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FUNCTIONAL STUDIES OF ANTI-GPI MONOCLONAL ANTIBODIES AND ANTI-CITRULLINATED PROTEIN ANTIBODIES (ACPAS)

李桃桃

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致恩师，好友和家人！

天行健，君子以自强不息；地势坤，君子以厚德载物。

——《易经》

POPULAR SCIENCE SUMMARY

Rheumatoid arthritis (RA) is a organ-specific chronic autoimmune disease that primarily affects the joints, causing symptoms such as pain, swelling, and stiffness. It affects about 1% of the global population and women are more prone to this disease. Due to the incomplete understanding of the disease's pathogenesis, there is currently no known cure for the condition. Furthermore, the incubation period of the disease is typically long. Therefore, early diagnosis and timely, effective treatment are crucial for helping patients manage and alleviate the symptoms of the disease. To early and accurate diagnose RA, the identification of new biomarkers that can be used to predict the onset and progression of RA has been an important item in the field of RA research.

Autoantibodies are a hallmark of many autoimmune diseases. The presence and levels of autoantibodies can be used as diagnostic and prognostic markers for these diseases, and targeted therapies aimed at reducing autoantibody production or activity are a promising approach for treatment. Glucose-6-phosphate isomerase (GPI) is one of many RA-associated antigens. GPI protein and peptide immunization can induce human RA-like arthritis in most mouse strains. Passive transer of anti-GPI antibodies induced arthritis in a short time. However, the major pathogenic B-cell epitopes of GPI, as well as the association of anti-GPI antibodies with human RA, are currently uncertain. Anti-citrullinated protein antibodies (ACPAs) is another autoantibody that appears above ten years before the clinical onset of RA. ACPAs have the highest specificity for RA compared to other antibodies. The production of ACPA is thought to be highly associated with a specific HLA-DRB1 allele, HLA-DRB1*0401. However, reliable experimental data to verify this association are lacking.

In this thesis, we take the perspective of autoantibodies and investigate the characteristics of specific antibodies and their roles in the development of RA. We also verify the relationship between ACPA and MHC gene risk factors using animal experimental data.

A summary of our main findings:

1. In the first two studies, we looked at how a harmful antibody that causes arthritis sticks to cartilage. We found that the part of the cartilage that the antibody attaches to can change and this change can make the antibody even more harmful. We also found that the levels of this antibody are higher in some RA patients and may be a reason why their disease is more severe. We think that discovering these epitopes can help doctors diagnose RA earlier in patients who have specific antibodies targeting these epitopes. Additionally, these epitopes can serve as effective targets for RA treatment.

2. In the third study, we investigated the association of ACPAs response and development of arthritis with HLA-DR4 alleles. In this study, we conclusively demonstrate an association between ACPA induction and HLA-DRB1*0401 by using a unique humanized mouse model. Interestingly, we found that the function of ACPAs is variable. ACPAs cross-reactive with cartilage can be pathogenic, whereas other promiscuous ACPAs can be protective or have no detectable impact on arthritis.

ABSTRACT

RA is an autoimmune disease that primarily impacts joints throughout the body. The disease can result in pain, stiffness, and reduced mobility. It is a chronic disease, meaning that it lasts a long time and can worsen over time if left untreated. As the disease progresses, it can lead to joint deformity and severe pain, which can significantly impact the patient's daily life. While there are treatments available to manage the symptoms of RA, there is currently no cure for the condition.

Glucose-6-phosphate isomerase (GPI) is an enzyme that plays a key role in glycolysis, the metabolic pathway that breaks down glucose to produce energy. Establishment of two GPI-dependent arthritis mouse models (K/BxN and GPI protein-induced arthritis mouse models), successfully identifying GPI as one of the RA-associated autoantigens. In human RA, elevated levels of free GPI protein and anti-GPI autoantibodies were found in the sera and synovial fluid of RA patients. Despite extensive research on the pathogenicity of GPI in RA, many questions remain unanswered, including what are the major pathogenic B-cell epitopes of GPI? What is the prevalence of anti-GPI antibodies in RA patients? Are anti-GPI antibodies specific for RA disease?

Cartilage Oligomeric Matrix Protein (COMP) is a glycoprotein that helps in the formation and maintenance of cartilage. It exists in the synovial fluid and serum of RA patients and the level of COMP have been found to be positively correlated with the severity of the disease. Studies have indicated that COMP might contribute to the breakdown of cartilage in RA by stimulating the production of inflammatory cytokines and activating enzymes known as matrix metalloproteinases.

Anti-citrullinated protein antibodies (ACPAs) are autoantibodies that have highest specificity for RA. During the autoimmune stage of RA, which occurs years before clinical symptoms appear, the immune system already begins to produce ACPAs. Studies have shown that the production of ACPAs is highly associated with the HLA-DRB1*0401 allele, which is one of the highest genetic risk factor for RA. Some studies believed that HLA-DRB1*0401 allele increases the likelihood of citrullinated peptides being presented to T cells, which can result in the production of ACPA by B cells. However, subsequent scientific research has disproved this hypothesis, indicating that HLA-DRB1*0401 does not exhibit a preference for presenting citrullinated peptides over non-citrullinated peptides. The current hypotheses and associated conclusions are either based solely on statistical analysis, so further experimental data must be obtained to test these hypotheses.

In the first study, we identified the arthritogenic B cell epitopes of GPI in mice. We showed that this pathogenic GPI B cell epitope is exposed exclusively on structurally modified GPI on the cartilage surface. Identification of these specific B cells by tetramer technology

showed that these cells were not negatively selected and present in naïve mice. These naturally autoreactive cells escaped tolerance mechanisms and could potentially play a pathogenic role in disease as shown by the association of the antibody response against this epitope with clinical disease parameters in human RA cohorts.

In the second study, we investigated the pathogenic role of anti-COMP antibodies in murine arthritis models in conjunction with studies on the autoantibody response to COMP in human RA cohorts.

In the third study, our findings demonstrate a link between the induction of ACPAs and the presence of HLA-DRB1*0401, as well as between the development of arthritis and this HLA allele. Interestingly, we found that the function of ACPAs is variable. ACPAs cross-reactive with cartilage can be pathogenic, whereas other promiscuous ACPAs can be protective or have no detectable impact on arthritis.

SCIENTIFIC PAPERS INCLUDED IN THIS THESIS

I. **Li, T**., Ge, C., Krämer, A., Sareila, O., Leu Agelii, M., Johansson, L., Forslind, K.,… … & Holmdahl, R. (2023). Pathogenic antibody response to glucose-6-phosphate isomerase targets a modified epitope uniquely exposed on joint cartilage. *Annals of the Rheumatic Diseases*.

II. Ge, C., Tong, D., Lönnblom, E., Liang, B., Cai, W., Fahlquist-Hagert, C., **Li, T**.,… & Holmdahl, R. (2022). Antibodies to cartilage oligomeric matrix protein are pathogenic in mice and may be clinically relevant in rheumatoid arthritis. *Arthritis & Rheumatology*, 0–3

III. **Li, T ,** Ge, C , Xu, B , Lundström , S, Krämer, A , … & Holmdahl, R.(2023) MHC class II associated immune response to citrullinated proteins and development of arthritis in humanised mice. *Manuscript* .

SCIENTIFIC PAPERS AND MANUSCRIPTS NOT INCLUDED IN THE THESIS

I. **Li, T ,** Ge, C , Luo, H , Krämer, A , Coelho, A … & Holmdahl, R.(2023) Citrullinated Type II collagen immunization breaks the physiological T cell tolerance to the immunodominant T cell epitope in P.266 E mice. *Manuscript.*

II. Krämer, A, **Li, T**, Lundström, S, Moreno Giró, À, Coelho, A, Xu, Z, Xu, B, Zubarev, R, Holmdahl, R.(2023) Fc Sialylation has no effect on pathogenicity of arthritogenic antibodies. *Manuscript*.

III. Ge, C , **Li, T**, Tong, D, Dobritzsch, D, Lönnblom, E, Cecilia Hagert, Holmdahl, R. (2023) Molecular basis for the recognition of triple helical collagen type II by pathogenic antibodies. *Manuscript.*

IV. Laura Romero-Castillo, **Li, T**, †, Nhu Nguyen D†, Outi Sareila, Bingze Xu, Viktoria Hennings, Ana Coelho, Zhongwei Xu, Carolin Svensson, Vilma Urbonaviciute1, Zeynep Sener, Johan Bäcklund, Olov Ekwall, Harald Burkhardt, Rikard Holmdahl. (2023) Autoimmune responses and arthritis in mouse models with human MHC class II and invariant chain knock-in. *Manuscript.*

V. Urbonaviciute, V , Romero-Castillo, L ,, Xu, B , Luo, H , Schneider, N , Weisse, S , Nhu-Nguyen, D , Do, A , Fernandez , G , **Li, T**, … & Holmdahl, R. (2023). A peptide-based tolerizing vaccine against autoimmune arthritis directly interacting with antigen-specific T cells. *Proceedings of the National Academy of Sciences*. Under revision.

