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DIVERSITY AND FUNCTION OF ANTI-MODIFIED PROTEIN AUTOANTIBODIES IN RHEUMATOID ARTHRITIS

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Diversity and function of anti-modified protein autoantibodies in rheumatoid arthritis

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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To my family

"It's in a world of its own" – Arthur Russel

POPULAR SCIENCE SUMMARY OF THE THESIS

Cells of the immune system are the body's army that attacks invading microbes such as viruses and bacteria. One type of immune cell is the B cell. The major function of the B cell is to produce large amounts of antibodies that target the invading pathogens by direct neutralization or by recruiting other cells of the immune system to help fight the invader. However, sometimes the immune system cannot distinguish self from non-self and harmless from harmful. Instead, the immune system starts attacking the body's own structure. This process is called autoimmunity and can lead to autoimmune disease. Rheumatoid arthritis is an autoimmune disease characterized by joints that are swollen and stiff from inflammation. It can eventually lead to joint destruction and disability. There is no cure for rheumatoid arthritis but medications can treat the symptoms. A sign of rheumatoid arthritis is the presence of a special type of antibodies called anti-citrullinated protein autoantibodies or ACPA. These antibodies can be measured in the blood in about two thirds of rheumatoid arthritis patients. ACPA bind to the body's own proteins, but only when the proteins are altered. Protein alteration is a normal activity that changes protein functions depending on the body's needs. It is therefore peculiar that some people develop antibodies that bind to the altered proteins. A type of protein alteration that lately has gained interest in rheumatoid arthritis research is that from oxidative stress. The body produces antibodies against these oxidized proteins to remove them. However, protein alteration from oxidative stress is more common in rheumatoid arthritis and patients can have specialized antibodies that will bind the oxidized proteins.

Scientists are not only interested in studying the self-reactive antibodies in rheumatoid arthritis, but also the responsible B cells that produce them. If B cells are found in the joints, it could be a sign of active inflammation and disease. A majority of antibodies in response to infections or vaccines are normally continuously produced by specialized cells in the bone marrow that have a long life span. It is therefore interesting to study if the harmful self-reactive antibodies are also produced in the bone marrow from long lived cells or if they come from B cells sitting locally in the joint.

In this thesis, we are identifying anti-altered protein antibody producing B cells in the joints of rheumatoid arthritis patients and for the first time, also in the bone marrow. We are using a specialized method in the laboratory to replicate the antibodies from patients' B cells. Our investigations determined both shared features and remarkable individual traits that B cells from different tissues have, in particular for rheumatoid arthritis. For example, the possible harmful self-targeting antibodies were individually able to bind a much greater amount of modified proteins than previously acknowledged. By investigating how antibodies can modulate the immune response by interacting with other immune cells, we learned that some of the antibodies have bone degrading effects and that some may amplify the inflammation.

With this thesis, we are broadening the understanding of the biology of rheumatoid arthritis which may lead to new treatment strategies and eventually a cure.

ABSTRACT

Rheumatoid arthritis (RA) is a chronic autoimmune disease particularly affecting synovial joints. Anti-citrullinated protein autoantibodies (ACPA) are detected in the serum of about 2/3 of RA patients and are being used to classify the disease. These autoantibodies may occur years before any signs of arthritis which implies that they are a cause rather than a consequence of disease. By using modified autoantigens to detect ACPAs in large patient cohorts and by purifying polyclonal autoantibodies from patients, the hunt for disease promoting autoantigens has been going on for decades. However, the relationship between ACPA specificity and any functional effects remains unclear. In order to understand the evolution, specificity, and function of autoreactive B cells in RA, the focus of this thesis is the generation of monoclonal antibodies (mAbs) from paired variable heavy- and light-chain immunoglobulin (Ig) sequences from identified single B cells from various RA tissue compartments.

We have identified citrulline-reactive autoantibody producing plasma cells in the synovium of RA patients with established disease. By generating single plasma cell derived mAbs, we learned that such autoantibodies may be directly involved in the pathogenesis of RA by promoting bone degrading osteoclasts. The mAbs were all multireactive to citrulline-peptides and citrullinated proteins, but with unique distinct binding patterns. We recognized glycine in +1 position to the citrulline and a fraction of the citrulline-reactive mAbs cross-reacted with carbamyl-peptides. In addition, the identified plasma cells displayed features of high somatic mutations and fragment antigen binding (Fab) variable N-glycosylation sites introduced by affinity maturation. By analyzing a selection of RA patient B cell derived mAbs for reactivity against apoptotic cells and activated neutrophils, we learned that a subset of the citrulline reactive mAbs bound nuclear antigens. Interestingly, a fraction of these mAbs could target nuclear histones independently of the citrullinating enzyme PAD by binding to acetylated histones. We explored the extent of the ACPA mAb multireactivity and cross-reactivity by acknowledging the importance of neighboring amino acids in addition to glycine in +1 to the citrulline. By analyzing the bone marrow plasma cell repertoire of RA patients, we observed differences in Ig-frequencies and variable Fab N-linked glycosylation sites between ACPA+ and ACPA- patients. We also found RA patient bone marrow plasma cell clonotypes. In addition, we identified citrulline-reactive bone marrow plasma cells that could bind activated neutrophils which strengthen previous reports of citrullinated histones as ACPA targets. Lastly, we identified autoantibodies against the oxidation-induced post translational protein modification adducts malondialdehyde (MDA) and malondialdehyde-acetaldehyde (MAA) in the bone marrow and lung of RA patients and individuals that also harbor ACPAs. A majority of these autoantibodies, that can promote osteoclastogenesis, need cross-linked MAA-protein for recognition independent of protein backbone.

Taken together, the generation of RA patient single B cell derived mAbs, have revealed remarkable features of the autoreactivity that increases the understanding of B cell involvement in the pathogenesis of RA.

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Manuscript

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LIST OF ABBREVIATIONS

ACPA	Anti-citrullinated protein antibodies
AA	Acetaldehyde
ACR	American college of rheumatology
ADCC	Antibody-dependent cellular cytotoxicity
AID	Activation-induced cytidine deaminase
ANA	Anti-nuclear antibody
anti-Carb	Anti-carbamylated protein autoantibodies
anti-KAc	Anti-acetylated protein autoantibodies
APC	Antigen presenting cell
BAL	Bronchoalveolar lavage
Bcl-6	B cell lymphoma-6
BCR	B cell receptor
cDNA	Complementary DNA
CDR	Complementarity-determining regions
CLP	Common lymphoid precursors
CMP	Common myeloid precursors
CpG	Cysteine-phosphate-guanine
CSR	Class switch rearrangement
CCP	Cyclic citrullinated peptide
D	Diversity
DADA2	Adenosine deiminase 2
DAMP	Damage-associated molecular patterns
DMARD	Disease Modifying Antirheumatic Drug
DUB	Deubiquitinase
EBV	Epstein-Barr virus
ELISA	Enzyme-linked immunosorbent assay
EULAR	European league against rheumatology
Fab	Fragment antigen binding
Fc	Fragment crystallizable
FcγR	Fragment crystallizable gamma receptor
FCS	Fetal calf serum
FDC	Follicular dendritic cells
FI	Fluorescence intensity
FOXP3	Forkhead box protein 3
FR	Framework region
GlialCAM	Glial cell adhesion molecule

HC	Heavy chain
HDAC	Histone deacetylase
His2B	Histone 2B
HLA	Human leukocyte antigen
Ig	Immunoglobulin
ITAM	Immunoreceptor tyrosine-based activation motif
J	Joining
LC	Light chain
LDL	Low-density lipoprotein
M-CSF	Macrophage colony-stimulation factor
MAA	Malondialdehyde-acetaldehyde
mAb	Monoclonal antibody
MCV	Mutated citrullinated vimentin
MDA	Malondialdehyde
MHC	Major histocompatibility complex
MS	Multiple sclerosis
MZ	Marginal-zone
NET	Neutrophil extracellular trap
NK	Natural killer
NSAID	Non-steroidal anti-inflammatory drug
PAD	Protein arginine deiminase
PAMP	Pathogen-associated molecular patterns
PTM	Post translational modification
RA	Rheumatoid arthritis
RAG	Recombination activating gene
RF	Rheumatoid factor
RU	Relative units
SHM	Somatic hypermutation
Syk	Spleen tyrosine kinase
TCR	T cell receptor
TLR	Toll-like receptors
TNFα	Tumor necrosis factor alpha
Treg	Regulatory T cells
V	Variable
VH	Variable heavy
WB	Western blot

1 INTRODUCTION

The immune system must have the ability to recognize a huge number of different foreign pathogens and be able to expand rapidly in order to fight the invading threat. At the same time, the immune system must discriminate the safe self from foreign harm. The production of large amounts of antibodies that can bind and neutralize invaders are key players in the immunologic attack. The development of antibody producing B cells is controlled by a process known as immunological tolerance, where the production of autoantibodies against self-structures are regulated. If such regulation fails, autoimmunity arises with the production of autoantibodies leading to autoimmune disease.

Rheumatoid arthritis is a chronic autoimmune disease characterized by joint inflammation and erosion, which left untreated leads to severe disability and premature mortality. Many patients have detectable autoantibodies against modified self-proteins in the blood stream. Autoantibodies are detected years before disease onset which suggests autoantibodies as the cause rather than the consequence of disease. However, the potential pathogenic impact of autoantibodies and autoreactive B cells are matters for investigation. Similarly, the relevant autoantigen(s) targeted is still controversial. Research on polyclonal serology responses gives limited insights into clonalities and specificities of autoantibodies and autoreactive B cells. By isolating single B cells from various RA patient tissue-compartments, identifying autoreactive cells and generating monoclonal antibodies for in-depth binding analysis, much can be learned about BCR phenotypes and breakage of tolerance. Furthermore, such monoclonal antibodies can be utilized for functional *in vitro* and *in vivo* studies of disease progression.

