From Medical Biochemistry and Biophysics
Karolinska Institutet, Stockholm, Sweden

Analysis of Antibodies to Cartilage Oligomeric Matrix Protein in Rheumatoid Arthritis and in Mouse Models

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Stockholm 2021

Published by Karolinska Institutet.
Printed by Universitetsservice US-AB, 2021

ISBN 978-91-8016-336-1
Cover illustration: A painting depicting a poem *Written on the Wall at West Forest Temple* by Su Shi in 1084: *It's a range viewed in face and peaks viewed from the side, assuming different shapes viewed from far and wide. In exploration we cannot make out the true face, for we are lost in the heart of the very place.*
(Translation modified from Xuyuanchong)

Analysis of Antibodies to Cartilage Oligomeric Matrix Protein in Rheumatoid Arthritis and in Mouse Models
THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Weiwei Cai

The thesis will be defended in public at Andreas Vesalius lecture hall, Karolinska Institutet, Solna, Stockholm, November 5th 9 am

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To my beloved family

POPULAR SCIENCE SUMMARY OF THE THESIS

In healthy individuals, the immune system protects against foreign invaders like bacteria and viruses. The key to a functioning immune system is that it can help an individual's body distinguish between parts of itself and parts from a foreign source. When the immune system fails to make this distinction, it can mistake its own body as foreign, as in autoimmunity. This may signal to the body to attack itself and thus lead to structural damage. Proteins called autoantibodies target the body's healthy cells or tissues, which is a common feature of many autoimmune diseases. However, their exact role in the development of autoimmunity remains unclear now.

There are more than 80 different known autoimmune diseases. Many of them share similar characteristics such as common symptoms including fever and pain, chronic disease process and a female to male preponderance. They also cause severe individual suffering and enormous socio-economic burdens. Their diagnosis can be difficult since most of them are complex diseases, which develop through a combination of genetic and environmental factors. Finally, most of them are incurable with treatment focusing on improving symptoms and prolonging lives.

One of the most common autoimmune diseases is rheumatoid arthritis (RA). Like many other autoimmune diseases, there is no cure. However, it is well-accepted that early diagnosis and treatment can improve the prognosis of this disease. Nowadays the most commonly used biomarkers in RA's diagnosis are autoantibodies such as rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPAs). The diagnosis criteria of RA include the presence of these autoantibodies. Despite their great importance in diagnosis of RA, the functions of these autoantibodies are not clear.

The work presented in this thesis attempted to identify the properties and potential role of autoantibodies in RA and in mouse models of RA. Study II, focused on antibodies against type II collagen (CII), a common and well-studied target in RA. Studies I, III and IV, shifted focus to cartilage oligomeric matrix protein (COMP), which is also a major protein in the joint cartilage but less studied than CII. A library of CII and COMP peptides was established and used to screen the sera of RA patients, healthy controls, and also mice immunized with certain antigens. The results revealed associations between some peptides to RA or certain animal models.

These findings increase our knowledge on the role of autoantibodies in RA and help identify potential biomarkers for earlier diagnosis and targeted treatment strategies.

ABSTRACT

Rheumatoid arthritis (RA), a common autoimmune disease, affects 0.5-1% of the world's population. It is not only an important health problem but also a socioeconomic problem considering that it can lead to joint destruction and disability. It is well known that the disease can actually start many years ahead of the clinical onset. The presence of autoantibodies observed in serum is a hallmark. These autoantibodies included antibodies reactive with immunoglobulin (rheumatoid factors, RF) and antibodies reactive to citrullinated proteins (ACPA). With time antibodies to joint proteins also appear. The functional importance of these autoantibodies is however not yet clear, and they could have both regulatory and functional importance in RA's development.

Type II collagen (CII), as the most abundant protein in joint cartilage, is a most common and well-studied target in RA. Thus, collagen induced arthritis (CIA) is the most commonly used model in which RA-like arthritis can be induced in susceptible mouse strains following immunization with heterogeneous CII emulsified in an adjuvant. Despite CIA's gold standard status, other models are needed to capture the entirety of this complex disease. Cartilage oligomeric matrix protein (COMP), as a major glycoprotein in the extracellular matrix (ECM) of cartilage and synovium, has recently become a candidate autoantigen in RA. COMP induced arthritis (COMPIA) is thus also developed to mimic RA conditions in susceptible mouse strains.

In **study I**, COMPIA was established in C57BL/6 mice and the major T and B cell epitopes involved in the development of this arthritis model were defined.

In **study II**, a library of native and citrullinated triple helical peptides (THPs) of CII was established. This library also contained some previously defined epitopes. Some monoclonal antibodies reactive against these defined epitopes, sera from RA patients, and sera from experimental mouse models showed a unique reactivity toward THPs as compared to cyclic ones. This suggested that the binding of epitopes to their autoantibody might be triple-helical-conformation-dependent. In addition, the responses of three identified THPs were observed at elevated levels in sera from RA patients when compared to healthy controls. These data gave support for the potential use of these epitopes as new biomarkers in RA.

In **study III**, arthritis was induced after immunization with an anti-COMP antibody, 15A, in naïve mice. Levels of antibodies specific to 15A's epitope, P6 peptide, and its citrullinated variants were increased significantly in RA patients compared to population controls, also correlating with a greater disease activity.

In **study IV**, despite an attempt to establish COMPIA in mouse strains that express the human major histocompatibility complex type II (MHCII) alleles DRB1*0401, DRB1*0402, and DRB1*0405, only mild arthritis developed in DR0401 mice with low incidence. All strains developed a strong IgG response to COMP after immunization though. Major B cell epitopes were identified in both sera from these immunized mice and from a RA cohort. One

citrullinated COMP peptide showed significantly elevated response in both RA and mice immunized with citrullinated COMP and was associated with disease activity in RA patients.

LIST OF SCIENTIFIC PAPERS

I. Zhao, Y., Urbonaviciute, V., Xu, B., **Cai, W.**, Sener, Z., Ge, C. and Holmdahl, R., 2021. Cartilage Oligomeric Matrix Protein Induced Arthritis—A New Model for Rheumatoid Arthritis in the C57BL/6 Mouse. *Frontiers in Immunology*, *12*, p.81.

II. Viljanen, J., Lonnblom, E., Ge, C., Yang, J., Cheng, L., Aldi, S., **Cai, W.**, Kastbom, A., Sjöwall, C., Gjertsson, I. and Holmdahl, R., 2020. Synthesis of an Array of Triple-Helical Peptides from Type II Collagen for Multiplex Analysis of Autoantibodies in Rheumatoid Arthritis. *ACS Chemical Biology*, *15*(9), pp.2605-2615.