CONTENTS

List of abbreviations

ACPAs	Anti-citrullinated protein antibodies
APCs	Antigen-presenting cells
bCOL2	Bovine Type II collagen
CII	Type II collagen
COL2	Type II collagen
COMP	Cartilage Oligomeric Matrix Protein
CIA	Collagen induced arthritis
CAIA	Collagen antibody induced arthritis
DPI	Days post immunization
DCs	Dendritic cells
FcgR	Fc gamma receptors
GPI	Glucose-6-phosphate isomerase
GPIA	GPI protein/peptide induced arthritis
HLA	human leukocyte antigen
JP2	Type II collagen
JP3	Cartilage Oligomeric Matrix Protein
JP5	Protein-arginine deiminase type-4
MHC II	major histocompatibility complex class II
mAbs	Monoclonal antibodies
OA	Osteoarthritis
PsA	Psoriatic arthritis
PADs	Peptidylarginine deiminases
PAD4	Protein-arginine deiminase type-4
PTPN22	Protein tyrosine phosphatase type 22
PCs	Plasma cells
Pg	Porphyromonas gingivalis
RA	Rheumatoid arthritis
RANKL	Receptor activator of nuclear factor kappa-B ligand
SLE	Systemic lupus erythematosus

SNP	single nucleotide polymorphism
SE	shared epitope
TCR	T cell receptor
Tfh	T follicular helper
TNF-a	Tumor necrosis factor-a

1 INTRODUCTION

Rheumatoid arthritis(RA) is a chronic autoimmune disease that, as its name suggests, primarily affects joints throughout the body. This disease affects 0.5-1% of the world's population. Despite significant advances in understanding the disease pathogenesis and the development of targeted therapies, the precise mechanisms underlying the initiation and progression of RA remain unclear.

The development of RA is attributed to a combination of various factors, with genetic and environmental risk factors being the most significant ones, which interact to trigger an autoimmune response that causes synovial inflammation and joint destruction. Various genetic factors, such as specific HLA alleles, lymphocyte receptor signaling genes, and other loci determining the activity of the immune system, have been associated with the development of RA. Additionally, environmental factors, like dietary factors, smoking, and infections, have been implicated as potential facilitator of RA development.

The presence of a high quantity of autoantibodies is a defining characteristic of autoimmune diseases. In the case of RA, different types of autoantibodies have been well characterized. Among all these autoantibodies, rheumatoid factor (RF) and ACPAs have been used as diagnostic and prognostic markers for RA with RF present in approximately 70% and ACPAs in 50-75% of RA patients. However, the exact targets of these autoantibodies and their contribution to disease pathogenesis are not fully understood.

The present thesis focuses on investigating the pathogenic antibody response to three different targets in RA, namely GPI, COMP, and citrullinated proteins. The first paper of this thesis (Paper 1) identified the major pathogenic B cell epitope of GPI and established a positive correlation between the antibody level and disease severity. The second paper (Paper 2) explores the pathogenic role of autoantibodies against another RA-related autoantigen, COMP protein and studies clinically relevant of RA. Finally, the third paper (Paper 3) investigates the MHC class II-associated immune response to citrullinated proteins and its role in the development of arthritis in our unique humanized mice model.

In general, this thesis presents novel research findings on the involvement of autoantibodies in the pathogenesis of RA by thoroughly discusses the characteristics and arthritogenic properties of three types autoantibodies. The insights provided by this paper may be valuable for the development of new diagnostic methods and anti-RA treatment strategies.

2 LITERATURE REVIEW

2.1 Rheumatoid arthritis (RA)

RA is a chronic autoimmune disease that primarily impacts the joints, leading to inflammation, swelling, pain, and ultimately joint deformity. In addition to joint symptoms, RA can also cause systemic symptoms such as fatigue, fever, and weight loss.[1] RA has a global prevalence of about 1%, with a higher incidence in women than men. Although the exact cause of RA is still not entirely clear, it is believed to be the consequence of a complicated mixture between genetic and environmental factors.[2]

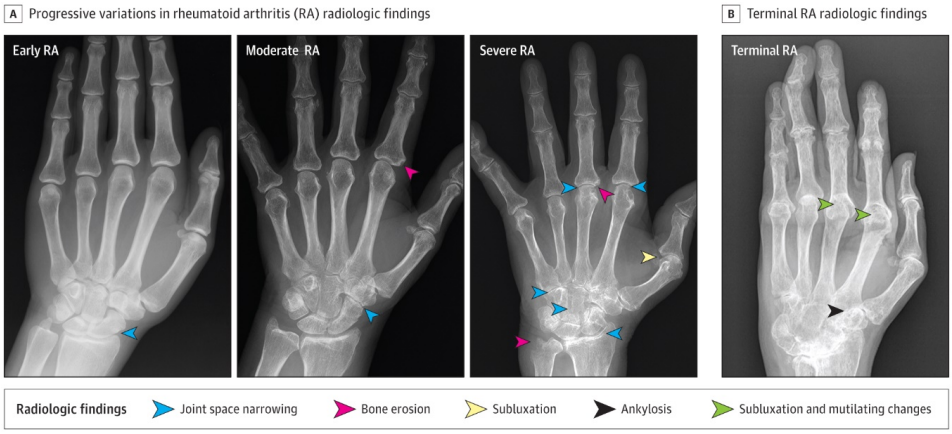


Figure 1 *Rheumatoid arthritis (RA) is a progressive disease that causes damage to an increasing number of joints over time, as evidenced by the worsening radiographic abnormalities from left to right. In* ***the early stages*** *of the disease (as shown in the left two images), bony or cartilage damage is either nonexistent or minimal, as can be observed in the nearly normal radiographs. In cases of* ***severe, established RA*** *(as shown in the second image from the right), joint damage can continue to worsen and affect additional joints. The image illustrates significant cartilage loss, resulting in joint space narrowing, as well as bone damage in the form of erosions. Malalignment is also apparent, particularly in the fifth digit. The image on the right depicts a case of* ***late-stage, terminal RA,*** *in which most of the joints commonly affected by RA have suffered significant damage. Notably, there is coalescence of carpal joints (indicated by the black arrowhead). These structural changes exemplify the stages of joint damage that can occur in RA when left insufficiently treated. The colored arrowheads in the image highlight specific abnormalities, but it is important to note that there are many other changes present in the right two radiographs.[3] Reprinted with permission from the American Medical Association (AMA).*

The pathogenesis of RA could be categorized into three phases[4]: **Autoimmunity stage:** At this stage, the immune system has already generated autoantibodies such as RF and ACPAs, which may lead to, or regulate, inflammation in the joints, but there are no noticeable symptoms yet. **Subclinical stage**: As the disease progresses, symptoms such as joint pain, stiffness, and swelling appear. This stage is sometimes referred to as early RA, and it can be difficult to diagnose because the symptoms are often vague and can be mistaken for other conditions. **Established RA stage**: Over time, the inflammation in the

joints can lead to damage to the cartilage and bone, causing deformities and making it difficult to move the affected joints.[5] This is known as established RA, and it can severely affect an individual's quality of life. Although there is no cure for RA, some people may experience periods of remission, during which their symptoms improve or even disappear completely. However, the disease can still be present and may flare up again in the future.

The process of diagnosing RA often entails a multifaceted approach, combining various methods such as clinical evaluation, laboratory analyses, and medical imaging.[6] The progression of RA begins several years before visible clinical symptoms appear, making early diagnosis and timely treatment crucial. The main medications currently available for the treatment of RA include nonsteroidal anti-inflammatory drugs (NSAIDs), disease-modifying antirheumatic drugs (DMARDs), and biologic therapies.[7] NSAIDs are effective in reducing pain and inflammation in RA patients, but they do not slow down the progression of the disease. DMARDs, have the potential to decelerate disease progression and protect the joints from damage in individuals with RA. The most commonly used DMARDs for RA are methotrexate, sulfasalazine, and hydroxychloroquine.[8]

In conclusion, RA is a chronic and debilitating disease that affects a significant portion of the global population, and while treatments are available to manage symptoms and slow disease progression, a cure for RA has yet to be discovered. Early diagnosis and intervention are essential to prevent joint damage and improve the prognosis of patients with RA. Developing new biomarkers that facilitate early diagnosis may improve the diagnosis and treatment of RA in the future.

2.2 Genetic factors

The occurrence and development of RA disease are closely related to genetic risk factors, with an estimated heritability of around 60%. Genome-wide association studies (GWAS) have revealed the association of around 100 loci with RA. The most consistently associated genetic factor with RA is the presence of certain human leukocyte antigen (HLA) alleles, specifically HLA-DRB1 alleles that encode the shared epitope (SE). [9–13]

The shared epitope (SE) is a sequence of five amino acids that is conserved in the third hypervariable region of the HLA-DRB1 gene, which is involved in the presentation of peptides to T cells.[14] The presence of SE alleles has been demonstrated to elevate the risk of developing RA, particularly in ACPA-positive individuals.[15,16] Certain variants of the HLA-DRB1 gene, such as HLA-DRB1*04:01, *04:04, *04:05, and *04:08 alleles are strongly associated with the development of RA.[17–19]