In this thesis, characteristics and functional properties of autoreactive B cells in rheumatoid arthritis were studied by the generation of monoclonal antibodies. The knowledge learned from this work increases the understanding of autoantibody reactivities in rheumatoid arthritis. By studying individual autoreactive B cells from different patients, tissue compartments and subsets, the knowledge of B cell involvement in the pathogenesis of rheumatoid arthritis increases.

2 LITERATURE REVIEW

2.1 THE IMMUNE SYSTEM

The body's resistance against infectious disease has evolved to protect us from invading pathogens. The immune system recognizes infectious agents such as virus and bacteria, toxins from bites or sting, physical damage from for instance a burn and alterations in cancer cells as well as plays roles in maintaining healthy cellular homeostasis [1]. The immune system consists of a network of tissues, cells, proteins and small molecules that interact in specific ways to counteract threats. The complex interplay to recognize harm, effectively neutralize damage and maintain homeostasis can be dysregulated causing imbalance which might lead to immune disorders. The immune system can be divided into innate and adaptive immunity. Innate is from birth and adaptive is acquired. Although the systems are divided, interplay occur [1, 2].

2.1.1 The Innate Immune System

The innate immune system together with the physical barriers such as skin, saliva and mucosal tissues, constitute the first line of defense against foreign harm. When defense barriers are broken, the innate immune cells including neutrophils, monocytes, macrophages, dendritic cells, mast cells, and the lymphoid natural killer (NK) cells mediate immediate responses. By their receptors recognizing pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) they react fast to different foreign threats. Important PAMP receptors include toll-like receptors (TLR) which are membrane and intracellular receptors that recognize microbial structures. In addition, liver produced plasma proteins known as the complement system target common structures in bacteria and viruses. The complement proteins circulate in the blood, lymph and other extracellular fluids and by binding to pathogens enables recognition and destruction by phagocytic innate cells. Other types of inflammation mediating soluble molecules are cytokines and chemokines, including interleukins, interferons, and tumor necrosis factors. All these soluble and cell-surface proteins mediate the immune response by interacting with innate and adaptive immune cells. Innate cells bridge the adaptive immune system by presenting digested and processed peptides to adaptive immune cells on the cellular membrane by the major histocompatibility complex (MHC) [2]. Professional antigen presenting cells such as dendritic cells present engulfed and processed extracellular antigens on MHC class II to helper T cells of the adaptive immune system [3].

2.1.2 The Adaptive Immune System

The adaptive immune system is activated within days of infection, it is specific, effective, expands rapidly and creates memory. This enables the immune system to not only intensify defense against current invading pathogen but will in addition recollect a possible recurring assault [1]. Since pathogens evolve constantly to avoid recognition, the immune system needs

to be trained. This process requires expansion and differentiation of the adaptive immune cells, the lymphocytes.

The adaptive immune response is specific since these lymphocytes are extraordinarily diverse and carry clonally unique antigen receptors. During an immune response, selected clones can proliferate to mount a specific immune reaction towards the pathogen. Essential for the adaptive immune system are the lymphocytes B cells and T cells and humoral responses i.e. antibodies. B cells mature in the bone marrow and T cells in the thymus. They share features in the ability to recognize their target molecules, i.e. antigens, by the surface receptors B cell receptor (BCR) and T cell receptor (TCR), both highly variable and specific. The unique variability is formed by random somatic rearrangements of the variable (V), joining (J) and, for the heavy chain BCR also diversity (D), gene segments [4].

2.1.3 T cells

Different subsets of T cells play roles in distinct types of immune responses, i.e. CD8+ cytotoxic cells are more essential in virus responses and in the recognition of cancer cells while CD4+ T cells are important for mediating antibody responses and activating other cell subsets. The TCR recognizes and become activated by short peptides bound to MHC. CD8+ cytotoxic effector T cells interact with intracellular peptides on MHC I and CD4+ helper T cells interact with extracellular peptides on MHC II on professional antigen presenting cells (APCs) [5]. T cells mediate functions by cell-cell interactions and by releasing cytokines. CD4+ T cells help B cells mature and some CD4+ cells are regulatory T (Treg) cells that downregulate an immune response. When a CD4+ T cell recognizes peptide on the TCR presented by MHC II it starts proliferating and release cytokines that prime the immune response. The activated CD4+ T cell help B cells mature against the specific antigen presented through the TCR-MHC II complex.

2.1.4 B cells

B cells produce circulating immunoglobulins, i.e. antibodies, which can recognize almost any foreign biomolecule without recognition of any self-structures. In contrast to the TCR, the BCR recognize soluble molecules and epitopes on intact proteins as well as in some cases carbohydrates, and lipids. Besides producing antibodies with effector functions and generate immunological memory, B cells also act as APCs and secrete immune regulatory cytokines. These fine-tuned functions are dependent on the B cell subset, its location in the body and the stimuli the specific B cell encounter.

B cell Development and Generation of Immunoglobulin Diversity

B cells develop in the bone marrow from bone marrow precursor cells to pro-B cells, pre-B cells and immature B cells. Central in the process is the rearrangement of the BCR, which starts with the heavy chain (HC) variable region (VH). The VH is constructed from combinations of variable (V), diversity (D) and joining (J) genes. One out of 40 V-, one out of 23 D- and one out of 6 J-genes are assembled [6]. Enabling more than 6,000 possible HC variable region rearrangements [7]. Recombination activating gene (RAG) 1 and 2 mediate VDJ

recombination by bringing flanking DNA together and cleaves at specific sites that are repaired by ligases. DJ genes are rearranged first in the early pro-B cell. If a productive rearrangement is made with all sequences in frame, recombination of the DJ-segment with a V-gene occur. If the B cell has made unproductive rearrangement, it will repeat the rearrangement on the second chromosome. If the repeated VDJ-recombination fails, the pro-B cell will go into apoptosis [1]. When VDJ-recombination of the HC is successful, the rearrangement of the light chain (LC) starts. LCs can be rearranged either from the κ or λ gene locus. LC-rearrangement always starts on the Igκ locus by rearrangement of Igκ VJ-genes. If the VJ-recombination results in two unproductive Igκ LCs, rearrangement will continue on the Igλ loci. LC recombination does not contain D-genes resulting in fewer possible LC rearrangements. Light chains from 30-38 Vκ- and Vλ-genes x 1-5 Jκ- and Jλ-genes. All together this results in about 2.1×10^6 different paired HC and LC variable gene segments. On top of that, non-template nucleotides, so called N-insertions, are randomly added enzymatically to the junctions of V, D and J-genes, resulting in over 10^{12} possible BCR combinations [8]. After successful LC rearrangement, the pre-B cell has made a BCR with a HC paired with either a Igκ or Igλ LC. The pre-B cell develops further into an immature B cell only if it has a functional BCR that can bind antigen (Figure 1). Immature B cells leave the bone marrow and enter the spleen where the final maturation step occurs in which IgD is co-expressed together with IgM.

B cell Activation and Germinal Center Responses

B cells circulate through follicles and lymph nodes where the first encounter with a microbial protein antigen occurs. By binding to the microbe, the BCR gets activated. Through the BCR, the B cell internalize and digest protein antigens, and presents peptides on to MHC II for interaction with CD4+ T helper cells. The primarily activated B cell has a low-affinity BCR with not so efficient effector functions. The activation results in B cell proliferation, possible differentiation into low-affinity and short-lived antibody secreting cells [9-11]. The activated B cell can migrate into the T cell zone in germinal centers for further maturation and proliferation in the dark zone. The B cell starts to proliferate with high turnover which enables Ig point mutations at a high rate in a process termed somatic hypermutation (SHM) [12] (Figure 1).

The somatic hypermutated cell can engage in binding to antigens presented by follicular dendritic cells (FDC) which leads to further helper T cell activation in a process referred to as affinity maturation. High affinity BCRs gets selected in this process in a Darwinian fashion. Low affinity B cells either go into apoptosis or can continue SHM to increase their antigen-specific affinity [13, 14]. Activated B cells in germinal centers enter circulation and can differentiate into memory B cells or antibody secreting plasma cells (Figure 1). Long lived plasma cells cycle back to the bone marrow were they reside as end stage differentiated antibody secreting cells that can survive for up to a lifetime. B cell subsets can be distinguished by cell surface markers and their transcription profile. Long lived plasma cells in the bone marrow have typically undergone rounds of T cell dependent SHM induced affinity maturations in germinal centers [15].

Classical B cells in germinal center responses are also denoted B2 cells. However, there are additional B cell subsets with more innate-like properties such as marginal-zone (MZ) B cells and B1 cells that are involved in T-cell independent responses. MZ B cells commonly recognize non-protein antigens such as polysaccharides and lipids. B-1 cells are first generated in the fetus and produce IgM that are present from birth[16]. These innate like IgM antibodies are called natural antibodies. They are germline encoded without SHMs, and have been hypothesized to be involved in the clearance of apoptotic cells and first-line of defense against bacterial pathogens [17].