III. Ge, C., Tong, D., Lonnblom, E., Liang, B., **Cai, W.**, Kastbom, A., Gjertsson, I., Doreen, D., Holmdahl, R. Antibodies to Cartilage Oligomeric Matrix Protein Are Pathogenic and Clinically Relevant in Rheumatoid Arthritis.
Manuscript

IV. **Cai, W.**, Xu, B., Lonnblom, E., Castillo, L., Pandey, R., Xu, Z., Cheng, L., Luo, H., Liu, M., Zhao, Y., Kastbom, A., Sjöwall, C., Gjertsson, I., Ge, C., Holmdahl, R. Antibody Response to Cartilage Oligomeric Matrix Protein in a Panel of HLA-DR4 Transgenic Mice and in Rheumatoid Arthritis.
Manuscript

CONTENTS

LIST OF ABBREVIATIONS

ACPA	Anti-citrullinated protein/peptide antibodies
ACR	American College of Rheumatology
AMPA	Anti-modified protein antibodies
B6N	C57BL/6NJ
BARFOT	Better Anti-Rheumatic PharmacOtherapy cohort
BCR	B cell receptor
CAIA	Collagen antibody induced arthritis
CCP	Cyclic citrullinated peptides
CFA	Complete Freund's adjuvant
CIA	Collagen induced arthritis
CII	Type II collagen
COMP	Cartilage oligomeric matrix protein
CXI	Type XI collagen
Fab	Antigen-binding fragment
Fc	Crystallizable fragment
FcgRs	Fc gamma receptors
GPI	Glucose-6-phosphate isomerase
HLA	Human leucocyte antigen
IgA	Immunoglobulin A
IgD	Immunoglobulin D
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL-1	Interleukin-1
IL-6	Interleukin-6
IFN-γ	Interferon gamma
IVIG	Intravenous immunoglobulin

i.d.	Intradermally
LPS	Lipopolysaccharide
MAb	Monoclonal antibody
MCP	Metacarpophalangeal
MHC	Major histocompatibility complex
MHCII	Major histocompatibility complex class II
MED	Multiple epiphyseal dysplasia
MFI	Median fluorescence intensity
MTP	Metatarsophalangeal
PAD	Peptidyl Arginase Deiminase
PIP	Proximal interphalangeal
PRR	Pattern recognition receptors
PSACH	Pseudo achondroplasia
RA	Rheumatoid arthritis
RF	Rheumatoid factor
SPF	specific pathogen-free
TCR	T cell receptors
THP	Triple helical peptides
TIRA-2	Second Swedish Early Intervention in RA Trial
TNF	Tumor necrosis factor
WINGA	Western Region Initiative to Gather Information on Atherosclerosis cohort

1. INTRODUCTION

1.1 THE IMMUNE SYSTEM

The immune system is a defense mechanism protecting us from harm caused by foreign organisms and materials such as viruses, bacteria, fungi, and toxins. The basic ability of immune system is to distinguish between self and non-self component and provide immune response by various mechanisms[1]. The immune system consists of 2 parts: innate immune system and adaptive immune system. The innate immune system is the first line of defense and is characterized by fast, broad, and non-specific immune responses. The adaptive immune system, also referred as the acquired immune system, is more complex and only present in relatively advanced species.

In the following section, the characteristics and functions of the innate and adaptive immunity, as well as the difference and connection between them, will be introduced in detail.

1.2 INNATE IMMUNITY

The innate immunity, an old and conserved defense strategy present in almost all forms of life, still plays essential role after millions of years' evolution. Various potential harmful microorganisms surround us every day from the time of our birth forward. Yet we only get infected or sick occasionally, indicating most such dangers are detected and defended by a quite efficient and consistent defense mechanism. This is exactly what the innate immune system provides. Characterized by non-specific and non-adaptive immune reaction, innate immune system can act immediately without requiring extra time for induction.

1.3 ADAPTIVE IMMUNITY

As mentioned above, the innate immunity is pre-programmed to react to more common and broader categories of pathogens while the adaptive immunity acquires specificity for a certain pathogen, requiring additional time to develop immune response.

Unlike innate immunity, in which the immune cells detect pathogens by recognizing certain structures of pathogens through pattern recognition receptors (PRR), which are already encoded in the genome[2], various pathogen-specific receptors in adaptive immunity are "acquired" with numerous encounters to various pathogens during our lifetime.

Another characteristic of adaptive immunity is memory. Once immunological memory is created after an initial response to a particular pathogen, a significantly enhanced response to that pathogen will be aroused in the possible encounters in the future. That's why people can get long-lasting—lifetime on some occasions—protection after recovery from some diseases. This is the theoretical basis of vaccination. Interestingly, the origin of immunology is actually attributed to a discovery of Edward Jenner in 1796. He observed that cowpox could induce protection against human smallpox[3], which was later developed into what we call vaccination now. Immunological memory plays pivotal role in the adaptive immunity and modern medicine.

The principal cells of our adaptive immune system are B lymphocytes and T lymphocytes. They take active roles in coordination in both humoral responses and cell-mediated immune responses. In humoral immunity, B cells can be activated by antigen alone or in a T cell-dependent manner. The latter involves somatic hypermutation of the B cell receptor variable region and leads to a highly flexible and adaptable immune response.

1.4 ANTIBODIES

Antibodies, also called immunoglobulin (IgG), are large, Y-shaped glycoproteins produced mainly by plasma cells in response to contact with a certain antigen, usually a foreign substance like bacteria or virus. Antibodies can recognize and bind to the specific antigen, activate the immune system, and finally remove them from our body. But in some cases, some antibodies are generated against self-components other than foreign substances, and this will possibly end up with the development of autoimmune diseases.

Antibodies can be found in free form or bound on the surface of B cells. The structure of antibodies mainly includes 2 identical chains and there are five isotypes of antibodies including IgG, IgA, IgM, IgE and IgD.

Antibodies can recognize various antigens by the antigen-binding fragment (Fab) with high affinity and activate leukocytes by interactions between its crystallizable fragment (Fc) and Fc gamma receptors (FcgRs) of immune cells which can further trigger a series of inflammatory response.

Paradoxically, some antibodies are found to contribute to the pathology of autoimmune diseases[4,5], while others are widely used in the treatment of inflammatory diseases[6]. High-dose intravenous immunoglobulin (IVIG), for example, is a therapeutic preparation of polyclonal IgG derived from healthy donors and has been used in the treatment of autoimmune diseases for decades[7]. Also, over the past decades monoclonal antibodies (mAbs) have been demonstrated to have the potential as drugs of various autoimmune diseases[8,9]. These examples suggest antibodies in autoimmunity act as a double-edged sword. This contributes to a complicated mechanism, which we do not fully understand.

1.5 RHEUMATOID ARTHRITIS

Rheumatoid arthritis(RA), one of the most prevalent and well-known autoimmune diseases, is characterised by persistent joint inflammation and in later stage, the damage of cartilage and even bone within the joint[10,11]. The prevalence of RA is believed to be around 1%, varying in different regions over the world[12,13].

Females are 2 to 3 times more likely to develop RA than males and the possible reason for the imbalance between genders hasn't been clarified precisely yet[14]. This disease impacts quality of life, leads to disability and shortens life expectancy[15], especially if patients cannot get early and efficient treatment.

The typical symptoms of RA are pains, inflammation, swelling and stiffness of synovial joints and systemic symptoms such as tiredness, fever, and a loss of appetite[16]. The commonly used classification criteria of RA include the 1987 American College of Rheumatology (Table 1) and 2010 ACR/European League Against Rheumatism criteria (Table 2)[17,18].

Like most other autoimmune diseases, RA is a polygenic and multifactorial syndrome[19,20,21]. It's widely agreed that both genetic and environmental factors contribute to the RA pathogenesis but the detailed mechanism how they interact with each other and contribute to the clinical outcome are poorly understood so far[22].