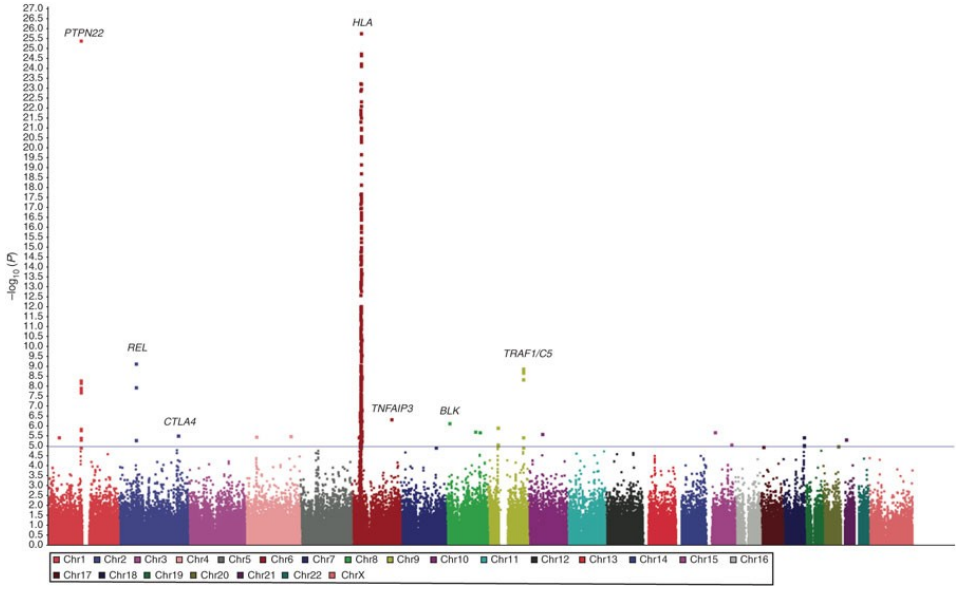


Figure 2 *Visualization of Genome-Wide Association Study (GWAS) Results with Correction for Inflation and Clustering: Identification of Significant SNPs in RA. The graph displays the position of each chromosome against the -log10 values of the trend-test P values after correcting for genome-wide inflation of χ2 and performing homogeneous clustering. Chromosomes are shown in alternating colors to enhance clarity, and a blue horizontal line is used to indicate SNPs with a P value of 1 × 10−5 or lower, which are considered significant. Note that the results for markers in the HLA region have been truncated.* Nature Genetics ***volume 41****, pages820–823 (2009). Reprinted with permission from Springer Nature.*

The precise mechanism by which SE alleles contribute to the development of RA is not yet fully understood, but it is believed to involve the presentation of autoantigens by HLA molecules to T cells, resulting in subsequent activation of the immune system and autoantibody generation. The SE alleles may affect the binding specificity of the HLA molecules, resulting in a greater ability to present certain autoantigens and thus leading to the production of autoantibodies and the development of RA. Additionally, SE alleles may contribute to the activation of pro-inflammatory pathways, further contributing to the pathogenesis of RA.

In addition to the HLA-DRB1 gene, other genes such as PTPN22[20,21], CTLA4, STAT4, CCR6, PADI4, and TRAF1-C5 have been identified as RA-associated SNPs as well. [17,22–24] Functionally characterization of all disease-associated SNPs is required to demonstrate causality of all disease-associated risk loci.

It is crucial to consider that genetic factors alone are not responsible for the development of RA, but they interact with environmental factors to increase the risk of developing the disease.[25,26][27,28]

2.2.1 Association of ACPA Production with SE-Encoding HLA Alleles

ACPAs recognize citrullinated peptides those are derived from various proteins such as collagen type II, fibrinogen, vimentin, and enolase, which are commonly found in the joints of RA patients.[29–35] As mentioned earlier, the presence of ACPAs is a unique characteristic feature of RA and is strongly associated with the presence of certain HLA-DRB1 alleles that encode the SE.[36–39] The association between ACPA production and HLA-DRB1 SE alleles has been extensively studied,[40] and it is estimated that approximately 60-70% of ACPA-positive RA patients carry at least one SE allele. The SE alleles associated with ACPA-positive RA include HLA-DRB1*0401, *0404, *0405, *0408,[41,42] In contrast, SE-negative RA patients are less likely to produce ACPAs.

The SE alleles are thought to contribute to ACPA production by influencing T cell responses to citrullinated peptides. Specifically, the SE alleles are associated with a reduced capacity to negatively select autoreactive T cells in the thymus, which leads to the generation of autoreactive T cells that can recognize citrullinated peptides. These T cells can then assist B cells to produce ACPAs, which contribute to the pathogenesis of RA.

Studies on transgenic mice expressing the HLA-DRB1*0401 SE allele have shown that they develop ACPAs and arthritis when exposed to citrullinated fibrinogen.[43] However, these studies are lacking proper arginine peptide controls to demonstrate that the ACPA response is specific to citrulline, and the evidence for arthritis is not convincing.

All in all, evidence exclusively demonstrating the dependence of ACPA induction on the HLA-DRB1*0401 allele is lacking, partly due to a lack of appropriate mouse strains as controls. To address this gap, Paper III in this thesis used a mouse model in which the DRB1*0401 SE allele was knocked in, providing a more naturalistic setting to investigate the effects of the HLA-DRB1*0401 allele on immune function and disease development.

2.3 B cells in the pathogenesis of RA

B cells are a type of immune cell that plays a crucial role in the body's defense system. In the context of RA, B cells have been implicated in the pathogenesis of the disease through several mechanisms:

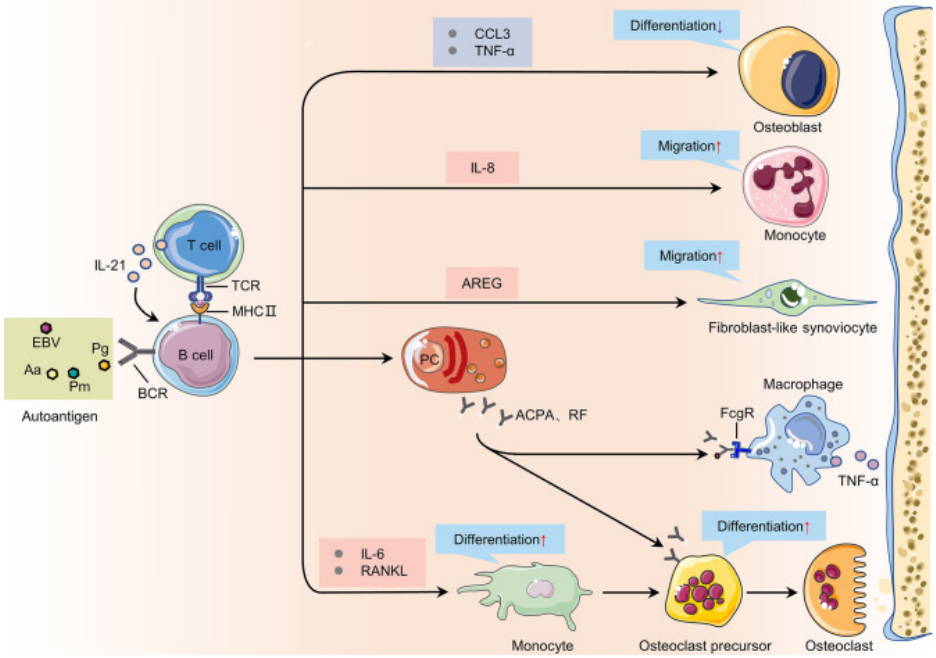


Figure 3 *Key Molecules and Pathogens Involved in B Cell-Driven Immunopathology in RA. AREG, amphiregulin;; Aa, Aggregatibacter actinomycetemcomitans; Pm, Proteus mirabilis; EBV, Epstein-Barr virus.[44] CC-BY 4.0.*

2.3.1 Antigen presentation

There are three primary types of antigen-presenting cells (APCs): B cells, DCs, and macrophages. These cells have the ability to efficiently take up, process, and present antigens to CD4+ helper T cells, eliciting an immune response. In RA, B cells are believed to play a critical role in the initiation and perpetuation of the immune response by presenting antigenic peptides to autoreactive T cells, which triggers their activation and proliferation, contributing to the inflammatory process in RA.[45] A specific T cell subset - T follicular helper cells (Tfh) cells - is essential for germinal center formation. During the germinal center reaction affinity maturation of B cells occurs, leading to the production of high-affinity antibodies and the formation of long-lived memory B cells. In RA patients, the number of Tfh cells in the peripheral blood and synovium significantly increased compared to healthy individuals.[46] The presence and proportion of Tfh cells has been correlated with ACPA serum titres, the hallmark antibody of RA. [47]

2.3.2 Production of autoantibodies

Autoreactive B cells differentiate into plasma cells which mainly secrete and produce autoantibodies. A wide variety of proteins are associated with RA and have been identified as RA-related autoantigens which can be recognized by the immune system and produce corresponding autoantibodies. The list of RA-associated autoantibodies is constantly growing, e.g. Rheumatoid factors, Anti-COL2, anti-GPI, anti-human cartilage

glycoprotein 39, ACPAs, anti-PAD, anti-immunoglobulin binding protein (BiP), and anti-histones antibodies. The presence of these autoantibodies can be helpful in diagnosing RA and may also be used as biomarkers for assessing disease severity and response to treatment.

Here, we have outlined the three main types of autoantibodies commonly associated with RA.

