From Department of Medical Biochemistry and Biophysics
Karolinska Institutet, Stockholm, Sweden

IDENTIFICATION AND FUNCTIONAL ANALYSIS OF ANTI-CITRULLINATED PROTEIN ANTIBODIES IN RHEUMATOID ARTHRITIS

Yibo He

何 一博

Karolinska Institutet

Stockholm 2023

All previously published papers were reproduced with permission from the publisher.
Published by Karolinska Institutet.
Printed by Universitetsservice US-AB, 2023

ISBN 978-91-8017-034-5
Cover illustration: The All-Seeing-Eye (immune system) gazing at the Tree of Knowledge (autoantibody) bearing the Fruit (autoantigen) in the ocean of chaos (autoimmunity). Originally illustrated for the cover artwork for the album of Black Reaper, *Celestial Descension* (2018), by Marcela Bolívar (Col). The illustration is approved to be used in this thesis by the artist and band.

Identification and Functional Analysis of Anti-citrullinated Protein Antibodies in Rheumatoid Arthritis

Thesis for Doctoral Degree (Ph.D.)

By

Yibo He

The thesis will be defended in public at Samuelssonsalen, Tomtebodavägen 6, KI, Stockholm. 15th June, 2023

Principal Supervisor:
Prof. Rikard Holmdahl, M.D., Ph.D.
Karolinska Institutet
Department of Medical Biochemistry and Biophysics
Division of Medical Inflammation Research

Co-supervisor:
Asst. Prof. Changrong Ge, Ph.D.
Karolinska Institutet
Department of Medical Biochemistry and Biophysics
Division of Medical Inflammation Research

Opponent:
Associate Prof. Felipe Andrade, M.D, Ph.D.
Johns Hopkins University School of Medicine
Department of Medicine
Division of Clinical and Molecular Rheumatology

Examination Board:
Prof. Helena Erlandsson Harris, Ph.D.
Karolinska Institutet
Department of Medicine
Division of Rheumatology

Prof. Carl Turesson, M.D., Ph.D.
Lund University
Department of Clinical Sciences
Division of Rheumatology

Prof. Carmen Fernández, Ph.D.
Stockholm University
Department of Molecular Biosciences
Division of Immunology

It’s just a ride.

- Bill Hicks

Popular science summary of the thesis

Have you ever heard for years the babushka next door complain about her joints and the hard life of being a rheumatoid arthritis (RA) patient? Yes, RA is an autoimmune disease that affects 0.5-1.0% of the global population, with the immune system going rogue and crusading within the body, somehow turning joints into battlefields of an eternal civil war. With time, joints swollen, bones destroyed, knees weak and arms are heavy, then you lose yourself.

It is now believed that the development of RA begins years before the clinical onset, as no civil war occurs without a prelude full of misunderstandings and warmongering rallies. One of the signs in this scenario is the appearance of autoantibodies, and one of the most interesting groups of these autoantibodies is the anti-citrullinated protein antibodies (ACPAs), which are by far the most specific autoantibodies in RA and included in the clinical diagnosis for RA. However, since the discovery of ACPA in 1964, we still have not been able to pinpoint the exact function of these antibodies.

Roses are red, violets are blue, we are still figuring out the role of autoantibodies in rheumatoid arthritis, while mankind has already landed on the Moon. Previous studies suggested that ACPAs are "warmongers," and recently ours and other groups showed that they perhaps could also be "peacekeepers." In Study I & II, we entered the realm of ACPAs and performed in-depth analysis of these antibodies from different angles. For **Study I**, which is the main focus of this thesis, we used various animal and cell models and demonstrated that certain ACPA could actually be protective in RA by interacting with macrophages, a type of innate immune cells. **In Study II**, we dived deeper into the molecular structures of these antibodies and revealed that sugars (variable domain glycans, VDGs) could have an intricate impact on either the specificity or biological function of ACPAs.

Apart from autoantibodies, in **Study III**, we investigated the regulatory role of two important genes (*Ncf1* and *Clec4b*) on neutrophils, an important player in inflammatory response in arthritis, and found that both genes together have an additive regulatory effect on neutrophils, subsequently regulating the severity of arthritis.

Together, although RA remains an incurable disease, we provide new perspectives not only for understanding, but also for the future development of potential therapeutics for RA.

Abstract

Rheumatoid arthritis (RA) is a complex autoimmune disease and typically manifested by joint inflammation and bone erosion with approximately 0.5% of the global population affected. To date, it is believed that genetic predisposition (e.g. *HLA-DRB1* alleles) and environment (e.g. cigarette smoking) are involved as risk factors for the development of RA. A hallmark of RA preceding the disease onset is the emergence of autoantibodies, including rheumatoid factors (RFs) and anti-citrullinated protein antibodies (ACPAs). Being the most specific (>90%) and sensitive (>60%) autoantibodies in RA, ACPAs have been included in the clinical criteria for the classification of RA. The function of ACPAs in RA is still unclear. Although patients with ACPA positivity are associated with more severe arthritis and in vitro studies have shown certain pathogenic effects of ACPAs, the in vivo evidence remains lacking. On the other hand, extensive but common N-glycosylation in the variable domain of ACPAs (90%) has been unveiled, questioning if these N-glycans serve a functional role.

In **Study I**, we expressed several monoclonal ACPAs derived from RA patients and identified their specificities using a panel of citrullinated peptides. We found one of the ACPAs, clone E4, could protect against collagen antibody induced arthritis in mice. The protection is joint-specific and depending on the interaction between E4 in complex with citrullinated alpha-enolase and FCGR2B on activated macrophages, enhancing the IL-10 secretion and supressing osteoclastogenesis by macrophages. In **Study II**, we focused on the variable domain glycans (VDGs) in ACPAs by employing crystallography, glycobiology and functional B cell assay. We showed that 1) VDGs are positioned in the vicinity of the paratope with an impact on the antigen-binding; 2) VDGs could enhance B cell activation, and 3) VDG-expressing B cell receptors stay longer on the cell surface. In **Study III**, we investigated the two most significant arthritis QTLs in inbred rats, *Ncf1* and *Clec4b*, and showed that Ncf1 and Clec4b together modulate the severity of arthritis in rats and their expression on neutrophils modulate the production of reactive oxygen species by neutrophils.

Taken together, the findings revealed a protective, rather than pathogenic effect of certain ACPAs in RA and elucidated the unique properties of VDGs in ACPAs and their functional impact on autoreactive B cells.

List of scientific papers

I. **Yibo He**, Changrong Ge, Àlex Moreno-Giró, Bingze Xu, Christian M. Beusch, Katalin Sandor, Jie Su, Lei Cheng, Erik Lönnblom, Christina Lundqvist, Linda M. Slot, Dongmei Tong, Vilma Urbonaviciute, Bibo Liang, Taotao Li, Gonzalo Fernandez Lahore, Mike Aoun, Vivianne Malmström, Theo Rispens, Patrik Ernfors, Camilla I. Svensson, Hans Ulrich Scherer, René E. M. Toes, Inger Gjertsson, Olov Ekwall, Roman A. Zubarev & Rikard Holmdahl. **A subset of antibodies targeting citrullinated proteins confers protection from rheumatoid arthritis**. *Nature Communications*, 14, 691 (2023).

II. Theresa Kissel, Changrong Ge, Lise Hafkenscheid, Joanneke C. Kwekkeboom, Linda M. Slot, Marco Cavallari, **Yibo He**, Karin A. van Schie, Rochelle D. Vergroesen, Arieke S.B. Kampstra, Sanne Reijm, Gerrie Stoeken-Rijsbergen, Carolien Koeleman, Lennard M. Voortman, Laura H. Heitman, Bingze Xu, Ger J.M. Pruijn, Manfred Wuhrer, Theo Rispens, Tom W.J. Huizinga, Hans Ulrich Scherer, Michael Reth, Rikard Holmdahl, Rene E.M. Toes**. Surface Ig variable domain glycosylation affects autoantigen binding and acts as threshold for human autoreactive B cell activation**. *Science Advances*, 8, eabm1759 (2022).

III. Mike Aoun, Xiaojie Cai, Bingze Xu, Gonzalo Fernandez Lahore, Michael Yi Bonner, **Yibo He**, Liselotte Bäckdahl and Rikard Holmdahl. **Glycan Activation of Clec4b Induces Reactive Oxygen Species Protecting against Neutrophilia and Arthritis**. *Antioxidants*, 11(1): 12 (2022).

Scientific papers not included in the thesis

I. Gonzalo Fernandez Lahore, Michael Förster, Martina Johannesson, Pierre Sabatier, Erik Lönnblom, Mike Aoun, **Yibo He**, Kutty Selva Nandakumar, Roman A. Zubarev & Rikard Holmdahl. **Polymorphic estrogen receptor binding site causes Cd2-dependent sex bias in the susceptibility to autoimmune diseases**. *Nature Communications*, 12, 5565 (2021)

II. Mike Aoun, Ana Coelho, Alexander Krämer, Amit Saxena, Pierre Sabatier, Christian Beusch, Erik Lönnblom, Manman Geng, Nhu-Nguyen Do, Zhongwei Xu, Jingdian Zhang, **Yibo He**, Bingze Xu, Johan Viljanen, Joanna Rorbach, Gonzalo Fernandez Lahore, Inger Gjertsson, Alf Kastbom, Christopher Sjöwall , Jan Kihlberg, Roman Zubarev, Harald Burkhardt, Rikard Holmdahl. **Antigen presenting autoreactive suppressor B cells**.
Manuscript.

CONTENTS

List of abbreviations

ACPA	Anti-citrullinated protein antibody
Anti-CarP	Anti-carbamylated protein antibody
APC	Antigen-presenting cell
BCR	B cell receptor
CAIA	Collagen-antibody induced arthritis
CCP2/4	Cyclic citrullinated peptides, version 2 or 4
CEP-1	Citrullinated human alpha-enolase peptide 1
CIA	Collagen induced arthritis
CLR	C-type lectin receptor
COL2	Collagen type 2
COMP	Cartilage oligomeric matrix protein
DC	Dendritic cells
Dcard	Dentritic cell immuno-activating receptor
EAE	Experimental autoimmune encephalomyelitis
ENO1	Alpha-enolase
FCGR2B	Fc gamma receptor IIb
GPI	Glucose-6-phosphate isomerase
IC	Immune complex
Ig	Immunoglobulin
ITAM/ITIM	Immunoreceptor tyrosine-based activation/inhibition motifs
LPS	Lipopolysaccharides
mAb	Monoclonal antibody
MHC	Major histocompatibility complex
NET	Neutrophil extracellular traps
NLR	NOD-like receptors
NOX2	NADPH oxidase 2
PAD	Peptidylarginine Deiminase
RF	Rheumatoid factor
ROS	Reactive oxygen species
SNP	Single nucleotide polymorphism
TLR	Toll-like receptor
VDG	Variable domain glycan

1 Introduction

1.1 Autoimmunity

In the late 19th century, the concept that the immune system could target against self-antigen was commonly unheeded since such phenomenon contradicted to the traditional understanding that immune system should only target non-self. In fact, a classic finding by Paul Erhlich that goat produced antibodies against the red blood cells from other goats but not its own was part of the basis to reject the existence of autoimmunity, expressed by Paul Ehrlich as *horror autotoxicus* (*1*). Regardless, he was the first to propose that the immune system is equipped with certain regulatory mechanism to prevent autoimmunity. It was until the 1950s, the dawn of modern science when technological breakthroughs and political upheavals were relentlessly concerting, the understanding of autoimmunity finally progressed into a new era. By demonstrating that the serum samples from patients with thyroiditis contained the autoantibodies against human thyroid extract, Ernest Witebsky, Ehrlich's student, postulated that "autoimmunization process within the patient" attacks the self-tissue and autoantibodies are involved in the pathological processes (*2*), which opened a new chapter in the field.