Many genetic factors have been found to be associated with increased or in some cases, decreased risk for RA. The strongest is a locus including a series of alleles called human leukocyte antigens (HLA)[23,24], which will be described in detail in later section. It is consistently associated with RA and actually by far the most prominent genetic factor. There are hundreds of additional loci with relatively minor effects, identified by genome-wide association studies, an important locus to mention PTPN22[25].

Environmental factors also seem to have relatively strong and consistent associations with increased risk of RA. Environmental risk factors for RA mainly include smoking, infection, exposure to air pollution, and some dietary factors[26,27,28,29,30,31].

Sadly, there is still no cure for RA yet and even no effective treatment for all patients, with RA's highly heterogeneous nature that variable phenotypes and prognosis in patients might indicate distinct pathological pathways.

Table 1. The 1987 ACR revised classification criteria for RA

CRITERIA	DEFINITION
Morning stiffness	Morning stiffness in and around the joints, lasting at least one hour before maximal improvement.
Arthritis of 3 or more joint areas	At least 3 joint areas simultaneously have had soft tissue swelling or fluid (not bony overgrowth alone) as observed by a physician. The 14 possible areas are right or left proximal interphalangeal (PIP), metacarpophalangeal (MCP), wrist, elbow, knee, ankle, and metatarsophalangeal (MTP) joints
Arthritis of hand joints	At least 1 area swollen (as defined above) in a wrist, MCP, or PIP joint.
Symmetric arthritis	Simultaneous involvement of the same joint areas (as defined above) on both sides of the body (bilateral involvement of PIPs, MCPs, or MTPs, without absolute symmetry).
Rheumatoid nodules	Subcutaneous nodules over bony prominences or extensor surfaces, or in juxta-articular regions as observed by a physician.
Serum rheumatoid factor	Demonstration of abnormal amounts of serum rheumatoid factor by any method for which the result has been positive in less than 5 percent of normal control subjects.
Radiographic changes	Radiographic changes typical of rheumatoid arthritis on posteroanterior hand or 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 diagnosed as RA if at least 4 of these 7 criteria are satisfied. The first four criteria must have been present for at least six weeks.	

Table adapted from Arnett, Frank C., et al. "The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis." Arthritis & Rheumatism: Official Journal of the American College of Rheumatology 31.3 (1988): 315-324.

Table 2. 2010 ACR/EULAR RA classification criteria

CRITERIA	SCORE
A. Joint involvement	
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 one small joint)	5
B. Serology (at least one test result is needed for classification)	
Negative RF *and* negative ACPA	0
Low positive RF *or* low positive ACPA	2
High positive RF *or* high positive ACPA	3
C. Acute phase reactants (at least 1 test result is needed for classification)	
Normal CRP and normal ESR	0
Abnormal CRP or normal ESR	1
D. Duration of symptoms	
< 6 weeks	0
≥ 6 weeks	1
The points from A to D should be added to obtain the total score. A score ⩾6 indicates the presence of definite RA.	

Table adapted from Aletaha, Daniel, et al. "2010 rheumatoid arthritis classification criteria: an American College of Rheumatology/European League Against Rheumatism collaborative initiative." Arthritis & rheumatism 62.9 (2010): 2569-2581.

1.6 AUTOANTIBODIES IN RA

Although the immunopathology of RA underlying disease development is not clearly understood, it is well agreed that RA has a preclinical stage. In this stage, only some biomarkers (mainly autoantibodies) indicating autoimmunity provocation can be identified[32, 33,34]. Among these biomarkers, rheumatoid factor (RF) and anti-citrullinated protein/peptide antibodies (ACPAs) are mostly described[35,36]. Cyclic citrullinated peptides (CCP2) assay detecting the reactivity to a set of synthetic cyclic citrullinated peptides is widely used in clinic for diagnosis of RA[37]. In seropositive RA patients, the increased level of these autoantibodies can be observed several years prior to the clinical onset, indicating that these factors may be used in prediction and prevention of RA[38,39,40].

To be noted, RA is a complex disease and has many different subtypes. For example, some of the patients are "seronegative" to the known biomarkers but still develop disease later[41]. On the other hand, the existence of these autoantibodies does not necessarily lead to the development of disease, either. RFs have been consistently associated with RA (60–80% sero-positivity), but its presence has also been reported in some non-RA patients suffering from other chronic inflammatory conditions as well[42]. Nevertheless, these factors are part of the diagnosis criteria[18].

Efforts have been made in identifying new biomarkers to help in the diagnosis of RA patients who test negative to the existing biomarkers and also in exploring potential biomarkers for disease activity and prognosis. This has been a difficult task. As previously noted, autoantibodies are highly heterogeneous and not immutable and can keep changing as the disease progresses through different stages.

There are mainly 2 types of autoantibodies related to RA or pre-RA patients according to current knowledge: 1) antibodies that bind to IgG, represented by RFs and 2) anti-modified protein antibodies (AMPAs), represented by ACPAs[43,44,45]. The former recognizes epitopes in the Fc region of IgG. The latter binds to anti-modified proteins such as ACPAs binding to proteins that are citrullinated and anti-carbamylated protein antibodies (anti-CarP Abs) binding to proteins in which lysine amino acid residues have been converted into homocitrullines[46,47,48].

The importance of autoantibodies in RA lies not only in that it can be risk factors of RA, but also in that they can possibly affect the pathogenesis of RA. The role of autoantibodies in preceding the onset of clinical disease has been confirmed in the animal experiments where autoantibodies can be used to induce arthritis in mouse models[49]. However, no definitive causal link with these autoantibodies has been made with the development of arthritis. Therefore, the question regarding the role these autoantibodies play in the development of disease remains unanswered.

1.6.1 Anti-CII Antibodies in RA

Type II collagen (CII), formed by homotrimers of alpha 1 chains, is the most abundant fibrillar

protein in the articular cartilage. In the previous decades, cartilage-specific molecules such as CII have long been regarded as candidate targets of pathogenic autoimmune responses in RA[50,51,52,53,54]. It has been reported in the literature that circulating autoantibodies against native and denatured CII have been detected in RA patients' sera[55]. Anti-CII antibody-producing B cells have also been identified in rheumatoid synovium and synovial fluid, while anti-CII antibodies are not detectable in healthy individuals[56]. Anti-CII antibodies are not only associated with the pathogenesis of RA but could also be of great value for prediction of the severity, activity and subtypes of disease and patients' responsiveness to the different treatment choices[57,58].

The role of anti-CII antibodies in RA has been supported by animal experiments in which arthritis can be induced in susceptible strains immunized with a combination of CII specific mAbs[59]. Arthritis produced by passive transfer of CII mAbs, so called collagen antibody induced arthritis (CAIA), is an RA animal model based on the function of anti-CII antibodies[60, 61]. And anti-CII antibodies are also an essential component in the pathogenesis of collagen induced arthritis (CIA)[62]. The potential role of CII-directed autoantibodies may be explained by the observation of forming immune-complex with the collagen in cartilage, followed by activation of the complement cascade and resulting in inflammation of the joints in the end[63].

