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CHARACTERISATION OF EPITOPE-SPECIFIC B CELLS AND THE ROLE OF IGG FC SIALYLATION IN MURINE ARTHRITIS MODELS

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Cover illustration: Drawing by my niece Luisa and my nephew Anton.

Characterisation of Epitope-specific B cells and the Role of IgG Fc Sialylation in Murine Arthritis Models

Thesis for Doctoral Degree (Ph.D.)

By

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To my family

An immune system of enormous complexity is present in all vertebrate animals. When we place a population of lymphocytes from such an animal in appropriate tissue culture fluid, and when we add an antigen, the lymphocytes will produce specific antibody molecules, in the absence of any nerve cells. I find it astonishing that the immune system embodies a degree of complexity which suggests some more or less superficial though striking analogies with human language, and that this cognitive system has evolved and functions without assistance of the brain.

Niels Kaj Jerne

Nobel Prize in Physiology or Medicine in 1984 “for theories concerning the specificity in development and control of the immune system and the discovery of the principle for production of monoclonal antibodies".

From his Nobel lecture: THE GENERATIVE GRAMMAR OF THE IMMUNE SYSTEM

Popular science summary of the thesis

B cells are an essential part of the immune system, as they are – among others - responsible for producing antibodies, providing defense and protection against infectious diseases. However, in autoimmune diseases, B cells become dysregulated, producing harmful antibodies against self-tissue, also called self-reactive. In the case of rheumatoid arthritis (RA), these antibodies are targeted against joint tissues, leading to inflammation and pain. Currently, there is no cure for RA.

The goal of this thesis was to investigate the dysregulation of B cells in arthritis.

First, we studied the effect of reactive oxygen species (ROS) on B cell differentiation (**study I**). ROS are reactive derivatives of molecular oxygen (O2), occurring naturally as a by-product of living cells. Historically, they were regarded as detrimental, leading to the rise of their opponents, "the antioxidants", as a food supplement to "maintain health". The role of ROS has however shifted in the last decades as now it is known that they have important functions in immune defence and cell signalling. In our study we show that intracellular ROS regulate the differentiation of B cells into antibody-producing plasma cells.

Next, we studied the role of B cells reactive against the self-protein GPI. There was a lack of knowledge about which part of the protein is attacked by antibodies and how this leads to inflammation in the joint (**study II**). Our study provides important clues to this, as we identified the specific targets (epitopes), a potential mechanism for how they start inflammation and the link of such antibodies with the mechanisms in the human disease RA. Paradoxically, we also found that a substantial proportion of self-reactive B cells can be found in healthy mice, providing hints for the initiation of this autoimmune response.

In the **third study**, we investigated Collagen type II-reactive B cells in mouse models, human healthy donors, and human RA patients. Col2 is a very abundant protein found in the joint cartilage. We found out that anti-Col2 B cells occur naturally under healthy conditions in rodents and human and we described their characteristics. Interestingly, they seem to play an important role in balancing other immune cells to not attack self-tissue containing Col2.

Finally, we looked at the sugars that antibodies carry (**study IV**). Antibodies are proteins, which means that they are mostly composed of protein building blocks, the amino acids. However, they do also contain many different sugars, whose role is much less known. We modified the sugars on antibodies that are known to cause arthritis in mice and tested their functions in different mouse arthritis models. Interestingly, some sugars can change the function of antibodies in a beneficial way, i.e. they are ameliorating the disease. Understanding the antibody sugars better might open new avenues for human RA therapy.

Populärwissenschaftliche Zusammenfassung der Doktorarbeit

B-Zellen sind ein essenzieller Teil des Immunsystems, da sie u.a. verantwortlich für die Produktion von Antikörpern sind. Antikörper leisten die Verteidigung und Schutz gegen Infektionskrankheiten (Erreger). Bei Autoimmunerkrankungen werden B-Zellen jedoch fehlreguliert, sie produzieren schädliche Antikörper gegen eigenes Gewebe, auch selbst-reaktiv genannt. Im Falle von Rheumatoider Arthritis (RA) zielen diese Antikörper auf Gelenkgewebe, was dort wiederum Entzündung und Schmerz verursacht. Gegenwärtig gibt es keine Heilung für Rheumatoide Arthritis. Die Zielstellung dieser Doktorarbeit war die Untersuchung der Fehlregulation von B-Zellen bei Arthritis.

Zuerst haben wir den Effekt von reaktiven Sauerstoffspezies während der B-Zell-Differenzierung studiert (**Studie I**). Reaktive Sauerstoffspezies (ROS) sind reaktive Abkömmlinge des molekularen Sauerstoffs (O2), die natürlicherweise als Nebenprodukt lebender Zellen auftreten. Historisch wurden sie als schädlich angesehen, was schön am Aufstieg derer Gegenspieler – den Antioxidantien – als Nahrungsergänzungsmittel zur Aufrechterhaltung der Gesundheit veranschaulicht werden kann.

In den letzten Jahrzehnten hat sich jedoch die Rolle von ROS verschoben, da mittlerweile bekannt ist, dass sie wichtige Funktionen bei der Immunverteidigung und bei der Zellensignalübertragung ausüben.

In unserer Studie zeigen wir, dass ROS innerhalb der B-Zelle die Differenzierung von normalen B-Zellen zu Antikörper-produzierenden Plasmazellen reguliert.

Als Nächstes haben wir B-Zellen reaktiv gegen das Selbstprotein GPI studiert. Dabei gab es eine Wissenslücke welcher Teil des Proteins von Antikörpern attackiert wird und wie dies zu einer Entzündung in den Gelenken führt (**Studie II**). Unsere Studie gibt Hinweise hierzu, da wir die genauen Antikörper-Ziele (Epitope), einen potenziellen Wirkmechanismus (d.h. wie Entzündung initiiert werden könnte) und die Verbindung solcher Antikörper mit der Krankheit RA identifiziert haben. Paradoxerweise haben wir auch eine substanzielle Zahl von selbst-reaktiven B-Zellen in gesunden Mäusen gefunden, ein Indiz für den Anfang der autoimmunen Reaktion.

In der **dritten Studie** haben wir Kollagen Typ II-selbst-reaktive B-Zellen in Mäusemodellen, gesunden Menschen (Spendern) und RA-Patienten untersucht. Col2 ist ein sehr häufiges Protein im Gelenkknorpel. Wir haben herausgefunden, dass Anti-Col2 B-Zellen natürlicherweise unter gesunden Bedingungen in Nagetieren und Menschen vorkommen und beschrieben deren Eigenschaften. Interessanterweise scheinen sie eine wichtige Rolle anderer Immunzellen auszugleichen, um die Attacke von Col2-enthaltendem Selbstgewebe zu verhindern.

Schließlich haben wir die Zucker, die Antikörper tragen, studiert (**Studie IV**). Antikörper sind Proteine, was bedeutet, dass sie hauptsächlich aus den Proteinbausteinen Aminosäuren bestehen. Jedoch enthalten sie auch viele verschiedene Zucker, deren Bedeutung weit weniger verstanden ist. Bei Antikörpern von denen bekannt ist, dass sie Arthritis in Mäusen verursachen können, haben wir die Zucker verändert und in verschiedenen Arthritis-Mäusemodellen getestet. Interessanterweise können bestimmte Zucker die Funktion der Antikörper auf eine vorteilhafte Art und Weise verändern, d.h. die Krankheit bessert sich. Ein besseres Verständnis der Antikörper-Zucker könnte neue Forschungsansätze für die RA-Therapie generieren.

Abstract

Autoimmune diseases are a major burden for society and economy. One of the most common autoimmune diseases is rheumatoid arthritis (RA) characterized by joint inflammation, swelling and pain. If left untreated, it ultimately leads to disability.

B cells play a major role in rheumatoid arthritis as shown by numerous studies and the success of anti-CD20 B cell depletion therapy in clinical use. In healthy individuals, B cells originate and develop in the bone marrow before they exit into the periphery where they mature and differentiate. B cells act in the body in a dual role, on the one hand as professional antigen-presenting cells (APCs), taking up antigen and presenting it to cognate T cells via MHC. On the other hand, B cells can generate and mature specific antibodies against almost any given antigenic target. This diversity is achieved by a process called V(D)J recombination. V, (D) and J segments are recombined during early B cell development, creating a vast diversity of unique sequences. Subsequently, the BCR diversity can be increased even further upon B cell activation by somatic hypermutation (SHM) and class switch recombination (CSR) in the germinal center (GC) reaction.