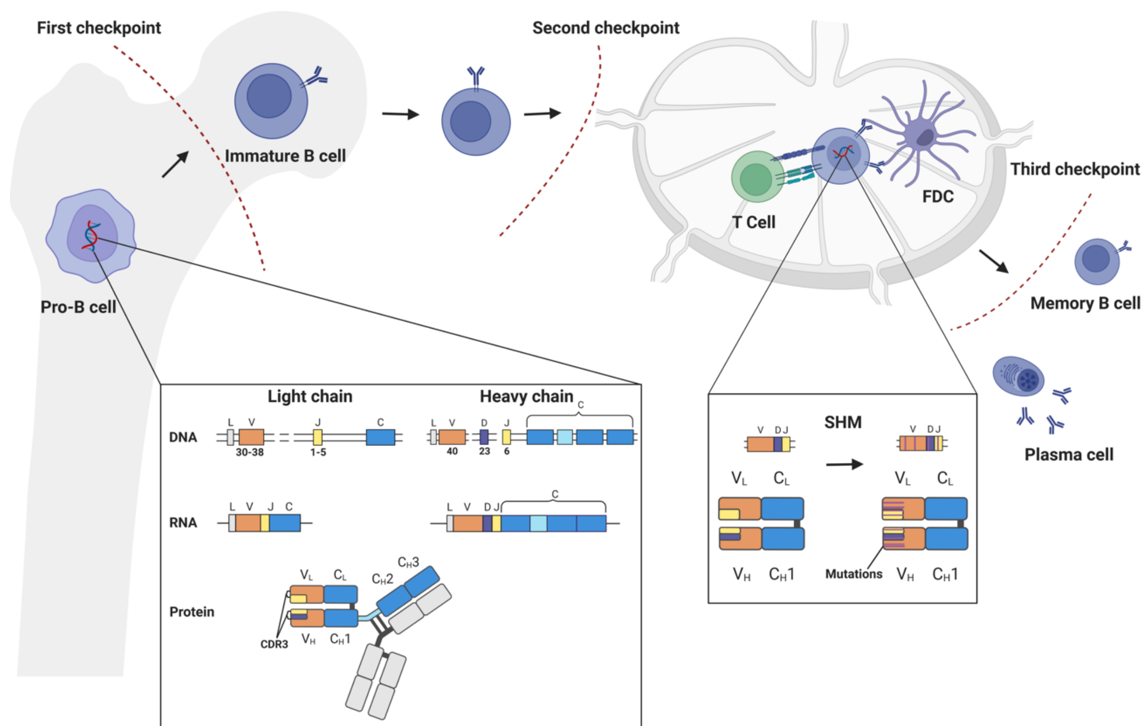

Figure 1. **B cells develop in the bone marrow and mature in germinal centers.** The generation of immunoglobulin diversity followed by tolerance checkpoints. Activated B cells undergo germinal center reaction where SHM leads to affinity maturation. Created with Biorender.com.

2.1.5 Antibodies

The secreted Igs, i.e. antibodies, play essential roles in protecting the host organism against foreign pathogens. Depending on the immunoglobulin constant region structure, the antibodies can be divided into different isotypes, IgM, IgD, IgG, IgA and IgE. The IgG and IgA can also be further subdivided into the IgG1-4 and IgA1-2 subclasses.

The IgG antibodies consist of two identical γ heavy chains (HC) with the size of around 50 kDa and two identical κ or λ light chains (LC) with the size of around 25 kDa, bound together by disulfide bridges. The secreted y-shaped antibody consists of a variable region for antigen recognition in the N-terminal region of the heavy chains linked to the light chains, which is also called fragment antigen binding (Fab) [18]. In contrast, the constant region or fragment

crystallizable (Fc) on the C-terminal end of the heavy chains are responsible for effector functions, including interaction with complement and fragment crystallizable gamma receptor (FcγR) (Figure 2).

Each chain holds a variable domain consisting of around 100 amino acids. This variable part of Fab consists of antibody stability framework regions (FRs) and regions typically involved in antigen recognition called complementarity-determining regions (CDR) [19]. The CDRs consists of in total six β-strand loops with very high sequence diversity. Three in the variable heavy domain, H1-H3, and three in the variable light domain, L1-L3 [2]. Somatic hypermutations are more common in the CDR regions and the AID enzyme responsible for mutations favors specific motifs known as AID hotspots. Moreover, replacement mutations resulting in amino acid changes are more common in CDRs in comparison with FRs as a sign of antigen selection and that mutations can result in changes in antibody binding [20, 21].

The part of the antibody that is involved in antigen recognition is called the paratope and the antibody binding site on the antigen is called epitope [22]. The majority of antibodies recognize conformational epitopes; hence they bind to the three-dimensional structure of the antigen, not linear sequences. These antibodies only bind proteins folded in a certain way. A computational analysis of available crystal structures of antibody-antigen complexes confirms this finding [23]. Nevertheless, linear epitopes, independent of protein folding unless the epitope becomes hidden, are also recognized by antibodies. Usually 4-7 amino acid residues are then critical for interaction [24].

Class Switch Rearrangement

During class switch rearrangement, the original IgM isotype can be transformed into other Ig isotypes and subclasses for different effector functions. It is an enzymatic process, again by the AID enzyme that rearranges the VDJ region to form a different Ig heavy chain isotype [25]. The V domain of an Ig μ heavy chain is moved to a downstream C region. Double stranded breaks followed by ligation and repair to form new heavy chain isotype. The process of class switch rearrangement is one way in that sense that a formed isotype can only develop further to the next one in a specific order: IgM, IgD, IgG1-4, IgA1-2 and IgE (Figure 2). Cytokines determine which isotype to be produced. IL-10 in humans gives IgG, IL-4 gives IgE, TGFβ gives IgA. The site of antigen exposure also effects isotype switching. For instance, IgA is produced mainly at mucosal sites and can be secreted through the mucosal epithelial.

In blood, the monomeric IgG is the most abundant isotype. Based on the hinge region structures, IgG has four subclasses, IgG1-IgG4, that bind FcγRs with different affinities [26].

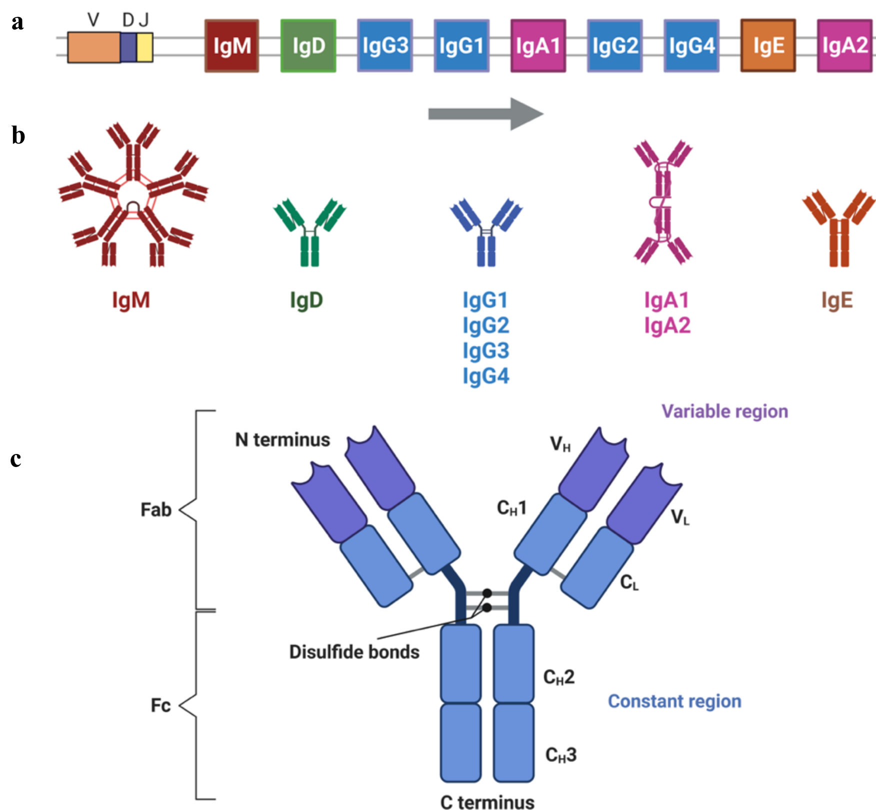

Figure 2. **Immunoglobulin structure and isotypes. a,** The Ig isotype is changed during CSR in an orderly fashion. The AID enzyme induce double stranded Ig heavy chain DNA breaks that enables formation of a new Ig isotype. **b**, Ig isotypes and subclasses have different structures that determine the different effector functions. IgA is depicted as dimer although can also be monomeric. **c**, Structure of the IgG molecule. Created with Biorender.com.

Antibody Effector Functions

In the protection against infections, antibody effector functions can be divided into four main categories. (i) By neutralization, antibodies prevent infection of cells by binding to the pathogens. (ii) By opsonization, antibodies bound to pathogens activate phagocytes by Fc binding to the Fc-receptor on the phagocyte. (iii) By complement activation, pathogens are killed through the complement system which also can activates phagocytes. (iv) By antibody dependent cell-mediated cytotoxicity (ADCC), Fc-binding to a Fc-receptor on macrophages, NK cells or neutrophils induces lysis of the antibody-coated cell [27]. Yet, autoantibodies may mediate functionalities in the disease pathogenesis that are not directly related to these classical pathways.

Monoclonal Antibodies

Individual B cells produce only one type of antibody molecule. While serological responses are always polyclonal and originating from multiple different B cells, antibodies produced by one B cell clone are defined as monoclonal antibodies (mAb). mAbs per definition bind exactly the same epitope whereas polyclonal antibodies may bind the same antigen but on different

epitopes and with different affinities. mAbs have been utilized extensively for a range of applications and has revolutionized both molecular biology when used as reagents in immunoassays and clinically as biological pharmaceuticals. Monoclonal antibodies can be produced by different means. Traditionally, mAbs are produced in rodents by immunization. An antigen is injected to provoke an immune response followed by isolation of splenic B cells that can be fused with a myeloma cell line to produce a stable mAb-secreting hybridoma cell line [28]. However, this method is relatively inefficient and difficult for human-derived B cells. Notably, generation of fully human derived monoclonals are important for investigations of clonal human B cell responses, as here within, or for development of therapeutic antibodies with lower immunogenicity. For human mAbs, the finding that Epstein-Barr virus (EBV) effectively immortalize B cells led to the development of human mAb producing cell lines [29]. The method have been optimized by pre-selecting antigen binding cells prior to EBV transformation [30]. While the addition of cysteine-phosphate-guanine (CpG)-dinucleotides during EBV-immortalization remarkably increased the efficiency [31]. Although, the efficiency of the method is still considered to be low which has stimulated alternative strategies for mAb production. Different method are used for isolation of Ig V regions from human B cells, cloning into mammalian expression vectors for expression in cell lines such as Hek293 or CHO, and thereby generating recombinant human mAbs.