According to our existing knowledge, some B cell epitopes of CII such as C1, J1, U1, D3, and F4 have been well defined so far and these CII epitopes are conserved across the various species including mice and humans[64, 65]. Interestingly, the antibodies specific to these different epitopes can have various functions. For example, antibodies to C1, J1, U1, D3 epitopes have been found to be pathogenic and be able to induce arthritis in mice[66] while antibodies to the F4 epitope is not arthritogenic and even show a protective effect in CIA and CAIA[66,67].

1.6.2 Anti-COMP Antibodies in RA

Cartilage oligomeric matrix protein (COMP), a homopentameric extracellular matrix glycoprotein, also known as thrombospondin 5, is a major non-collagen component of cartilage[68,69]. It is synthesized by chondrocytes, and it is localized extracellularly[70]. It presents mainly in articular cartilage but also can be detected in other parts of the body such as the nasal, tracheal and meniscal cartilage[71]. COMP has been found to play an important role in formation and assembly of the collagen fibril and stabilization of the cartilage matrix[72,73,74]. The biological importance of COMP is also verified by the fact that some mutations of the COMP gene are related to two different diseases, pseudo achondroplasia (PSACH) and multiple epiphyseal dysplasia (MED)[75,76,77].

Just as CII, COMP is also a candidate autoantigen in RA. It has been reported that an elevated COMP level in serum has been found in a high proportion of RA patients[78], indicating a possible diagnostic value of COMP in RA.

Despite its status of a major joint protein, there are not as much research on the role of COMP and antibodies against COMP in RA as CII until now. But there is still some evidence showing

the importance of anti-COMP antibodies in RA. The presence of anti-COMP antibodies in synovium and sera of RA patients, for instance, possibly indicates a joint local B cell immune response to this cartilage protein[79]. Also, in previous study, a series of mAbs against COMP were produced and characterized. Some of these COMP-specific mAbs can bind to native COMP in cartilage in vivo and enhance arthritis after co-immunization with a pathogenic anti-CII mAb[80], indicating the potential arthritogenic role of anti-COMP antibodies.

1.7 ANIMAL MODELS FOR RA

Due to the complexity of autoimmune diseases, no single model can depict the entirety of the human condition, but each of them can enable us to study certain aspects of the disease. The models of RA have been developed in various animal species, but rats and mice are most widely used in studying the progression and pathogenesis of RA[81,82]. Mouse models for RA can either be spontaneous or induced. Spontaneous models mainly include the TCR transgenic K/BxN model, the TNF-alpha overproducing transgenic mice, and the SKG model while induced models mainly include glucose-6-phosphate isomerase (GPI)-induced arthritis, COMP induced arthritis mice model (COMPIA), CAIA, and CIA[83,84].

1.7.1 CIA

CIA is a most commonly used model for RA, in which RA-like arthritis can be induced in naïve mice following an intradermal immunization with heterogeneous CII emulsified in an adjuvant[85,86]. CII as a major constituent of joint cartilage, is regarded as a main target of autoantibodies in the onset and development of RA. Heterogeneous CII emulsified in an adjuvant will provoke an autoimmune response that attacks the joints. Based on the existing knowledge, both T and B cells play a role in the pathogenesis of CIA[87,88] but how they play a role and interact with each other in the priming or later stage remains unclear.

It was found that B cells might play a role by production of autoantibodies which target joint proteins especially CII, and its role was confirmed by the report that B cell-deficient mice are resistant to CIA[87]. The role of T cells in CIA is more complex and can include two main pathways. Firstly, T cells help B cells to produce arthritogenic antibodies. Secondly, T cells themselves may also function by activating other immune cells leading to joint inflammation. T cells' role can also be confirmed by the fact that synovitis can be induced by transfer of T cells[89], and development of arthritis can be ameliorated after blockage of T cell function[90]. Both Fc receptor and complement system are vital for disease induction[91,92].

1.7.2 CAIA

CAIA is also an extensively used mouse model for RA[93]. It is quite different animal model compared with CIA, despite sharing several characteristics with it. It relies on the injection of a cocktail of mAb directed against CII, followed by a single injection of LPS to boost the disease. It is characterized by symptoms of cartilage erosion and deposition of IgG antibodies[94].

Interleukin-1 (IL-1) and tumour necrosis factor (TNF), but not interleukin-6 (IL-6), are required in CAIA[95].

1.7.3 COMPIA

Just as mentioned earlier, besides CII, COMP is also a large protein attracting interest of rheumatologist and researchers[96]. So, an animal model in which arthritis was induced in rats or mice by immunization with COMP has been established[97,98]. The COMPIA was performed in susceptible strains of mice with similar protocol to CIA, except that the antigen used for immunization is COMP instead of CII. This mice model is characterized by the appearance of erythema and swelling of the front and rear paws[99]. The key role of T cell in the development of arthritis in this model has been confirmed by the association of COMPIA with certain MHC haplotype[99] but the detailed immunopathology in COMPIA has not been clarified yet.

COMPIA can be used as an appropriate and alternative model for studying the pathogenesis of arthritis and it has some advantages over other various models.

1.8 MHC AND RA

Although the detailed immunopathology of RA remains poorly understood, it is well-accepted that RA is a complex disease with both genetic and environmental factors involved in its pathogenesis. The major histocompatibility complex (MHC) class II (MHCII), for example, is a genetic risk factor with strong association to RA[100,101, 102].

MHCII molecules are a class of MHC molecules playing key roles in antigen-presenting and initiating immune responses. MHCII protein complex is encoded by a complex of genes on chromosome 6, the human leukocyte antigen gene complex (HLA) in humans. HLA is responsible for encoding cell-surface proteins involved in the regulation of the immune system and mutations in HLA have been correlated to some autoimmune diseases[103]. MHCII proteins encoded by the HLA mainly include HLA-DR, HLA-DQ, HLA-DP, HLA-DM and HLA-DO. Some HLA-DR alleles like DRB1*0401 and DRB1*0405 have been identified as risk factors for RA, while DRB1*0402 has been related to the protection to RA[104,105].

2. RESEARCH AIMS

In study I, the main aim was to establish a new model of RA in C57BL/6 (B6N) mice by immunization with COMP.

In study II, the main aim was to establish a library of high-quality triple helical peptides as a tool for future studies including characterizing CII as an autoantigen in RA, investigating the potential role of anti-CII antibodies as biomarkers and pathogenic factors in arthritis.

In study III, the main aim was to explore the structure, function, and relevance of anti-COMP antibodies in RA.

In study IV, the main aim was to investigate whether COMP can induce arthritis in mouse strains which express humanized MHCII alleles DRB1*0401, DRB1*0402, and DRB1*0405, and to find out the specificity of the antibody response to COMP in these mice and in RA patients.

3. MATERIALS AND METHODS

3.1 PATIENT MATERIAL

3.1.1 TIRA-2

The prospective cohort second Swedish Early Intervention in RA Trial (TIRA-2)[106] were recruited from 6 rheumatology units in mid- or southeast Sweden between 2006-2009. The inclusion criteria of this cohort are: Early RA patients with a symptom duration from 6 weeks to 12 months. Most patients (84%) fulfilled the 1987 criteria from ACR. Two smaller groups were recruited, with the symptom of morning stiffness lasting over 60 minutes, symmetric arthritis, and small-joint engagement of the hands or feet (5% patients), or with palpable synovitis involving $\geqslant$1 joint and anti-CCP positive (11% patients). At the initial sera sampling, all patients hadn't been treated with any disease-modifying antirheumatic drugs.