However, the stochastic process of gene rearrangement and mutations also leads to autoreactive BCRs that upon encounter of self-antigen could initiate an autoreactive immune response. This fundamental dogma of autoimmunity has been postulated in many autoimmune diseases including RA. Therefore, it is important to understand the role and regulation of self-reactive B cells in arthritis.

In **study I**, we investigated the role of Ncf4 dependent intracellular reactive oxygen species (ROS) in controlling the differentiation from B cells into antibody-producing plasma cells. By employing Ncf4 mutated (R58A) mice we could show that lower levels of intracellular ROS induce plasma cell formation, increased CXCR3 and decreased CXCR4 expression. Increased plasma cell formation led to higher self-reactive anti-Col2 antibody production and enhanced disease severity. We concluded that intrinsic ROS can substantially modulate self-reactive B cell differentiation and thereby the disease phenotype.

In **study II**, we mapped the pathogenic B cell epitopes on glucose-6-phosphate isomerase (GPI), an autoantigen in human RA and mouse models of arthritis. We identified a single peptide (GPI293-307) as the most dominant epitope targeted both in human and mouse models. Following the observation of the early emergence of antibodies against these epitopes, we found that GPI293-307-specific B cells are not deleted during B cell development and activated in the pathogenesis of arthritis due to encounter of a structurally modified form of GPI protein on the articular cartilage surface exposing the GPI293-307 neoepitope. We concluded that this finding has important implications about the understanding of the role of naturally existing self-reactive B cells in GPI-mediated arthritis.

In **study III**, we studied the B cell response to Collagen type II, another important autoantigen in human RA and mouse models of arthritis. The focus was on the C1 epitope, a triple-helical peptide sequence originally identified in collagen-dependent mouse models. Using both B cell receptor knock-in mouse models, healthy human donors, and RA patients, we could identify and characterise for the first time a population of physiologically occurring self-reactive B cells that exhibit an immune-suppressive phenotype. We conclude that B suppressor cells (Bsups) represent a new subpopulation of B cells tolerizing T cells against self-antigens.

Finally, in **study IV**, we changed the focus from the specificity of self-reactive BCRs as conferred by the Fab fragment of immunoglobulins (Igs) to the importance of the Fc fragment. More specifically, we addressed the question of the interactions between the Immunoglobulin G (IgG) isotype, its Fc glycosylation, and the consequent effector functions in different murine arthritis models. To this end, we made use of available well-characterized pathogenic monoclonal antibodies (mAbs) causing arthritis in susceptible mouse strains. A glycoengineering method was applied on these antibodies to yield an array of different glycovariants to be tested in murine models. We showed that while the Fc glycan does not play a role in the collagen antibody-induced arthritis (CAIA) model, there seems to be an isotype -/and sialylation-dependent function in the regulation of arthritis severity in the collagen-induced arthritis (CIA) model.

In conclusion, we contributed towards the understanding of naturally existing autoreactive B cells in arthritis autoimmunity.

List of scientific papers

I. Chang He, Huqiao Luo, Ana Coelho, Meng Liu, Qijing Li, Jing Xu, **Alexander Krämer**, Stephen Malin, Zuyi Yuan, Rikard Holmdahl
NCF4 dependent intracellular reactive oxygen species regulate plasma cell formation. Redox Biology. https://doi.org/10.1016/j.redox.2022.102499

II. Taotao Li, Changrong Ge, **Alexander Krämer**, Outi Sareila, Monica Leu Agelii, Linda Johansson, Kristina Forslind, Erik Lönnblom, Min Yang, Bingze Xu, Qixing Li, Lei Cheng, Göran Bergström, Gonzalo Fernandez, Alf Kastbom, Solbritt Rantapää-Dahlqvist, Inger Gjertsson, Rikard Holmdahl
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III. Mike Aoun, Ana Coelho*, **Alexander Krämer***, Amit Saxena*, Pierre Sabatier, Christian Michel 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 A. Zubarev, Harald Burkhardt, Rikard Holmdahl *Authors contributed equally.
Antigen presenting autoreactive suppressor B cells [Manuscript]

IV. **Alexander Krämer**, Susanna L Lundström, Àlex Moreno Giró, Taotao Li, Ana Coelho, Zhongwei Xu, Bingze Xu, Roman A. Zubarev, Rikard Holmdahl
Fc Sialylation has no effect on the pathogenicity of arthritogenic antibodies [Manuscript]

Scientific papers not included in this thesis

I. Ana Coelho*, Mike Aoun*, Amit Saxena, Christian M. Beusch, Pierre Sabatier, **Alexander Krämer**, Chang He, Jaime James, Roman A. Zubarev, Stephen Malin, Rikard Holmdahl. **Bursting B cells as a regulator for autoimmunity**. *Authors contributed equally. [Manuscript]

II. Ana Coelho, Mike Aoun, Christian M. Beusch, Pierre Sabatier, Chang He, **Alexander Krämer**, Roman A. Zubarev, Stephen Malin, Rikard Holmdahl. **Oxidative regulation of germinal center B cells by *Ncf1***. [Manuscript].

III. Taotao Li, Bingze Xu, **Alexander Krämer**, Changrong Ge, Susanna L. Lundström, Lei Cheng, Huqiao Luo, Ana Coelho, Rikard Holmdahl. **Citrullinated human alpha-Enolase immunization induces DR4-restricted anti-citrullinated protein antibody (ACPA) response with protective effect**. [Manuscript]

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List of abbreviations

(m)Ab	(monoclonal) Antibody
ACPA	Anti-citrullinated protein antibody
APC	Antigen-presenting cell
ASC	Antibody secreting cell
BCR	B cell receptor
CD	Cluster of differentiation
CIA	Collagen-induced arthritis
Col2	Collagen type II
DHR	Dihydrorhodamine
ELISA	Enzyme-linked immunosorbent assay
GPI	Glucose-6-phosphate isomerase
HEK	Human embryonic kidney
Ig	Immunoglobulin
IL	Interleukin
IMAC	Immobilized metal ion affinity chromatography
LPS	Lipopolysaccharide
Ncf	Neutrophil cytosolic factor
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PE	Phycoerythrin
RA	Rheumatoid arthritis
ROS	Reactive oxygen species
RT	Reverse transcription; room temperature

1 Introduction

1.1 Rheumatoid arthritis

Rheumatoid arthritis is a chronic autoimmune disease affecting 0.5-1 % of the world population[1]. The disease causes inflammation and pain in the joints. It is clinically diagnosed based on the 2010 ACR/EULAR criteria, a composite clinical score considering a set of clinical parameters: "presence of synovitis in at least 1 joint, absence of an alternative diagnosis that better explains the synovitis, and achievement of a total score of 6 or greater (of a possible 10) from the individual scores in 4 domains: number and site of involved joints (score range 0–5), serologic abnormality (score range 0–3), elevated acute-phase response (score range 0–1), and symptom duration (2 levels; range 0–1)"[2].

As for many other autoimmune diseases the aetiology of RA is unknown. Based on epidemiological data it has been postulated that genetic risk factors as well as environmental factors play a role in the origin of disease[3]. The strongest association between genetics and RA has been shown for certain HLA alleles[4]. The so called shared epitope is the strongest genetic risk factor[5,6]. Furthermore, it has been described that smoking is associated to RA[7]. However, the underlying functional mechanism is unknown.

The discovery that specific antibodies can be detected in the serum already many years before clinical disease onset and diagnosis has led to the formulation of a consent hypothesis on the disease course among researchers in the field[8–10] (Figure 1). In the first phase, self-tolerance of the immune system is broken and autoantibodies specific for self-proteins appear in the blood. These autoantibodies have been subclassified into two major groups: anti-citrullinated protein antibodies (ACPAs) and rheumatoid factors (RF). ACPAs are specific against the post-translational protein modification citrulline, while rheumatoid factors show a specificity against the Fc region of immunoglobulin G (IgG). Both antibody classes are used as diagnostic markers, especially the presence of ACPA as tested by the anti-CCP2 test offers a high specificity for RA[11,12].