1. **Rheumatoid factor (RF):** RF binds to the Fc portion of IgG antibodies. The isotype of RF can vary, but the most commonly detected isotype is IgM. However, RF can also be of other isotypes such as IgG, IgA, and IgE. RF is found in up to 80% of patients with RA, and its presence is considered to be a diagnostic criterion for the disease. Nevertheless, RF is not exclusive to RA, but exist in other autoimmune and infectious diseases as well, like primary Sjögren's syndrome (60%–95%), SLE (15%–35%) and systemic sclerosis (20%–30%).[48,49] Furthermore, RF is also present in healthy subjects, so it is not specific for RA, and this greatly reduces its value as a diagnostic marker for RA.[50]
2. **Anti-citrullinated protein antibodies (ACPAs):** ACPAs are antibodies that target citrullinated proteins.[51] The sensitivity of ACPAs for RA is around 70% and the specificity for established RA rangs from 90% to 98%.[51–54] Same as RF, ACPA can be detetced up to 10 years before the disease onset. ACPAs can be detected most conveniently by the commercially available anti-CCP2 (cyclic citrullinated peptide) assay. Together with RF, the anti-CCP2 test is a clinically widely used marker for the early and specific diagnosis of RA. [55]
3. **Anti-carbamylated protein antibodies (anti-CarP):** Anti-CarP antibodies target carbamylated proteins, which undergone a post-translational modification that results in the conversion of lysine residues to homocitrulline(hCit) residues.[56] Both HCit and citrulline(Cit) are amino acids that undergo post-translational modification, and they have a similar structure except for the fact that hCit has an additional methylene group. The primary distinction between hCit and Cit is their method of formation. Specifically, hCit is produced through a chemical process involving cyanate and lysine, whereas Cit is generated enzymatically from arginine by PAD4.[56] Anti-CarP antibodies exist in up to 45% of individuals with RA and the antibody is correlated with radiological progression and worse clinical outcomes.[57,58] Several studies have shown that combining tests for anti-CCP, RF, and anti-CarP antibodies, particularly when all three are positive (known as triple positivity), can significantly increase the likelihood of diagnosing RA.[56,59,60]

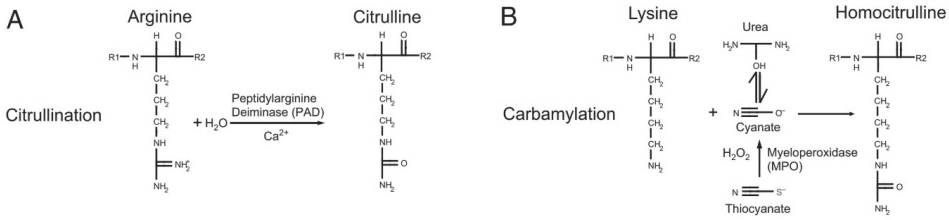


Figure 4*. This figure depicts citrullination (A) and carbamylation (B), which are two distinct processes that involve the modification of different amino acids in a similar manner, leading to the formation of comparable end-products.[56] Proc Natl Acad Sci U S A. 2011 Oct 18; 108(42): 17372–17377.*

2.3.3 **Cytokines Secretion**

The synovial tissue of RA patients harbors a diverse range of cytokines that are intricately linked to the development of the disease. Meanwhile, B cells present in the peripheral blood of these individuals are capable of producing and secreting various cytokines. These cytokines include tumor necrosis factor-alpha (TNF-α), interleukin-1 (IL-1), interleukin-6 (IL-6), and others.[61,62] B cells can also produce cytokines, which are signaling molecules that help to regulate the immune response. Maintaining a balance of these cytokines is crucial for preserving joint function, but an imbalance can lead to the development and progression of RA.

Because of their involvement in these processes, B cells have become an important target for RA treatments. One approach is to use medications that specifically target B cells, such as rituximab, which can deplete B cells by targeting CD20 located on the surface of B cells.[63] Another approach is to target the cytokines produced by B cells, such as interleukin-6 (IL-6).[61,64–67] Several medications that target IL-6, such as tocilizumab, are now available for the treatment of RA.

2.4 RA-related autoantigens

2.4.1 Glucose 6-phosphate isomerase (GPI)

GPI is an enzyme that plays a vital role in the glycolytic pathway, which is the metabolic pathway that breaks down glucose to produce energy in the form of ATP (adenosine triphosphate).[68] In addition to its role in the glycolytic pathway, GPI has been found to have other functions. For instance, GPI can also be called as angiogenic factor (AMF) which serves as a secreted cytokine to promote endothelial cell motility.[69] Moreover, GPI has been identified as as a neurotrophic factor for spinal and sensory neurons, and as a lymphokine that is produced by T-cells stimulated by lectins. Furthermore, research has shown that GPI can induce the secretion of immunoglobulins by human mononuclear cells.[68,70,71]

The discovery of the spontaneous arthritis mouse model, K/BxN, revealed the close association between gpi protein and RA. As a result, gpi has been categorized as one of

the autoantigens linked to RA. The pathogenesis of arthritis in mice has also been extensively investigated.[72,73] Purified anti-GPI polyclonal antibodies obtained from the sera of diseased K/BxN mice, as well as the sera themselves, have been shown to efficiently passively transfer RA into naïve mice. This transfer of arthritis has been shown to be independent of both B and T cells. GPI protein and peptide-induced arthritis (GIA and GPIA) have been widely used as arthritis mouse models. These two models are T and B cells dependent. Due to the therapeutic responses to anti-CD4 mAb,[74] anti-IL-17mAb,[75] TNFa and CTLA-4 Ig,[76] an IL-6 inhibitor,[77] and JAK-kinase inhibitor[78], the GPI-induced model has been demonstrated to be a vaulable tool for evaluating the efficacy of new drugs for treating the disease.

While GPI protein is typically found in the cytoplasm, studies have suggested that levels of both GPI protein and antibodies to GPI elevated in the serum of RA patients.[79,80] However there is ongoing debate about the prevalence of anti-GPI antibodies in the sera of RA patients,[81–83] as well as their specificity to the disease. Additional research is necessary to clarify these findings.

2.4.2 Cartilage Oligomeric Matrix Protein (COMP)

COMP is a large extracellular matrix protein that is predominantly expressed in cartilage and other connective tissues such as tendon and ligament.[84,85] It plays a vital role in maintaining the structural integrity and mechanical properties of these tissues. COMP is composed of five identical subunits, each consisting of four domains: a coiled-coil domain, a calcium-binding domain, and two type III repeats.[86] Mutations in the COMP gene have been associated with several skeletal disorders, including pseudoachondroplasia and multiple epiphyseal dysplasia.[87,88]

Increased concentrations of COMP in the bloodstream have been linked to joint pathology and cartilage damage in different medical conditions, including RA and osteoarthritis.[89–91] In fact, in RA, synovial fibroblasts within the joint produce high levels of COMP. This protein acts as a chemoattractant for immune cells, such as T cells and macrophages, leading to increased inflammation and joint damage. Additionally, COMP promotes angiogenesis, which is also associated with the inflammation seen in RA. Therefore, measuring COMP levels in the blood can help monitor disease activity and progression, and targeting COMP may hold promise as a therapeutic approach for RA.

2.4.3 Type II collagen (COL2)

Type II collagen is a vital component of cartilage, a connective tissue that provides cushioning and support to joints. This fibrillar collagen forms long, thin fibers that provide structural support to tissues. Composed of three protein chains, or alpha chains, wound together in a triple helix configuration, type II collagen is also present in other connective

tissues such as the vitreous humor of the eye, the nucleus pulposus of the intervertebral disc, and the inner ear.[92]

As a major component of cartilage, COL2 can be mistaken by the immune system and be recognized as a foreign invader, triggering an autoimmune response. This immune response can cause inflammation and damage to the joints, as well as the production of autoantibodies against CII. Studies have linked the presence of anti-CII antibodies to more severe joint damage and worse clinical outcomes in RA.[93–95] These antibodies can directly damage cartilage by inducing an inflammatory response and activating immune cells such as T cells and macrophages. Emulsifying CII with complete Freund's adjuvant (CFA) has been demonstrated to be a reliable method for inducing arthritis models in various mouse strains that are genetically predisposed to the disease. The resulting mouse disease exhibits characteristics similar to the pathogenesis of human RA.[96–98] And heterologous CII (e.g., rat CII, human CII) immunization induced severer and higher incidence arthritis in mouse than homologous CII.[99,100] The mouse study yielded important insights into the immune system's recognition of critical structures. Specifically, the Aq MHC class II molecule presents a glycopeptide from position 260-270 of CII to T cells for recognition.[101–103]. Notably, the DR4 and DR1 class II molecules in mice, which are linked to RA susceptibility, recognize an almost identical glycopeptide (CII261-273) that can cause arthritis.[104,105] T cells respond to the same peptide in human RA, specifically showing a preference for the galactosylated form. The lysine side chain at position 264 is the main target for both mouse and human T cells.