Phage display is a specialized method to produce high affinity human mAbs against specific epitopes *in vitro* [32]. In phage display combinatorial methods, gene libraries of antibody fragments either synthetic or isolated after immunization are expressed on the surface of a bacteriophage that is infecting *E. coli*. The expression of antibody fragments on the surface of the phage particle is accomplished by fusing the nucleotide sequence of the antibody fragment for display to a gene encoding a phage coat protein. [33]. The best binder can then be fused with an antibody backbone of choice to be expressed recombinantly. An advantage is the generation of high affinity antibodies but a disadvantage is that the natural heavy and light chain pairing is not maintained in the libraries. Moreover, nowadays a common method to make human mAbs for therapeutic use is also to utilize transgenic mice carrying human immunoglobulin loci followed by immunization and hybridoma mAbs production [34].

A method for studying B cell antigen specificity and functionality is single cell cloning. The BCR can be expressed as recombinant mAb for determination of target antigen and function. Single B cells of interest are first isolated followed by complementary DNA (cDNA) production of the variable Ig heavy and light genes by reverse transcription polymerase chain reaction (RT-PCR), amplification and cloning into expression vectors followed by recombinant mAb expression [35, 36].

Working with recombinant antibodies also gives the possibility for different antibody engineering strategies. The first generation of therapeutic antibodies were chimeric antibodies with mouse variable regions and human constant regions while subsequent strategies included humanized antibodies where the original murine CDR loops mediating antigen binding are

grafted into human variable region scaffold. Moreover, engineering of the constant regions can be performed to increase stability or to alter effect functions such as Fc receptor binding [37].

2.1.6 B cell Tolerance

The process that prevents the immune system from being autoreactive by tolerating self-antigens is referred to as immunologic tolerance. Different sites in the body participate in this regulation during the development and activation of B cells and T cells [38].

Central Tolerance

For B cells, the first checkpoint, also referred to as central tolerance, occurs in the bone marrow when the immature B cell with a newly rearranged functional BCR undergo negative selection. In the negative selection process, the immature B cell exposed to a self-antigen with high affinity receives signals that trigger cell death by apoptosis in a process referred to as clonal deletion [39] (Figure 1). However, the autoreactive immature B cell is not necessarily deleted. If the immature B cell binds to a self-antigen with high affinity in the bone marrow, it might instead of being deleted, proceed to a process called receptor editing [40]. The autoreactive B cell may re-express *RAG* genes and resume light-chain Igκ gene recombination to express a new Ig LC [41]. The new Vκ gene deletes the pre-existing VκJκ gene by recombination with downstream Jκ. It is only the LC that can be rearranged with receptor editing. The HC cannot recombine since some gene segments are lost during the initial recombination [7]. If soluble proteins are recognized by B cells in the bone marrow with low avidity then the B cell survive but the BCR is not expressed properly and the B cell become non-functional and unresponsive in a process called anergy. In a study with human B cells, pre-B cells without functional light chain transcripts, early immature B cells with functional Igκ or Igλ chain transcripts and immature B cells with cell surface IgM expressed were isolated from the bone marrow and blood of healthy individuals. Ig-transcripts were amplified and cloned into expression vectors for subsequent recombinant monoclonal IgG expression in a mammalian cell line. The expressed antibodies were tested by ELISA for DNA, RNA, LPS and insulin antigens, cytoplasmic antigens by Hep-2 cell extracts on slides and a standard assay for anti-nuclear antibodies (ANA). Results show a clear decrease in autoreactivity and polyreactivity between pre-B cells and the immature B cells [36].

Peripheral Tolerance

When B cells leave the bone marrow to the circulation and starts differentiating, they undergo a second check point for autoreactivity against additional self-antigens. Regulatory T (Treg) cells can suppress self-reactive B cells at this stage [42] (Figure 1). In the third B cell check point, self-reactive B cells that have reached the periphery and encounters self-antigens without co-stimulation from helper T cells becomes anergic or apoptotic by downstream BCR signaling [43-45] (Figure 1). These processes are referred to as peripheral tolerance. Yet another suggested peripheral tolerance mechanism is that of receptor revision. Similar to receptor editing, RAG-gene induced rearrangement of the BCR is suggested in the periphery

[46]. Deficiencies in B and or T cell tolerance are factors leading to autoimmunity. However, more than 30% of class-switched B cells are autoreactive in healthy individuals [47].

2.2 AUTOIMMUNITY

The immune system is developed to recognize and target foreign pathogen structures. Autoimmunity is the process where components of the immune system target self-structures such as proteins, phospholipids and nucleic acids. The development to autoimmune disease occurs when autoimmunity cause damage [48]. The autoimmunity often occurs years before onset of disease symptoms, which makes it difficult to understand the cause and to cure. While there are different diseases that can involve a dysregulation of the immune system, autoimmune diseases are traditionally classified as disease where you have the presence of autoantibodies targeting self-molecules and autoreactive B cells and T cells. Altogether there are over 80 different autoimmune diseases which affects 3-5% of the population. These range from more organ specific disease such as myasthenia gravis, pemphigus vulgaris and Grave's disease to systemic diseases such as systemic lupus erythematosus and systemic sclerosis. In organ specific diseases autoantibodies are generally targeting cell surface bound structures and have a direct effect, leading to functional impact or organ destruction. However, the rheumatic autoimmune diseases, including rheumatoid arthritis, have a more systemic component, with autoantibodies targeting more widely expressed antigens and where the role for autoantibodies in the pathogeneses may be more complex. Rheumatic diseases are systemic diseases with different clinical manifestations that often affects the joints [49].

2.3 RHEUMATOID ARTHRITIS

RA is a chronic, systemic and autoimmune inflammatory syndrome particularly affecting synovial joints [50, 51]. Prevalence is about 0,5-1% worldwide and higher in women compared to men. In addition, incidence of disease increases with age [52]. Primary symptoms are joint pain, stiffness and swelling as well as cartilage and bone degradation. When left untreated, the disease may eventually lead to severe disability and premature mortality. Secondary symptoms are fatigue, malaise and weight loss [53]. There is no cure for RA, instead, treatment aims at minimizing joint damage and maximizing physical functionality. Conventional Disease Modifying Antirheumatic Drugs (DMARDs), such as methotrexate, reduce inflammation whereas Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) and glucocorticoids reduce pain. If conventional DMARDs are not effective enough, biological agents that block inflammatory cytokines i.e. therapeutic antibodies or decoy receptors can be used [54]. Furthermore, the fusion protein abatacept (CTL4) that blocks T cell activation by binding to CD80 are utilized to modulate the adaptive immune system. B cell depletion by targeting CD20 with the monoclonal antibody rituximab also have therapeutic effect [55].

A. Joint involvement (swollen or tender)	
1 large joint	0
2-10 large joints	1
1-3 small joints	2
4-10 small joints	3
>10 joints and 1 small joint	4
B. Serology	
RF- and ACPA- (seronegative)	0
RF low or ACPA low	2
RF+ or ACPA+ (seropositive)	3
C. Acute-phase reactants	
Normal CRP and ESR	0
Abnormal CRP or ESR	1
D. Duration of symptoms	
<6 weeks	0
>6 weeks	1

Table 1. **The American College of Rheumatology/European League Against Rheumatism (ACR/EULAR) classification criteria.** Patient classified as RA if sum of A-D ≥ 6/10 and at least one joint with clinical sign of synovitis that cannot be explained by other disease.

2.3.1 Seropositive RA

Detection of autoantibodies in patient sera are included in the American College of Rheumatology (ACR)/European League Against Rheumatology (EULAR) classification of RA together with a scoring system for number of involved joints, inflammatory markers, and symptom duration [51] (Table 1). Antibodies against the Fc-part of IgG, known as rheumatoid factor (RF), have been used in the classification since 1987 and anti-citrullinated protein autoantibodies (ACPA) have been included since 2010 [50, 51]. Up to 70% of RA patients have ACPA and/or RF antibodies [56, 57]. ACPA have 95% specificity for RA compared to 85% for RF [58, 59]. About 30% of RA patients are seronegative i.e. without detectable IgG cyclic citrullinated peptide 2 (CCP2) and RF IgM antibodies. By including measurements of ACPA fine specificities, RF IgA and IgG and CCP2 IgA, the number is smaller [60-62]. Seropositive and seronegative RA differ from each other concerning genetic background, environmental risk factors, as well as pre-clinical and clinical symptoms including development of joint destruction. [63-67]. Presence of RF and/or ACPA thus predicts more severe disease outcome when it comes to joint erosions over time and this difference also affects choice of treatment strategies [68-70]. However, the diagnostic tests used in the clinic may not detect certain ACPA-antigens and RF-isotypes which complicates the definition of seropositivity [61, 62]. While much is still unknow about the etiology of RA, it is a multifactorial disease with both genetic and environmental risk factors contributing.