In **Study II**, **Study III** and **Study IV,** serum samples from this cohort were screened and analyzed for their reactivity to our established peptide library.

3.1.2 BARFOT

Better Anti-Rheumatic PharmacOtherapy (BARFOT) cohort[107] was recruited between 1992-2006 for an observational prospective multicenter study of patients with early RA. The inclusion criteria of this cohort are: All patients fulfill the revised 1987 criteria from ACR with a disease duration up to 1 year.

In **Study II**, antibody reactivity in serum samples from this cohort were analyzed.

3.1.3 Population controls

For both TIRA-2 and BARFOT cohorts, no healthy controls were recruited in the original study design. Instead, healthy controls from WINGA or Malmö Diet and Cancer Study were used.

In **Study II**, age and gender matched population controls for BARFOT and TIRA-2 were obtained from the Malmö Diet and Cancer Study and individuals with rheumatic diseases were excluded.

In **Study III** and **Study IV,** serum samples from healthy controls recruited to be used in the Western Region Initiative to Gather Information on Atherosclerosis cohort (WINGA) were used. In addition, controls with a rheumatic disease were excluded. One thing to be noted is that these controls were originally recruited to match atherosclerosis patients which have a different gender and age distribution compared to RA, a limitation of using this population as a control in our RA study.

3.2 EXPERIMENTAL ANIMALS

In **Study I** and **IV**, mice strains carrying H-2b or human MHCII alleles were used to find out their susceptibility to COMPIA. In **Study III,** B10Q mouse strain was used to study cartilage

antibody-induced arthritis, and the Cia9i congenic mouse strain for immunohistochemical staining. All mice were bred and kept under specific pathogen-free (SPF) conditions and with individually ventilated cages containing wood shavings and folded paper strips.

3.3 EXPERIMENTAL ARTHRITIS

3.3.1 Cartilage Oligomeric Matrix Protein Induced Arthritis (COMPIA)

In **Study I** and **IV**, COMPIA model was used. Mice were immunized intradermally (i.d.) at the base of the tail with 100 μg hCOMP/denatured hCOMP/hCOMP_F95S/citrullinated hCOMP_F95S emulsified 1:1 in Freund's complete adjuvant (CFA) or in Freund's incomplete adjuvant (IFA) containing Mycobacterium Tuberculosis H37Ra in a total volume of 100 μL on day 0. Most of mice involved were boosted on day 35 except mice involved in experiment 2 of Study **IV** were boosted on day 21 with 50 μg of the same antigen with the one used in the first immunization emulsified 1:1 in IFA in a total volume of 50 μL. Scoring was done regularly to check the sign of inflammation in the peripheral joints. The arthritis was evaluated in a blinded manner following clinical scoring protocol on a 60-point scale[108]. To briefly describe the scoring system, clinical arthritis is defined as seeable swelling and redness in mice joints and score are given as follows: for each swollen or red toe, 1 point is given; for each swollen joint (MTP joints, metacarpal phalangeal joints, PIP joints, and distal interphalangeal joints), 1 point is given; for a swollen ankle 5 points are given. So maximum score per limb is 15 and maximum score per mouse is 60. Blood was sampled at various time points for later analysis.

3.3.2 Collagen Antibody-Induced Arthritis (CAIA)

In **Study III**, CAIA model was used to evaluate the arthritogenicity of the mAbs. Three groups of B10Q mice were injected intravenously with M2139 (9 mg), 15A (9 mg) or a combination of M2139 and 15A (4.5 mg for each), respectively. On day 5, all mice were boosted with 25μg LPS intraperitoneally to enhance the severity of arthritis. Arthritis development was monitored daily in a blinded manner for 12 days using scoring protocol described earlier.

3.4 BEAD-BASED MULTIPLEX ASSAY

In **Study I-IV**, antibody responses were analyzed using a bead-based multiplex assay, the Luminex platform. In **Study I**, **Study II** and **Study III**, a platform established at Medical Inflammation Research with a Bio-plex 200 was used. In **Study IV**, both a platform established at the Plasma Profiling division at Sci-Life and a platform established at Medical Inflammation Research were used.

In this assay, magnetic beads with unique dyes were first coated with Neutravidin, followed by coupling with biotinylated peptides. Next, human or mice serum samples were diluted and incubated in a blocking buffer (3% BSA, 5% non-fat dried milk powder, 100ug/ml Neutravidin diluted in PBS-T) for 1 h at RT on a shaker with a speed of 850rpm. Optimal serum concentrations, blocking buffers and blocking time were evaluated in earlier protocol

optimization. After pre-blocking, the serum samples were mixed with the beads coupled with different peptides and incubated for 75 minutes at RT on a shaker with a speed of 850rpm. Beads were washed on a plate washer and then resuspended in 3% BSA/PBS-T containing the secondary anti-human or anti-mouse IgG Fcγ-PE. After 40mins of incubation with the secondary antibody, the plates were read, and the fluorescence intensity was measured. The median fluorescence intensity (MFI) was used to quantify the response of the antibody to the given peptides.

4. RESULTS AND DISCUSSIONS

4.1 STUDY I: CARTILAGE OLIGOMERIC MATRIX PROTEIN INDUCED ARTHRITIS—A NEW MODEL FOR RHEUMATOID ARTHRIIS IN THE C57BL/6 MOUSE

In this study, a new model of RA, COMPIA, was established in B6N mice. Severe arthritis with high incidence was induced after COMP immunization in B6N mice. This finding is valuable because this strain has a standard background in immunology research which is commonly used for genetically modification. However, it carries the H-2b haplotype and there is no collagen II peptide associated with H-2b so far, which means this strain is resistant to CIA, the most widely used animal model for RA, due to the restriction by the MHCII haplotype[109,110]. Now it is found that immunization with another joint protein, COMP, can induce arthritis in this strain, providing a novel and useful model for RA. The main advantage of this model is that it saves tremendous amount of time and resources spent on mice backcrossing to introgress a proper MHCII haplotype[111].

This new model shares a lot of characteristics with the well-known CIA model. For example, it is also MHCII associated, and T cell recognition of an antigen-derived peptide bound to the Ab molecule is required for arthritis development.

The result that mice immunized with denatured COMP only developed quite mild arthritis with low incidences compared with mice immunized with native COMP showed that native structure of COMP is required to induce severe arthritis in mice.

Both T cell and B cell epitope were investigated relying on a peptide library containing overlapping human COMP sequences. The COMP-derived peptide recognized by T cells was detected by interferon gamma (IFN-γ) ELISPOT assay of lymphocytes or splenocytes from COMP immunized B6N mice and a peptide named as P9 with its sequence APGFCFPGVACIQTESGA was proven to be important in arthritis development. And to identify the key amino acid in the immuno-dominant epitope, some mutated peptides were synthesized and tested in the ELISPOT. P9-1 with a mutation of phenylalanine at position 95 failed to induce IFN-γ secretion, suggesting that the phenylalanine is critical for the immunogenicity of COMP in this model. Later a new recombinant COMP protein with mutation of phenylalanine at position 95 to serine instead, named as COMP_F95S, were produced and used to immunize B6N mice, resulting in that only 1 out of 8 mice developed mild arthritis, confirming the critical role of this amino acid in arthritis development in this model.