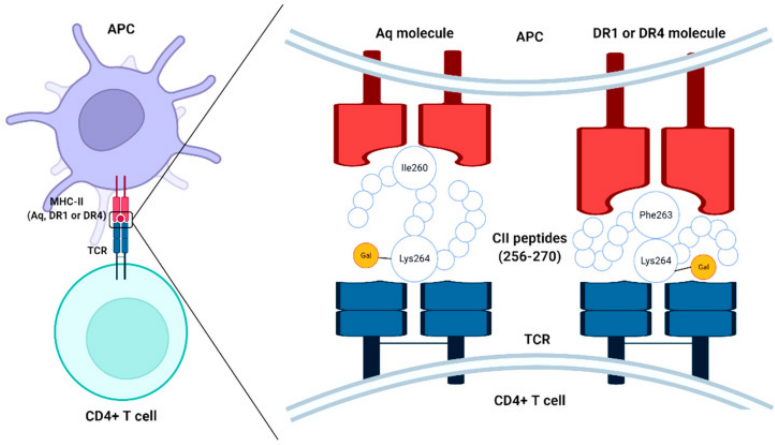


Figure 5 *This diagram illustrates how type II collagen (COL2) peptides (256–270) become fused with MHC-II molecules and the T cell receptor (TCR) of CD4+ T cells, through a process known as chimerism. APC-antigen-presenting cell.[92] CC-BY 4.0*

In the CIA model, B cells target epitopes located in the triple helical structure of CII, specifically C1, J1, U1, and D3. These epitopes have a conserved sequence that is also recognized in RA.[106–109] Mice injected with monoclonal antibodies (mAbs) targeting

these dominant epitopes developed arthritis, indicating that antibodies binding these epitopes are pathogenic functionally. The triple helical epitopes on the CII molecule, are characterized by a conserved motif consisting of an arginine and a hydrophobic amino acid in the sequence G-X-R-G. Notably, certain specific epitopes, such as the "C1" epitope located at position 359-370, contain tandem repeats of these conserved motifs.[110]

2.5 Citrullination of autoantigen enhances its arthritogenicity.

Several autoantigens related to RA, such as vimentin, fibrinogen, α-enolase, filaggrin, and COL2 can be citrullinated and are known to be targeted by ACPAs. [111–118] Several studies have compared the arthritogenicity of citrullinated and non-citrullinated proteins in mice. For example, one study found that immunization with citrullinated fibrinogen induced more severe arthritis in DR4-transgenic mice than immunization with non-citrullinated fibrinogen.[119] Another study demonstrated that citrullinated vimentin peptide are more cable of activating T cells compared to non-citrullinated vimentin in HLA-DRB1*0401 transgenic mice and HLA-DR$^+$ patients [120,121]

The increased arthritogenicity of citrullinated proteins/peptides is thought to be due to their increased immunogenicity. Citrullination creates a neoepitope that is perceived as foreign by the immune system, leading to the production of ACPAs, which plays a crucial role in the pathogenesis of RA. The conversion of positively charged arginine to neutral citrulline leads to altered peptide interactions with the shared epitope and an increase in peptide-MHC affinity, which is considered a key step in the pathogenesis of RA.[121] Citrullination also affect protein structure and function.[122,123] For example, citrullination of vimentin has been shown to alter its polymerization and increase its susceptibility to proteolytic degradation, which may contribute to the development of arthritis.

Overall, citrullination of proteins can increase their arthritogenicity in certain MHC-II restricted mice and is thought to play a role in the development of RA in humans. The mechanisms underlying the increased immunogenicity and altered structure and function of citrullinated proteins are an active area of research and may provide new insights into the pathogenesis of RA.

2.6 B cell epitope mapping

B cell epitope mapping is a technique used to identify specific regions or sequences on a protein antigen that are recognized by B cells and induce the production of antibodies. B cell epitope mapping is especially important in the development of vaccines, as it can help to identify the most immunogenic regions of a protein antigen.[124] This information can then be used to design more effective vaccines that induce stronger and more specific immune responses. B cell epitope mapping is also used in the diagnosis of autoimmune diseases, such as rheumatoid arthritis, where specific antibody responses are used as diagnostic markers.

There are several methods used for B cell epitope mapping, including peptide scanning, alanine scanning, and phage display. In peptide scanning, overlapping peptides covering the entire protein sequence are synthesized and tested for reactivity with antibodies. This helps to identify the precise amino acid residues that contribute to antibody binding. In alanine scanning, individual amino acid residues within the antigen are mutated to alanine, and the resulting peptides are tested for antibody binding. This allows for the identification of critical amino acids in the epitope. In phage display, bacteriophages displaying random peptide sequences are screened with antibodies to identify the specific peptides that bind to the antibody. In this thesis, a series of linear/cyclic/ triple helical peptides (THPs) peptides derived from several RA-rerated autoantigens were synthesis based on the positions of arginine. On both sides of the arginine or citrulline residue, seven consecutive amino acids were added. The peptide was modified to include biotin at the N-terminus, using the flexible linker amino hexanoic acid (Ahx).[125] Bead-based flow immunoassay was applied for the B cell epitope screening.[126]

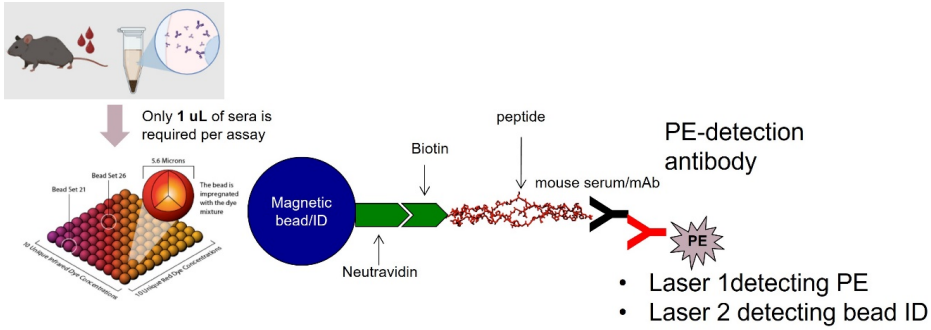


Figure 6: *Bead-based flow immunoassay for detection of antibodies in the serum. One bead population with specific ID carries one specific peptide. Maximum 1ul serum was needed for each biological sample.*

2.7 Mouse models used in RA research.

Animal models have proven to be crucial in investigating the cellular and molecular mechanisms that cause RA arthritis. Even though mice and humans are evolutionarily distant, the clinical characteristics of arthritis in mouse models frequently exhibit similarities to those observed in human RA.[127] In RA research, mouse models have been extensively utilized to investigate the underlying mechanisms of the disease and to develop and test new treatments. Each type of mouse models has its own strengths and limitations, but all contribute to advance our understanding of RA's pathophysiology. Here are some additional details about the different types of mouse models used in RA research:

Table 1 Models for rheumatoid arthritis.[127]

Model type	Disease name	Induction agent / target gene for genetic manipulation	Disease characteristics	MHC dependence
Human disease	Rheumatoid arthritis	Unknown	Chronic	HLA-DR4/DR1
Mouse models				
Induced arthritis models	*Staphylococcus aureus*-induced arthritis[128]	Toxic shock syndrome toxin-1-producing *S. aureus* LS-1 strain	Acute	$H2^k > H2^p$, $H2^q$
	Borrelia burgdorferi-associated arthritis[129][130] (model for Lyme disease)	Borrelia burgdorferi	Acute	Nd
	Collagen-induced arthritis	Heterologous collagen type II (COL2)[131]	Acute/chronic	$H2^q$ (A^q), $H2^r$
	Collagen-induced arthritis	Homologous collagen type II[99]	Chronic	$H2^q$ (A^q)
	COMP-induced arthritis[132]	Heterologous Cartilage Oligomeric Matrix Protein[132]	Chronic	$H2^b$
	GPI protein-induced arthritis[75]	Heterologous GPI protein	Chronic	$H2^q$ (A^q)
	GPI peptide-induced arthritis[134–136]	Peptide $GPI_{325-339}$	Chronic	$H2^b$
	Proteoglycan-induced arthritis[136]	Human cartilage proteoglycan	Acute to chronic	$H2^d$, $H2^k$
	Pristane-induced arthritis[137,138]	Pristane (2,6,10,14-tetramethylpentadecane)	Chronic	B^f, B^a, B^u[139]
	Anti-COL2 antibody-induced arthritis	Transfer of collagen type II specific antibodies[107,140]	Acute	MHC independent
	Anti-GPI antibody transfer-induced arthritis	Transfer of GPI-specific polyclonal antibodies[141]	Acute	MHC independent
Genetically Manipulated Models	TNF-α transgenic[142]	Human tumor necrosis factor	Acute	Nd
	IL-1Ra[143]	IL-1 receptor antagonist	Chronic	Nd
	HTLV-1 env-pX transgenic[144]	Human T-Cell Leukemia Virus Type 1 env-pX	Chronic	MHC independent
	K/BxN TCR transgenic[145]	$H2^k$ restricted bovine ribonuclease (KRN) peptide specific TCR	Chronic	$H2^{g7}$
Environment-induced model	Stress-induced arthritis[146]		Chronic	Nd

Nd = not determined or not clarified. Table adapted from: DOI: https://doi.org/10.1016/S0168-9525(02)02684-7

Overall, mouse models have played a valuable role in fostering our understanding of the pathophysiology of RA and have provided a valuable tool for the development and testing of new therapies. While it's worth noting that no model can exactly simulate the intricacies of human disease, and each model has its own strengths and limitations. The use of different mouse models in combination with clinical studies has allowed for a more comprehensive understanding of RA and has led to the identification of several promising therapeutic targets.