2.3.2 Risk Factors

Genetic Risk

A large number of genetic loci are associated with seropositive RA [71, 72]. However, genes encoding certain HLA class II molecules, specifically HLA-DR, holds the strongest genetic risk associated with seropositive RA [73]. In the third hypervariable region of the HLA-DRB1 chain there is common amino acid sequence (QKRAA or QRRAA or RRRAA) known as "the shared epitope" [74]. Positions 70-74 in the third hypervariable region of the HLA-DRB1 chain contain the positively charged P4 pocket. The positively charged shared epitope would favor binding to the neutrally charged citrulline over the positively charged arginine [75, 76]. Lately, additional amino acids at positions 9, 11 and 13 were shown to associate with RA risk [77]. Notably, the strong MHC-II association of RA emphasizes the importance of T-cells and the adaptive immune system in the RA pathogenesis.

Environmental Risk

In addition to genetic risk factors, environmental risk factors have been described for seropositive RA. A major environmental risk factor for seropositive RA is harmful airway exposure such as cigarette smoke and silica dust. It was shown that the genetic HLA-DR association together with airway exposures from cigarette smoke or silica dust particles gave increased risk for obtaining seropositive RA. Hence, it has been hypothesized that the pre-joint inflammatory ACPA response is initiated in lungs or airways [73]. This concept was recently fueled by the finding of ACPA in sputum and bronchoalveolar lavage together with visible lung changes of early ACPA positive RA patients [78, 79]. This might be explained by increased levels of citrullinated proteins in lung and airways due to smoking [80]. Another potential site for triggering is the gum. Here, the link to periodontitis caused by *Porphyromonas gingivalis* has gained attention [81, 82]. Since *P gingivalis* has the ability to citrullinate proteins with an enzyme, a molecular mimicry mechanism has been suggested in which the autoimmune response in RA originate from infectious immunity against *P gingivalis* [83]. In addition, the intestinal tract has been connected with the development of RA by observed changes in the gut microbiota [84].

Studies on genetic and environmental risks are useful for patient classification and prevention but clinically relevant treatment strategies are developed by investigations of immunological mechanisms.

2.4 AUTOANTIBODIES IN RA

2.4.1 Rheumatoid Factor

In the late 1930s, RF was the first autoantibody discovered in RA [85] and later the first autoantibody included in the ACR/EULAR classification [50]. Not all RA patients have serum levels of RF and the antibody is present in the healthy population as well as other rheumatological syndromes and some infectious diseases, such as syphilis and hepatitis C [86, 87]. RF bind the Fc part of IgG when that IgG bind antigen in immune complexes [88] and

although IgM and IgA are the most studied isotypes, RF can also be of IgG isotype [89]. In RA, RF is suggested to have pro-inflammatory properties by amplification of the inflammatory properties of immune complexes. Furthermore, RF epitope recognition has been suggested to differs in RA compared to other diseases [90]. RFs are reported to have lower levels of somatic mutations compared to ACPA [91] and the transcriptomics profile suggests a separate B cell development profile that is more responsive to innate stimuli for RF B cells compared to ACPA B cells in RA [92, 93].

2.4.2 Autoantibodies Against Post Translational Modifications

Posttranslational modifications (PTM) are chemical changes of the protein backbone. The changes range from addition or replacement of small chemical groups to large fatty acids, sugars or proteins. These modifications can either be catalyzed by enzymes that recognize specific targets or can be chemical. Physiological PTMs regulate protein folding, subcellular localization, ligand interactions and have immunogenic properties. However, PTMs might also break tolerance to self-proteins and consequently, autoantibodies reactive to modified self-proteins have been described in RA and other diseases [94]. In addition to citrullination, autoreactivities to carbamylation, acetylation and malondialdehyde/malondialdehyde-acetaldehyde-modifications have gained attention in the field of RA [95] (Figure 3).

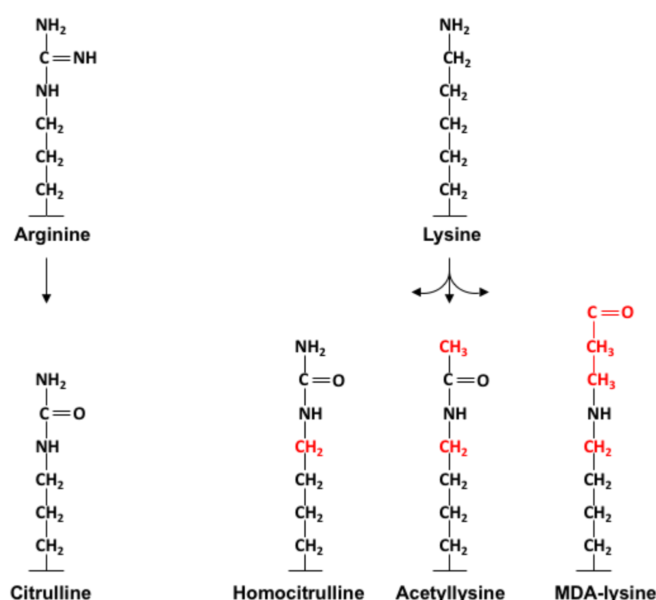

Figure 3. **AMPA targets in RA.** Citrulline modified from arginine. Homocitrulline, acetyllysine and MDA-lysine modified from lysine. Differences to citrulline depicted in red.

Anti-Citrullinated Protein Autoantibodies

The amino acid citrulline in proteins is not formed during protein translation, instead it is introduced during PTM by deamination of the amino acid arginine in a calcium dependent reaction catalyzed enzymatically by peptidylarginine deiminase (PAD) [96] (Figure 4). Citrullination is involved in several physiological processes such as skin keratinization, gene

regulation and tissue differentiation. The change from positively charged arginine to the neutrally charged citrulline affects intramolecular and intermolecular interactions, causing protein unfolding, loss of function and eventually protein degradation. Four isoforms of PAD have been characterized in humans. All of them dependent on Ca^{2+} for activity. PAD2 and PAD4 are the only PADs expressed in immune cells. PAD2 is ubiquitously expressed throughout the body whereas PAD4 is expressed primarily in granulocytes and monocytes [97]. The native PAD4 enzyme has been described as a target for autoantibodies in RA [98, 99]. Furthermore, the prokaryotic PAD enzyme from *Porphyromonas gingivalis* that can citrullinate human antigens in a Ca^{2+} independent manner is suggested to be associated with RA [82, 100]. Human PADs have extensive mutual sequence homologies and seem to have the ability to citrullinate most proteins with accessible arginines *in vitro* but the enzymatic efficiency may differ as does tissue specific expressions. It is also possible that amino acids adjacent to arginine are important for citrullination to occur. A study describes the preference for the amino acid Glycine in +1 position to arginine [101]. Other factors important for citrullination are subcellular localization of the enzyme, microenvironment and physiochemical features of the target protein [102-104]. A wide range of citrullinated proteins well beyond those citrullinated peptides and proteins traditionally used to detect ACPA in RA patients have been identified [62, 105, 106].

In 1964, anti-perinuclear factor (APF) antibodies were described in sera of RA patients [107] and in 1979, anti-keratin antibodies (AKA) were described [108]. These antibodies were used as bio-markers for RA [109]. It was later discovered that these antibodies recognized the same antigen, namely epidermal filaggrin and pro-filaggrin-related proteins on epithelial cells which endorsed a re-naming to anti-filaggrin antibodies (AFA) [110]. These filaggrin proteins were later found to be citrullinated. In the late 1990s, it was found that these reactivities were against citrulline and the first anti-cyclic citrullinated peptide (CCP) antibody test was developed using cyclic citrullinated filaggrin peptides [111-113]. Updated versions of the CCP test, containing other cyclic cit-peptides than filaggrin peptides, is since 2010 used to classify RA in the ACR/EULAR classification [51]. ACPAs from RA patients are known to recognize a broad range of citrullinated proteins and peptides; both ubiquitous antigens together with inflammatory and joint specific targets including vimentin, collagen type II, fibrinogen, α-enolase, hnRNPs and histones [114-119]. ACPAs can be present long before disease onset and about ~1% of the healthy population harbor detectable serum ACPA levels [56, 57]. Substantial amount of research has been dedicated at finding the “RA autoantigen” by studying patient autoantibodies in sera from large well-defined cohorts together with purified patient autoantibodies [62, 120-125].

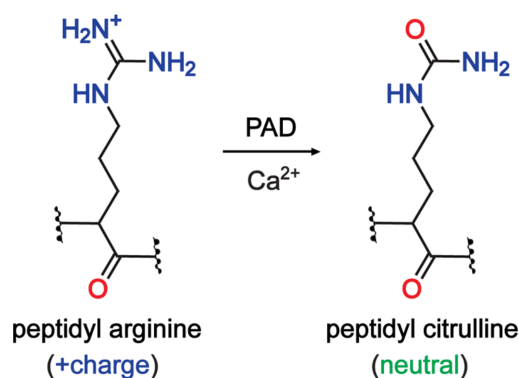

Figure 4. **Citrullination.** The primary ketimine group in the positively charged arginine is PAD enzymatically replaced by a ketone group, forming neutral citrulline in a Ca^{2+} dependent manner.

Anti-carbamylated Protein Autoantibodies

Carbamylation is a non-enzymatic PTM where cyanate mediates the conversion of lysine into carbamyl-lysine or homocitrulline (Figure 5). Cyanate is in equilibrium with urea and normally cyanate levels are too low to induce carbamylation but during special conditions such as uremia, inflammation and cigarette smoke exposure, cyanate levels increase [126, 127]. Structurally, homocitrulline is similar to citrulline but with the addition of a methylene group.

The prevalence of anti-carbamylated protein autoantibodies (anti-Carb) in RA overlap well with that of ACPA, although there are differences depending on study and antigen used [48, 128-130]. When carbamylated fetal calf serum (FCS) was used as antigen in a RA serum screening, it resulted in association with more severe disease [131]. Interestingly, anti-Carb titers can, in similarity with ACPA, be present years before disease onset [132-134].