Autoimmunity by definition is an aberrant immune response towards against self-antigen, it arises when the checking mechanisms in the immune system fails to distinguish between self and non-self-antigens, ultimately leading to the breaking of tolerance (*3*). A typically hypothesis is that genetic predisposition, environmental stimuli and defective regulation synergistically direct the initiative stage of autoimmune diseases, resulting in inflammatory responses and the generation of autoantibodies. Depending on the course of disease and treatment, autoimmune diseases can lead to fatal outcome or chronic burden, their clinical manifestations greatly differ among each other, some are limited to certain organs while others being more dispersive and systemic (*4*). To date, over 100 inflammatory disorders have been collectively defined as 'autoimmune diseases.' However, with numerous subtypes, they can be challenging to define based on knowledge and criteria. Typical systemic autoimmune diseases include rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), multiple sclerosis (MS) and Sjögren's syndrome etc., whereas examples like type I diabetes (T1D) and inflammatory bowel disease (IBD) are more organ-specific. Aside from being the standing burden for the society and economy, autoimmune diseases cause long-term physical and psychological sufferings to the individuals, leading to a dramatic decrease of living quality, if not death. Notably, many of them have significantly higher incidence in females and in the western population.

1.2 Rheumatoid arthritis

Rheumatoid arthritis (RA) is one of the most common autoimmune diseases, a systemic and complex inflammatory condition with 0.5% of worldwide incidence and higher frequency in Caucasian. It is clinically manifested by inflammation of joints, arthralgia and bone erosion, eventually leading to bone destruction following the lack of medical attention, sometimes also accompanied by complications in tissues that are not restricted to joints. Multiple risk factors, including predisposing genes such as *HLA-DRB1* alleles, and environmental stimuli such as smoking and estrogen drops, have been associated with the disease development (*5*–*8*). By combining them, the development of RA has been characterized to three stages (Fig.1) (*9*). The autoimmune responses direct the first phase of disease course that can last for decades before the clinical onset. In this priming phase, without symptoms, activation of autoreactive B cells allows the production of autoantibodies, for example, rheumatoid factors (RFs) and anti-citrullinated protein antibodies (ACPAs), which usually play as strong indicators. With time and constant exposure to autoantigens, more autoantibodies specifically targeting joint autoantigens emerge along with RFs and ACPAs, their titers dramatically increase - most likely a consequence of epitope spreading and T cell-dependent responses (*10*). Together with innate immune responses and extensive secretion of pro-inflammatory cytokines, the course reaches to the point where symptoms such as joint pain and swelling that can be perceived by patients. Shortly after the subclinical arthritis, the development enters the clinical stage triggered by inflammatory cascades and dysregulated immune responses, acquiring more severe and chronic manifestations such as cartilage degradation and bone erosion/destruction.

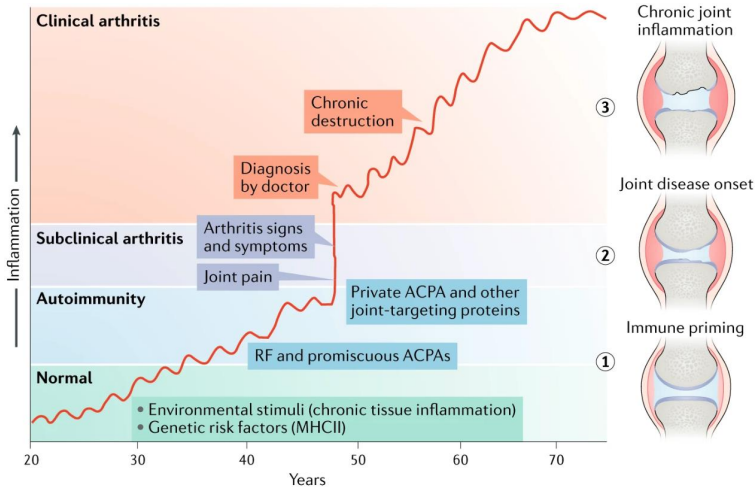

Fig.1: The proposed courses of rheumatoid arthritis. The illustration shows the hypothetical course of RA characterized by three stages: 1) immune priming; 2) joint disease onset; and 3) chronic joint inflammation, with age at the x-axis and degree of inflammation at the y-axis. *Illustration adapted from (9) with permission from Springer Nature.*

1.3 Risk factors of RA

The players that lead to the breaking of tolerance in RA are still to be understood, no conclusion could yet be drawn from either genetic or environmental analysis. Regardless, lots of progress have been made throughout the decades. One of the classic hypotheses of genetic risk factors in RA is the 5-aa motif in position 70-74 of HLA-DRβ chain coded by *HLA-DRB1* alleles, designated "shared epitope" (SE) (*11*). The presence of SE, for example, the *HLA-DRB1*04* allele group (**0401, *0404, *0405* etc.) is often associated with severe RA (*12*, *13*). This strong association remained unchallenged for long, with the exception that an aspartic acid in position 70 of the SE sequence DERAA (encoded by *HLA-DRB1*0402* allele), instead of arginine or glutamine, could confer protection in RA (*14*, *15*). However, the SE-coding alleles have not been shown to be a risk factor in seronegative RA, it is suggested that the presentation of autoantigens to T cells may be primarily driven by SE-containing HLA molecules, inducing the T cell-dependent responses and contributing to the expansion of autoreactive B cells and ACPA occurrence.

Smoking is by far the most significant risk factor from the environment. The association is especially stronger with seropositive RA vs. seronegative RA, although it may be irrelevant to ACPA or RF status (*16*, *17*). Smoking, and potentially airway pollutants from different sources, such as silica and coal dust, have been linked to increased level of peptidyl-arginine deiminases (PADs) and citrullinated antigens in lungs, and thus were hypothesized to induce ACPA production as a consequence of citrullination and HLA-SE alleles possession (*18*, *19*). However, analysis using multicenter cohort showed that only double-positive RA (ACPA + RF), but not single-positive RA (ACPA/RF) is associated with smoking (*20*), supported by another study that in RF negative patients, no relationship between smoking and ACPA was found (*21*).

Taken together, the contributions from the genetic or environmental risk factors to RA development are complex and still largely unknown, it is clear that RA is a multi-factorial disease driven by both genetics and environment (*22*), all leading to the eventual emergence of autoantibodies such as ACPAs.

1.4 Autoantibodies in RA

1.4.1 Rheumatoid factor (RF)

One of the sets of the autoantibodies that were first identified in RA is rheumatoid factors (RFs). In 1940, RF was first described by Dr Erik Waaler, who was later acknowledged as the discoverer of RF, based on an observation on an unusual agglutination of sheep blood caused by the serum from a patient with RA (*23*, *24*). It was E.C. Franklin and his colleagues who were then able to identify RFs in RA patients that were reacting to antibody instead of antigen (*25*, *26*).

RFs are characterized as the autoantibodies that react with the Fc part of IgG, forming immune complex (IC) to participate in the process of immune responses. The hypotheses suggest that the formation of IgG IC may expose the antigenic determinants on the Fc gamma region that permits the recognition by RF, as opposed to monomeric native IgGs (*27*, *28*), implicating that native IgGs are present in a form where the Fc epitopes are shielded from RF and explaining why circulating RFs do not normally bind to IgGs in blood. In RA, RFs and ACPAs are frequently found together, RFs have been shown to enhance monocyte activation by ACPA ICs in vitro (*29*). Furthermore, crosslinking of RF and ACPA may lead to an enhanced binding of ACPA to citrullinated antigens (*30*). However, RFs do not exhibit preferential binding to ACPAs over native IgGs, and the galactosylation level in the Fc region does not seem to affect the binding of RF to IgGs (*30*). Therefore, it is possible that the inflammation is not promoted by the ACPA-RF interaction, but rather by an increase titer of autoantibodies during the progression of the disease.

Although called "rheumatoid factors," RFs are not exclusive to rheumatic diseases such as RA, SLE or Sjögren's syndrome. They can also be found in non-rheumatoid conditions such as leprosy, syphilis, pulmonary tuberculosis, chronic liver disease and sarcoidosis, all of which involve immune responses to pathogen infection (*31*). This has been a longstanding question if infection is responsible for the generation of RFs and contributing to the development of RA. One logical assumption is that, by binding to the Fc gamma region, RFs could participate in the removal of ICs that are formed by IgGs and captured foreign pathogens and therefore facilitate the clearance of pathogens. Nonetheless, due to the relatively lower specificity in RA, the origin and function of RF are still to be understood (*32*).

1.4.2 Anti-citrullinated protein antibody (ACPA)

One of the hallmarks for RA is the autoantibodies recognizing citrullinated antigens, designated anti-citrullinated protein antibodies (ACPAs). They were initially reported by Nienhuis *et al* (*33*) in 1964 as a type of antibodies against perinuclear factor and antibodies against keratin in RA by Young *et al* (*34*), which were later proved to be a highly specific family of autoantibodies against citrullinated filaggrin. Nowadays, the targets of ACPAs have been widely extended, such as citrullinated alpha-enolase, vimentin, fibrinogen and collagen type etc. ACPAs have a sensitivity around 70% and by far the highest specificity (>90%) in RA (*35*), typically tested by anti-cyclic citrullinated peptides (CCP) ELISA. Together with RFs, ACPAs have been included in the ACR/EULAR 2010 classification criteria for RA (*36*).

Citrullination is a post-translational modification (PTM) controlled by PADs with the presence of Ca^{2+}. It enzymatically converts the positively charged arginine on the epitope to the non-natural amino acid citrulline, which is neutral and more hydrophobic. Normally, citrullination is a physiological and necessary PTM that widely takes place within the body, especially in maintaining

the skin and mucosal tissues. However, how the tolerance to citrullinated proteins is breached is still unknown. Hypothetically, this could be due to the constant exposure to environmental risk factors that induce hypercitrullination, which inevitably expands the pool of citrullinated autoantigens. One typical example is smoking, which is correlated to the over-expression of PAD2 and increased citrullination level in lung tissues (*18*). Another example is the bacterial infection by *Porphyromonas gingivalis* (*P.gingivalis*) causing periodontitis, which expresses a unique PAD enzyme (*P*.PAD) with capability of citrullinating proteins such as alpha-enolase and fibrinogen (*37*). Regardless, although the implications from both examples are supported by certain epidemiological and experimental data, there is still a lack of conclusive evidence as a link to their direct contribution in RA etiology. On the other hand, the development of ACPAs has not been fully understood. According to some current perspectives, incomplete elimination of autoreactivity during both central tolerance in the bone marrow and peripheral tolerance in secondary lymphoid tissues may enable certain autoreactive B cells to evade elimination and persist. These citrullinated autoantigen-driven B cells then receive help from T cells in association with HLA-SE, resulting in extensive somatic hypermutation, epitope spreading, isotype switch and increased titers within the time window around the onset of RA, and further maturated in established RA (*38*). Together, RA patients or healthy individuals may possess mature autoreactive B cells to citrullinated proteins, but whether these elusive B cells are directly involved in the pathogenesis of RA requires further investigation.