This first phase is called autoimmunity phase and can last for several years. In the next phase the patient starts to feel mild to severe pain in the joints, escalating to an inflammatory clinical arthritis. This stage is called the subclinical phase of rheumatoid arthritis, as first clinical symptoms occur but the patient is not diagnosed yet. Possibly, first self-medication starts.

In the 3rd stage the disease is diagnosed clinically by a physician. Non-steroidal anti-inflammatory drugs (NSAIRD) are the standard first line treatment. The second line treatment are disease-modifying antirheumatic drugs (DMARDs), among them Methotrexate (MTX) being the "gold standard" in RA treatment. If NSAIRD and standard DMARDs fail to improve the disease course or are not well tolerated by the patient anti-inflammatory biologic treatment is initiated. Most of the biologics used in RA treatment belong to the anti-TNF-α antibody class[13]. The antibodies bind the inflammatory cytokine TNF-α and thus neutralize its biological effector functions.

They are injected single or in combination with MTX and have a significant beneficial effect on the disease. As an alternative to NSAIRD the classical anti-inflammatory drug class glucocorticoids are used for quick relief, however they have an undesirable side effect profile. Thus, the use of it in the clinic decreases.

There is a wide spectrum of clinical disease courses, some patients develop only a mild disease that requires only first-line treatment, other patients (especially ACPA+ RF+ seropositive patients) develop a more severe disease phenotype that requires intervention with immunomodulatory treatment[3]. There is no cure for rheumatoid arthritis.

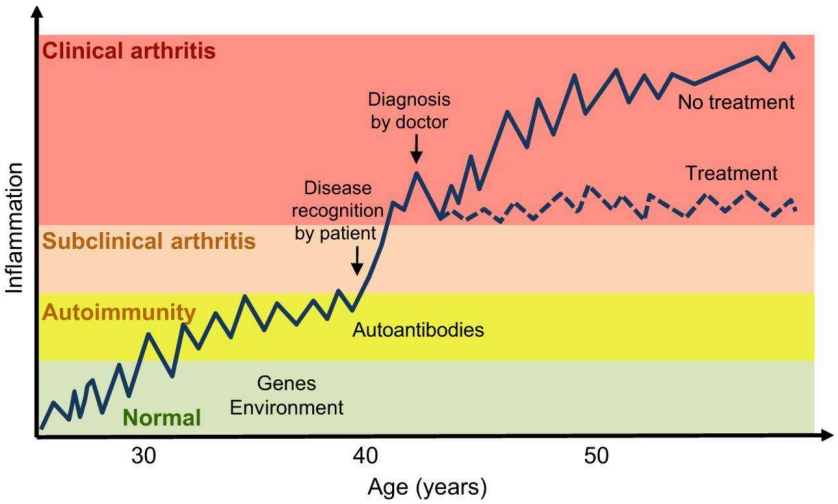

Figure 1: The key stages of rheumatoid arthritis *(reprinted from Yau & Holmdahl, 2016 CC-BY 3.0* [14]*)*

1.2 B lymphocytes

B lymphocytes are a crucial component of the adaptive immune system, capable of tailoring specific antibodies against almost any given antigen during an immune response. In contrast to the second arm of adaptive immunity (T cells), B cells are not major histocompatibility complex- (MHC-) restricted[15].

B cells are true-allrounders as they possess dual capabilities. On the one hand they can directly attack the antigen by red flagging them for the innate immune system with antibodies. On the other hand, they act as professional antigen-presenting cells (APCs) presenting antigenic peptides to T cells via MHC. Each B cell possesses a unique B-cell receptor (BCR) specific for one or several target antigens[15]. All unique BCRs are initially generated by a somatic genomic recombination mechanism called V(D)J recombination[16].

It occurs during early B-cell development in the bone marrow and rearranges gene segments to generate a vast diversity of BCRs. Upon antigen encounter, B cells undergo affinity maturation in the germinal center (GC) reaction. In the GC, somatic hypermutation (SHM) and class-switch recombination (CSR) occur to boost antigen affinity of the BCR and to mediate effector functions of secreted BCRs (resp.), known as immunoglobulins (Ig) or antibodies (Ab). The GC reaction has two outputs: long-lived memory B cells responsible for "reactive memory" and plasma cells[17]. The entirety of all unique BCRs/Ab expressed by B cells is called the BCR/Ab repertoire.

1.2.1 B lymphocyte tolerance

During early B-cell development, as well as later in the GC reaction, B cells recognizing self-antigens can produce harmful consequences, if not controlled. The BCR repertoire must be "instructed" to be tolerant against self-antigens. Self-reactive B cells are prevented from contributing to the repertoire by three processes: receptor editing, deletion, and anergy[18,19]. During receptor editing, functionally arranged BCRs recognizing self-antigens undergo further rearrangement to remove autoreactivity[20,21]. In this way, self-reactive B cells are rescued from deletion, i.e. induced cell death[22]. Anergy is characterized by a resting, unresponsive state of the B cell. Receptor editing and deletion are important to establish so-called central tolerance during early-stage B-cell development. The counterpart of central tolerance is peripheral tolerance, taking place out of the bone marrow[23]. In the case of rheumatoid arthritis, it is largely unknown why B cells escape from tolerization and produce ACPA even years before disease onset. One study employing single B cell cloning has shown that central and peripheral B cell tolerance checkpoints are defective in RA[24].

1.3 Autoantibodies in RA

1.3.1 Rheumatoid factors

RFs are the first type of autoantibody described in RA, discovered in 1940 by Waaler[25]. Their name implies specificity for rheumatic disease, yet they can be found in a variety of other diseases, like infectious disease and even in healthy individuals[26]. As they were the first detectable autoantibodies in RA, they were included in the 1987 ACR classification criteria[27].

RFs are specific for the Fc region of IgG, as described before. They can be of IgM, IgG and IgA isotype with IgM representing the most studied one[28]. Even though the fine specificity of RF (epitopes) has been mapped in recent years[28,29], the functional role in RA remains largely unknown. One function was attributed to RF in the context of joint inflammation in RA, as IgM-RF - ACPA immune complexes could induce macrophage activation and a subsequent proinflammatory cascade[30].

Intriguingly, it has been shown recently that rheumatoid factors bind only to structurally modified IgG[31]. This structural modification can be induced among others by antigen-binding and physico-chemical alterations.

This evidence suggests a role for RF as physiologic antibodies in clearing immune complexes at the site of inflammation or infection, which is further supported by the mucosal location of IgA-RF[32].

1.3.2 Anti-citrullinated protein antibodies (ACPAs)

ACPAs are a diagnostic marker with high specificity for rheumatoid arthritis. The standard clinical practice test to detect ACPAs is the cyclic citrullinated peptide 2 (CCP2) test, detecting ca. 70 % of RA patients as ACPA-positive[33,34].

Interestingly, ACPAs can detect the post-translational amino acid modification citrulline on several different proteins, the most prominent described antigens are the extracellular matrix protein collagen type II (Col2)[35–37], the blood clotting protein fibrinogen[38,39], the structural proteins vimentin[40] and filaggrin[41] and the glycolytic enzyme α-enolase[42]. Blood serum biobank studies have shown that ACPA titres rise in pre-symptomatic RA (pre-RA) individuals[43] and that the polyclonal ACPA in the serum show a pattern of epitope spreading[44], i.e. the serum recognizes the citrulline side chain on different autoantigen-derived peptides and full proteins. This has led to the formulation of the attractive hypothesis of a direct association of ACPA with disease pathology, as discussed below.

Studies using monoclonal antibodies of human (RA) and mouse (arthritis models) origin have shown that the spectrum of fine specificities of monoclonal Abs is broad[10]. Based on their protein crystal structures showing the precise binding mechanism it has been proposed that ACPAs can be classified into three groups: promiscuous ACPAs, private-specific ACPAs and private cross-reactive ACPAs[10] (Figure 2).