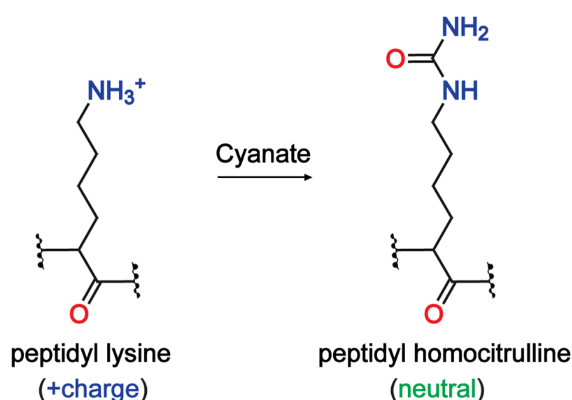

Figure 5. **Carbamylation**. A carbamoyl group is non-enzymatically added to the positively charged lysine by cyanate, forming the neutrally charged honocitrulline.

Anti-acetylated Protein Autoantibodies

Acetylation in eukaryotes is a reversible enzymatic PTM where an acetyl group is added to amine of lysine residues by lysine acetyltransferases [135] (Figure 6). In bacteria, acetylation can occur non-enzymatically in the presence of acetyl-coenzyme A, a citric acid cycle fatty acid metabolism intermediate [136]. Acetylation is involved in gene transcription, cell division, signal transduction and metabolism. Acetylated lysine is similar to homocitrulline except for replacement of the terminal amine to a methyl group [137, 138]. Histone acetylation is a well-known epigenetic process. The acetylated histones relaxes the chromatin which leads to increased transcription [139]. Interestingly, bacteria is known to acetylate not only own proteins but also host proteins [136, 140]. It has been described that the gut biome can acetylate proteins [141].

By using acetylated vimentin-like peptides for detection of anti-acetylated protein autoantibodies (anti-KAc) in RA patients, similar prevalence to ACPA and anti-Carb with ACPA overlap were detected [142, 143]. Recently, KAc have been detected in ACPA positive individuals without RA [134].

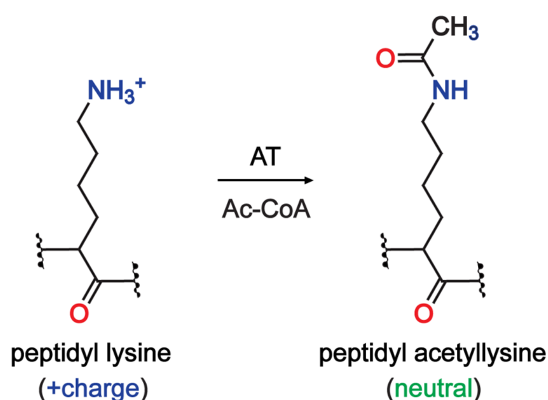

Figure 6. **Acetylation**. An acetyl group is acetyl transferase enzymatically added to the positively charged lysine in the presence of acetyl-coenzyme A, forming the neutrally charged acetyllysine.

Anti-malondialdehyde and Anti-malondialdehyde-acetaldehyde Autoantibodies

Reactive oxygen species lead to lipid peroxidation and subsequent production of the highly reactive malondialdehyde (MDA) which can covalently modify amino acids in proteins. Originally, MDA modification has been especially highlighted in the context of cardiovascular diseases and oxidized low-density lipoprotein (LDL) [144, 145]. MDA has also been described to cause nucleotide modification of DNA. Proteins are typically modified on the ε-amino group of lysine side chains by a Schiff base reaction forming a PTM that alter protein structure and functionality leading to epitopes targeted by autoantibodies [146]. Interestingly, MDA-protein adducts are detected in the RA synovium [147, 148]. MDA together with free acetaldehyde (AA) form an additional autoantibody epitope *in vitro*, namely malondialdehyde-acetaldehyde (MAA) characterized by a fluorescent dihydropyridine (DHP)-lysine ring structure [149]

(Figure 7). It is difficult to control the MDA/MAA-modification in experimental settings and detection with immunoassays have been suggested not to be sufficient [150, 151]. Suggested exogenous source of acetaldehyde required for MAA modification *in vivo* are smoking, alcohol and infection [152-157]. Citrullination and MAA-modification have been shown to co-localize [158].

Anti-MDA antibodies of the IgM isotype are prevalent in the natural IgM pool and have been suggested to have beneficial properties in homeostasis maintenance by clearing apoptotic cells and modified proteins [16]. On the contrary, anti-MDA/MAA IgG antibodies are elevated in RA and SLE [147, 148, 159, 160]. One such study detects anti-MAA autoantibodies prior to RA diagnosis [161]. Interestingly, MDA- and MAA-epitopes can form inter- and intramolecular protein crosslinks which might serve as autoantibody epitopes themselves [147, 162].

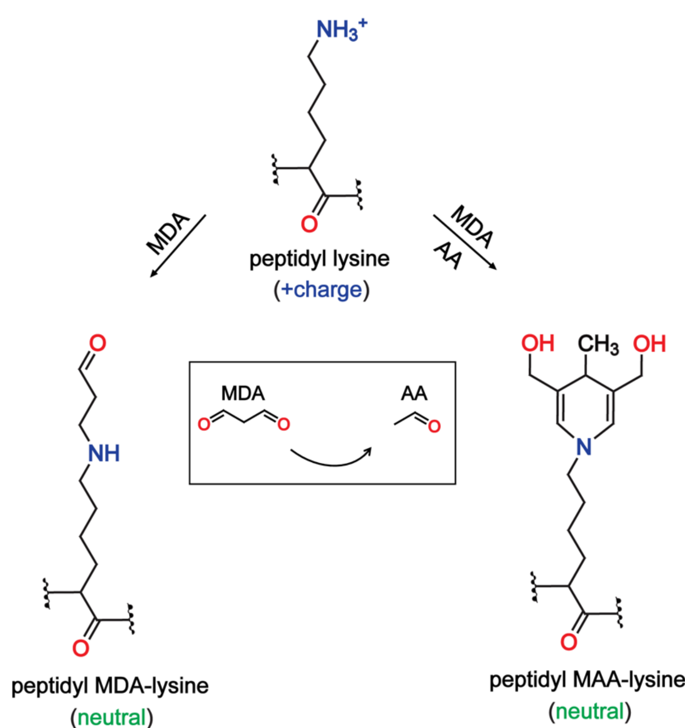

Figure 7. **MDA- and MAA-modification**. MDA-adduct is formed on the positively charged lysine by ROS, forming the neutrally charged MDA-lysine. MAA-adduct is formed by MDA and acetaldehyde.

2.4.3 Etiology

RA often manifests clinically in the sixth decade of life [163]. However, the development of RA is often described as a long evolutionary process were genetically pre-disposed individuals exposed to environmental factors develop autoantibodies years before disease onset [57] (Figure 8). Self-tolerance is thereby broken and autoimmunity occur. The timing is not precise but autoantibodies can be measured in individuals decades before disease onset [164, 165]. The autoimmune period can last for years and individuals might never develop arthritis. This initial breakdown of self-tolerance occurs outside the joints and the term "second hit" is sometimes used to describe the next stage towards arthritis were inflammation in the joints by immune cell infiltrates eventually leading to RA [70]. The environmental risk-factors have been shown to increase the presence of PTMs and thereby the availability of modified

autoantigens. Smoking accumulate citrullinated proteins in the lung [80], *P.gingivalis* contains the citrullinating enzyme pPAD [81, 166] and the gut microbiome can acetylate proteins [141]. Furthermore, these factors cause inflammation at mucosal sites, leading to recruitment of innate cells and subsequent adaptive immune activation. The concept that RA is initiated at mucosal sites is known as the "mucosal origin hypothesis" [167]. In addition, features of the autoantibody response are suggested to evolve during the "second hit". Results from RA patient serology investigations indicate rising autoantibody levels [168], isotype switching [169] and increase in the number of antigens detected by autoantibodies known as "epitope spreading" [62, 120, 170, 171].

The presence of serum RF, ACPA and anti-Carb autoantibodies in individuals years before disease onset is well studied [57, 132-134, 168]. In addition, the presence of anti-acetylated autoantibodies have been detected in ACPA+ individuals without RA [134]. Anti-MAA autoantibodies are detected prior to RA diagnosis but after the development of other anti-PTM autoantibodies [161].

Studies have shown that autoantibody levels increase dramatically before disease onset [171, 172], followed by a slight decrease after disease onset and eventually a long-term stabilization [173]. Interestingly, RF concentrations have been shown to decrease more prominently compared to ACPA levels which suggest that ACPA but not RF may be produced by long lived plasma cells in the bone marrow [174].

Antibodies analyzed in sera and from large well-defined patient cohorts are polyclonal. In order to study individual autoreactive B cells from different tissue compartments, RA patient single B cell derived recombinant mAbs are used to study characteristics and functionalities involved in the RA joint pathogenesis.