ACPAs as such are autoantibodies against citrullinated epitopes. Based on the different binding patterns and structural analysis, three typical subgroups of ACPAs have been classified, namely promiscuous, private specific and private cross-reactive ACPAs (Fig.2) (*9*). The promiscuous ACPAs, for example the E4 or "L" antibodies, are highly specific to the citrulline side chain but have weak or no affinity to the surrounding amino acids and bind not only to citrullinated collagen type 2 (COL2) but also other citrullinated proteins (*39–41*); the private specific ACPAs, for example the ACC4 antibody, specifically recognizes the citrullinated C1 epitope on COL2 in a fixed form of an alpha chain peptide, but not other epitopes (*42*); and the private cross-reactive ACPAs, for example the ACC1 antibody, binds to either unmodified or citrullinated triple-helical epitopes on COL2, but not with other collagens or proteins except for a few citrullinated peptides (*43*). During the last years, the promiscuous, or "citrulline-specific" ACPAs, have been extensively studied in regard of their crystal structure, specificity and function (*39–41*), which will be extended in the next subsections. Monoclonal ACPAs isolated from patients with RA are likely to have a promiscuous nature and possess a small binding pocket that is well-suited for accommodating the citrulline side chain. This promiscuity is attributed to the fact that the ACPAs have limited interactions with the side chains of neighboring residues (*40*). However, in many cases, these ACPAs are only able to bind to a limited number of citrullinated epitopes, most likely because of

the disturbance caused by the neighboring amino acids or certain variable domain glycans (VDGs) (*40*, *41*).

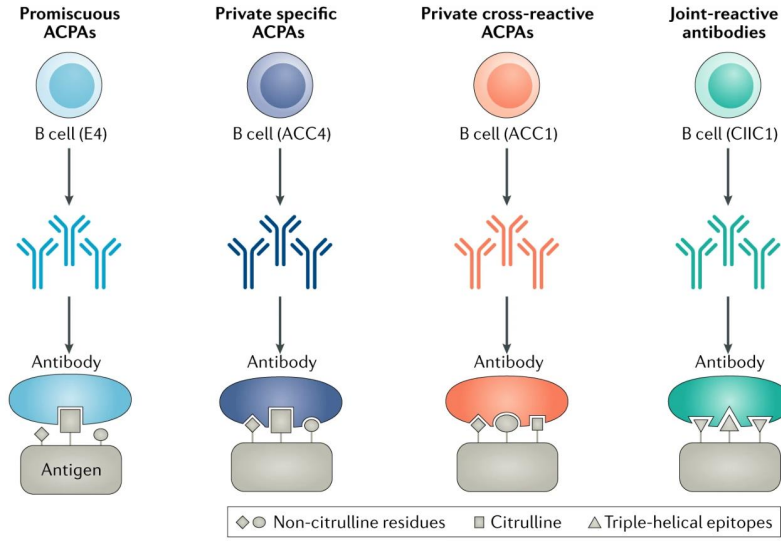

Fig.2: Examples of different subgroups of ACPAs and joint-specific autoantibodies in RA. Illustration of the epitope-binding by different subgroups of autoantibodies in RA. Each example is given based on the crystallized antibody-peptide complex. 1) Promiscuous ACPAs: the E4 antibody are widely reactive with citrullinated collagen type 2 (COL2) and other citrullinated proteins owing to citrulline-specificity. 2) Private specific ACPAs: the ACC4 antibody only reacts with the citrullinated C1 epitope with a form of COL2 alpha chain peptide, but not other epitopes. 3) Private cross-reactive ACPAs: the ACC1 antibody cross-reacts with triple-helical and cyclic epitopes on COL2, either in native or citrullinated forms. 4) Joint-reactive antibodies: the CIIC1 antibody is specific to the native triple-helical C1 epitope on COL2, but not other epitopes. *Illustration adapted from* (*9*) *with permission from Springer Nature*.

1.4.3 Function of ACPA

Since long time ago, topics surrounding the functions of ACPAs in RA have been extensively discussed. The presence of ACPAs in RA patients is associated with a more erosive and severe disease course (*44*), and it is also statistically evident that *HLA-DRB1* alleles encoding the SE are involved in such scenario (*45–47*). In terms of these strong associations, lots of efforts have been made to investigate the potential pathogenic role of ACPAs, in which ACPAs were shown to be instrumental in causing pathogenic effects on various models, supported by their interaction with innate immune system by activating complement system, or inducing pro-inflammatory cytokine secretion by interacting activating Fc receptors (*48–50*). Meanwhile, human polyclonal ACPAs were reported to induce osteoclastogenesis in vitro and in vivo (*51*), likely dependent on the Fc-glycans of human autoantibodies (*52*). By far, only certain subgroups of joint-specific ACPAs have been shown to be pathogenic in vivo, such as ACC1 and ACC4 antibody (*42*, *43*). Nonetheless,

although it has become rather reasonable to assume that ACPAs are pathogenic in RA based on the previous data, it is still a putative notion since direct evidence from the application of patient-derived monoclonal ACPAs with proper controls, animal models and characterization, were lacking. In retrospect, interestingly, ACPAs were detected in approximately 1% of healthy individuals who never developed arthritis symptoms with time (*53, 54*). It is intriguing to think that a part of the ACPA response prior to the development of RA is to prevent the disease by either mediating the inflammation with immune complex formation or contributing to the clearance of citrullinated autoantigens induced by aberrant hypercitrullination.

Although it remains difficult to conclude the function of ACPAs in RA, emerging evidence suggests a protective, rather pathogenic role of ACPAs. In 2020, a study using engineered monoclonal ACPA specific to citrullinated histone 2A and 4, which were characterized in 2013, showed that these antibodies could inhibit collagen antibody-induced arthritis (CAIA) and collagen-induced arthritis (CIA) in mice, with postulated mechanisms such as by inhibiting the release of neutrophil extracellular traps (NETs) or initiating the phagocytosis of NETs by macrophages, either way diminishing NETosis (*55, 56*). However, the role of NETs in the pathogenesis of RA has been elusive and rather inconclusive, and the used antibodies did not display a promiscuity resembling typical promiscuous ACPAs. As a matter of fact, a patent concerning protective monoclonal ACPA derived from RA patients had been documented in 2018 (PCT/EP2018/082236), which was in fact the E4 ACPA and its variants in **Study I**. The protection had been carefully investigated since 2016 and recently published (*39*), demonstrating that the protective effect of E4 ACPA in arthritis is exerted through the formation of immune complex (e.g. with citrullinated ENO1) and interaction with FCGR2B on macrophages, increasing IL-10 secretion and suppressing osteoclastogenesis. Around the same time, two independent studies also observed protective effects of monoclonal ACPAs in derived from RA patients (*57, 58*), these recombinantly expressed ACPAs showed distinct reactivities towards a variety of citrullinated proteins/peptides, displaying typical features of promiscuous ACPAs and profound protective effect in CAIA. Similarly, the protective effect seemed to be dependent on the interaction between antibody and FcγRs (*39, 58*), and was more potent when the antibody was injected in the early stage of CAIA (*57*). The antigen specificities of these ACPAs are likely distinct among each other, but since the ACPA-FcγRs interaction is important for the protection, it is likely an interaction with the low affinity FcγRs and the formation of immune complex is preferred in this scenario, which was suggested with the example of E4 ACPA (*39*).

Together, the implications from recent data open a new door to pinpoint the functional role of ACPAs in RA. An optimal study design including the application of mutated antibodies (mutations on the paratope of ACPAs), extended peptides with unmodified controls (arginine peptides) and

careful selection of animal models could be largely helpful, and more efforts are still required to investigate the effect of ACPAs on pain and the functions of ACPA-VDGs in RA.

1.4.4 Variable domain glycans in ACPA

Immunoglobulins G (IgGs) are glycoproteins with conserved glycans linked to the asparagine (N297) in the Fc tail, which are crucial for FcR and C1q binding, whereas in the serum from healthy individuals, only 15-25% of IgGs are glycosylated in the variable domain (*59*). Strikingly, in contrast, approximately 90% of ACPA-IgGs in serum from RA patients possess variable domain glycans (VDGs), these VDGs are highly sialylated not only in serum, but also more enhanced in synovial fluid from RA patients (*60*, *61*). Since only a few heavy chain variable region genes carry germline-encoded *N*-glycosylation sites, the high degree of VDGs in ACPAs could theoretically be the consequence of somatic hypermutation in germinal centres, supported by B cell receptor (BCR) sequencing of ACPA-expressing B cells (*62*).

The questions surrounding the occurrence of ACPA-IgG-VDGs and their function are still to be experimentally addressed. ACPAs are low-avidity antibodies (*63*), suggesting that the affinity maturation of BCRs in the process of B cell selection is incomplete or absent to some extent. It was then hypothesized that the VDGs in these BCRs could enable the bypassing of selection mechanisms, thus conferring to the breach of tolerance by these B cells (*38*). Moreover, recent data also implicated that VDGs could provide advantages for the survival and activation of the ACPA-expressing B cells (*41*). In crystallographic and binding analysis, VDGs are shown to be positioning near the paratope/citrulline-binding pocket, forming hydrogen bond with the CDR structures, and possibly disrupting the antigen-binding and further influenced by sialylation (*41*). However, it may be more dependent on the epitopes and the positioning of the VDGs, as not all bindings are disrupted (*40*). Notably, selective point-mutations on the paratope are sufficient to abolish the citrulline-specificity of ACPAs (*39*).

1.4.5 Other autoantibodies in RA

Besides RFs and ACPAs, other autoantibodies have also been identified with various degrees of sensitivity and specificity. One such family is the anti-carbamylated protein (anti-CarP) antibodies, which recognize carbamylated proteins/peptides and are found in approximately 44% of RA patients (*64*). Carbamylation is another PTM that involves the conversion of lysine to homocitrulline by cyanate, and is common in patients with renal diseases caused by high blood concentration of urea, as well as in individuals who smoke or have conditions that increase cyanate intake (*65*, *66*). Homocitrulline is structurally similar to citrulline, with the addition of one methylene group in the side chain. ACPAs and anti-CarP antibodies are often present together in RA, with single or double positivity (*65*, *67*). However, it is unclear whether this is due to true

cross-reactivity of monoclonals or a polyclonal response in RA, and further testing of isolated antibodies is required. Although the increase of anti-CarP antibodies is associated with the progression of RA (*68*), the mechanism underlying the breach of tolerance to carbamylated antigens and the function of these antibodies are still unclarified.

Another identified group of autoantibodies in RA is the anti-PAD4 antibodies. As described in previous sections, the PAD enzyme family (PAD1-4 and 6) is attributed to citrullination in humans. Each PAD enzyme differs in the substrate selection and location of activity (*69*). In RA, 30-50% of patients were detected with anti-PAD4 antibodies, and the association between anti-PAD4 antibodies and ACPA was also observed (*70–72*). The effect of anti-PAD4 antibodies upon binding to PAD4 is unclear, one obvious thinking is that it depends on the epitopes and the substrates. For example, the PAD4 inhibitors suppress the PAD4 activity by binding to the active site C645 (*73*), and theoretically, PAD4 bound by antibodies may confer a more efficient conformation for the enzymatic citrullination. In addition, PAD4 can also undergo autocitrullination (*74*), more data is needed to understand whether this self-modification will change or maintain the enzymatic activity, but it is possible that citrullinated PAD4 itself could also be a target for ACPAs.

Collagen type 2 (COL2) is the most abundant constituent in joint cartilage and an important autoantigen in experimental arthritis. The COL2 antibodies show clear arthritogenicity in classic CIA and CAIA models and are associated with acute onset of RA (*75–81*). However, the detection of these antibodies are not well-established mainly due to the lack of standards/well-defined reagents, leading to a highly deviating prevalence of 3-88% that is difficult to interpret (*82*, *83*). So far, COL2 antibodies have not been included as a biomarker for RA diagnosis,

1.5 Animal models in experimental arthritis

Mice and rats are the most commonly used animals for experimental arthritis, owing to their low cost, homogeneous genetics and convenience of handling. Although there is no single animal model that can completely mimic the conditions in human diseases, the models with maximal similarity are usually preferred, with various interventions to supplement the dissimilarities. Another advantage of using rodent models is the availability of selective modifications of the genes, generating congenic, knockout or transgenic strains (*84*). In this section, representative animal models for RA research will be discussed.