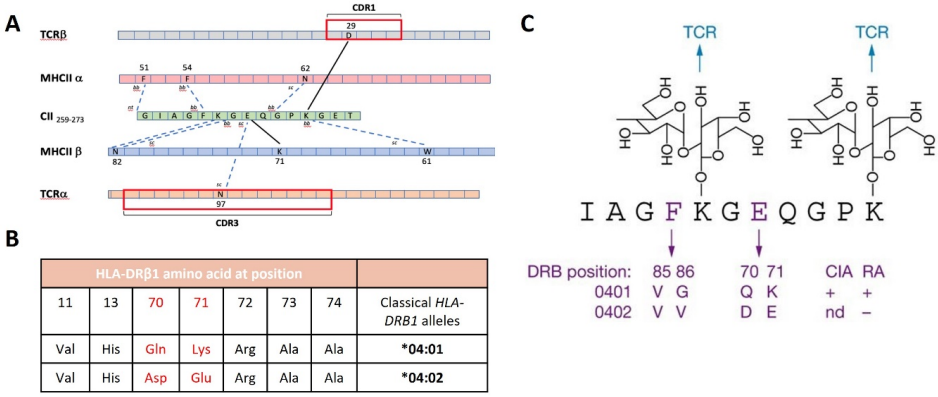
2.7.1 Humanized mouse model

Humanized mouse models are critical to progressing our knowledge of human RA. HLA-DR1/DR4 transgenic mice have been widely used to study the immune response to specific antigens and RA development.[119,147,148] However, using transgenic mice in research comes with some potential disadvantages. For instance, the level of transgene expression can vary greatly depending on the number of transgene copies and the site of transgene integration in the genome, which can lead to unpredictable gene dosage effects that may affect experimental results. Moreover, using a promoter to drive transgene expression can introduce variability in expression levels and patterns, as different promoters can drive expression in different cell types, and the promoter's strength can affect the level of expression. This can complicate data interpretation.

In comparison, HLA-DRB1*0401 allele knock-in mice offer several advantages over transgenic mice. Knock-in mice express the HLA-DRB1*0401 allele in the context of the endogenous mouse MHC molecules, which better mimics the natural human immune system and makes the results obtained from these mice more applicable to human disease. The expression of HLA-DRB1*0401 in knock-in mice occurs in a physiological and balanced manner that more accurately reflects the normal immune response seen in humans. In contrast, transgenic mice may show overexpression of the HLA gene, leading to abnormal immune responses. Additionally, knock-in mice have the transgene inserted into the endogenous locus, enabling expression under the control of the natural regulatory elements, which leads to consistent expression levels and avoids potential artifacts from using a promoter. Using knock-in mice allows for a direct comparison with wild-type mice that express the endogenous mouse MHC molecules, providing a better control group for experiments. In contrast, transgenic mice can be more difficult to interpret due to the overexpression of the transgene and potential confounding effects.

HLA-DRB1*0401/0402 allele and human invariant chain (Ii) double knock in mouse were used for the research included in the thesis. The Ii is a protein that plays a crucial role in the immune system by regulating the trafficking and presentation of antigens by major MHCII molecules. Human Ii increase the efficiency of HLA-DRB1*0401 folding and enhance its stability compared to the mouse Ii. This results in a higher yield of properly folded and stable HLA-DRB1*0401 molecules, which are important for accurate antigen presentation.

Our group's studies have identified the crucial interactions within the trimolecular complex, which includes the DRB1*04:01 molecule, the MHC II peptide, and the TCR.(Figure 7).



HLA-DRβ1 amino acid at position							
11	13	70	71	72	73	74	Classical *HLA-DRB1* alleles
Val	His	Gln	Lys	Arg	Ala	Ala	***04:01**
Val	His	Asp	Glu	Arg	Ala	Ala	***04:02**

Figure 7 The Binding of COL2 Peptide to the DR4 Molecules. *(A) This figure provides a comparative summary of intermolecular bonds present in trimolecular complexes formed by T-cell receptor TCR#16 and MHC molecule HLA-DRB1*04:01, which are associated with naked collagen type II (COL2). Amino acids are labeled with their one-letter code and their position within the corresponding protein sequence is indicated by a number. Salt bridges are represented by bold lines, and hydrogen bonds are represented by dashed lines. Additionally, aromatic interactions (ai), backbone (bb) interactions, complementarity determining regions (CDRs), side chain (sc) interactions, and other important features are also shown.[149] Reprinted with permission from BMJ Publishing Group Ltd. (B) Amino acid residues present at positions 11 (or 13), 70 to 74 within classical alleles of HLA-DRB1. (C). The collagen II (COL2) 256–270 peptide predominantly binds to the DRB10401 molecule through the phenylalanine(F) residue at position 263 and glutamic acid(E) residue at position 266. Notably, this peptide selectively binds to the 'shared epitope' (SE) structure within the peptide pocket of DR4 molecules that is linked to RA rather than the peptide binding pocket of other major histocompatibility complex class II molecules lacking the SE motif, such as the DRB10402 molecule. The important COL2-binding residues in the DR molecule are shown in the figure. T-cell receptors recognize galactosylated side chains present on lysine (K) residues at positions 264 and 270 of the COL2 peptide.[105] Reprinted with permission from Springer Nature.*

Mice expressing the HLA-DRB1*0401 allele (referred to as "Parker") is more susceptible to collagen-induced arthritis than both HLA-DRB1*0402 allele-expressing mice (referred to as "Dunder") and MHC-II H2b-expressing mice (referred to as "B6N") (Figure 8).

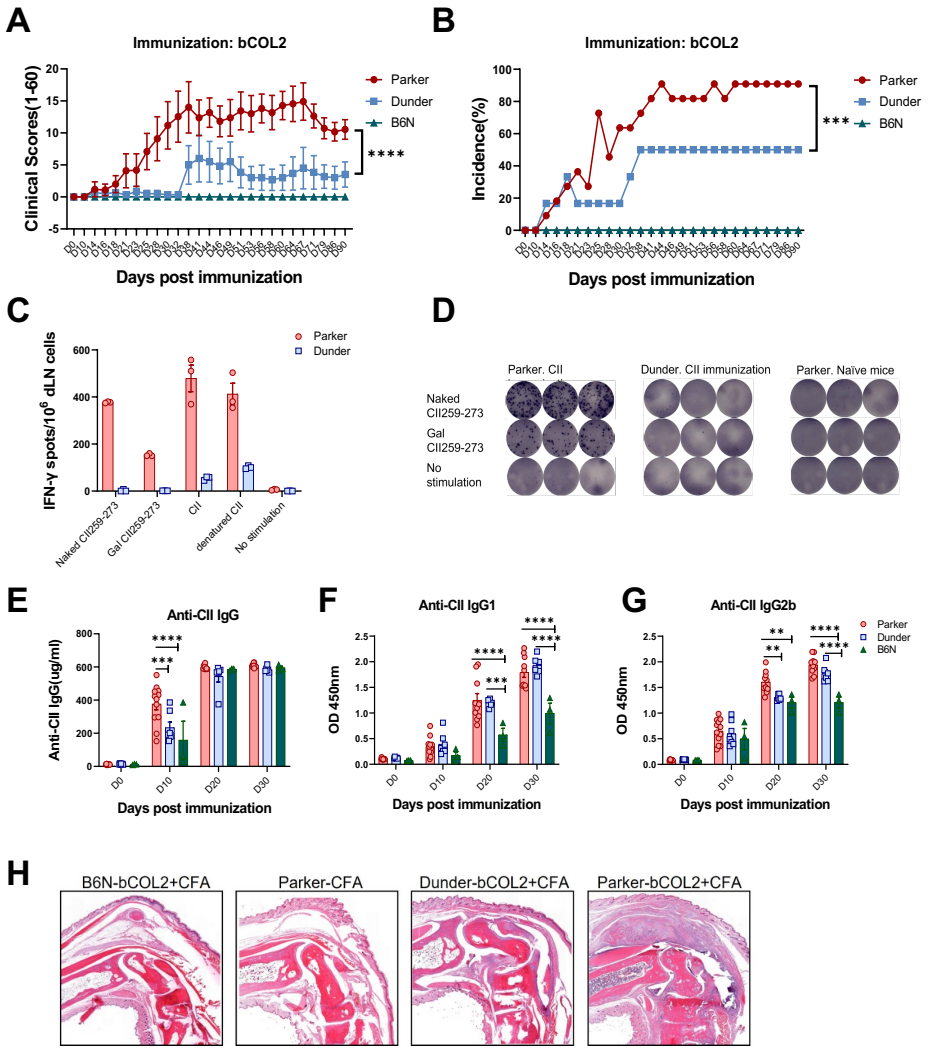


Figure 8 *(A-B) The severity (A) and incidence (B) of arthritis were measured in mice with different MHC-II backgrounds immunized with native bovine COL2(bCOL2) protein. (C-D) At DPI14, lymphocytes were recovered and re-stimulated in vitro with the naked/Gal CII259-273 peptide or native and denatured COL2. The numbers of cytokine (IFN-γ) secreting cells were determined by ELISPOT (C). Representative triplicate wells show the typical appearance of IFN-γ positive spots after cells were incubated overnight either with naked-CII259-273 peptide (top), gal-CII259-273 (middle), or medium (bottom) as a no-stimulus control (D). Serum anti-COL2 IgG (E)/IgG1(F)/IgG2b(G) antibodies at different time points after COL2 immunization were detected by ELISA. (H) Ankle joint histological examination of B6, Dunder, and Parker mice immunized with bCOL2 was performed, and the paws were collected at the end of the CIA experiment and sections were stained with H&E.*

In summary, HLA-DRB1*0401/0402 and human invariant chain knock-in mice are valuable tools for studying the genetic and immunological factors that contribute to RA pathogenesis. By mimicking the human immune response in a mouse model, these mice

allow for the investigation of the mechanisms underlying the disease and the development of new therapeutic interventions.