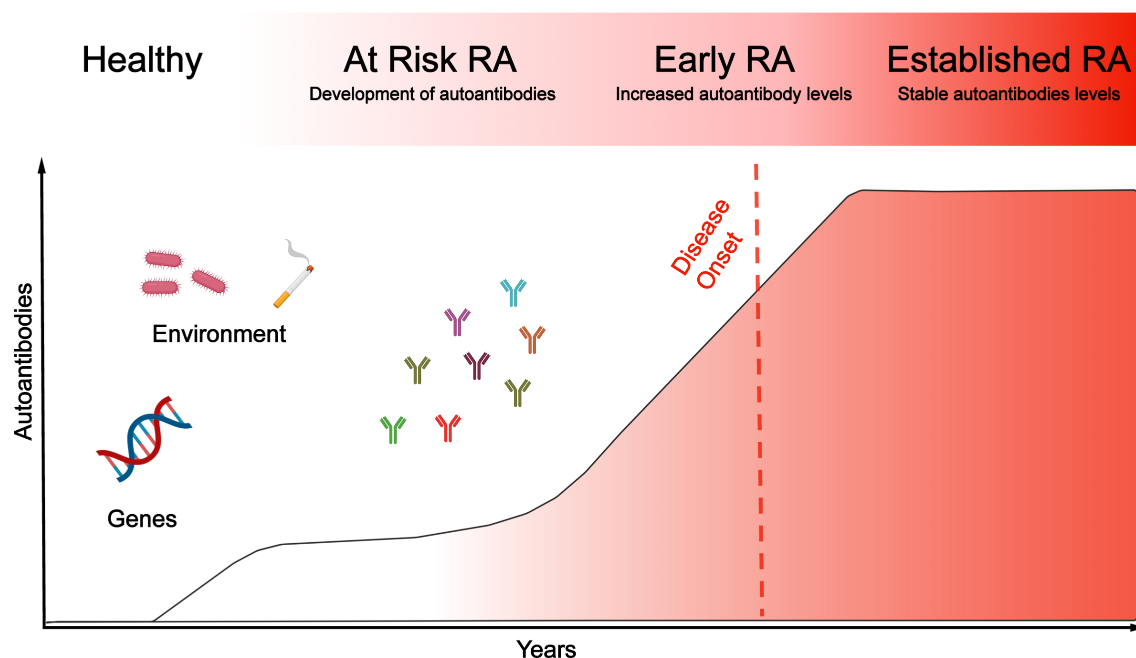

Figure 8. Progression to RA. Genes and environment are risk factor for the development of autoantibodies years before disease onset. Antibody levels increase dramatically before disease onset followed by stabilization during established disease . Made with Biorender.com.

2.4.4 AMPA in Joint Pathogenesis

RA joint pathogenesis is a complex interplay between different cellular and inflammatory components. When the healthy thin mesenchymal membrane in the synovium gets inflamed, it expands through fibroblast proliferation and extracellular matrix deposition. The inflamed synovial membrane is vascularized with infiltrating leukocytes in the synovium, expression of pro-inflammatory cytokines, dysregulation of synovial fibroblasts, osteoclasts and proteinases digesting the extracellular matrix [175]. The synovial immune cell infiltration is characterized by cells from both the innate and adaptive immune system. These cells form ectopic lymphoid structures in the inflamed joints similar to germinal centers [176]. The cellular infiltrate profile is somewhat different between patients which can be described with the term pathotypes. By using single-cell RNA sequencing and mass cytometry, a variety of cell populations have been identified in the synovial tissue of joints with established disease including subsets of fibroblasts, monocytes, T cells and B cells [177]. Effector CD8+ T cells are functionally relevant [178] and the helper CD4+ T cells are together with the macrophages responsible for the osteoclast-promoting TNF-signature seen in RA [179]. In addition, CD4+ T cells stimulate functional B cells that are of main interest in this thesis [70].

Various functions for autoreactive B cells such as antigen presentation to CD4+ T cells [180], cytokine secretion [67] and antibody production may contribute to the pathogenesis of RA. B cell-depletion therapy by the anti-CD20 mAb rituximab has positive effects in seropositive RA [181-183]. However, not all B cells are depleted and ACPA levels are stabilized over time [184]. Both RF and ACPA are suggested for involvement in the RA pathogenesis. However, RF is not specific to RA and RF B cells are suggested to have a more innate like activation pathway and function compared to ACPA [92, 93]. Consequently, the ACPA response and possible ACPA functions have gained interest in the field of RA. Although, one cannot rule out the possibility that the ACPA response is a consequence of the inflammation and thereby not promoting it. Since RA pathogenesis involves joint destruction, efforts have been made to study B cell involvement in the RA synovium where ACPA are enriched and possibly produced [185, 186]. Antibody functions can be studied by isolating patient polyclonal ACPA. However, to better understand more precise the B cell involvement in RA. ACPA B cells have been isolated for the generation of mAbs utilized for functional *in vitro* and *in vivo* studies.

The RA synovium is rich in macrophages [187]. The resident macrophages are replaced by tissue-adaptive bone marrow derived macrophages and monocytes during chronic inflammation [188]. The macrophage heterogeneity give rise to both pro-inflammatory and protective roles in the synovium. For instance, a macrophage sub-population promote fibroblast invasiveness in an epidermal growth factor dependent manner [189] whereas another sub-population lining the synovium have anti-inflammatory properties [190]. Activated macrophages are a major source of cytokines and by using affinity isolated RA polyclonal antibodies it has been shown that ACPA-containing plate bound immune complexes enhance the production of pro-inflammatory cytokines from macrophages through FcγR IIa, TLR-4 and MyD88 [191, 192]. Although the plate bound immune complexes does not necessarily reflect conditions *in vivo* where immune complexes are most likely soluble. However, the addition of

RF fuels the immune complex function [192]. These polyclonal studies have been confirmed by using blood and synovial single B cell derived mAbs [193, 194]. In addition, polyclonal ACPA alone activated macrophages resulting in cytokine release and differentiation towards a pro-inflammatory phenotype [195, 196].

Osteoclasts are highly specialized bone resorption cells of the myeloid lineage. Bone resorption that leads to joint erosions, periarticular bone loss and systemic osteopenia is prevalent in RA [165]. In this context, ACPA have been shown to induce bone destructive effects by promoting osteoclasts. Several studies show that polyclonal autoantibodies reactive with anti-citrullinated vimentin, among other citrullinated autoantigens, can enhance osteoclastogenesis and bone destruction *in vitro* [197-199]. Lately, dendritic cell differentiation into osteoclasts have been suggested to be promoted in studies using ACPA mAbs [200]. Interestingly, osteoclasts express Fcγ receptors that bind IgG Fc in immune complexes. ACPA are thereby suggested to induce osteoclast promoting effects by forming immune complexes with soluble antigens not necessarily present on the osteoclast itself. Recently, IgA immune complexes have been shown to induce osteoclast-mediated bone resorption [201]. Furthermore, the Fcγ-receptor facilitated immune complex effect on osteoclasts have been shown to be mediated by Fc-glycosylation [197, 202]. In addition to ACPA, anti-MDA/MAA mAbs derived from RA synovial B cells have osteoclast promoting effects *in vitro* [147, 203].

Fibroblasts in the RA synovial supportive connective tissue release cytokines and chemoattractants that erode cartilage [204]. Furthermore, ACPA mAbs have been shown to facilitate fibroblast migration. In this study, ACPA mAbs were suggested to directly bind RA synovial fibroblasts and induce expression of PAD-enzyme and cytokine release [205].

Neutrophils are innate immune cells that upon activation can exert an anti-microbial function by extrusion of cellular DNA and protein complexes that form neutrophil extracellular traps (NETs) through a form of cell death coined NETosis [206]. This process is pro-inflammatory and linked to RA [207]. Purified polyclonal antibodies have been shown to bind antigens in NETs and to stimulate NETosis [208, 209]. These studies have been confirmed by using ACPA mAbs [20]. However, a study using polyclonal ACPA failed to completely confirm these observations [210]. PAD4 has a unique role in the formation of NETs and citrullinated histones and other nuclear proteins in NETs have been suggested as autoantigens in RA [211]. Links between genetic RA risk associations of PAD4, deubiquitinase (DUB) domain of the A20 protein, *PTPN22* and adenosine deiminase 2 (DADA2) which all can be connected to NETosis have been discussed [212-216]. However, this concept has been a matter of debate with demands of a more correct nomenclature for NETosis since several DNA releasing mechanisms are not PAD-dependent [217]. Interestingly, PAD4 independent NETosis opens up for involvement of alternative PTMs in histone remodeling [218]. On the other hand, therapeutic ACPA mAbs have been suggested to inhibit NETosis [219].

The involvement of ACPA in the development of arthritis in mice have been extensively studied. An early study shows how ACPA mAbs induces inflammation in the RA joint *in vivo*. mAbs against citrullinated fibrinogen did increase the severity of arthritis mice when co-

injected with anti-collagen II antibodies [220]. Several citrullinated antigens such as histones, fibrinogen, vimentin, collagen and albumin have been shown to increase joint inflammation but not necessarily arthritis in mice [118, 221-224]. *In vivo* studies of pain in mice have shown that polyclonal affinity purified RA patient ACPA induce non-inflammatory pain-like behavior [225]. Effects that recently have been confirmed with ACPA mAbs [226]. Mechanisms that might be explained by Fc-mediation [227]. However, ACPA alone does not induce arthritis which makes it yet impossible to rule out immune-modulating effects of ACPA given that ACPAs are secondary to inflammation [219].

2.4.5 AMPA Characteristics

Traditionally, AMPA investigations are based on the polyclonal serological response to selected modified target antigens or alternatively by utilizing affinity-purified patient polyclonal autoantibodies. Such studies have revealed ACPA, anti-Carb and anti-KAc cross-reactivity patterns with competition and inhibition experiments [134, 142, 228-230].

By isolating citrulline-reactive B cells from different RA patient tissue compartments, a lot can be learned from the B cell phenotypes. Isolation by surface receptor staining can subgroup the B cells and determine activation status. Additionally single cell sequencing adds information of the variable Ig genes and by amplifying the paired heavy and light Ig-genes for subsequent vector cloning, mAbs can be expressed. The ACPA mAbs can be used by different means to investigate antigen specificity. The paired variable heavy and light sequences provides information on affinity maturation, class switch rearrangement and clonal relationships. The procedure was originally developed in the Nussenzweig's laboratory [35, 36] and is now being utilized in several laboratories. The procedure of isolating single B cells can be performed with several different techniques. Flow cytometry can be used to divide B cells in sub-populations for selection of interesting B cells. By "snap-shooting" the B cell repertoire with single cell sequencing enabled by the droplet 10X sequence technology, researchers can capture the B cell repertoire transcription profile at a given time point [231].