1.5.1 Collagen induced arthritis

As a gold standard, collagen induced arthritis (CIA) is more extensively studied, it shares large similarities in regard of pathological and immunological features with human RA (*85*, *86*). The CIA model is typically achieved by injecting COL2 from a variety of sources (bovine, human, or chick), inducing the breach of tolerance and generation of antibodies against collagen. COL is

usually emulsified in complete Freund's adjuvant (CFA) (*78*), a mixture of mineral oils, heat-killed mycobacteria and emulsifying agent, which enables enhanced cell-mediated and humoral immune responses to COL2. In addition, the model is later boosted by injection of COL2 emulsified with incomplete Freund's adjuvant (IFA), in which the mycobacteria is not present. CIA is dependent on T and B cell responses, in the A^q-expressing mice it has been found that both cell types share the epitopes almost identical to RA patients with HLA-SE alleles (*87*), resembling the RA patients with HLA-SE alleles.

1.5.2 Collagen antibody induced arthritis

Collagen antibody induced arthritis (CAIA) is an acute and simple mouse model for the investigation of pathogenic mechanisms of RA and screening of therapeutic agent, it has strikingly similar pathogenic features that are in human RA, including synovitis with various cell infiltrations, pannus formation, cartilage degradation and bone erosion (*88*). CAIA is induced by administration of monoclonal antibodies against COL2, typically a cocktail of antibodies against the defined epitope C1, J1, D3 and U1 (*80*), together with the TLR ligand lipopolysaccharide (LPS) as a booster. Because of direct injection of pathogenic antibodies against COL2, CAIA could mimic an effector phase of arthritis with minimal involvement of adaptive immune responses, providing advantages over CIA such as significantly shorter time for arthritis induction (few days vs. weeks in CIA) and wider selection of mouse strains (*89*). The major limitations of CAIA are the economic applicability due to the large amount of antibodies needed, making it a costly model; and its incapability to be used to study the chronic phase of arthritis because of its rapidity. Nonetheless, CAIA has become a highly useful model to investigate the functional roles of autoantibodies in RA (*39*, *43*, *57*, *58*).

1.5.3 Pristane induced arthritis

Pristane induced arthritis (PIA) is one of the most studied models in experimental arthritis. In rats, one single injection of the mineral oil, pristane, could induce severe and chronic arthritis within 2 weeks, with almost 100% of incidence and outstanding reproducibility (*90*). The rats develop severe and chronic arthritis restricted to the joints, involving pannus formation, MHC class II expression and T cell infiltration(*90*, *91*) that are similar to human RA. It has become an ideal model to investigate the mechanisms of joint inflammation and to test new drugs against arthritis (*92*). However, although the production of antibodies against COL2 has been detected in PIA (*93*), there is no evidence showing that these antibodies are driving the pathogenesis.

2 Research aims

The development of RA is initiated by complex genetic and environmental risk factors, autoantibodies play a critical effector role in the downstream. The role of ACPAs in RA has been a subject of debate. Even though ACPAs have been associated with negative impact on RA development and implicated in different models to have a rather pathogenic role, a lot of questions surrounding the origin, specificity, VDGs, origin, specificity and most importantly, the exact function in RA, have not been fully addressed and understood. **Study I & II** in this thesis are primarily to understand the specificity and functions as well as the role of VDGs of ACPAs, whereas in **Study III**, the focus shifted to two regulatory genes, *Ncf1* and *Clec4b*, to demonstrate how interactions between two genes could regulate the immune responses.

In **Study I**, we aimed to identify the reactivities of monoclonal ACPAs derived from RA patients to a panel of citrullinated peptides, and to investigate their functions in vivo using various mouse models including the evoked pain-like behaviour model and CAIA, further detailed by histological staining with different tissues from mice and humans. To propose a mechanism, we employed the knockout mice and in vitro models, while using the proteomic analysis to identify the targets of ACPAs.

For **Study II**, we combined structural analysis, glycobiology and functional B cell assays to dissect the key characteristics of VDGs on ACPA-expressing B cells and tried to understand the impact of VDGs on antigen-binding and their effect on B cell behaviour.

Study III aimed to demonstrate the epistasis of two major regulators in rats, Ncf1 and Clec4b, in arthritis, and to understand how their interactions could affect the severity and susceptibility of arthritis.

3 Materials and methods

This section provides descriptions for the representative experiments mainly for **Study I**. Full details of methodology in all experiments are referred to the original papers included in **Study I-III.**

3.1 Expression and purification of antibodies and Fab fragments

Chimeric antibodies containing human variable and mouse constant domain were designed. The mouse IgG2b constant region sequence was obtained from UniProtKB with accession number P01867 and the mouse lambda-1 P01843. First, vectors containing the mouse IgG2b heavy chain (HC) constant region and the lambda light chain (LC) constant region were created. The variable regions of HC and LC from RA patients were then inserted in the frame before the mouse constant region. Four DNA fragments were synthesized with restriction sites at the 5′ and 3′ ends. The synthesized genes (constant regions of both HC and LC) were digested using FastDigest™ restriction enzymes. The digested DNA fragments were cloned into the mammalian expression vector pCEP4 that was digested using the same restriction enzymes. After ligation, two vectors containing mouse IgG2b and lambda constant regions were obtained, designated pCEP4-mIgG2b and pCEP4-mL, respectively. The HC and LC plasmids were co-transfected into Expi293F cells with FectoPRO™ DNA transfection reagent. The supernatants were harvested 6 days post-transfection. The chimeric antibodies were purified using a 5 mL HiTrap Protein affinity column, or protein G-based affinity chromatography with the ÄKTA™ system. The purified antibodies were dialyzed to PBS solution and the endotoxin was determined with <0.1 EU/mg protein. The antibodies that were traditionally expressed by hybridomas were similarly purified following the hybridoma subcloning and single-clone expansion. For Fab fragment preparation, chimeric antibodies were produced similarly as above, and dialyzed against PBS. The Fab fragments were prepared using the ImmunoPure Fab Preparation Kit (Pierce) following the manufacturer's instructions. Cleavage was evaluated by SDS-PAGE. Fab fragments were further purified by SEC on a HiLoad 16/600 Superdex 200 column.

3.2 Multiplex bead-based array (Luminex)

The antibody responses to peptides were performed using the multiplex bead-based array (Luminex). In short, MagPlex® beads with unique IDs were activated by sulfo-NHS combining with EDC, NeutrAvidin was conjugated to the beads, and each biotinylated peptide was coupled to designated bead ID. The beads were mixed and incubated with indicated antibodies, followed by incubation with 1:750 diluted goat anti-mouse IgG secondary antibody conjugated with R-Phycoerythrin. The antibody reactivity to the peptides was detected by Bio-plex 200 system (Bio-

Rad) and processed by Bio-plex Manager 6.2 (Bio-Rad), and the raw values of median fluorescence intensity (MFI) were used for analysis.

3.3 Collagen-antibody induced arthritis

For the functional study of ACPA in collagen antibody-induced arthritis (CAIA), indicated dose (2, 3 or 4 mg/mouse) of each monoclonal antibody were intravenously or intraperitoneally injected into designated mice on day 0. The mice received a boost by intraperitoneally injecting 25 μg of lipopolysaccharide (LPS, 055:B5) on day 3 or 5. The development of arthritis was monitored in different time points using an extended scoring system. Briefly, each inflamed (swollen or red) toe or knuckle was given 1 point, and each inflamed wrist or ankle was given 5 points, resulting in a maximum score of 15 (5 toes + 5 knuckles+ 1 wrist/ankle) per limb and 60 in total for each mouse.

3.4 Bone marrow derived macrophages

For in vitro differentiation of BMDMs, depending on the purpose, bone marrow cells were obtained from Balb/c, B10.Q or FCGR2B knockout mice, and (2–3) × 10^6 cells/well were seeded in 6-well culture plates for protein extraction, 10^6 cells/well in 24-well plates for cytokine detection, or 10^4 cells/well in 96-well black μ-plate (ibidi) for IF assay. The RPMI 1640 GlutaMAX™ medium containing 10% FBS, 1% penicillin-streptomycin and 20 ng/ml of recombinant murine M-CSF was used for the differentiation, the medium was changed every 2–3 days, cells were cultured for ≥5 days at 37 °C in a conventional CO2 incubator until adherent BMDMs were fully differentiated.

3.5 Immunoprecipitation

To precipitate the targets of given antibodies, 10 μg of each indicated biotinylated antibodies were immobilized to streptavidin-coated magnetic beads (Dynabeads™ MyOne™ Streptavidin C1/T1, Thermo Fisher Scientific) according to the manufacturer's instructions. Extracted proteins from macrophages or human synovial fluid were subjected to incubation with the beads for 2 h at room temperature with gentle rotation. The beads were washed between each step 3–4 times by sterile PBS. The proteins that were captured by the beads were then eluted and stored at −80 °C before further analysis.

3.6 Ethical considerations

All experiments using human/animal samples were approved by local ethical committee with valid permits.

Undoubtedly, animal experiments should be performed under the framework of modern ethical consideration. It is factual that even if we use animals to only evaluate the drug toxicity in pre-clinical trial, it is still vastly different when it comes to clinical trials, thus the validity of animal

experiments are of concern, at least from the public. However, the public must be made aware that in the field of biomedicine as well as in any other scientific research, only continuing trial-and-error to understand errors will show the best way to reach a goal. Indeed, there is always a room for improving the animal experiments when it comes to the welfare and validity, guidelines exist based on the continuous evolution of our understanding between humans and other species, which pave a path for us to balance our modern value and need for survival. On the other hand, the benefit of animal experiments is not limited to translation to human, in fact, a large number of drugs applied to humans are same as those used in animals, for example, the antibiotics.

Currently, animal models are still irreplaceable for drug testing before trials on humans, it is plausible that with the rapid development of AI and bioinformatics, much could be extracted for use in the areas such as diagnosis, epidemiology and drug predictions, but none by far could change the superiority of animal experiments before anything could be applied to human body, whereas human samples remain difficult to access in different ways. That being said, it is unquestionable that increasing restrictions on animal experiments will jeopardize and slow down the research. More efforts surly need to be made to improve the conduction of animal studies, but it should be based on more rational discussions with the support of science and balancing of needs. It has always been a matter of communication between the scientific community and the public, the politics comes after.

4 Results and discussion

4.1 STUDY I: A SUBSET OF ANTIBODIES TARGETING CITRULLINATED PROTEINS CONFERS PROTECTION FROM RHEUMATOID ARTHRITIS

The functional role and reactivity of ACPAs in vivo are not well understood, but their strong association with RA suggests a potential pathogenic role. However, our findings in this study suggest that certain ACPA may not contribute to arthritis or bone erosion, and may even provide protection. This protective effect appears to be specific to joints and is facilitated by the formation of local immune complexes, potentially involving proteins such as citrullinated ENO1, COL2 and other unidentified citrullinated proteins. Additionally, we observed that some antibodies, including certain ACPAs with a more targeted interaction with cartilage, can exacerbate arthritis and pain-like behavior. The extent to which injected antibodies are pathogenic or protective may be determined by the function of the local immune complexes they form, and these complexes are usually depositing in the joint compartments such as cartilage, synovium and phagocytes (*94–97*). By far, several, but not all cartilage-targeting antibodies such as antibodies against COL2, cartilage oligomeric matrix protein (COMP) and glucose-6-phosphate isomerase (G6PI) have been shown to be pathogenic in experimental arthritis (*42, 81, 98–101*). It is also important to note that the protective effects of antibodies may depend on their preparation and tendency to form non-specific immune complexes or aggregates. To address this issue in our studies, we included well-defined controls such as antibodies with critical amino acids mutated, exemplified by E4m in our present work. On the other hand, visualizing the precise and dynamic interactions in vivo can be difficult, so we conducted in vitro tissue staining and examined the binding of monoclonal antibodies that were injected. It is important to note that the monoclonal antibodies produced in vitro may not have the full range of variability in Fab or Fc-glycosylation profiles compared to those produced naturally in vivo. We compared the specificity and function of E4 with and without Fab or Fc glycans and did not observe any variation. However, it is possible that the dynamic expression of more complex carbohydrates could fill the space and interfere with targeting in vivo, which cannot be ruled out (*41*).