The E4 monoclonal antibody cloned from a human ACPA$^+$ RA patient is classified into the promiscuous ACPA subclass showing a broad reactivity spectrum towards a set of citrullinated autoantigens[45]. Private specific ACPAs do not recognize exclusively the citrulline, but also interact with other non-citrulline residues. ACC4 is a hybridoma monoclonal antibody cloned from a collagen-induced arthritis mouse model showing a private specific reactivity pattern against the triple-helical C1 epitope of Col2[36]. Private cross-reactive antibodies are antibodies showing a specificity towards several triple-helical epitopes on Col2 including the C1 epitope. The ACC1 antibody is an example of a private cross-reactive antibody[36]. Recent evidence suggests that ACPA *per se* are not necessarily pathogenic[46,47] but that it is rather joint protein-cross-reactive antibodies binding cartilage *in vivo* that trigger arthritis[48,49]. This is exemplified by the pathogenic CIIC1 antibody that recognizes the triple-helical C1 epitope on collagen type II specifically and binds to cartilage in vivo without the need of citrullination[50–52].

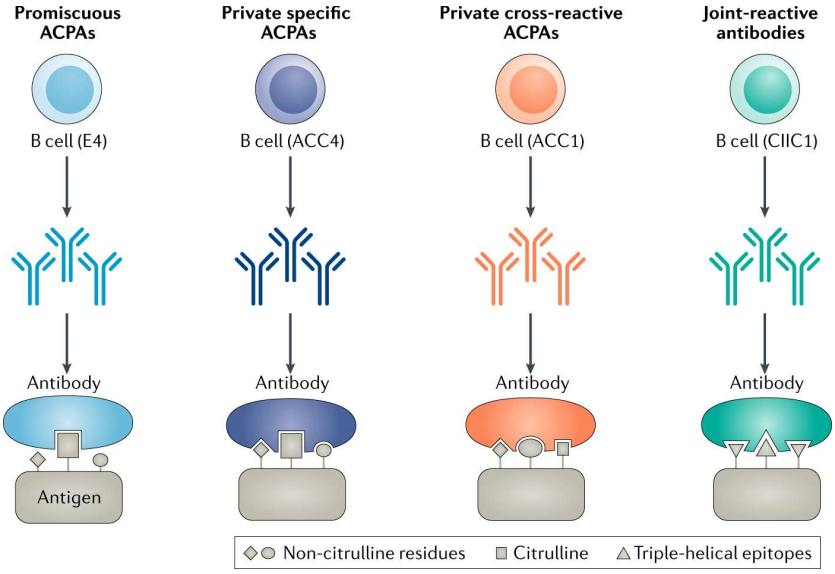

Figure 2 Examples of different types of ACPAs and joint-reactive autoantibodies in RA *(Figure from Ge and Holmdahl, 2019[10], reprinted with permission from Springer Nature).*

1.4 Reactive oxygen species

Reactive oxygen species is a family of derivatives from molecular oxygen (O_2)[53]. Examples include superoxides ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($HO^{\bullet}$). The common denominator is the high reactivity of ROS, oxidizing other (bio-)molecules[53]. ROS are generated intracellularly by respiration in the mitochondria and by the action of NADPH oxidases (NOX). In mitochondria, electrons escape from the electron transport chain during the process of oxidative phosphorylation, reducing molecular oxygen (O_2) to superoxide anion ($O_2^{\bullet-}$)[54].

NADPH oxidase (NOX) complexes are expressed by phagocytic leukocytes and other cell types[55]. The NOX2 complex expressed in the phagocytes plays a crucial role in the so-called oxidative burst, a defense mechanism against pathogens[56,57]. In this process pathogens, e.g. bacteria, are killed by the release of ROS by phagocytes. Additionally, ROS has been shown to be important in the regulation of the immune response[58].

The inactive NOX2 complex consists of membrane-bound proteins $P22^{phox}$ (Cyba) and $GP91^{phox}$ (Cybb). $P47^{phox}$ (Ncf1), $P67^{phox}$ (Ncf2), $P40^{phox}$ (Ncf2), and the small G-protein Rac are found in the cytosol[59]. In the activated state, neutrophil cytosolic factor 1 (Ncf1) is phosphorylated and the Ncf1 heterotrimer is recruited to the Cyba/Cybb complex at the cell membrane.

The active NOX2 complex transfers electrons from the donor nicotinamide adenine dinucleotide phosphate ($NADP^+$) to molecular oxygen, resulting in superoxide anions. The superoxide is converted into O_2 and H_2O_2 by the action of the superoxide dismutase (SOD) enzyme[60].

1.5 Immunoglobulin G N-glycosylation

N-glycosylation is a physiological process adding carbohydrates to proteins. N-glycosylation occurs in the endoplasmic reticulum (ER) and Golgi apparatus of the cell[61]. For successful N-glycosylation, the consensus amino acid motif N-X*-S/T (X* being any amino acid except proline) is required. A conserved N-glycosylation site can be found at position Asparagine (N) 297 in the CH2 of the fragment crystallizable (Fc) region of immunoglobulin G (IgG)[62,63]. In contrast to the Fc fragment, only a small share of IgG have N-glycosylation sites in the fragment antigen-binding (Fab)[64]. It has been shown that only about 15-25 % of serum IgG carry Fab glycans[65]. Only few V gene germline alleles contain the N-X-S/T motif for N-glycosylation[64], most of the N-glycosylation sites in the variable region of an immunoglobulin are introduced through SHM during GC reaction[66,67].

The core structure of a complex type biantennary glycan found on the Fc of immunoglobulins is composed of N-acetylglucosamine (GlcNAc), and mannose (Man) that can be extended with fucose, galactose, and/or sialic acid (N-acetylneuraminic acid) sugar groups[65]. The glycan profile can be highly variable, comprising different structures and composition (Figure 3).

1.5.1 IgG Fab glycosylation

Interestingly, it has been found that ACPA in RA have a higher frequency of Fab glycans in the variable domain compared to antibodies in healthy individuals[68]. This finding has led to the hypothesis that there is a positive selection pressure on the generation of *de novo* Fab N-glycosylation sites in the GC reaction under disease conditions. Another study has found that the Fab glycan can change the binding to citrullinated antigens[69]. Recent evidence shows that the presence of a Fab glycan results in an altered antigen binding, thereby modulating the BCR signalling and eventually changing the threshold for B cell activation[70]. Another hypothesised effector role of Fab glycans is via binding to lectins. Lectins are proteins recognizing and binding to carbohydrate structures[71]. Lectins are expressed on a wide variety on immune cells and can regulate their activation and differentiation[72–74]. However, evidence on how Fab glycans exert their *in vivo* functions via lectins is currently lacking. The knowledge on Fab glycans remains mostly descriptive and scarce, as reviewed by Kissel *et al.*[75].

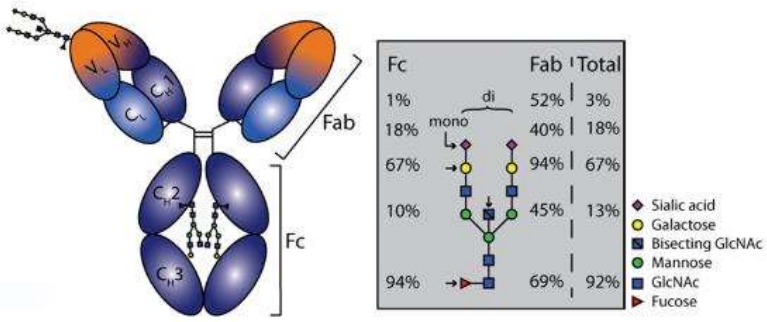

Figure 3 Glycoprofiles of IgG Fab and Fc glycans *(Figure from van de Bovenkamp et al., 2016[65], reprinted with permission from The American Association of Immunologists)*

1.5.2 IgG Fc glycosylation

As described before Fc glycosylation is a conserved process, however it has been shown that the composition of the Fc glycan repertoire changes in health and disease. For example, Fc glycan changes are associated with age and sex in healthy individuals[76–79]. Changes in the IgG Fc glycan repertoire have also been reported in several inflammatory diseases including inflammatory bowel disease (IBD)[80,81], systemic lupus erythematosus[82], multiple sclerosis[83] and rheumatoid arthritis (RA)[84,85]. Focussing on RA, Ohmi *et al.* [86] show that the IgG Fc fragment is desialylated and agalactosylated in RA patient serum and serum-derived purified polyclonal ACPA compared to healthy donor. Furthermore, the Fc is less sialylated in arthritis mouse models[86]. These findings go in-line with literature showing an anti-inflammatory effect of intravenous immunoglobulin (IVIg), a therapy used for the treatment of several inflammatory conditions. Studies suggest that this effect is mediated by sialylation on the Fc, as the effect of IVIg is recapitulated by Fc glycoengineered IgG[87] and sialylated IVIg preparations more potent in inflammation suppression than its native counterpart[88].