Besides T cell response, which could probably play a key role in the development of arthritis in these mice, a strong antibody response was also aroused after COMP immunization, likely contributing to the disease development further[112]. A specific B cell epitope was also defined by screening serum samples from mice previously immunized with COMP.

4.2 STUDY II: SYNTHESIS OF AN ARRAY OF TRIPLE-HELICAL PEPTIDES FROM TYPE II COLLAGEN FOR MULTIPLEX ANALYSIS OF AUTOANTIBODIES IN RHEUMATOID ARTHRITIS

In this study, a library of triple helical peptides containing the major B-cell epitopes on CII was established as a platform to screen anti-CII antibodies and search for potential biomarkers.

Despite collagen's status as a proposed autoantigen in RA for decades[113] and a large amount evidence supporting the association between anti-CII antibodies and RA[114], anti-CII antibodies is not widely used as a biomarker for diagnosis or prognosis in clinic. The clinical criteria only include traditional biomarkers, RF and ACPA[18]. A standardized assay for anti-CII antibodies is needed to include anti-CII antibodies as a biomarker in RA, which is what we attempted to do in this study.

Each chain of these THPs were designed as a 24-amino-acid sequence with 5 GPO repeats at both ends. The sequence contains both the native and citrullinated form of some earlier defined epitopes of CII, such as C1, U1, E10, and F4. Triple helical structure was formed by assembly of three identical CII chains relying on a covalent linkage of three strands at the C-terminal branch. Besides, for later usage of streptavidin-mediated capture of these THPs in immune assays, the biotin moiety was introduced to these THPs as an affinity tag.

After the design, these peptides were synthesized and purified, followed by chemical characterization and purity examination using analytical reverse-phase high-performance liquid chromatography (HPLC), mass spectrometry and circular dichroism spectroscopy. The evaluation results showed that these THPs were synthesized in desired form with high purity. A thermal study also demonstrated that most THPs had melting temperatures ranging between 46 °C and 57 °C, indicating a relatively high stability.

In previous studies, it was found that various anti-CII antibodies could have various roles in RA. For example, mAbs directed against the C1 and U1 epitopes were found to be able to induce arthritis in mice[66], the F4 epitope, however, has been reported to be protective[115] and suppress development of arthritis in CAIA mice[67]. The result confirmed earlier report by the observation of a significantly elevated level of antibody specific to C1, U1 but not F4 epitope in RA patients (n= 2075) compared to controls (n= 935). Finally, a novel finding that epitope E10 is also related to RA.

In addition, the results of this study also demonstrate that the binding of anti-CII antibodies to antigen is conformation dependent. This means a triple helical structure is required for the binding[116], highlighting the need to have a library of triple helical peptides synthesized as a tool for detailed research on anti-CII antibodies as potential biomarkers in RA.

4.3 STUDY III: ANTIBODIES TO CARTILAGE OLIGOMERIC MATRIX PROTEIN ARE PATHOGENIC AND CLINICALLY RELEVANT IN RHEUMATOID ARTHRITIS

In this study, the properties and functions of anti-COMP antibody 15A were investigated in detail. The results demonstrated that immunization with 15A alone could induce arthritis in naïve mice. This is quite interesting finding considering that although it has been well known that anti-CII antibodies can induce arthritis in naïve mice there is not much evidence showing that anti-COMP antibodies alone can have similar effect[80]. Histology staining showed that 15A could specifically bind to cartilage and COMP both in vivo and in vitro, partly explaining why it can induce joint inflammation in mice.

To further explain the mechanism of 15A specifically recognizing COMP in joint cartilage and to establish the correlation between its molecular structure and function, the 3D structure of complex of 15A Fab and its epitope, P6 peptide, was characterized. The findings revealed the molecular basis of that 15A antibody could bind to the surface of the native protein in vivo and efficiently induce arthritis in mice.

Another interesting finding is the effect of Ca^{2+} ions on the interaction of 15A and COMP and this effect was dose dependent. It is earlier reported that presence of Ca^{2+} ions can influence the conformational changes of COMP[117,118] and *in silico* docking of 15A Fab and the P6 epitope in the 3D structure of (truncated) COMP demonstrated that the epitope would not be accessible to the 15A mAb in the absence of Ca^{2+} ions. So, it is possible that presence of Ca^{2+} ions in joints could result in increased accessibility of some epitopes of COMP and also citrullination of these epitopes, further leading to the formation of local immune complexes in joints and increasing joint pains and inflammation.

An even more interesting finding obtained in the screening of RA cohort with established COMP peptide library is that the levels of serum IgGs specific to the 15A epitope, P6 peptide, as well as its citrullinated variant were significantly elevated in a RA cohort compared to the healthy controls. They also correlated with a higher disease activity score, implicating the potential role of anti-COMP antibodies as a diagnostic and prognostic marker in a subset of RA patients.