4.2 STUDY II: SURFACE IG VARIABLE DOMAIN GLYCOSYLATION AFFECTS AUTOANTIGEN BINDING AND ACTS AS THRESHOLD FOR HUMAN AUTOREACTIVE B CELL ACTIVATION

In this study, we demonstrate that the presence of VDGs has a significant impact on B cell function, including antigen binding, BCR signaling, and downregulation, and may therefore play a crucial role in determining the outcome of B cell responses.

It is noteworthy that VDGs are highly abundant in ACPA response in RA. The study provides evidence supporting the specific and extensive presence of VDGs on autoreactive B cells in RA, which may confer an advantage for B cell signaling, potentially explaining the improved stability and survival of these B cells and the increased levels of autoantibodies that eventually lead up to the onset of RA. The findings could improve our understanding of B cell mediation in autoimmune diseases, as they provide insight into how the possession of VDGs may be attributed to the evasion of immune checkpoints in humans, leading to the breaking of tolerance..

4.3 STUDY III: GLYCAN ACTIVATION OF CLEC4B INDUCES REACTIVE OXYGEN SPECIES PROTECTING AGAINST NEUTROPHILIA AND ARTHRITIS

In this study, we found that the activation of Clec4b with glycans may lead to the induction of ROS and inhibition of neutrophilia, these effects may play a crucial role in the profound regulation over autoimmune arthritis. Additionally, we found that *Clec4b* expression is responsible for the correlation between neutrophil infiltration and acute inflammation observed in the carrageenan air-pouch model. Our investigation on recombinantly expressed rat Dcar proteins revealed their specificity to Zymosan, a macromolecule from the yeast cell wall, through the binding to the carbohydrate recognition domain, which triggered potent Ncf1/NOX2-derived ROS production. Furthermore, carbohydrate profiling analysis of wild type and mutated Dcar suggested a preference for binding to galactose-attached sialic acids.

5 Concluding marks

To conclude, **Study I & II** provide in-depth analysis on the specificity, function and structure of monoclonal ACPAs in RA, revealing the protective, rather than pathogenic effect of certain ACPAs in experimental arthritis. Moreover, the observed impact of VDGs on antigen-binding, B cell activation and survival deepens the current understanding regarding the functional role of these VDGs. In brief, these two studies address the haunting questions highlighting the function and glycosylation of ACPAs and have meaningful implications for the pathogenesis and therapeutics of RA. **Study III** identifies a new function for *Clec4b* in regulating arthritis via the Ncf1/NOX2 pathway, resulting in increased ROS production and a significant impact on experimental arthritis. the findings represent the first characterization of the epistasis between two positionally cloned SNPs associated with an autoimmune disease in eukaryotes.

6 Acknowledgements

My harvest today would not have been possible without the amazing people that took part in this journey with me over the years, many thanks are merely the smallest expression of my gratitude.

Rikard, thank you for accepting me as your PhD student and letting me become a part of **MIR**. In science, you are like a perpetual motion engine, emitting knowledge and discoveries nonstop, your dedication to immunology will always be a huge inspiration for my future career. During the years you have given me the maximal freedom to explore in science, supported and motivated me throughout my ups and downs, the immense faith and support you bestowed upon me have been the major fertilizer of the fruitfulness of my PhD which honestly changed my life.

Changrong, as my co-supervisor, you taught me a lot not only in science, but also in life. I am constantly in awe of your insane amount of knowledge in biochemistry, structural biology and bioinformatics. Thank you for your kindness, encouragement and patience for me, and more importantly, your help during my transition to an independent PhD student. **Bingze**, every single protein you produce is textbook perfection, and sorry that my antibody production is still not as satisfying as yours (but I am not the only one). I miss the time we worked together and our small talks, and I am truly indebted to your help in my PhD years. **Angel**, thank you for taking care of us and being supportive all the time, you are still the only person who I can speak Hokkien with in this country so far, which since the first day I arrived in Sweden has made me feel like home.

As a big family, MIR is where I hatched my PhD, none of my achievements could have come true without the companionship and reinforcement from previous and current members of MIR.

Ana, for being the best neighbor ever! You've been sitting right next to me since the first day of your PhD and there were so much great time we've spent together, well you know sometimes I am more or less a heavy type but the vibe in our corner is perfect. Thank you for bearing with me and your unconditional support whenever I had difficult situations, especially in life, I forever will cherish this friendship. **Mike**, for your rather intense personality, the great smoking breaks and topics from politics to life we've shared since the downstairs of Scheeles väg, thanks for always standing by my side like a bro (but on certain level of intensity). **Gonzalo**, the "Gonzi" or "Gonzo," for our normal conversations in life and science and the bananas in some weird topics, I miss when we were just repeating dumb and nonsense phrases to each other in the office. Have a great time in Harvard and come visit in the future. **Jaime**, "Jamo," for the nearly 5 years of being a fantastic neighbor of mine since Scheeles väg. I enjoyed the time when we shared common topics in sci-fi/fantasy and that time we went to the fantasy concert and so many other activities, enjoy Köpenhamn and come visit us from time to time! **Alexander**, for being an alternative culture. You always give me a typical German vibe (it's a compliment) through the years, thanks for always being so kind and helpful. **Laura,** for injecting the wild Spanish energy to MIR, and always working hard and partying hard! You brought my understanding of Spanish to the next level! **Erik**, for being the supportive elder in MIR and the Santa Claus for my miniature collection. Your insightfulness in science benefits me a lot. **Michael**, "Daddy B," for the Zen and great conversations we had in politics and other general topics, I'm still waiting for your seminar with Southern accent. **Kejsi**, for being such a wonderful neighbor! You've worked hard and congrats on your new position. Looking forward to hanging out again after your return from Italy. **Zhongwei**, for your potent IT support for MIR, nice Sichuan cuisine and great discoveries in the F4 project. I hope you master the guitar someday. **Ulrika**, for the time we spent together in the lab and Västra Skogen, especially when sometimes you came and scared me by knocking on my window. I still keep the

playlist on Spotify you created for me. **Vilma**, for always being so nice and helpful. I like every single photo you took with your camera, you are certainly a natural. **Outi**, for the short period we shared in the beginning of my PhD and now a more exciting opportunity to work together. Thank you for trusting me and being supportive as a role model leader, and I promise I will fix my procrastination. **Alex M,** for the substantial work on the animal models for the E4 project and for always being so modest and helpful, I hope you spend more time with us in Stockholm in the future. **Caroline**, In Flames are meh, Entombed are cool, and it's an amazing to realize that in this kingdom of death metal we have an old schooler in the lab too. **Jianghong**, for your stubbornness in a good way in science and the lunch time we had before. **Rajan**, for always being a nice person, and always offering your help without hesitation. Thank you for accepting to be the chairperson in my defence. **Xiaojie,** for being the cutie pie and always hanging out with us. **Liselotte**, for the essential work on taking care of the animal house and managing the lab. **Zeynep**, for the short period we had together and now you're a cool mom. **Anne-Laure**, for your short-term co-supervision for my PhD. I still remember the time you tried to teach me how to use LSRII. **Lei**, for being so quiet and helpful all the time. Thank you for your contributions to diagnostic project, I've learnt a lot. **Huqiao**, one of the most hard-working people in MIR. You deserve every success then and now, and in your future career. **Taotao**, I wish you success in the future. **Weiwei**, for being neighborly and helpful. **Yanpeng**, for your curiosity and always being smiley. **Clara**, the previous Spanish energy in MIR, my first skål in MIR was with you. **Amit**, for always teasing Johnny and that classic "how will you manage." **Dani**, for always being energetic and passionate about everything. **Carlos and Kristina,** for taking care of our animals**. Emma & Biborka**, for the generous help in lab and animal house. **Chang, Meng, Jing X, Qijing, Atakan, Maëlys & Julian**, I wish you a bright and successful future in either academia or the industry.

MIR retreat, September 2020.

This part is dedicated to my friends at Biomedicum and KI in general. **Christian**, "Kiki," the unspoken MIR member. An eternal thank for the big contribution to the proteomics in my project. I love the time and beer we shared together and I will hunt you down if you don't visit when you

come back from the US. **Max**, for being a trve old school metalhead in Biomedicum and your dedication to guitars. It's shame that we didn't manage to jam together, now enjoy your new career as a guitar crafter in the UK! **Pierre**, for always being classy and kind, you still owe me a Warhammer miniature! **Amir**, you reminded me of Bill Hick's joke "I don't smoke two packs of cigs per day, I go through two lighters." Thanks for the interesting topics and breaks over the years. **Keying & Feng**, thanks for the activities and heartwarming time we spent together, we should hang out more often.

Many thanks to our collaborators, **Inger Gjertsson** and **Olov Ekwall** from Göteborg, **René Toes** and **Theo Rispens** from Leiden, **Roman Zubarev** from MBB and **Vivianne Malmström** from CMM, a large part of my project was from your valuable collaborations. **Helena H** and **Mia O** for the absolutely fantastic organization of NCRSCID and all the amazing people in there. In addition, many thanks to different funding bodies from Sweden and the China Scholarship Council for the financing in various ways.

Apart from people from work, I would like to thank **Daniel Dindsdale**, for being a stubborn goa gubbe. Thanks for always being a supportive bro to everything in my life, I know we had some disagreement but hope this page could be flipped somehow. Sacrilege is forever the best Swedish melodic death metal band in my heart. **Christopher Brown**, it's nice to finally see you in person during this 10 years of acquaintance and collaboration in music. Salve 218! **Alexander Sokolov**, one of the most rational and reasonable people I have ever met and a fanatic in gaming and Warhammer. Hope to hang out again in Prague or Stockholm. **Mei Huang**, my supervisor during my master's, I would not have been able to pursue my PhD without your full-support. Thanks for the trust and guidance you and **Lihong Nan** gave me during my college life and many other things. **Lan Yang**, for being a good roommate and friend during my master's and these years when I am in Sweden.

This paragraph is by request dedicated to **Gini, Weijie & Allen**, the three brothers of mine since 2003. It has been a while since I hung out with you last time, but it has always been fun talking with you guys.

Tanya, for the love and support you gave me since the day we knew each other, and thanks for allowing me to put a ring on your finger and for deciding to spend the future with me. **Alla**, for your shyness and loving personality, and accepting me to your family. **Yuri,** for emptying vodka with me in Kyiv and reminding me to make your daughter happy by letting me play with your shotgun. **Gabi**, I know you are literally a dog and I made fun of your sad looking face, but you are a truly good boy and I love you for tackling me down and licking my face the first moment we met.

Lastly, I want to thank my family for the unbreakable support and encouragement. It was my **parents** who sacrificed so much for me since the first day I was born, nothing I have achieved today could have been possible without you. To my **grandparents** and all other **relatives** in the big family, thank you for always having faith in me and supporting me in your loving ways. **Turtle**, I know you are literally a cat and I don't know why I gave you this name, even though you love my parents more than me now, you are still my good orange boy, thanks for the friendship since 2011, hope your kidneys are still kicking well.