Even *per se* arthritogenic antibodies specific against collagen II and citrulline (resp.) used in the collagen antibody induced arthritis mouse model have been shown to inhibit murine arthritis upon enforced Fc sialylation[86]. This regulatory sialylation effect has been shown to be dependent on antigen-specificity[86].

Mechanistic explanations for the sialylation effect include conformational changes of the immunoglobulin resulting in modulated binding to Fcγ receptors, regulation of binding to lectins (e.g. Sialic acid-binding immunoglobulin-type lectins, SIGLECs) and/or regulation of complement[63].

2 Research aims

B cells play a crucial role in the pathogenesis of rheumatoid arthritis. The main goal of the thesis was to investigate the fine-specificities and regulation of self-reactive B cells. An extension of this goal was the investigation of the regulation of antibody (IgG) function by Fc glycosylation.

In **study I**, our goal was to investigate the regulation of B cell activation and differentiation by intracellular ROS produced by Ncf4.

In **study II**, we aimed to map and characterise the epitopes of the B cell response against the model autoantigen GPI in GPI-dependent mouse models as well as in human RA.

In **study III**, we aimed to investigate the regulation of the self-reactive anti-Col2 B cell response in both genetically engineered mouse models and in humans.

In **study IV**, we aimed to understand the role of Fc glycosylation of self-reactive monoclonal antibodies in murine arthritis models.

3 Material and Methods

3.1 Experimental arthritis models

3.1.1 Collagen-induced arthritis

Arthritis can be induced in the mouse model by immunization of genetically susceptible mouse strains with heterologous collagen type II. The collagen is emulsified in Complete Freund's adjuvant (CFA) and injected intradermally at the base of the tail. Three to four weeks after the first injection, a booster immunization is administered with a lower dose of CoI2 emulsified in incomplete Freund's adjuvant (IFA). Dependent on the susceptibility of the used mouse strain, arthritis (swelling of paws) starts to develop three to four weeks after the first injection, increasing in disease severity over a time course of two to three weeks after the boost. The CIA model is considered a gold standard model in preclinical arthritis research. It models the chronic disease phase of human rheumatoid arthritis and is dependent on both a specific self-reactive B and T cell response.

3.1.2 Cartilage-antibody induced arthritis (CAIA)

In CAIA, disease is induced by administration of one or a mix of several cartilage-specific monoclonal antibodies (mAbs) into genetically susceptible mouse strains. Commonly, the mice are boosted three to five days after primary injection with lipopolysaccharide (LPS). In contrast to CIA, the disease onset appears very rapidly after boosting, during the 2nd week after the primary injection of antibody.

Originally, the mix of mAbs for arthritis induction contained only antibodies specific for collagen-type II, thus the disease is called collagen-antibody induced arthritis. Later, the mix was extended by mAbs specific for other cartilage proteins (collagen type XI, COMP) to induce disease in less susceptible mouse strains. CAIA is used as a model for the acute phase of disease, with a strong disease severity developing within only a few days. The model has a strong component involving the innate immune system, while it is independent of B and T cells.

3.1.3 GPI-dependent arthritis mouse models

Two well-established GPI-dependent mouse models exist, the K/BxN and the GPI-induced arthritis model. The K/BxN model was discovered in a moment of serendipity when the cross of non-obese diabetic mice (NOD) mice with TCR-transgenic KRN mice yielded spontaneous joint inflammation in the offspring [89]. The joint inflammation is mainly mediated by GPI-specific autoantibodies. K/BxN serum transfer can induce disease in standard laboratory mouse strains, giving a rapid disease onset within only a few days modelling the effector phase of human RA [90]. An alternative to serum-transfer induced arthritis is the injection of heterologous full recombinant GPI protein emulsified in CFA, followed by boost with GPI+IFA 12 days later in genetically susceptible mouse strains.

A third GPI-dependent mouse model is induced by injection of the major T cell epitope ($GPI_{325-339}$) identified in GPIA [91]. This model was not used in this thesis.

3.2 Antibody production

In this thesis, mAbs were produced from two sources: B cell hybridoma cell line or Human Embryonic Kidney (HEK) cell line.

B cell hybridoma mAb clones were generated and maintained as described earlier [92].

Antibodies with no available corresponding hybridoma clone were produced recombinantly in HEK-derived suspension culture-adapted Expi293 cells. Corresponding gene segments (dsDNA) were ordered and cloned into a mammalian expression vector plasmid. The plasmid was produced in large scale and transiently transfected into Expi293 cells using chemical-based transfection.

Both hybridoma and HEK-derived mAbs are purified from culture supernatants using Protein G-coupled resins on a professional chromatography system.

3.3 Recombinant protein production

Recombinant proteins in this thesis were either purchased commercially or produced in HEK cells. The procedure is analogous to the described antibody production in HEK and differs only in terms of the purification method used. All recombinant proteins were purified using immobilized metal ion affinity chromatography (IMAC) using Nickel (Ni)-based resins.

3.4 Flow cytometry

Spleen, lymph nodes and bone marrow were harvested from euthanized mice and mashed to obtain single cell suspensions. The cells were resuspended in PBS and counted. Equal number of cells were plated into a V-bottom 96-well plate followed by Fc block (clone 2.4G2) to avoid nonspecific antibody binding to FcγRs. Cells were stained with LIVE/DEAD dead cell stain (Thermo Fisher Scientific) followed by resuspension in FC buffer (PBS + 1% BSA). Next, cells were stained with an optimal (titrated) concentration of commercial fluorochrome-conjugated mAbs against cell surface antigens. After 30 min staining, cells were washed with FC buffer, followed by analysis using a BD LSRII or Invitrogen Attune NxT flow cytometer.

In experiments involving fluorescence-activated cell sorting (FACS), the same staining procedures were followed. The cells were sorted on a BD FACSAria III into prefilled 96-well U-bottom cell culture plates. Data was analysed using the latest version of FlowJo.

3.5 Tetramer stains

Fresh tetramers were prepared by mixing biotinylated peptides (antigens) with commercial fluorochrome-conjugated streptavidin in a molar ratio of 4.5:1. Tetramers were titrated to determine the optimal staining concentration. Cells were stained and analysed following the procedure in the Flow cytometry chapter.

3.6 ROS detection

Intracellular ROS was detected by incubation of cells with the dihydrorhodamine (DHR) 123 probe and cell activation by phorbol 12-myristate 13-acetate (PMA). Readout was done by fluorescence detection by flow cytometry.

Extracellular ROS was detected by incubation of cells with PMA, isoluminol and horseradish peroxidase (HRP). PMA is a cell activator, isoluminol is a ROS probe exhibiting chemiluminescence in the presence of horseradish peroxidase (HRP). Chemiluminescent signal was detected with a BioTek Synergy 2 plate reader. Signal was detected and plotted in relative light units (RLU).

3.7 Magnetic bead cell sorting

Total B cells and epitope-specific B cells were isolated from mouse lymphoid organs and human PBMCs using magnetic bead cell sorting kits from Miltenyi Biotec. Total B cells were isolated by negative "untouched" selection using B cell isolation kit mouse/human, while epitope-specific B cells were isolated by positive selection using Anti-PE MicroBeads according to the manufacturer's instructions. After magnetic bead cell sorting, cells were subjected to further analysis by flow cytometry, mass spectrometry (proteomics), sequencing, cell transfer into mouse (*in vivo*) or cell culture (*ex vivo*).

3.8 Cell culture

Bulk or single cell B cells from mouse and human were sorted using FACS or magnetic bead cell sorting procedures and seeded into prefilled 96-well U bottom cell culture plates. In both study I and III the basic culture medium was composed of RPMI +10 % fetal bovine serum (FBS) + penicillin/streptomycin (P/S), designated as "complete medium". Dependent on the study (I & III), the stimulation cocktail contained further ingredients listed below.

In study I, complete medium was supplemented with different molecules dependent on the scope of the experiment: LPS, hydrogen peroxide, GSK2795039, rmIL-4, rmIL-21, Goat Anti-Mouse IgM ($F(ab')_2$).