3 RESEARCH AIMS

RA is a long-term autoimmune disorder that is characterized by inflammation and degradation of the synovial joints. Antibodies targeting glucose-6-phosphate isomerase (GPI), cartilage oligomeric matrix protein (COMP), and citrullinated proteins have been implicated in the pathogenesis of RA, but the mechanisms underlying their pathogenicity remain unclear. Therefore, the aim of this thesis is to investigate the role of these antibodies in the development and progression of RA, and to identify new therapeutic targets for this debilitating disease. Specifically, this thesis will address the following research questions:

1. What is the prevalence and clinical relevance of antibodies targeting GPI and COMP in RA patients?
2. What is the molecular mechanism by which GPI-specific antibodies target joint cartilage and contribute to joint destruction in RA?
3. What is the pathogenic role of GPI/COMP-specific antibodies in the development and progression of RA?
4. How does the MHC class II associated immune response to citrullinated proteins contribute to the development of arthritis in humanized mice, and what are the implications for RA pathogenesis and treatment?

To answer these research questions, this thesis will use a combination of in vitro and in vivo approaches, including immunoassays, imaging techniques, animal models, and humanized mice. The findings of this thesis could aid in enhancing our comprehension of RA's pathogenesis and the creation of novel treatments for this incapacitating condition.

4 METHODS

4.1 GPI protein induced arthritis (GPIA)

To induce human glucose-6-phosphate isomerase protein-induced arthritis (GPIA), animals were immunised with 300 μg of hGPI protein emulsified 1:1 in CFA (Difco) at the base of the tail in a total volume of 100 μl. 12 days later, the mice were given a booster injection of 50 μg of hGPI protein emulsified 1:1 in IFA (Difco) in a total volume of 50 μl. Development of clinical arthritis was followed through visual scoring of the animals based on the number of inflamed joints in each paw, starting day 5 postimmunisation. Mice were examined 2-4 times per week for a maximum of 90 days after immunisation. An extended scoring protocol ranging from 1 to 15 for each paw with a maximum score of 60 per mouse was used. Each arthritic toe and knuckle were scored as 1, an arthritic ankle or wrist was given a score of maximum 5. All the mice were age-matched, randomized to an experimental group, and investigators were blind to the groups until the end of experiments. In each experiment, 3 to 10 mice per group were used.

4.2 Collagen type II induced arthritis (CIA)

Mice were immunised with 100 μg of bovine COL2 emulsified 1:1 in CFA (Difco) at the base of the tail in a total volume of 100 μl. 21 days later, the mice were given a booster injection of 50 μg of bovine COL2 protein emulsified 1:1 in IFA (Difco) in a total volume of 50 μl. Development of clinical arthritis was followed through visual scoring of the animals based on the number of inflamed joints in each paw,

4.3 Passive transfer of antibodies

BQ.Cia9i and BQ.Fcgr2b-/- mice were assigned into different injection groups randomly and were given different monoclonal antibodies cocktails by intravenous injection at day 0. Only BQ.Cia9i mice were boosted with 100 μl lipopolysaccharide (LPS) (0.25 mg/ml) from Escherichia coli serotype O55:B5 (Sigma-Aldrich) intraperitoneally at day 5 after antibody injection. Arthritis development was checked daily blinded to the experimenter for 21 days. Clinical arthritis was scored as described above in GPI protein induced arthritis. All the mice were age-matched, randomized to an experimental group, and investigators were blind to the groups until the end of experiments. In each experiment, 3 to 10 mice per group were used.

4.4 Generation of monoclonal antibodies

All monoclonal antibodies (mAbs) were generated by B cell hybridoma technology following a protocol described earlier.[150,151] Lymphocytes from inguinal lymph nodes and splenocytes of mice with the highest antibody titers were taken out at peak time based on the serum antibody ELISA result. Inguinal lymphocytes and splenocytes were

fused with NSO-bcl2 myeloma cells and cultured in a complete DMEM medium (DMEM+10%FCS) containing Hypoxanthine, Aminopterin, Thymidine (HAT). 10-14 days after the fusion procedure, the culture supernatant was tested for antigen specificity by ELISA. Protein or peptide-positive clones further subcloned by limiting dilution. After 4-5 rounds of subcloning, positive single clones producing highest antibody titers were picked for expansion and antibody production. After being cultured in DMEM medium containing 4% ultra-low IgG fetal bovine serum for three weeks, the supernatant was collected and monoclonal antibodies were purified by using Protein G Sepharose (Cytiva, Uppsala, Sweden). The procedure for mAbs purification was described earlier.[151] 0.1 M glycine (pH 2.7) was used as elution buffer and antibodies were neutralized with a one-eighth volume of 1 M Tris–HCl (pH 9.0) immediately after being eluted. The concentration of monoclonal antibodies was quantified spectrophotometrically at 280 nm after three times dialysis in phosphate-buffered saline (PBS) (pH 7.0). Antibody solutions were sterilized by passing through 0.2 µm syringe filters (#4612 Pall) and were stored at 4°C or -20°C (for long time storage) until use.

4.5 Enzyme-linked immunosorbent assay (ELISA)

For protein coating, 96-well flat-bottom MaxiSorp ELISA plates (Nunc) were coated with hGPI protein (native or denatured form) at a concentration of 5 µg/ml in PBS at 4°C overnight. For peptide coating, ELISA plates were coated with Neutravidin (10 µg/ml) at 4°C overnight, followed by incubation with biotinylated peptides (5 µg/ml) for 1h at RT. Plates were blocked with 3% non-fat milk in PBS at 37°C for 1h. Mouse serum was diluted 1:100 (v/v) into 3% non-fat milk. Culture supernatants and purified monoclonal antibodies were diluted following a fixed dilution series (1:5) in 3% non-fat milk. Diluted mouse serum, culture supernatants, and monoclonal antibodies were added into the blocked plate (100 µl/well) and incubated at 37°C for 1h. For the measurement of total IgG, F(ab')2 Goat anti-Mouse IgG-Fc Fragment horseradish peroxidase (HRP) Conjugated (Bethyl Laboratories) was used at 1:5000 dilution.

For monoclonal antibody isotype characterization, HRP-conjugated goat anti-mouse–IgM, -IgG1, -IgG2a, -IgG2b, -IgG2c or -IgG3 reagents (Southern Biotech) were used. Between each step ELISA plates were washed 5x with PBS + 0.05 % Tween20 (PBS-T). For signal detection, ABTS (Roche) or alternatively TMB (Seramun) + 0.16 M sulfuric acid (stop solution) were used. Absorbance was read at OD405nm and OD450nm (Synergy 2, BioTek) for ABTS and TMB (resp.).

4.6 Luminex immunoassay

The detection of autoantibody responses using Luminex technology has been described previously. Briefly, all biotinylated peptides were captured on beads via recognition of NeutrAvidin (ThermoFisher Scientific), which was immobilized on the beads with amine

coupling. Human serum samples were diluted 1:100 (v/v) in assay buffer (3% bovine serum albumin, 5% milk powder, 0.1% ProClin 300, 0.05% Tween 20, 100 μg/ml NeutrAvidin in phosphate buffered saline [PBS]) and incubated for 60 minutes at room temperature. Then the serum samples were transferred to a 384-well plate containing peptide-coated beads, with a liquid handler (CyBio Selma). After incubation at room temperature on a shaker for 75 minutes, all beads were washed with PBS-Tween (PBST) on a plate washer (no. EL406; BioTek) and resuspended in a solution containing secondary anti-human phycoerythrin-conjugated IgG Fcγ (Jackson ImmunoResearch). After 40 minutes of incubation, the beads were washed with PBST and subsequently measured in a Flexmap 3D system (Luminex). The median fluorescence intensity was used to quantify the interaction of serum antibody with given peptides.

5 RESULTS AND DISCUSSION

5.1 Study I - Pathogenic antibody response to glucose-6-phosphate isomerase targets a modified epitope uniquely exposed on joint cartilage.

In this study, we conducted an investigation into the mechanisms behind the development of arthritis in both K/BxN and GPI-immunised mice, as well as a subset of RA patients. Through this investigation, we identifed the peptide GPI293-307 as the dominant B cell epitope in both the murine studies and the human RA sera. Next, monoclonal antibodies to this epitope were generated and were shown to be able to induce arthritis in mice by targeting structurally modified GPI on the joint cartilage. Based on our results we hypothesized that structurally modified GPI protein deposits on the cartilage surface are bound by naturally occurring antibodies, creating inflammatory GPI-antibody immunocomplexes. Under genetically or environmentally susceptible conditions, these immune complexes could then initiate or reinforce local inflammation.

We further noted that GPI293-307-specific IgG can be found in the sera of control subjects and that these specific antibody titers gradually rise in pre-RA subjects before clinical RA onset. More specifically, the antibody titers were significantly higher in pre-RA patients one year before clinical onset compared to control subjects. In the sear of established human RA patient cohorts, the anti-GPI293-307 response was positively associated with more severe radiographic joint damage. Taken together this evidence, it points towards a role of anti-GPI293-307 IgG in RA disease pathogenesis. To address the question whether the anti-GPI response is specific for RA, we investigated sera titer in other arthritic disease cohorts (osteoarthritis, psoriatic arthritis, ankylosing spondylitis). The anti-GPI293-307 response was significantly higher in RA compared to the control disease cohorts. Histological analysis confirmed that anti-GPI293-307 monoclonal antibody binds exclusively to RA cartilage. However, due to limited sample sizing, additional studies are required to draw the definite conclusion whether or not anti-GPI293-307 is specific to RA or not.

