA1003.3 specifically bound citrullinated fibrinogen and carbamylated α-enolase. The lack of binding to citrullinated α-enolase despite strong binding to CEP-1 could be due to a lack of citrullination at the CEP-1 epitope, or a lack of accessibility of the CEP-1 epitope in ELISA. Finally, we proceeded to verify the ability of RA1003.3 to bind citrullinated fibrinogen on the cell surface. RA1003.3 cells were incubated with either unmodified or citrullinated fibrinogen coupled to Alexa-594, washed and subjected to confocal fluorescence microscopy. RA1003.3 exhibited specific binding of citrullinated fibrinogen, and did not bind native fibrinogen (Figure 6).

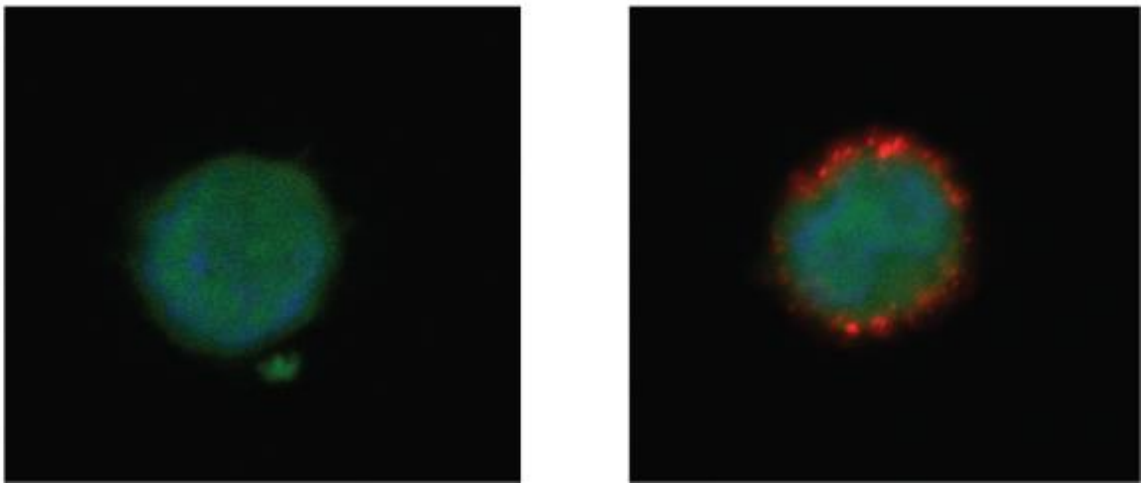

Figure 6: Immortalized B cell clone RA1003.3 binds citrullinated fibrinogen (right panel), but not native fibrinogen (left panel). Green GFP indicates the cytoplasm; red Alexa-595 shows fibrinogen; blue DAPI indicates the cell nuclei. Figure from **Study IV**.

We have yet to demonstrate internalization, and that surface binding of citrullinated fibrinogen indeed is mediated by the B cell receptor. Still, our results confirm the utility of this method for deriving antigen-specific autoreactive B cells from patient material. It also supports our findings of cross-reactivity between ACPA and anti-CarP antibodies in **Study II**, as RA1003.3 was able to bind both citrullinated and carbamylated epitopes.

5 CONCLUSIONS

The main findings from the projects presented in this thesis are as follows:

- The anti-CCP2 test detects ACPAs against a varity of *in vitro* citrullinated proteins and peptides, as well as antigens present *in vivo* in the rheumatoid joint **(Study I).**
- Cross-reactivity is a common component of the ACPA response - between different citrullinated antigens, and between citrullinated and carbamylated (i.e. homocitrullinated) antigens **(Studies II** and **IV)**.
- In light of the cross-reactivity between citrullinated and carbamylated antigens, the existence of an independent anti-CarP antibody system is questionable (**Study II**).
- Seronegative RA is not entirely a seronegative disease subset: ACPA and/or IgA RF and/or IgG RF are present in a proportion of CCP2-negative / IgM RF-negative patients, and this subset resembles seropositive RA in terms of associations with risk factors **(Study III).**
- While HLA-DRB1 SE also associates with the presence of ACPA in CCP2-negative disease, smoking showed a significant association with RF, but not with ACPA, highlighting the importance of investigating ACPA together with RF when elucidating disease pathways in RA (**Study III**).
- The ACPA-negative/RF-negative subset of RA still requires better biomarkers; no major association could be detected for autoantibodies present in other autoimmune diseases **(Study III).**
- ACPA-producing B cell clones can be isolated and expanded from CCP2-positive RA patients, and are able to bind citrullinated proteins *in vitro*, providing a tool for the future investigation of the role of ACPA-producing B cells in RA **(Study IV).**