In study III, complete medium was supplemented with sodium pyruvate, MEM non-essential amino acids, β-mercaptoethanol, rhIL-2, rhIL-21, human holo-Transferrin, and the TLR agonist CpG ODN2006 [93].

In both studies, cell culture plate were pre-seeded with irradiated CD40L expressing fibroblasts to simulate physiological CD40 activation on the B cells. This was essential to support growth in single B cell cultures required for mAb cloning.

Concentrations and manufacturers of the reagents can be retrieved from the corresponding Materials & Methods sections of the constituent papers. The cells were cultured in humidified incubators at 37 °C and 5 % CO_2.

3.9 Immunoglobulin cloning

In study III, C1-specific B cells were cloned from human healthy donor and RA patient PBMCs. Single epitope-specific B cells were isolated and sorted into cell culture plates as described in the previous paragraphs. The immunoglobulin cloning protocol was adapted from Lindner *et al.*[93]. After 12 days of culture, culture supernatants were aspirated carefully, leaving the cell cluster behind at the bottom of the plate. The cell clusters were lysed using appropriate lysing buffer and snap-frozen on dry ice, followed by storage at -80 °C. The cell supernatants were screened for successful B cell expansion (anti-IgM/IgG) and specificity against the desired antigen (anti-C1) by enzyme-linked immunosorbent assay (ELISA). Positive hits (wells) were then picked from the plate containing the cell lysates and immunoglobulin cloning was performed. Briefly, first mRNA was isolated from the lysates using Oligo-dT beads, followed by cDNA synthesis and specific amplification by polymerase chain reaction (PCR). In the first PCR round, a multiplex primer set was used to amplify the IGH, IGK and IGL genes separately, followed by a 2nd round of nested PCR introducing restriction sites on both ends of the primary PCR product. The PCR product was cloned into a mammalian expression vector plasmid already containing the constant segments of IGHG1, IGKC or IGLC to yield the expression of a full heavy and light chain, resp. The plasmids were sequenced and in case more than one pair of heavy and light chain was obtained, all possible combinations were co-transfected into Expi293 cells as described before. More than one pair of heavy and light chain could occur if one well contained more than one cell, caused by inaccurate FACS.

Expi293 culture supernatants' specificity was validated by ELISA and mAbs with validated specificity (designated as "MAK clones") were purified by Protein G affinity chromatography, as described.

3.10 Ethical considerations

This thesis is mostly based on studying mouse models of arthritis. To a minor extent also samples of human origin were used to study the self-reactive immune response in humans both in healthy conditions and in RA patients.

In Sweden, the use of animal models for research is tightly regulated by the requirement of an ethical permit to be applied for beforehand and by the constant supervision of the animals by specialised animal technicians and veterinarians. All staff working at the animal house at Karolinska Institute is independent from the researcher performing the experiments. This ensures that the practices specified in the ethical permit are followed and the animal house staff even has extensive rights to enforce compliance with the permits and the law. Misconduct of the researcher can even be reported to higher authorities, ultimately leading to a ban to conduct animal experiments for the researcher.

All that said, I believe that applicable regulations ensure that the mice are treated as humane and ethical as possible under the given circumstances. Animal research remains indispensable for the development of new therapies and progress in medical research, also in the field of RA. Without mouse studies, most of the currently approved medicines could have not been tested and their toxicity profile determined before being administered to humans for the first time.

Given that, I would also like to state my opinion on the reproducibility of animal models. Especially in the field of arthritis research many different mouse models are used with different protocols to induce disease. In my opinion, murine arthritis models should be more standardized by following common protocols. The research community should agree on standard protocols, reagents, and best practices to make research results consistent and comparable between different publications and research groups. Also, the characteristics of the mouse models used in each publication need to be justified and discussed more extensively to avoid that wrong conclusions are drawn from a mouse model that was inappropriate for the research question tackled.

Many published research findings cannot be reproduced, leading to unnecessary suffering of mice and a waste of research funds.

Efforts are ongoing to replace mouse studies with *in vitro/ex vivo* models involving 2D/3D cell culture, organs on a chip and – since recently – the development of organoid cultures. These models provide helpful clues to study the effect of medicines, however they also face limitations regarding the interactions between different organ systems when it comes to the metabolism of drugs. Another quite recent and emerging field of study is the development of in silico clinical trial using artificial intelligence (AI). However, these efforts suffer from incompleteness and lack of publicly available data.

In the framework of this study, PBMCs and cartilage explant samples were obtained from human healthy donors and RA patients. The donors gave informed consent and the sample and data processing is regulated by an ethical permit approved by the local ethical committees. Blood samples are taken by common procedures and cartilage samples were obtained as a by-product during common planned joint surgeries. Given this "minimally invasive" nature of sample collection and the ethical sample and data processing I believe that basically no harm is inflicted.

4 Results

4.1 Study I: NCF4 dependent intracellular reactive oxygen species regulate plasma cell formation

Ncf4 is a component of the NOX2 complex responsible for ROS production in phagocytes. Previously, it was shown that Ncf4 shows a specificity towards phospholipids found in endosomal and phagosomal membranes, thereby mediating intracellular ROS production. To study the role of intracellular ROS production, the amino acid R58 responsible for the interaction with the endosomal membrane phospholipid phosphatidylinositol-3-phosphate was mutated and studied in a mouse model. In an earlier study, our laboratory could show that the R58A mutation promotes susceptibility to the collagen-induced arthritis model [94].

In the current study, we investigated the effect of lack of intracellular ROS production on the immune response in collagen-induced arthritis with a focus on the effects on B cell activation and differentiation.

First, the Ncf4^{R58A} model was validated by showing that only intracellular but not extracellular ROS production in B cells was affected by the mutation. To make sure that this effect was independent of secondary effects of the Ncf4 mutation on other NOX2 components, the expression of Ncf1 and Ncf2 was proved to be unaffected. Next, the phenotypic effects of the Ncf4^{R58A} were investigated by asking the question what role it plays in regulating the disease severity of CIA. We could show that the mutation enhanced both the disease severity and increased the anti-Col2 antibody titres. To pinpoint a source for the increase in anti-Col2 IgG titres, the frequency of anti-Col2-specific antibody secreting cells was shown to be increased in inflamed synovia of mice.

To test whether the increase in anti-Col2 IgG levels was partially mediated by changes in T cell co-activation, the ability of B cells to present antigen via MHC and T cell activation was checked. Neither antigen presentation nor T cell activation and differentiation was affected by the Ncf4 mutation.

Following up on the observation of ASC in synovia, plasmablast and plasma cell formation was followed during the priming stage of CIA and a higher frequency and absolute number of antibody secreting cells were found in the lymph nodes. To prove that the effect of Ncf4^{R58A} on ASCs is not dependent on the antigen and disease model used, the pristane-induced lupus (PIL) was induced and showed consistent results. B cell transfer experiments into B cell deficient recipient mice were performed to demonstrate that the effect on B cells was not exerted by extrinsic stimuli or other cell types. Instead, we could show that intracellular ROS via Ncf4 acts and regulates B cell intrinsically. This result was further corroborated by *in vitro* culture of isolated B cells using a set of stimuli to induce B cell differentiation into plasmablasts.

Finally, we could show that ASCs from Ncf4^{R58A} show distinct expression patterns of CXCR3 and CXCR4 compared to wildtype, consistent with a plasmablast/plasma cell migration (homing) pattern to inflamed synovia compared to bone marrow.

4.2 Study II: Pathogenic antibody response to glucose-6-phosphate isomerase targets a modified epitope uniquely exposed on joint cartilage

Glucose-6-phosphate isomerase (GPI) is a ubiquitously expressed protein involved in a variety of functions, the most prominent being glycolysis. Increased levels of anti-GPI antibodies and free GPI protein was found in a subset of human RA patients, however no direct association with the disease could be established. Evidence about the pathogenicity of GPI in arthritis comes from two well-established mouse models, K/BxN and GPI-induced arthritis (GPIA).

In the current study, we dissected the antibody response to GPI both in mouse models and in human RA patients. We gained novel insights into the unique structural nature of the dominant GPI epitope and associated the antibody response against this epitope to human RA.