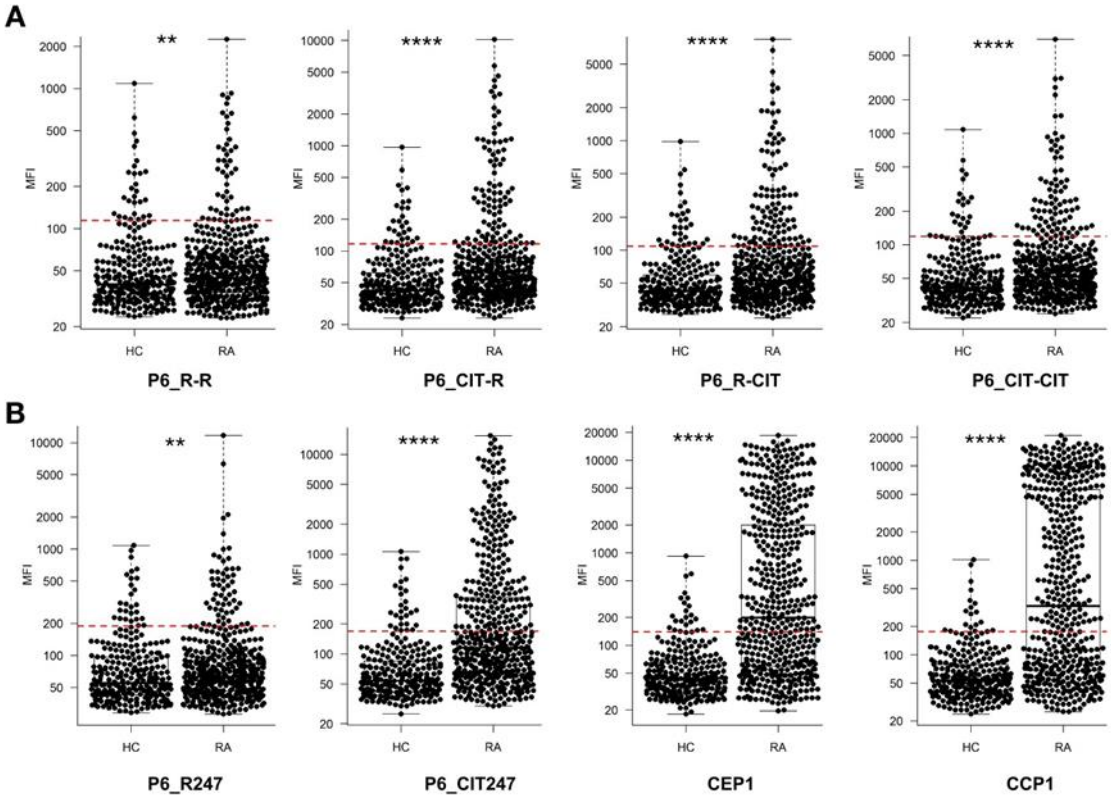

Figure 1 The levels of antibody reactivity to all P6 variants were increased with statistical significance ($p < 0.05$) in RA patients compared to population controls. Among the peptides of linear form, compared with the unmodified P6-R-R ($p = 8.24\times10^{-3}$), the autoantibody responses to the three citrullinated variants, P6-R-CIT ($p = 1.13\times10^{-10}$), P6-CIT-R ($p = 6.37\times10^{-10}$), and P6-CIT-CIT ($p = 4.11\times10^{-9}$) were obviously more significant. Similarly, such difference can also be observed in the peptides of cyclic form: citrullinated P6 epitope P6-CIT247 ($p = 2.47\times10^{-33}$) showed much higher reactivity in RA patients than its unmodified form P6-R247 ($p = 5.72\times10^{-3}$)). Among all peptides, P6-CIT247 showed the highest prevalence (38%). In comparison, the prevalence of antibody response to two classical citrullinated peptides CCP1 (57%) and CEP1 (56%) were also analyzed in the same cohort, which are comparable to previous report[119, 120].

4.4 STUDY IV: ANTIBODY RESPONSE TO CARTILAGE OLIGOMERIC MATRIX PROTEIN IN A PANEL OF HLA-DR4 TRANSGENIC MICE AND RHEUMATOID ARTHRITIS

In this study, the original attempt was mainly to investigate whether COMP can induce arthritis in mouse strains that express human MHCII alleles DRB1*0401, DRB1*0402, and DRB1*0405. To deplete the possible influence of B6N background (see **Study I**), hCOMP_F95S instead of native hCOMP was used in immunization of these humanized mice. In order to increase the susceptibility for arthritis, the Ncf1 (m1j/m1j) mutation was

introduced in DR0401 mice. The results showed that only mild arthritis developed in about 20% of the DR0401 mice and no arthritis was observed in the DR0402 or DR0405 mice.

To further test the atherogenicity of citrullinated antigen, citrullinated hCOMP_F95S was also prepared and used in immunization of these humanized strains with similar protocol compared to the mice immunized with hCOMP_F95S. No severe arthritis was observed either.

But all strains developed a strong anti-COMP IgG response. To find out the major B cell epitope in COMPIA in these humanized strains, screening of serum samples from immunized mice with the established COMP peptide library described before was done. The most prominent epitope P-C-22-R, with the sequence LVRNPDQRNT, was proved to be quite conservative and shown to occur in all the humanized mouse strains and the B6N strain, known to be susceptible to COMPIA. Besides, P-C-22-Cit, the citrullinated version of P-C-22-R, also have a significantly elevated response level in most mice. Another interesting finding correlated with **Study III** is that P-C-14-R, which is the epitope of one pathogenic anti-COMP antibody named as 15A, also showed an enhanced antibody response in a few mice. There are relatively mild reactivities to some other peptides such as P-C-5-R, P_C_339, and P_C_418, which gave a response in about half of these mice with a relatively low level. When comparing different strains, it is obvious that the antibody response appeared earlier and was more robust in the DR0401.Ncf1 mice than in other strains, possibly because the Ncf1 mutation increases the susceptibility of these mice. However, the antibody response pattern is distinctive in mice immunized with citrullinated hCOMP_F95S, with P-C-15-CIT, the epitope of 15A, gave a strong response, while only a few arginine peptides showed a weak response in these mice.

Later similar screening was also done using sera from an early RA cohort, TIRA-2. The results showed that the antibody response to COMP was mainly directed to the citrullinated form in RA patients compared with healthy controls, though there is also some elevation in the response levels to arginine peptides identified in COMPIA in mice. The level of P-C-15-CIT was significantly increased in both RA patients and mice immunized with citrullinated COMP. It was also associated with disease activity in RA patients.

5. CONCLUSIONS

In **Study I**, a new model for RA, COMPIA, was established in B6N mice and the major B and T cell epitopes involved in the arthritis development were defined.

In **Study II**, a library of high-quality THPs covering the major B-cell epitopes of CII was established, affirming the possibility of investigating potential biomarkers for RA using multiplex analysis of autoantibodies. The screening result using this library confirmed the roles of C1 and U1 as the two immuno-dominant CII-epitopes for antibodies in RA clarified in previous reports.

In **Study III**, an anti-COMP antibody 15A was found to be pathogenic and be able to induce arthritis in mice alone. The 3D structure of 15A complex with its epitope, P6 peptide, was clarified. The conformational mimicry of a peptide with the same sequence occurring in the full-length COMP protein explains how such antibodies can cross-react with the corresponding protein, bind specifically in vivo and be pathogenic. The response levels of antibodies to P6 peptides in native or modified forms are elevated in RA patients and correlate with higher disease activity.

In **Study IV**, despite a strong IgG response to COMP and similar B epitope recognition as in mice known to be susceptible to COMPIA, the mice expressing human MHCII did not develop severe arthritis. The B cell epitope in mice immunized with COMP was an arginine peptide; however, in RA, the most significantly elevated responses appear against citrullinated peptides, indicating citrullinated COMP peptides might function as useful biomarkers in RA.

6. POINTS OF PERSPECTIVE

Tremendous efforts have been made by researchers all over the world to investigate RA, yet a detailed mechanism of this disease remains unclear and thus no cure for this disease is available currently. Now people realize it could take possibly another century worth of work to solve RA's problem. However, it is always possible to improve patients' condition by early diagnosis and intervention. Also, it is well known that RA patients can include very distinct subsets with different biomarkers and varying response to the same treatment. Therefore, biomarkers garner wide attention in RA research. Although the current diagnostic criteria only include RF and ACPA, a lot of autoantibodies specific to joint proteins, such as CII or COMP, are all potential candidates for an updated biomarker list for RA. In addition, identification of these autoantibodies could shed light on the development of new classification system and improvements in individualized treatment of RA. To achieve this, different mice strains with genetic modifications were used to mimic humans and various experimental models were established to grasp the different aspects of this heterogeneous disease. The efforts and findings in studies present in this thesis may not give answers to the most concerned question, but providing an attractive path to further investigation and finally solution of RA.

7. ACKNOWLEDGEMENTS

At the end of my PhD student journey, I am glad to express my gratitude and respect here. It has been my privilege to have had people as intelligent, hardworking, cooperative as you surrounding me during my PhD studies, and I would not have survived it without all of your support.

To my main supervisor, **Rikard**: Thank you for giving me the opportunity to come here and meet all the other great people at MIR! I was a naïve student knowing nothing about lab work and it was your patient guidance that led me on this scientific road. Thank you for always being so enthusiastic when listening to my ideas, so encouraging when I got frustrated and so supportive when I met difficulties. It has been my honor to be your student and your impact, the strict attitude to science, will be with me during all my life.