7 References

1. F. Himmelweit, The Collected Papers of Paul Ehrlich. *London: Pergamon* (1956).

2. E. Witebsky, CHRONIC THYROIDITIS AND AUTOIMMUNIZATION. *J. Am. Med. Assoc.* **164**, 1439 (1957).

3. P. J. Delves, "Autoimmunity" in *Encyclopedia of Immunology* (Elsevier, 1998; https://linkinghub.elsevier.com/retrieve/pii/B0122676560007 73), pp. 292–296.

4. M. D. Rosenblum, K. A. Remedios, A. K. Abbas, Mechanisms of human autoimmunity. *J. Clin. Invest.* **125**, 2228–2233 (2015).

5. A. J. Silman, J. E. Pearson, Epidemiology and genetics of rheumatoid arthritis. *Arthritis Res.* **4 Suppl 3**, S265-72 (2002).

6. P. Stastny, Association of the B-cell alloantigen DRw4 with rheumatoid arthritis. *N. Engl. J. Med.* **298**, 869–71 (1978).

7. T. Tuomi, M. Heliovaara, T. Palosuo, K. Aho, Smoking, lung function, and rheumatoid factors. *Ann. Rheum. Dis.* **49**, 753–756 (1990).

8. M. Heliövaara, K. Aho, A. Aromaa, P. Knekt, A. Reunanen, Smoking and risk of rheumatoid arthritis. *J. Rheumatol.* **20**, 1830–5 (1993).

9. C. Ge, R. Holmdahl, The structure, specificity and function of anti-citrullinated protein antibodies. *Nat. Rev. Rheumatol.* **15**, 503–508 (2019).

10. J. Sokolove, R. Bromberg, K. D. Deane, L. J. Lahey, L. A. Derber, P. E. Chandra, J. D. Edison, W. R. Gilliland, R. J. Tibshirani, J. M. Norris, V. M. Holers, W. H. Robinson, Autoantibody epitope spreading in the pre-clinical phase predicts progression to rheumatoid arthritis. *PLoS One*. **7**, 1–9 (2012).

11. P. K. Gregersen, J. Silver, R. J. Winchester, The shared epitope hypothesis. an approach to understanding the molecular genetics of susceptibility to rheumatoid arthritis. *Arthritis Rheum.* **30**, 1205–1213 (1987).

12. C. Turesson, D. J. Schaid, C. M. Weyand, L. T. H. Jacobsson, J. J. Goronzy, I. F. Petersson, G. Sturfelt, B.-M. Nyhäll-Wåhlin, L. Truedsson, S. A. Dechant, E. L. Matteson, The impact of HLA-DRB1 genes on extra-articular disease manifestations in rheumatoid arthritis. *Arthritis Res. Ther.* **7**, R1386-93 (2005).

13. I. Moreno, A. Valenzuela, A. García, J. Yélamos, B. Sánchez, W. Hernánz, Association of the shared epitope with radiological severity of rheumatoid arthritis. *J. Rheumatol.* **23**, 6–9 (1996).

14. D. L. Mattey, P. T. Dawes, M. A. Gonzalez-Gay, C. Garcia-Porrua, W. Thomson, A. H. Hajeer, W. E. Ollier, HLA-DRB1 alleles encoding an aspartic acid at position 70 protect against development of rheumatoid arthritis. *J. Rheumatol.* **28**, 232–9 (2001).

15. N. Carrier, P. Cossette, C. Daniel, A. de Brum-Fernandes, P. Liang, H. A. Ménard, G. Boire, The DERAA HLA-DR alleles in patients with early polyarthritis: protection against severe disease and lack of association with rheumatoid arthritis autoantibodies. *Arthritis Rheum.* **60**, 698–707 (2009).

16. D. Sugiyama, K. Nishimura, K. Tamaki, G. Tsuji, T. Nakazawa, A. Morinobu, S. Kumagai, Impact of smoking as a risk factor for developing rheumatoid arthritis: A meta-analysis of observational studies. *Ann. Rheum. Dis.* **69**, 70–81 (2010).

17. S. Y. Bang, K. H. Lee, S. K. Cho, H. S. Lee, K. W. Lee, S. C. Bae, Smoking increases rheumatoid arthritis susceptibility in individuals carrying the HLA-DRB1 shared epitope,

regardless of rheumatoid factor or anti-cyclic citrullinated peptide antibody status. *Arthritis Rheum.* **62**, 369–377 (2010).

18. L. Klareskog, V. Malmström, K. Lundberg, L. Padyukov, L. Alfredsson, Smoking, citrullination and genetic variability in the immunopathogenesis of rheumatoid arthritis. *Semin. Immunol.* **23**, 92–8 (2011).

19. L. Klareskog, P. Stolt, K. Lundberg, H. Källberg, C. Bengtsson, J. Grunewald, J. Rönnelid, H. E. Harris, A.-K. Ulfgren, S. Rantapää-Dahlqvist, A. Eklund, L. Padyukov, L. Alfredsson, A new model for an etiology of rheumatoid arthritis: smoking may trigger HLA-DR (shared epitope)-restricted immune reactions to autoantigens modified by citrullination. *Arthritis Rheum.* **54**, 38–46 (2006).

20. T. J. van Wesemael, S. Ajeganova, J. Humphreys, C. Terao, A. Muhammad, D. P. M. Symmons, A. J. MacGregor, I. Hafström, L. A. Trouw, A. H. M. van der Helm-van Mil, T. W. J. Huizinga, T. Mimori, R. E. M. Toes, F. Matsuda, B. Svensson, S. M. M. Verstappen, D. van der Woude, Smoking is associated with the concurrent presence of multiple autoantibodies in rheumatoid arthritis rather than with anti-citrullinated protein antibodies per se: a multicenter cohort study. *Arthritis Res. Ther.* **18**, 285 (2016).

21. D. Murphy, D. Mattey, D. Hutchinson, Anti-citrullinated protein antibody positive rheumatoid arthritis is primarily determined by rheumatoid factor titre and the shared epitope rather than smoking per se. *PLoS One*. **12**, e0180655 (2017).

22. A. H. M. van der Helm-van Mil, K. N. Verpoort, F. C. Breedveld, T. W. J. Huizinga, R. E. M. Toes, R. R. P. de Vries, The HLA–DRB1 shared epitope alleles are primarily a risk factor for anti–cyclic citrullinated peptide antibodies and are not an independent risk factor for development of rheumatoid arthritis. *Arthritis Rheum.* **54**, 1117–1121 (2006).

23. C. Ragan, Role of Hypersensitivity in the Pathogenesis of Rheumatoid Arthritis. Heberden Oration, 1958. *Ann. Rheum. Dis.* **18**, 1–7 (1959).

24. E. Waaler, ON THE OCCURRENCE OF A FACTOR IN HUMAN SERUM ACTIVATING THE SPECIFIC AGGLUTINATION OF SHEEP BLOOD CORPUSCLES. *Acta Pathol. Microbiol. Scand.* **17**, 172–188 (2009).

25. G. M. Edelman, H. G. Kunkel, E. C. Franklin, INTERACTION OF THE RHEUMATOID FACTOR WITH ANTIGEN-ANTIBODY COMPLEXES AND AGGREGATED GAMMA GLOBULIN. *J. Exp. Med.* **108**, 105–120 (1958).

26. E. C. Franklin, H. R. Holman, H. J. Müller-Eberhard, H. G. Kunkel, AN UNUSUAL PROTEIN COMPONENT OF HIGH MOLECULAR WEIGHT IN THE SERUM OF CERTAIN PATIENTS WITH RHEUMATOID ARTHRITIS. *J. Exp. Med.* **105**, 425–438 (1957).

27. J. L. Abruzzo, C. L. Christians, THE INDUCTION OF A RHEUMATOID FACTOR-LIKE SUBSTANCE IN RABBITS. *J. Exp. Med.* **114**, 791–806 (1961).

28. S. L. Maibom-Thomsen, N. H. Trier, B. E. Holm, K. B. Hansen, M. I. Rasmussen, A. Chailyan, P. Marcatili, P. Højrup, G. Houen, Immunoglobulin G structure and rheumatoid factor epitopes. *PLoS One*. **14**, e0217624 (2019).

29. J. Sokolove, D. S. Johnson, L. J. Lahey, C. A. Wagner, D. Cheng, G. M. Thiele, K. Michaud, H. Sayles, A. M. Reimold, L. Caplan, G. W. Cannon, G. Kerr, T. R. Mikuls, W. H. Robinson, Rheumatoid Factor as a Potentiator of Anti-Citrullinated Protein Antibody-Mediated Inflammation in Rheumatoid Arthritis. *Arthritis Rheumatol.* **66**, 813–821 (2014).

30. W. J. J. Falkenburg, A. C. Kempers, G. Dekkers, P. Ooijevaar-de Heer, A. E. H. Bentlage, G. Vidarsson, D. van Schaardenburg, R. E. M. Toes, H. U. Scherer, T. Rispens, Rheumatoid factors do not preferentially bind to ACPA-IgG or IgG with altered galactosylation.

Rheumatology. **56**, 2025–2030 (2017).

31. E. M. Tan, J. S. Smolen, Historical observations contributing insights on etiopathogenesis of rheumatoid arthritis and role of rheumatoid factor. *J. Exp. Med.* **213**, 1937–1950 (2016).

32. D. N. Posnett, J. Edinger, When Do Microbes Stimulate Rheumatoid Factor? *J. Exp. Med.* **185**, 1721–1723 (1997).

33. R. L. F. Nienhuis, E. Mandema, C. Smids, New Serum Factor in Patients with Rheumatoid Arthritis: The Antiperinuclear Factor. *Ann. Rheum. Dis.* **23**, 302–305 (1964).

34. B. J. Young, R. K. Mallya, R. D. Leslie, C. J. Clark, T. J. Hamblin, Anti-keratin antibodies in rheumatoid arthritis. *BMJ*. **2**, 97–99 (1979).

35. L. De Rycke, Rheumatoid factor and anticitrullinated protein antibodies in rheumatoid arthritis: diagnostic value, associations with radiological progression rate, and extra-articular manifestations. *Ann. Rheum. Dis.* **63**, 1587–1593 (2004).

36. D. Aletaha, T. Neogi, A. J. Silman, J. Funovits, D. T. Felson, C. O. Bingham, N. S. Birnbaum, G. R. Burmester, V. P. Bykerk, M. D. Cohen, B. Combe, K. H. Costenbader, M. Dougados, P. Emery, G. Ferraccioli, J. M. W. Hazes, K. Hobbs, T. W. J. Huizinga, A. Kavanaugh, J. Kay, T. K. Kvien, T. Laing, P. Mease, H. A. Ménard, L. W. Moreland, R. L. Naden, T. Pincus, J. S. Smolen, E. Stanislawska-Biernat, D. Symmons, P. P. Tak, K. S. Upchurch, J. Vencovský, F. Wolfe, G. Hawker, 2010 Rheumatoid arthritis classification criteria: An American College of Rheumatology/European League Against Rheumatism collaborative initiative. *Arthritis Rheum.* **62**, 2569–2581 (2010).

37. N. Wegner, R. Wait, A. Sroka, S. Eick, K.-A. Nguyen, K. Lundberg, A. Kinloch, S. Culshaw, J. Potempa, P. J. Venables, Peptidylarginine deiminase from Porphyromonas gingivalis citrullinates human fibrinogen and α-enolase: Implications for autoimmunity in rheumatoid arthritis. *Arthritis Rheum.* **62**, 2662–2672 (2010).

38. H. U. Scherer, T. W. J. Huizinga, G. Krönke, G. Schett, R. E. M. Toes, The B cell response to citrullinated antigens in the development of rheumatoid arthritis. *Nat. Rev. Rheumatol.* **14**, 157–169 (2018).