First, we identified the dominant B cell epitope of GPI in the two GPI-dependent mouse models K/BxN and GPIA. An antibody response of IgM isotype against the GPI$_{293-307}$ peptide could be already detected in naïve mice and a corresponding class-switched anti- GPI$_{293-307}$ IgG response appeared at a very early timepoint shortly after disease initiation (immunization with GPI).

Next, several mAbs against GPI were cloned to investigate the structural features of the newly defined epitopes. While some antibodies bound to GPI epitopes exposed on the protein surface, the GPI$_{293-307}$ – specific mAb clones could bind only to denatured GPI protein. By using a combination of solid/liquid phase binding assays and a crystal structure of the GPI$_{293-307}$ – specific mAb clone TL1 we could show that the epitope is hidden within the protein structure under native conditions, while it is exposed only when the protein changes its conformation due to external cues. As the requirements for the conformational change are unknown, the structure modification of GPI was induced experimentally by chemical-induced denaturation.

Given the intriguing observation of natural autoreactive GPI$_{293-307}$ -specific IgM in naïve mice and that the antibody response to this epitope switched isotype within only one-week post-immunization led us to hypothesize that corresponding GPI$_{293-307}$ – specific B cells must have been already existent in naïve mice. To prove this, we applied B cell tetramer stains. Tetramers are prepared by conjugating biotinylated peptides of interest with fluorochrome-conjugated streptavidin.

Using this methodology, we could identify $GPI_{293-307}$ – specific B cells in all major lymphoid organs (spleen, lymph nodes, bone marrow) known to accommodate B cells. After immunization the frequency of epitope-specific B cells increased further and a decrease in IgM expression could be observed, suggesting class switch.

In the next step, we wanted to study which role the $GPI_{293-307}$ epitope plays in the pathogenic process by infusing the generated mAb clones in disease-susceptible mouse strains. Antibody-induced arthritis allows direct investigation of the pathogenicity of a set of antibodies. Both mAbs uniquely specific for $GPI_{293-307}$ and other mAbs reactive against other GPI epitopes were tested. To enhance disease severity and incidence, the antibodies were co-injected with the known arthritogenic anti-Col2 antibody M2139. Only the $GPI_{293-307}$-specific clones were pathogenic in the mouse models, while mAbs reactive against other GPI epitopes showed no pathogenic activity.

Joint cartilage stains with healthy and inflamed tissues of both mouse and human origin proved furthermore that only $GPI_{293-307}$ – specific but no other mAbs bound to inflamed arthritic mouse and human cartilage *in vitro*, providing a potential explanation for their pathogenic effect.

Finally, a link to the human disease RA was established by screening clinical cohorts of pre-RA patients, established RA patients and corresponding (healthy) population controls against the $GPI_{293-307}$ peptide. An increased antibody response against the peptide could be associated with pre-symptomatic, early, and established RA patients. Furthermore, an elevated titre against $GPI_{293-307}$ was associated with radiographic joint erosion.

4.3 Study III: Antigen presenting autoreactive suppressor B cells

During the stochastic process of generating BCR diversity by V(D)J recombination, self-reactivity emerges. While T cells are predominantly educated and selected in the thymus, the process of counter selecting self-reactive B cells has been unclear for a long time. The current dogma is that autoreactive B cells are selected at two checkpoints, one in the bone marrow, the birthplace of B cells, and another one in the periphery. The ultimate goal of the entire process is to establish healthy immune tolerance, i.e. no attack of self-antigens. The mechanisms of tolerance were described in the literature review.

In the current study, we identified Col2-specific self-reactive B cells in healthy mice, rats, and humans. We applied a genetically engineered mouse model to study the fate and function of these B cells *in vivo*, ultimately leading to the description of a new B cell subset coined B suppressor cells.

First, we developed the methodology to identify and trace Col2-specific B cells by the tetramer technology already described in study II with one modification: Instead of cyclic peptides, triple-helical peptides were used, mimicking the natural structure of the Col2 protein. Using this methodology and enrichment of this rare population with beads we could identify C1 epitope-specific B cells in the three mammalian species mouse, rat and human. To validate the specificity of the tetramer stain used, mAbs were cloned from positively stained B cells. The mAbs panel cloned from human healthy donors and RA patient showed a monospecificity to the triple-helical C1 epitope.

Next, we wanted to investigate how the autoreactive C1-specific B cell population is selected *in vivo*. To this end, we applied a genetically engineered mouse model with an IgH knock-in encoding for a heavy chain specific for the C1 epitope (ACB mouse). Only the heavy chain was knocked-in to allow free recombination of the heavy chain with different light chains. The purpose was to see if autoreactive B cells are counter-selected during B cell selection and the B cell pool shifts away from autoreactivity by means of receptor editing and somatic hypermutation (SHM). Interestingly, we could show that self-reactive C1-specific B cells are not negatively selected during development and bypass all known mechanisms of B cell tolerance. To understand this phenomenon the metabolic signature of C1-B cells was analysed, showing a metabolically dormant phenotype.

In the following step, we investigated the question of the functional role of this B cell population *in vivo*. B cell transfer experiments proved that they suppress inflammation in an antigen-specific way. C1 B cells transferred into recipient mice immunized with Col2 (CIA model) suppressed inflammation, while this effect was not seen in a transfer into an experimental autoimmune encephalitis model. Anti-inflammatory B cells have been described in literature as regulatory B cells (Bregs) and their function has been shown to be IL-10-dependent. To investigate whether C1 B cells show a Breg phenotype, we performed experiments with IL-10 knockout mice. Intriguingly, the suppressive effect of C1-specific B cells was IL-10 independent and thus we coined them as B suppressor cells, distancing them from the previously described Bregs.

To define the properties of Bsups we next performed a molecular analysis of this population by bulk mass spectrometry and single cell sequencing to characterize the proteome and the transcriptome, resp.

We determined that the Bsups exhibit an antigen-presentation signature with the strong upregulation of CCR7 and CD72 compared to the control B cells. Based on this finding we hypothesized that C1-Bsups might present antigen to T cells and induce regulatory T cell (Treg) differentiation. Indeed, Bsups induced Tregs in both *in vivo* and *ex vivo* experimental settings in a contact-dependent way.

Finally, we investigated the role of CD72 which was identified in the previous molecular analysis. By blocking CD72 with an antibody, we could demonstrate that the observed Treg induction is partially mediated by CD72.

4.4 Study IV: Fc Sialylation has no effect on the pathogenicity of arthritogenic antibodies

All Immunoglobulin G molecules carry a conserved glycan at Asparagine 297 (N297). The composition of Fc glycans among the polyclonal IgG pool shows an enormous complexity, yet the function of different glycan types and their association with antibody specificity is poorly studied. Monoclonal antibodies in combination with inbred mouse studies with a homogenous genetic background offer a unique opportunity to study the relationship between Fc glycan composition and inflammation.

In the current study, we dissected the role of Fc sialylation of pathogenic mAbs in the murine arthritis models CAIA and CIA.

First, we analysed the Fc glycan composition of mAbs produced by B cell hybridoma by mass spectrometry. The analysed clones showed a distinct glycosylation pattern with varying degrees and abundances of different galactosylated and sialylated glycoforms. Common pattern includes an intermediate level of basic nongalactosylated glycoforms, while a high level of galactosylated glycoforms and a very low to low level of sialylated glycoforms were detected.

In the literature, an anti-inflammatory effect of sialylated Fc glycan on polyclonal IgG preparations was described. To test if this effect also operates on a monoclonal level on pathogenic antibody clones, we tested different combinations of pathogenic antibody cocktails containing mAbs against Col2, Col11 and COMP were tested in the CAIA mouse model. No difference in terms disease severity nor disease incidence could be observed.

To find out if this applies also to another common collagen-dependent mouse model of arthritis (CIA) we injected sialylated anti-Col2 mAb M2139 both prophylactically and therapeutically during disease onset. We could observe an anti-inflammatory effect with a reduction in disease severity for sialylated M2139-mIgG1, but not for sialylated M2139-mIgG2b, showing a dependency on the antibody isotype.