To my co-supervisor, dear **Bingze**: Thank you for accepting me as a student and teaching me from the first day I arrived in MIR. It is difficult to describe your importance to my PhD studies with words. You have always selflessly offered to me help when I needed it. Sometimes I avoided asking you for help because I don't want to bother you too often. But still, you would come and help me in different ways. Thank you also for being a model to follow and I guess everyone in MIR will agree with this.

To **Erik**: Thank you for actually being my other co-supervisor, if not officially. You taught me the basic principles in designing experiments and you endlessly gave me valuable suggestions and ideas for my research. You also showed me how important it is to improve my presentation skills, which I will continue to work on in my future life.

To lovely **Laura**: Thank you for generously sharing your mice with me. It was a pleasure to collaborate with you. I usually feel awkward easily, so I hate to ask people for anything, but you are so nice and never let me feel any embarrassment while giving exactly what I need.

To **Rajan**: I feel lucky to have you come to our group and make a pleasing cooperation with me in the end of my PhD journey. Thank you for lots of inspiring discussions and technical supports!

To **Zhongwei**: Thank you, firstly, of course, for the HE staining you did for me. It was great work especially considering that you were quite busy with your own things but still made time for my slides. Thank you, secondly, for your IT support. When students defend, they only experience the busy period once, while you as the person in charge of all our IT things, experienced it 5 times this year! Thank you, finally, for your accompany as a neighbor and friend. I may not talk much but your jokes and dialect really made me smile a lot.

To sweet **Huqiao**: We actually didn't have much chance to talk—scientifically or non-scientifically—until you moved to Mike's old place. It was originally the hardest time during my PhD study, with serious homesickness. You changed my situation in a way you may not

realize. Of course, you gave many smart and novel ideas for my research. But I would prefer to say thank you, just for being sweet and considerate, just for having been there with me.

Jaime, our baking genius, thank you for being a sweet friend of mine and a good Swedish translator when I struggled with customer service. I will never forget the taste of your home-made cakes and I believe your talent is not only in baking but a lot of other things! **Mike**, thanks for being a humorous neighbor. And please forgive me for sometimes failing to respond to your great sense of humor. I didn't mean to, and I actually appreciate you for always being energetic and fresh. **Michael**, thank you for always being so helpful and friendly. I really enjoyed time spent with you in the research school and I can still remember how you shared your own life story, your family and also your political views with us during the supper. Sometimes you gave me a feeling of the old gentlemen's style and it really impressed me. **Taotao**, thanks for always being so optimistic and athletic! And also, for the interesting memory of sharing a room with me in Holland. **Yibo**, our MIR musician, thanks for your excellent music. I am by no means an expert, but I really admire your talent in art. **Anna**, our Miss Fashion, your work in your project is as excellent as your fashion taste! Lovely **Xiaojie**, thanks for your delicious pudding and nice smiles every day! **Alex K**, thanks for your high-quality presentations in our project meetings. **Alex M**, thanks for the good discussion on F4's mechanism. **Gonzalo**, thanks for always making the beneficial remarks and suggestions in seminars. **Meng**, thanks for the valuable 0402 mice. **Guanzhi**, for participation in protective group. **Thomas**, for bringing some fresh air to our group. **Qijing and Chang**, for a lot of discussions and joy time spent together!

Outi, you are my original co-supervisor and pitifully I missed the chance to be your student. Luckily in the last year of my PhD study, I regained the opportunity to work with you. I would say that you are a great researcher and a project leader! **Lei**, thanks for a lot of support in the Luminex and thanks for inviting me to your home. **Changrong**, thanks for giving me a lot of tips on both my project and my daily life. It was my pleasure to collaborate with you. **Silvia**, thanks for a lot of work to optimize the Luminex protocol. **Vilma**, thanks for always being so patient and helpful. We didn't have too many discussions but every time I learnt something from you. And of course, thanks for the nice photos you made for me! **Amit** and **Ulrika**, for some nice talks when I just arrived in Sweden. **Liselotte**, thanks for the excellent work as a lab manager. Your positive attitude in the movement to Biomedicum really helped the whole group a lot. **Angel**, as the core of MIR, you have done amazing organization work. You also guided me and Yibo on how to prepare a workshop and it was extremely valuable experience.

Besides MIRs, I would also say thanks to our collaborators: **Alf Kastbom, Christopher Sjöwall** for TIRA-2 cohort samples, **Inger Gjertsson** for BARFOT cohort samples, **Jan Kihlberg and Johan Viljanen** for triple helical peptides design and synthesis. These are the basis of my research and none of my research could have been done without your involvements.

Here are some special friends to be appreciated now. My research has mainly 2 parts: one part was on human cohorts, and another was on animal models. Therefore, I would like to show my

gratitude and respect to all the patients involved, mice sacrificed in my project and people working in animal house.

Also special thanks to The National Clinical Research School in Chronic Inflammatory Diseases, to its organizer **Jan Alvar Lindencrona** and **Helena Erlandsson Harris**, and to the friends I made during all the workshops and seminars. It is great experience and unforgettable memory.

Finally, my family and friends in China. Thank my parents, for being perhaps the best couple I have met and giving me a most enlightened and harmonious family. Thank you for unconditional love and trust. Thank you for telling me it is ok to be less competitive to my peers and just enjoy what I am. Especial thanks to my mom, who started and achieved a master's degree in her 50's for nothing but interest, it was her actions that gave me the motivation to persist and explore and never give up. And also especial thanks to my dad, who always find positive things in life, it was his attitude giving me the feeling PhD is not a stressful task but an interesting game to win or something to learn. And my friends, **Yujingya, Zhushan, Xiaolin, Wangting, Cuican, Guomin, Chenhua, Chenxinxia, Cuiyanhua, Gaojiwei, Guocheng, Gengkeyi, Hanhuirong, Hejingyan, Huxiaomeng, Jiaotong, Jinming, Konglinghua, Liangxiuming, Lidanyang, Lilin, Lixia, Lishuijie, Liuhui, Liuxinyuan, Liuyaxuan, Renlipeng, Songdawei, Sunshihua, Tongdongmei, Wuyujiao, Xingxiangling, Xujing, Xushanshan, Yaozeyu, Yinweiyao, Zhangji, Zhangyujie, Zhengjiaojiao, Zhengyunjuan, Zhongjianghong, Zhoubaoyi, Zhouqianqian**. As a start of the story, I have decided to go to another university to get a MD degree in 2017 and it was Jingya, who was working in Karolinska Institute then, suggested to me to come to Karolinska and get a PhD instead. So, she changed my mind and my life. Thank Zhushan for always waiting for me no matter how many times I disappoint you. Thank Xiaolin for the thousands of pages of chat records we made together in the past 10 years. And thanks all the friend I made after coming to Sweden. Without their friendship, I would feel lost in a land far away from home. And finally, thanks to my homeland. It was my country that provides support for my study, so I have the opportunity to study here. And my feelings towards homeland is always my source of power during all the times.

It is really hard to acknowledge everyone who gave me help and support in the past 4 years and there will be always details missing in the list. Here I would like to sincerely thank everyone I met along the way over the years and wish you best luck!

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