39. Y. He, C. Ge, À. Moreno-Giró, B. Xu, C. M. Beusch, K. Sandor, J. Su, L. Cheng, E. Lönnblom, C. Lundqvist, L. M. Slot, D. Tong, V. Urbonaviciute, B. Liang, T. Li, G. F. Lahore, M. Aoun, V. Malmström, T. Rispens, P. Ernfors, C. I. Svensson, H. U. Scherer, R. E. M. Toes, I. Gjertsson, O. Ekwall, R. A. Zubarev, R. Holmdahl, A subset of antibodies targeting citrullinated proteins confers protection from rheumatoid arthritis. *Nat. Commun.* **14**, 691 (2023).

40. C. Ge, B. Xu, B. Liang, E. Lönnblom, S. L. Lundström, R. A. Zubarev, B. Ayoglu, P. Nilsson, T. Skogh, A. Kastbom, V. Malmström, L. Klareskog, R. E. M. Toes, T. Rispens, D. Dobritzsch, R. Holmdahl, Structural Basis of Cross-Reactivity of Anti–Citrullinated Protein Antibodies. *Arthritis Rheumatol.* **71**, 210–221 (2019).

41. T. Kissel, C. Ge, L. Hafkenscheid, J. C. Kwekkeboom, L. M. Slot, M. Cavallari, Y. He, K. A. van Schie, R. D. Vergroesen, A. S. B. Kampstra, S. Reijm, G. Stoeken-Rijsbergen, C. Koeleman, L. M. Voortman, L. H. Heitman, B. Xu, G. J. M. Pruijn, M. Wuhrer, T. Rispens, T. W. J. Huizinga, H. U. Scherer, M. Reth, R. Holmdahl, R. E. M. Toes, Surface Ig variable domain glycosylation affects autoantigen binding and acts as threshold for human autoreactive B cell activation. *Sci. Adv.* **8** (2022), doi:10.1126/sciadv.abm1759.

42. H. Uysal, R. Bockermann, K. S. Nandakumar, B. Sehnert, E. Bajtner, Å. Engström, G. Serre, H. Burkhardt, M. M. G. M. Thunnissen, R. Holmdahl, Structure and pathogenicity of antibodies specific for citrullinated collagen type II in experimental arthritis. *J. Exp. Med.* **206**, 449–462 (2009).

43. C. Ge, D. Tong, B. Liang, E. Lönnblom, N. Schneider, C. Hagert, J. Viljanen, B. Ayoglu, R. Stawikowska, P. Nilsson, G. B. Fields, T. Skogh, A. Kastbom, J. Kihlberg, H. Burkhardt, D. Dobritzsch, R. Holmdahl, Anti-citrullinated protein antibodies cause arthritis by cross-reactivity to joint cartilage. *JCI Insight*. **2** (2017), doi:10.1172/jci.insight.93688.

44. A. H. van der Helm-van Mil, K. N. Verpoort, F. C. Breedveld, R. E. Toes, T. W. Huizinga, Antibodies to citrullinated proteins and differences in clinical progression of rheumatoid arthritis. *Arthritis Res. Ther.* **7**, R949 (2005).

45. A. H. M. van der Helm-van Mil, K. N. Verpoort, S. le Cessie, T. W. J. Huizinga, R. R. P. de Vries, R. E. M. Toes, The HLA–DRB1 shared epitope alleles differ in the interaction with smoking and predisposition to antibodies to cyclic citrullinated peptide. *Arthritis Rheum.* **56**, 425–432 (2007).

46. T. W. J. Huizinga, C. I. Amos, A. H. M. van der Helm-van Mil, W. Chen, F. A. van Gaalen, D. Jawaheer, G. M. T. Schreuder, M. Wener, F. C. Breedveld, N. Ahmad, R. F. Lum, R. R. P. de Vries, P. K. Gregersen, R. E. M. Toes, L. A. Criswell, Refining the complex rheumatoid arthritis phenotype based on specificity of the HLA-DRB1 shared epitope for antibodies to citrullinated proteins. *Arthritis Rheum.* **52**, 3433–3438 (2005).

47. E. Lundström, H. Källberg, L. Alfredsson, L. Klareskog, L. Padyukov, Gene-environment interaction between the DRB1 shared epitope and smoking in the risk of anti-citrullinated protein antibody-positive rheumatoid arthritis: All alleles are important. *Arthritis Rheum.* **60**, 1597–1603 (2009).

48. C. Clavel, L. Nogueira, L. Laurent, C. Iobagiu, C. Vincent, M. Sebbag, G. Serre, Induction of macrophage secretion of tumor necrosis factor α through Fcγ receptor IIa engagement by rheumatoid arthritis–specific autoantibodies to citrullinated proteins complexed with fibrinogen. *Arthritis Rheum.* **58**, 678–688 (2008).

49. H. Ji, K. Ohmura, U. Mahmood, D. M. Lee, F. M. . Hofhuis, S. A. Boackle, K. Takahashi, V. M. Holers, M. Walport, C. Gerard, A. Ezekowitz, M. C. Carroll, M. Brenner, R. Weissleder, J. S. Verbeek, V. Duchatelle, C. Degott, C. Benoist, D. Mathis, Arthritis Critically Dependent on Innate Immune System Players. *Immunity*. **16**, 157–168 (2002).

50. L. A. Trouw, E. M. Haisma, E. W. N. Levarht, D. van der Woude, A. Ioan-Facsinay, M. R. Daha, T. W. J. Huizinga, R. E. Toes, Anti-cyclic citrullinated peptide antibodies from rheumatoid arthritis patients activate complement via both the classical and alternative pathways. *Arthritis Rheum.* **60**, 1923–1931 (2009).

51. U. Harre, D. Georgess, H. Bang, A. Bozec, R. Axmann, E. Ossipova, P.-J. Jakobsson, W. Baum, F. Nimmerjahn, E. Szarka, G. Sarmay, G. Krumbholz, E. Neumann, R. Toes, H.-U. Scherer, A. I. Catrina, L. Klareskog, P. Jurdic, G. Schett, Induction of osteoclastogenesis and bone loss by human autoantibodies against citrullinated vimentin. *J. Clin. Invest.* **122**, 1791–1802 (2012).

52. U. Harre, S. C. Lang, R. Pfeifle, Y. Rombouts, S. Frühbeißer, K. Amara, H. Bang, A. Lux, C. A. Koeleman, W. Baum, K. Dietel, F. Gröhn, V. Malmström, L. Klareskog, G. Krönke, R. Kocijan, F. Nimmerjahn, R. E. M. Toes, M. Herrmann, H. U. Scherer, G. Schett, Glycosylation of immunoglobulin G determines osteoclast differentiation and bone loss. *Nat. Commun.* **6**, 6651 (2015).

53. T. Tasliyurt, B. Kisacik, S. U. Kaya, B. Yildirim, Y. Pehlivan, F. Kutluturk, H. Ozyurt, S. Sahin, A. M. Onat, The frequency of antibodies against cyclic citrullinated peptides and rheumatoid factor in healthy population: a field study of rheumatoid arthritis from northern turkey. *Rheumatol. Int.* **33**, 939–942 (2013).

54. A. van Zanten, S. Arends, C. Roozendaal, P. C. Limburg, F. Maas, L. A. Trouw, R. E. M. Toes, T. W. J. Huizinga, H. Bootsma, E. Brouwer, Presence of anticitrullinated protein

antibodies in a large population-based cohort from the Netherlands. *Ann. Rheum. Dis.* **76**, 1184–1190 (2017).

55. R. G. S. Chirivi, J. W. G. van Rosmalen, M. van der Linden, M. Euler, G. Schmets, G. Bogatkevich, K. Kambas, J. Hahn, Q. Braster, O. Soehnlein, M. H. Hoffmann, H. H. G. van Es, J. M. H. Raats, Therapeutic ACPA inhibits NET formation: a potential therapy for neutrophil-mediated inflammatory diseases. *Cell. Mol. Immunol.* **18**, 1528–1544 (2021).

56. R. G.S. Chirivi, Anti-Citrullinated Protein Antibodies as Novel Therapeutic Drugs in Rheumatoid Arthritis. *J. Clin. Cell. Immunol.* **01** (2013), doi:10.4172/2155-9899.S6-006.

57. A. M. Gomez, R. C. Brewer, J.-S. Moon, S. Acharya, S. Kongpachith, Q. Wang, S. Jahanbani, H. H. Wong, T. V Lanz, Z. Z. Love, G. Min-Oo, A. Niedziela-Majka, W. H. Robinson, *bioRxiv*, in press, doi:10.1101/2023.03.08.531560.

58. B. Raposo, M. Afonso, L. Israelsson, H. Wähämaa, R. Stålesen, F. Wermeling, A. H. Hensvold, C. Grönwall, B. Rethi, L. Klareskog, V. Malmström, Divergent and dominant anti-inflammatory effects of patient-derived anticitrullinated protein antibodies (ACPA) in arthritis development. *Ann. Rheum. Dis.* **82**, 724–726 (2023).

59. F. S. van de Bovenkamp, L. Hafkenscheid, T. Rispens, Y. Rombouts, The Emerging Importance of IgG Fab Glycosylation in Immunity. *J. Immunol.* **196**, 1435–1441 (2016).

60. Y. Rombouts, A. Willemze, J. J. B. C. van Beers, J. Shi, P. F. Kerkman, L. van Toorn, G. M. C. Janssen, A. Zaldumbide, R. C. Hoeben, G. J. M. Pruijn, A. M. Deelder, G. Wolbink, T. Rispens, P. A. van Veelen, T. W. J. Huizinga, M. Wuhrer, L. A. Trouw, H. U. Scherer, R. E. M. Toes, Extensive glycosylation of ACPA-IgG variable domains modulates binding to citrullinated antigens in rheumatoid arthritis. *Ann. Rheum. Dis.* **75**, 578–585 (2016).

61. L. Hafkenscheid, A. Bondt, H. U. Scherer, T. W. J. Huizinga, M. Wuhrer, R. E. M. Toes, Y. Rombouts, Structural Analysis of Variable Domain Glycosylation of Anti-Citrullinated Protein Antibodies in Rheumatoid Arthritis Reveals the Presence of Highly Sialylated Glycans. *Mol. Cell. Proteomics*. **16**, 278–287 (2017).

62. R. D. Vergroesen, L. M. Slot, L. Hafkenscheid, M. T. Koning, E. I. H. van der Voort, C. A. Grooff, G. Zervakis, H. Veelken, T. W. J. Huizinga, T. Rispens, H. U. Scherer, R. E. M. Toes, *Ann. Rheum. Dis.*, in press, doi:10.1136/annrheumdis-2017-212052.

63. P. Suwannalai, H. U. Scherer, D. van der Woude, A. Ioan-Facsinay, C. M. Jol-van der Zijde, M. J. D. van Tol, J. W. Drijfhout, T. W. J. Huizinga, R. E. M. Toes, L. A. Trouw, Anti-citrullinated protein antibodies have a low avidity compared with antibodies against recall antigens. *Ann. Rheum. Dis.* **70**, 373–379 (2011).

64. M. K. Verheul, S. J. H. van Erp, D. van der Woude, E. W. N. Levarht, M. J. K. Mallat, H. W. Verspaget, J. Stolk, R. E. M. Toes, A. E. van der Meulen-de Jong, P. S. Hiemstra, C. van Kooten, L. A. Trouw, Anti-carbamylated protein antibodies: a specific hallmark for rheumatoid arthritis. Comparison to conditions known for enhanced carbamylation; renal failure, smoking and chronic inflammation. *Ann. Rheum. Dis.* **75**, 1575–1576 (2016).

65. M. A. M. van Delft, T. W. J. Huizinga, An overview of autoantibodies in rheumatoid arthritis. *J. Autoimmun.* **110**, 102392 (2020).