5 Discussion and Conclusions

The role of intracellular ROS mediated by Ncf4 was already investigated in earlier studies, showing that a defect in Ncf4 promotes arthritis[94]. In **study I**, we have extended these findings by studying the effect of intracellular ROS in B cells. Our study was focused on the study of the phenotypic effect of intracellular ROS on B cells, as it is largely unknown how the different components of the NOX2 complex regulate B cell differentiation mechanistically. It is well known that B cells differentiate into short-lived plasma blasts and long-lived plasma cell as a response to antigen. This activation can occur extrafollicularly, e.g. in an inflamed joint, or in lymphoid organs in the course of a germinal center reaction. Data from mostly knockout studies have shown that NOX2 derived-ROS regulates B cell signalling, antigen presentation and antibody production[95]. The intracellular targets of ROS in immune cells have been poorly mapped, studies suggest that ROS exert their secondary messenger function by oxidizing thiols on proteins extracellularly and intracellularly. Our lab has shown that T cells can be regulated by ROS via thiol oxidation, thereby ameliorating arthritis in the mouse model[96]. A recent study from our laboratory also proved the effect of ROS directly oxidizing PTPN22, an important regulator of TCR signaling[97]. However, so far evidence on the ROS protein targets in B cells is missing. Further studies are required to identify these targets.

In **study II** we investigated the long-standing question of the role of GPI in autoimmune arthritis. Even though the role of GPI in autoimmune arthritis disease models is known since more than two decades[89,98], the dominant GPI B cell epitope in mouse and human had not been mapped yet. Our study fills this gap, proposing that GPI causes a pathogenic response by deposits of structurally modified GPI protein on the articular cartilage surface. We suggest that the GPI deposits expose a neo-epitope to naturally occurring B cells which in turn initiates a local inflammatory response. Furthermore, we provided convincing evidence of the association of the antibody response to this epitope with human RA. Given the surprising presence of naturally occurring GPI-peptide-specific B cells we speculated that they might escape the negative selection mechanisms to establish B cell tolerance. Regarding the peculiar nature of the structurally modified epitope in GPI, we propose the antibodies derived from these B cells as a new class of autoantibodies found in RA, in addition to ACPA, the hallmark antibodies of RA.

In **study III**, we continued to work on B cell selection mechanisms. As mentioned earlier, it was proposed in B cell tolerance pillar studies[23,24,19] that B cell tolerance is established in the bone marrow and the periphery by three mechanisms: receptor editing, clonal deletion and anergy. Our study points towards the existence of a new anti-inflammatory B cell subset. These B cells had a very narrow epitope specificity as shown by tetramer stains and mAb cloning.

It is noteworthy that the cloned mAbs (MAK clones) showed a unique monospecificity towards the C1 R-R epitope, as opposed to the cross-reactivity of many other pathogenic Col2-specific mAbs cloned and described earlier by our lab[92,99]. We hypothesize that low affinity/high avidity (IgM) autoreactive B cell clones might escape negative selection mechanisms through a very limited and narrow binding profile to discrete epitopes. However, upon inflammation in the joint, these B cells might get activated, undergo SHM and CSR in the germinal center reaction, finally leading to clones with increased affinity and IgG-mediated effector functions. These clones then might show pathogenic activity, as shown for the previously mentioned mAb clones in the mouse model. Taken together all data generated, it argues for a B cell subset with a defined toolset. It is conceivable that under healthy physiological conditions these B cells play an important role in cellular immune homeostasis with a limited humoral component. This means that under healthy conditions these B cells probably show limited antibody secretion as opposed to pathogenic plasmablast/plasma cell B cell clones induced upon inflammation, as studied in paper I.

Integrating **study II** and **III**, it is conceivable that similar mechanisms govern the fate of naturally occurring autoreactive B cells in both studies. A more precise immunophenotyping of the autoreactive anti-GPI B cells was unfortunately beyond the scope of study II.

Finally, in **study IV** we investigated the ambiguous role of the IgG Fc glycan in murine arthritis models. IgG is an essential component of humoral immunity, and it has been shown early how crucial the Fc glycan is in mediating IgG Fc effector functions. The study of IgG Fc glycans was boosted in 2006 when a study by the Ravetch lab attributed the anti-inflammatory effect of intravenous immunoglobulin (IVIg) to Fc sialylation[100]. Since then, some glycoforms have been associated with a pro-inflammatory role, while others have been associated with an anti-inflammatory role. These attributions were largely made in descriptive studies using polyclonal IgG preparations, derived from sera of healthy and diseased individuals. Many studies were also undertaken using IVIg preparations, derived from pooled healthy donor plasma.

After IgG Fc sialylation was broadly recognized as the major determinant of the anti-inflammatory effect of polyclonal IgG preparations, significant efforts have been made to pinpoint the mechanism and more specifically the cellular receptors for the terminal sialic acids. Some studies[87,101] reported the C-type lectin receptor DC-SIGN, which was challenged later[102]. In our study, we aimed to study the role of sialylation on pathogenic mAbs. We conclude that the anti-inflammatory role of pathogenic mAb Fc sialylation depends on IgG isotype and the immunology of the mouse model used, thereby adding up also the results of previous studies[86,103].

6 Points of perspective

This thesis studies many facets of B cell biology and many follow-up studies about their role in autoimmunity are conceivable.

Building on the results of **study I** it will be necessary to study the protein targets of intracellular ROS, which can be achieved by modern mass spectrometry methods. Based on broad target screening and identification, new research avenues can be opened to study the master ROS effectors in B cells. The study design could be analogue to previous studies about ROS regulation of T cells published by our laboratory[96,97]. Deepening the knowledge of B cell ROS regulation will be particularly important in the context of any disease with a strong B cell component. This knowledge would allow the development of medicines to modulate ROS levels specifically in pathogenic activated B cell subsets, thereby inhibiting their potential to differentiate into pathogenic plasma blasts/plasma cells. Under other circumstances, it might be necessary to do the opposite, using ROS to activate B cell differentiation.

In **study II**, we elucidated the role of GPI in arthritis. More evidence of the pathogenic role of antibodies against the dominant GPI B cell epitope could be collected by cloning mAbs derived from healthy donor and RA B cells from different tissues. In our study we have developed the methodology that can be easily transferred to stain this B cell subset in humans. MAb cloning combined with testing these antibodies in (humanised) mouse models could provide important clues on how pathogenic B cell clones develop. The tetramer stain technology developed could also be modified to target pathogenic autoreactive B cells *in vivo* by coupling the tetramer with a tolerogenic signal instead of a fluorochrome. Testing such an approach in preclinical models could open exciting opportunities for the targeted silencing of pathogenic B cells in human RA.

Similar lines of study are feasible for **study III** in which we have described a suppressive B cell subset based on epitopes derived from the model antigen Col2 being studied in our laboratory since several decades. However, it is very likely that naturally occurring physiological autoreactive B cell specificities against other epitopes and antigens exist. It can be speculated that such a B cell population could have a very heterogenous repertoire, dependent e.g. on the immunoglobulin genetic diversity[104] or other genetic and environmental factors. With the data of paper III in the background it will be also very tempting to follow the fate and modulate the suppressive B cells *in vivo*. One big open question is for example, how these B cells act in different experimental models and settings. Could it be possible, that aberrant activation of such B cells could ultimately lead to disease? To achieve this goal, the first step will be to validate markers for this subset. Then the antigen specificity repertoire can be screened, followed by functional studies in different disease settings.

In **study IV**, we focused on IgG antibodies as the major humoral component of the B cell effector response. Here, precise glycoengineering of recombinant therapeutic mAb might offer new opportunities to expand the repertoire of IgG effector responses *in vivo*. This is currently already underway, as reviewed elsewhere[105]. One example is the development of afucosylated mAbs.

Another attractive, but arguably technically more challenging perspective is *in vivo* glycoengineering of beneficial and pathogenic antibodies. Even though there is evidence for a prophylactic and therapeutic effect of injected recombinant glycoenzymes in some inflammatory models[106], specific IgG targeting and supply of nucleotide-sugar donors to the enzymes *in vivo* might be limiting factors. We touched upon on this aspect in study IV, however more detailed studies might open new avenues for glycoenzyme therapy.

Another way forward for glycotherapy could be the approach to target sialic acid receptors directly. This can be achieved by designing small molecules ligands (glycomimetics) to Siglecs, thereby inhibiting immune cell activation[107]. One promising candidate is CD22 expressed by B cells, which is a well-studied inhibitory co-receptor[108,109]. Currently, CD22 is targeted clinically for the treatment of B cell malignancies[110].

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