Taken together, these results provide a basis for future investigation into the putative specificity and mechanism of the ACPA response. From **Study I**, it is clear that the CCP2 assay comprehensively and directly captures the ACPA response. Work with purified ACPA IgG in **Study I and II** confirmed that cross-reactivity is likely a common component of the ACPA response. In particular, investigation of the crossreactivity of anti-CEP-1 antibodies with carbamylated epitopes in **Study II** suggests that the anti-CarP antibody response is far from discrete and may be a subset of ACPAs. The results of **Study III** further underline the serological differences between ACPA-positive and ACPA-negative RA and supports the idea that disease in ACPA-negative RA might arise through mechanisms disparate to ACPA-positive RA. Finally, the results of **Study IV** provide a methodological basis for the mechanistic interrogation of ACPA-producing B-cells in RA.

6 FUTURE DIRECTIONS

While the “ACPA hypothesis” of RA is intriguing, the lack of any definitive study supporting the pathogenicity of either human ACPAs or ACPA-specific lymphocytes is troubling. One possible explanation is that the ACPAs present in established disease may have lost their specificity and affinity due to epitope-spreading in the chronic inflammatory environment in the synovium. In this case, the isolation and characterization of ACPAs or ACPA lymphocytes present in the preclinical stage of the disease would be imperative to provide answers as to the true antigenic target of ACPAs (if one exists) as well as their ability to initiate disease. However, the fact that ACPAs in pre-clinical stages are present at such low levels, combined with the practical difficulty of prospectively recruiting pre-symptomatic individuals, makes this approach difficult. That said, a number of efforts are currently being made to recruit ACPA-positive individuals with arthralgia but before the onset of synovitis, in order to address precisely these questions.

At least two published studies (149,150) have demonstrated that healthy individuals also commonly possess immature B cells in peripheral circulation capable of producing ACPAs upon activation (though they do not spontaneously secrete antibody in non-RA individuals). It has also been observed that non-RA B-cell lymphoma patients have significantly increased prevalence of ACPAs compared to healthy controls, albeit at low titres (151). It would seem likely then that control of B cell tolerance to (abnormally) citrullinated proteins is poorly regulated, and that the generation of ACPA-specific immature B cells is a common event. If so, this would underscore the importance of HLA-dependent T-cell help in the activation of these immature ACPA-positive B cells in RA. It would be interesting to explore the characteristics of these ACPA, either through isolation of B cell clones and/or molecular cloning of the immunoglobulin sequences from this "healthy" B cell population.

However, there is also the possibility that ACPAs themselves are not pathogenic. Rather, they could be indicative of the immunological characteristics and/or anatomical location of inflammation in RA. Notably, low levels of ACPA are present in approximately 2% of the general population (59), and the majority of these individuals will never develop RA. Moreover, while the transfer of ACPA to experimental animals can induce pain behavior and changes in bone structure, it does not seem to induce arthritis (103,104). Identifying the etiology of ACPAs would be crucial in elucidating their role in RA pathogenesis. Studies on pre-clinical serum samples have revealed that the ACPA response matures over time, with increased Ig levels, Ig usage, Fc glycosylation and epitope-spreading, before onset of clinical synovitis (63,152-154). However, no single ACPA fine-specificity have been identified as

being the first to appear, and no single ACPA fine-specificity have been shown to eventually cause arthritis. A broader approach to identify *in vivo* targets is therefore necessary. Additionally, while the use of peptide antigens derived from different candidate autoantigens is convenient for screening purposes, it is questionable if they have any relationship to *in vivo* B cell targets, as B cell epitopes are typically conformational, not linear.

However, the findings of this thesis will hopefully allow these questions to be answered in the future. Through **Study I,** we have generated ACPA pools that are now being used for the identification of *in vivo* antigen targets, as well as effects on cell populations, as well as their capability to induce arthritis in animal models. Through **Study II,** we can confirm that ACPA cross-reacts with carbamylated antigens. This has now been shown for three candidate autoantigens in RA (i.e. fibrinogen (114), α-enolase and vimentin (118)), but whether this phenomenon has any relevance *in vivo* requires further investigation. **Study III** highlights the need for new biomarkers, better classification of seronegative RA, and better clinical tests for seropositive RA. And finally, the results in **Study IV** - while very preliminary - lay the ground for high-throughput, in-depth studies of the characteristics of the ACPA lymphocyte population, specifically in regards to HLA-DRB1 SE-mediated antigen presentation, and theoretically could even serve as a test bed for ACPA-specific therapeutics.

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