66. J. Shi, P. A. van Veelen, M. Mahler, G. M. C. Janssen, J. W. Drijfhout, T. W. J. Huizinga, R. E. M. Toes, L. A. Trouw, Carbamylation and antibodies against carbamylated proteins in autoimmunity and other pathologies. *Autoimmun. Rev.* **13**, 225–230 (2014).

67. S. Turunen, P. Hannonen, M.-K. Koivula, L. Risteli, J. Risteli, Separate and overlapping specificities in rheumatoid arthritis antibodies binding to citrulline- and homocitrulline-containing peptides related to type I and II collagen telopeptides. *Arthritis Res. Ther.* **17**, 2 (2015).

68. M. Brink, M. K. Verheul, J. Rönnelid, E. Berglin, R. Holmdahl, R. Toes, L. Klareskog, L. A. Trouw, S. Rantapää-Dahlqvist, Anti-carbamylated protein antibodies in the pre-symptomatic phase of rheumatoid arthritis, their relationship with multiple anti-citrulline peptide antibodies and association with radiological damage. *Arthritis Res. Ther.* **17**, 25 (2015).

69. K. L. Bicker, P. R. Thompson, The protein arginine deiminases: Structure, function, inhibition, and disease. *Biopolymers*. **99**, 155–163 (2013).

70. E. H. Halvorsen, S. Pollmann, I.-M. Gilboe, D. van der Heijde, R. Landewe, S. Odegard, T. K. Kvien, O. Molberg, Serum IgG antibodies to peptidylarginine deiminase 4 in rheumatoid arthritis and associations with disease severity. *Ann. Rheum. Dis.* **67**, 414–417 (2007).

71. Z. Reyes-Castillo, C. A. Palafox-Sánchez, I. Parra-Rojas, G. E. Martínez-Bonilla, S. del Toro-Arreola, M. G. Ramírez-Dueñas, G. Ocampo-Bermudes, J. F. Muñoz-Valle, Comparative analysis of autoantibodies targeting peptidylarginine deiminase type 4, mutated citrullinated vimentin and cyclic citrullinated peptides in rheumatoid arthritis: associations with cytokine profiles, clinical and genetic features. *Clin. Exp. Immunol.* **182**, 119–131 (2015).

72. M. L. Harris, E. Darrah, G. K. Lam, S. J. Bartlett, J. T. Giles, A. V. Grant, P. Gao, W. W. Scott, H. El-Gabalawy, L. Casciola-Rosen, K. C. Barnes, J. M. Bathon, A. Rosen, Association of autoimmunity to peptidyl arginine deiminase type 4 with genotype and disease severity in rheumatoid arthritis. *Arthritis Rheum.* **58**, 1958–1967 (2008).

73. H. D. Lewis, J. Liddle, J. E. Coote, S. J. Atkinson, M. D. Barker, B. D. Bax, K. L. Bicker, R. P. Bingham, M. Campbell, Y. H. Chen, C. Chung, P. D. Craggs, R. P. Davis, D. Eberhard, G. Joberty, K. E. Lind, K. Locke, C. Maller, K. Martinod, C. Patten, O. Polyakova, C. E. Rise, M. Rüdiger, R. J. Sheppard, D. J. Slade, P. Thomas, J. Thorpe, G. Yao, G. Drewes, D. D. Wagner, P. R. Thompson, R. K. Prinjha, D. M. Wilson, Inhibition of PAD4 activity is sufficient to disrupt mouse and human NET formation. *Nat. Chem. Biol.* **11**, 189–191 (2015).

74. F. Andrade, E. Darrah, M. Gucek, R. N. Cole, A. Rosen, X. Zhu, Autocitrullination of human peptidyl arginine deiminase type 4 regulates protein citrullination during cell activation. *Arthritis Rheum.* **62**, 1630–1640 (2010).

75. K. S. Nandakumar, R. Holmdahl, "Collagen Antibody Induced Arthritis" in (2007; http://link.springer.com/10.1007/978-1-59745-402-5_16), pp. 215–223.

76. K. S. Nandakumar, E. Bajtner, L. Hill, B. Böhm, M. J. Rowley, H. Burkhardt, R. Holmdahl, Arthritogenic antibodies specific for a major type II collagen triple-helical epitope bind and destabilize cartilage independent of inflammation. *Arthritis Rheum.* **58**, 184–196 (2008).

77. K. S. Nandakumar, L. Svensson, R. Holmdahl, Collagen Type II-Specific Monoclonal Antibody-Induced Arthritis in Mice. *Am. J. Pathol.* **163**, 1827–1837 (2003).

78. R. HOLMDAHL, R. BOCKERMANN, J. BACKLUND, H. YAMADA, The molecular pathogenesis of collagen-induced arthritis in mice—a model for rheumatoid arthritis. *Ageing Res. Rev.* **1**, 135–147 (2002).

79. I. Lindh, O. Snir, E. Lönnblom, H. Uysal, I. Andersson, K. Nandakumar, M. Vierboom, B. 't Hart, V. Malmström, R. Holmdahl, Type II collagen antibody response is enriched in the synovial fluid of rheumatoid joints and directed to the same major epitopes as in collagen induced arthritis in primates and mice. *Arthritis Res. Ther.* **16**, R143 (2014).

80. K. S. Nandakumar, R. Holmdahl, Efficient promotion of collagen antibody induced arthritis (CAIA) using four monoclonal antibodies specific for the major epitopes recognized in both collagen induced arthritis and rheumatoid arthritis. *J. Immunol. Methods.* **304**, 126–136 (2005).

81. R. Holmdahl, K. Rubin, L. Klareskog, E. Larsson, H. Wigzell, Characterization of the antibody response in mice with type II collagen–induced arthritis, using monoclonal anti–type II collagen antibodies. *Arthritis Rheum.* **29**, 400–410 (1986).

82. H. K. Beard, R. Ryvar, J. Skingle, C. L. Greenbury, Anti-collagen antibodies in sera from rheumatoid arthritis patients. *J. Clin. Pathol.* **33**, 1077–1081 (1980).

83. V. A. Manivel, D. van der Woude, R. Toes, A. Filer, K. Raza, J. Rönnelid, *Ann. Rheum. Dis.*, in press, doi:10.1136/annrheumdis-2017-211974.

84. R. Di Paola, S. Cuzzocrea, Predictivity and sensitivity of animal models of arthritis. *Autoimmun. Rev.* **8**, 73–75 (2008).

85. K. Kannan, R. A. Ortmann, D. Kimpel, Animal models of rheumatoid arthritis and their relevance to human disease. *Pathophysiology*. **12**, 167–181 (2005).

86. D. L. Asquith, A. M. Miller, I. B. McInnes, F. Y. Liew, Animal models of rheumatoid arthritis. *Eur. J. Immunol.* **39**, 2040–2044 (2009).

87. J. Bäcklund, C. Li, E. Jansson, S. Carlsen, P. Merky, K.-S. Nandakumar, S. Haag, J. Ytterberg, R. A. Zubarev, R. Holmdahl, C57BL/6 mice need MHC class II Aq to develop collagen-induced arthritis dependent on autoreactive T cells. *Ann. Rheum. Dis.* **72**, 1225–1232 (2013).

88. R. HOLMDAHL, M. E. ANDERSSON, T. J. GOLDSCHMIDT, L. JANSSON, M. KARLSSON, V. MALMSTROM, J. MO, Collagen induced arthritis as an experimental model for rheumatoid arthritis. *APMIS.* **97**, 575–584 (1989).

89. L. M. Khachigian, Collagen antibody-induced arthritis. *Nat. Protoc.* **1**, 2512–2516 (2006).

90. J. Tuncel, S. Haag, M. H. Hoffmann, A. C. Y. Yau, M. Hultqvist, P. Olofsson, J. Bäcklund, K. S. Nandakumar, D. Weidner, A. Fischer, A. Leichsenring, F. Lange, C. Haase, S. Lu, P. S. Gulko, G. Steiner, R. Holmdahl, Animal Models of Rheumatoid Arthritis (I): Pristane-Induced Arthritis in the Rat. *PLoS One*. **11**, e0155936 (2016).

91. C. Vingsbo, P. Sahlstrand, J. G. Brun, R. Jonsson, T. Saxne, R. Holmdahl, Pristane-induced arthritis in rats: a new model for rheumatoid arthritis with a chronic disease course influenced by both major histocompatibility complex and non-major histocompatibility complex genes. *Am. J. Pathol.* **149**, 1675–83 (1996).

92. N. Choudhary, L. K. Bhatt, K. S. Prabhavalkar, Experimental animal models for rheumatoid arthritis. *Immunopharmacol. Immunotoxicol.* **40**, 193–200 (2018).

93. A. C. Y. Yau, E. Lönnblom, J. Zhong, R. Holmdahl, Influence of hydrocarbon oil structure on adjuvanticity and autoimmunity. *Sci. Rep.* **7**, 14998 (2017).

94. P. A. Monach, W. Hueber, B. Kessler, B. H. Tomooka, M. BenBarak, B. P. Simmons, J. Wright, T. S. Thornhill, M. Monestier, H. Ploegh, W. H. Robinson, D. Mathis, C. Benoist, A broad screen for targets of immune complexes decorating arthritic joints highlights deposition of nucleosomes in rheumatoid arthritis. *Proc. Natl. Acad. Sci.* **106**, 15867–15872 (2009).

95. A. A. Vetto, M. Mannik, E. Zatarain-Rios, M. H. Wener, Immune deposits in articular cartilage of patients with rheumatoid arthritis have a granular pattern not seen in osteoarthritis. *Rheumatol. Int.* **10**, 13–19 (1990).

96. T. D. Cooke, E. R. Hurd, H. E. Jasin, J. Bienenstock, M. Ziff, Identification of immunoglobulins and complement in rheumatoid articular collagenous tissues. *Arthritis Rheum.* **18**, 541–551 (1975).

97. H. S. Luthra, F. C. McDuffie, G. G. Hunder, E. A. Samayoa, Immune complexes in sera and synovial fluids of patients with rheumatoid arthritis. Radioimmunoassay with monocylonal rheumatoid factor. *J. Clin. Invest.* **56**, 458–466 (1975).

98. H. Burkhardt, T. Koller, Å. Engström, K. S. Nandakumar, J. Turnay, H. G. Kraetsch, J. R. Kalden, R. Holmdahl, Epitope-specific recognition of type II collagen by rheumatoid arthritis

antibodies is shared with recognition by antibodies that are arthritogenic in collagen-induced arthritis in the mouse. *Arthritis Rheum.* **46**, 2339–2348 (2002).

99. C. Ge, D. Tong, E. Lönnblom, B. Liang, W. Cai, C. Fahlquist-Hagert, T. Li, A. Kastbom, I. Gjertsson, D. Dobritzsch, R. Holmdahl, Antibodies to Cartilage Oligomeric Matrix Protein Are Pathogenic in Mice and May Be Clinically Relevant in Rheumatoid Arthritis. *Arthritis Rheumatol.* **74**, 961–971 (2022).

100. M. Maccioni, G. Zeder-Lutz, H. Huang, C. Ebel, P. Gerber, J. Hergueux, P. Marchal, V. Duchatelle, C. Degott, M. van Regenmortel, C. Benoist, D. Mathis, Arthritogenic Monoclonal Antibodies from K/BxN Mice. *J. Exp. Med.* **195**, 1071–1077 (2002).

101. B. T. Wipke, Z. Wang, W. Nagengast, D. E. Reichert, P. M. Allen, Staging the Initiation of Autoantibody-Induced Arthritis: A Critical Role for Immune Complexes. *J. Immunol.* **172**, 7694–7702 (2004).