Intriguingly, anti-GPI293-307 could be found already in naïve mice with an IgM isotype. After GPI immunization (GPIA), the anti-GPI293-307 response underwent class switch and rose in titers over the experimental arthritis disease course. This observation has led us to formulate the question, why the specific antibody response was already detectable in naïve mice. We hypothesized that autoreactive B cells must be present in naïve mice, producing low-titre/affinity antibodies against the epitope. However upon initiation of an anti-GPI immune response, these B cells get activated and produce high-titre/affinity antibodies against the structurally modified epitope. To address this issue, we applied tetramer technology to identify anti-GPI293-307 B cells in both naïve and immunized mice in several lymphoid organs. Interestingly, we could identify such B cells in a

significantly higher frequency compared to B cells against a control peptide. After this result the unsolved question remains why naturally autoreactive anti-GPI293-307 exist in naïve conditions and why they are not counterselected according to the known mechanisms of B cell tolerance. To study the fate of such B cells experimentally, genetically modified VDJ knock-in mice were generated in literature. It is conceivable to generate anti-GPI293-307 knock-in mice and study the fate of such B cells in vivo.

Furthermore, it would be interesting to translate our findings in mice into humans, i.e. tracking and tracing such B cells in human healthy donors and RA patients. The identification of such B cells in humans would pave the way for more detailed characterization of this B cell subset by single B cell antibody cloning or single cell sequencing. Such natural human autoreactive antibodies could improve our understanding of the pathogenesis of human RA.

Overall, this study provides new unique insights about anti-GPI antibodies and unravels their relevance in RA.

5.2 Study II - Antibodies to Cartilage Oligomeric Matrix Protein Are Pathogenic in Mice and May Be Clinically Relevant in Rheumatoid Arthritis.

In this study, we explored the characteristics of a COMP-specific antibody 15A11, which targets the native COMP protein present in joint cartilage and induces arthritis in mice upon injection. The antibody 15A11 cross-reacts with human COMP, which implying that the pathogenicity of anti-COMP antibodies might be clinically relevant in humans as well. Additionally, 15A11 react with both native and citrullinated forms of COMP. To gain insights into the mechanism underlying the specific recognition of COMP in joint cartilage by mAb 15A11, we conducted a crystallographic study of the Fab fragment of the antibody in complex with the P6 epitope. Our analysis of the crystal structure revealed that the interaction between the 15A11 antibody and the P6 epitope is mainly governed by paratope shape-complementarity. Previously, we had identified this epitope as spanning residues 232-252 of mouse COMP in an epitope mapping study.

In addition, our study has shown that a single injection of monoclonal antibody 15A11 can induce experimental arthritis. Furthermore, histological analysis revealed that 15A11 binds strongly to the cartilage surface of joint tissue in naïve mice. RA patients have been found to show significantly elevated levels of antibody response against both native and citrullinated versions of the P6 epitope compared to healthy controls which make the P6 epitope holds clinical significance as a biomarker for disease activity and joint damage in RA.[152]

In summary, this study provides a structural basis for understanding the role of anti-COMP antibodies in RA pathology and their potential use as biomarkers. However, more

research is needed to fully explore their potential as both biomarkers and therapeutic targets in RA.

5.3 Study III - MHC class II associated immune response to citrullinated proteins and development of arthritis in humanised mice.

In this study, we demonstrate the dependence of ACPA induction on the HLA-DRB1*0401 allele. By immunizing mice with different MHC-II backgrounds with native and citrullinated forms of various RA-associated autoantigens, we found that ACPA responses were only present in citrullinated protein-immunized DRB1*0401 allele knock-in mice, but not in DRB1*0402 allele knock-in mice or control B6N mice. To determine that ACPAs responses were truly citrulline-specific, we used arginine and corresponding citrullinated peptides in our screen, and only unilateral positive for citrullinated peptide were defined as ACPAs response. The findings suggest that the DR4 allele plays a critical role in the pathogenesis of RA by promoting the production of ACPA.[153,154] However, further studies are needed to investigate the underlying mechanism of ACPA production in response to immunization with citrullinated proteins.

Additionally, DRB1*0401 allele knock-in mice developed more severe arthritis compared with other two strains when they were immunized with native COL2 or citrullinated COL2. The arthritis phenotype observed in this study is in agreement with human research indicating that the HLA-DRB1*0401 genotype confers a significantly increased risk for developing RA, particularly in its more severe manifestations.[155]

It may not be surprising that citrullinated COMP/PAD4 immunization induces ACPA responses against citrullinated peptides derived from the immunized antigen, but we were really excited when we found that citrullinated COL2 induced a very promiscuous ACPA response, highly specifc for the citrulline sidechain but which could also bound to citrullinated peptides from GPI and Fibrinogen. Similarly, citrullinated αEnolase immunization induced ACPA response that could bind peptide GPI_C_19_Cit which RA patients have extremely high antibody response against with. This peptide has been recognized as a new promising diagnosis marker for RA.[156] Further we revealed that monoclonal ACPAs generated from these mice displayed diverse functions, indicating the unpredictable nature of ACPAs' functions. But Fc sialic acid content levels were associated with the function of ACPAs in antibody-induced mouse arthritis as previous report.[157]

The arthritic properties of citrullinated αEnolase remain controversial. The results of a research group demonstrated that administration of citrullinated αEnolase can induce arthritis in DR4 transgenic mice [36]. However, the arthritic properties of citrullinated αEnolase were not replicated by our group.[158] Moreover, the exact mechanism by which citrullinated αEnolase contributes to the pathogenesis of RA has not yet been revealed. Our results showing that immunization with citrullinated αEnolase induces a protective

ACPA response in DRB1*0401 allelic knock-in mice, which led us to hypothesize that the presence or absence of GPI_C_19_Cit-specific antibodies in mice affected citrullinated αEnolase immunization outcome, leading to differences in the results between different study groups.

It is also important to note the limitations of our study, including the use of a mouse model that may not entirely replicate the human disease and the lack of exploration of the mechanisms underlying the pathogenic and protective effect of monoclonal ACPAs used in this study. Nonetheless, our study underscores the diagnostic and therapeutic potential of targeting citrullinated epitopes in RA and provides a basis for future research in this area.

6 CONCLUSIONS

Overall, the three papers presented in this thesis shed light on the pathogenic mechanisms underlying the development of arthritis, a chronic inflammatory disease affecting millions of people worldwide.

Study I highlights the critical role of glucose-6-phosphate isomerase (GPI) as a target for pathogenic antibodies in arthritis. Specifically, the study identifies a modified epitope on joint cartilage that is uniquely exposed and targeted by these antibodies. This finding underscores the importance of understanding the molecular basis of autoimmune responses in arthritis, which may lead to the development of more effective therapeutic strategies targeting specific antigens.

In **Study II**, the pathogenic role of antibodies to cartilage oligomeric matrix protein (COMP) is further investigated in mice, suggesting the clinical relevance of this molecule in rheumatoid arthritis. These outcomes provide additional support for COMP as a potential biomarker and therapeutic target for arthritis.

Finally, **Study III** describes the MHC class II-associated immune response to citrullinated proteins and its effect in the progress of arthritis in humanized mice. This study provides insight into the interplay between genetic and environmental factors in arthritis pathogenesis, highlighting the complex nature of this disease.

Together, these papers provide valuable insights into the molecular and immunological mechanisms underlying arthritis, which may pave the way for the development of more targeted and effective therapeutic interventions. Overall, this thesis contributes to our understanding of the pathogenesis of arthritis and has important implications for the diagnosis and treatment of this debilitating disease.

7 POINTS OF PERSPECTIVE

Clinical implications: Rheumatoid arthritis is a complex and heterogeneous disease that poses significant challenges for diagnosis and treatment. The identification of new biomarkers, such as GPI and COMP-specific antibodies, may offer several clinical benefits. These antibodies may aid in the early diagnosis of RA, particularly in cases where traditional diagnostic criteria are inconclusive. Furthermore, the detection of these antibodies may help to identify patients who are at increased risk of joint damage and disease progression, allowing for more targeted and aggressive treatment strategies. Additionally, the development of therapies that target the underlying mechanisms of these antibodies may improve outcomes for RA patients.

Pathogenicity of antibodies: The pathogenicity of antibodies in RA is a complex and evolving field of study. While the role of autoantibodies in RA pathogenesis is well-established, the exact pathogenesis of joint destruction caused by these antibodies is not fully understood. The identification of pathogenic antibodies, such as GPI and COMP-specific antibodies, provides an opportunity to further investigate the molecular mechanisms underlying this disease. Specifically, studies may investigate the downstream effects of these antibodies on joint tissues, the participation of other immune cells in the development of RA, and the interactions between genetic and environmental factors in the development of autoantibody responses.

Therapeutic implications: The identification of new targets for RA therapy, such as GPI and COMP, is an important step towards developing more effective treatments for this disease. Therapies that target these antigens may provide a more targeted approach to treating RA, with fewer side effects than current treatments. Additionally, the use of humanized mice as a preclinical model for testing new therapies may improve the translation of preclinical findings to the clinic, ultimately benefiting RA patients.

Future directions: The results of this thesis provide a foundation for further research into the role of antibodies in RA pathogenesis. Future studies may investigate the use of GPI and COMP-specific antibodies as biomarkers for predicting disease progression and response to therapy. Additionally, the development of novel immunotherapies that target these antigens may improve outcomes for RA patients. Furthermore, the use of humanized mice as a model for studying RA pathogenesis may lead to the identification of additional targets for therapy and provide new insights into the mechanisms underlying this disease.

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