B cells have been isolated from different tissue compartments by different means. Circulating plasmablasts have been isolated by flow cytometry surface staining [193, 232]. Notably, in these cases the cells are selected based on B cell subset and not on antigen specificity. Similarly, synovial B cells isolated on surface expression of CD19+ by flow cytometry have been used for generation of mAbs [20]. By using a similar strategy, single B cell derived mAbs recognizing cit-peptides and bacterial antigens have been derived from the gum of RA patients [233]. In contrast, pre-selection of autoreactive antigen specific B cells can be accomplished with the use of modified-peptide tetramers to isolate single B cells by flow cytometry [234-237]. One such study compare cit-reactive ACPA B cells with RF [92]. In addition, synovial memory B cell derived mAbs have been generated using B cell immortalization techniques and screening for CCP2 reactivity [238]. Another approach is to first sequence B cell variable Ig repertoires and select interesting clones for mAb generation based on interesting Ig sequence features or clonality. ACPA mAbs from synovial tissue plasmablasts have been identified with

such techniques [194, 239]. Furthermore, the sequence analyze of the variable Ig genes of the produced ACPA mAbs reveal that ACPA carry high numbers of SHM [193, 234-236, 240].

IgG Fc glycosylation patterns with reduced levels of galactosylation and sialylation is a well characterized feature of autoantibodies in RA [241, 242]. The heavily glycosylated autoantibody variable domain is another feature that seem to appear before RA disease onset [243-245]. Sites encoding N-glycosylation of the variable Fab IgG region can be introduced during SHM [246-248]. ACPA mAb investigations have revealed that ACPA are extensively Fab glycosylated on both heavy and light chain variable domains [245, 249]. Introduction of the N-glycosylation motif, N-X-S/T, where X is any amino acid except proline, give rise to N-glycosylation [250, 251]. The function of this N-linked glycosylation has been studied using ACPA mAb. Such glycans could in general affect antibody binding which has been showed for weak binding ACPA mAbs as well as the ability to activate downstream BCR signaling in a B cell line [252]. However, ACPA IgG is primarily glycosylated outside CDR3s and hence presumably not in the antigen binding region [253] which suggests that N-glycosylation is not directly involved in antigen binding. However, the glycosylation may stabilize the IgG and thereby mediate Fc-effector functions [254]. The glycosylation sites are introduced during B cell affinity maturation and hypothesized to give an advantage during B cells selection and repeated rounds of T cell help. Notably, ACPA IgM are less glycosylated [255].

Affinity purified polyclonal antibodies have been used to study multireactivity and cross-reactivity with PTM-antigens. CCP-purified antibodies are multireactive with cit-peptides [229]. Similar results have been obtained from multireactive carbamylated antigen purified polyclonal antibodies [228]. A study show the ability of ACPA mAb to react with multiple citrulline targets by molecular structural studies [256]. ACPA mAb affinity maturation have been suggested to be T cell dependent [92] and ACPA mAb affinity maturation and multireactivity studies have been performed by producing predicted germline reversions [193, 235].

Recently cross-reactive AMPA were detected before RA disease onset [134]. By investigating ACPA mAb antigen recognition, several independent research groups have learnt that some ACPA but not all are cross-reactive with carbamylated and acetylated antigens [193, 240]. Furthermore, by immunizing mice with one AMPA, the production of other AMPAs were induced [257]. Isolation of IgM-expressing B cells from PBMC by tetramers and expressing the IgM-receptor reveal cross-reactivity to citrulline-, carbamyl- and acetyl-antigens [258]. Furthermore, a B cell line was used to get activation of the BCR with different PTM-antigens [236].

To summarize, further studies of autoreactive B cells and AMPA in RA is a step closer to the discovery of inciting autoantigens, the break of tolerance towards self-antigen and the mechanisms involved in disease onset. Findings which could improve treatment strategies by the development of specific immunomodulatory drugs. By generating AMPA mAbs, investigators aim at finding clues on the RA autoantigen and possibly discover AMPA that either promote or inhibit disease. Another approach is to explore the subset of B cells

responsible for functional AMPA in order to develop treatment targets. Investigations of AMPA in various compartments and tissues give hints to how B cells influence RA pathology. Lastly, to study AMPA B cells early in ACPA+ and AMPA+ individuals without disease would deepen knowledge of when functional AMPA+ B cells are present.

3 RESEARCH AIMS

- Evaluate unique characteristics of AMPA in RA.
- Dissect the functional subgroups of AMPA in RA.
- Identify AMPA in unexplored tissue compartments in RA.

Specific Aims

Paper I

Analyze antigen specificities and mutation patterns of RA patient derived synovial plasma cell mAbs for further investigations of antigen cross-reactivity.

Paper II

Examine the interaction between RA patient derived mAbs and nuclear antigens, in order to understand the triggering mechanisms of autoreactivity and pathogenic roles of AMPA.

Paper III

Utilizing patient derived mAbs to characterize the multireactivity and cross-reactivity to different protein modifications to reveal autoantibody subsets in RA.

Paper IV

Identification and characterization of citrulline-reactive RA patient bone marrow plasma cells by generating single cell derived mAbs.

Paper V

Identification and characterization of MDA/MAA-reactive B cells from the lung and bone marrow of RA patients and their potential preference to different MDA/MAA-modifications.

4 MATERIALS AND METHODS

4.1 PATIENT MATERIAL

Biologic materials from RA patients and healthy controls were used in **Paper I-V**. All studies utilize recombinantly expressed mAbs derived from human B cells from various compartments. In **Paper I-III** and **V**, peripheral blood and synovial fluid samples were collected from patients fulfilling the ACR 1987 [259] and or the EULAR/ACR 2010 [51] criteria at the Rheumatology Clinic at Karolinska University Hospital. In **Paper IV** and **V**, bone marrow samples from RA patients fulfilling the 2010 ACR RA classification [51] and undergoing hip arthroplasty were used. In **Paper V**, bronchoalveolar lavage (BAL) fluid from RA patients fulfilling the 2010 ACR RA classification [51] and healthy controls were used. In **Paper II** and **IV**, human neutrophils were isolated from peripheral blood from healthy donors. Informed consent to use the cells for research was obtained from the patient in accordance with a protocol approved by the Ethical Review Committee North of Karolinska University Hospital in compliance with the Helsinki declaration. In **Paper I,II** and **III**, human peripheral blood samples were also taken from patients in Minnesota [234]. In **Paper III**, human peripheral blood samples were taken from patients in Amsterdam [238]. In **Paper II**, serum samples from 243 RA patients fulfilling the 1987 ACR RA [259] and/or the 2010 ACR/EULAR classification criteria [51] and 157 population-based controls from the Epidemiological Investigation of Rheumatoid Arthritis (EIRA) study [260] were used. Thymus tissue was obtained from children undergoing cardiac surgery. Parents gave informed written consent and the study was approved by the Regional Ethical Board at the University of Gothenburg.

4.2 IDENTIFICATION AND ISOLATION OF SINGLE CELLS

RA patient single B cells from peripheral blood, synovial fluid, bone marrow and BAL were identified and isolated for subsequent mAb production.

Fluorescent Foci Method

To capture IgG secreting cells from RA patient synovial fluid in **Paper I**, we made use of the fluorescent foci method [261]. Mononuclear synovial cells were incubated on a solid phase of magnetic streptavidin beads coupled to biotin-F(ab)2 anti-human IgG and a fluorescent-labeled anti-human Fcγ specific antibody. After incubation on glass slides, a halo of fluorescent IgGs surrounding a single antibody secreting cells was visible under the microscope. The single B cell was picked manually using a micromanipulator. In this way we were able to collect single antibody secreting cells from RA synovial samples without any bias towards antigen recognition. This method has its limitations by being time and labor intensive.

Flow Cytometry Sort

In **Paper I-III,** analysis of mAbs generated from citrulline-reactive peripheral blood B cells isolated using a flow cytometry tetramer-sorting technique developed with collaborators [234]. Citrulline-alpha enolase and filaggrin peptide tetramers were used to capture citrulline-specific

memory B cells for the generation of mAbs. Furthermore, we utilized flow cytometry for analysis and to single sort interesting cell populations. In **Paper IV** and **V**, B cells were first isolated using negative magnetic bead selection, followed by staining with surface markers and single-sorted on general B cell marker CD19 and plasma cell marker CD138 in **Paper IV**. In **Paper V**, surface staining of CD19 was used to isolate all B cells. Index sorting was used to backtrack cells of interest for further B cell subset analysis.

10X

In **Paper IV**, we utilized droplet 10X sequencing technology for B cell repertoire single cell sequencing. Bone marrow B cell suspension were enriched by negative magnetic bead selection. Single cell barcoded droplet RT-PCR generated gene expression and paired VDJ cDNA libraries were generated by sequencing.

B cell Ig Sequencing

Single B cell paired heavy and light chain variable sequencing was performed. Double light chains were excluded with exception of cells expressing both IgM and IgD where the IgM was used in the analysis. Interesting genotypes such as >15 miss-match mutations in heavy and/or light variable regions, carried the N-glycosylation consensus motif N-*X*-S/T (whereby *X* is any amino acid except proline) and clonality defined by paired VH-VL CDR3 amino acid sequences were used for selection of interesting B cell clones for subsequent mAb expression. Sequences were annotated using VQuest towards the international ImMunoGeneTics (IMGT) database [262].

To revert the variable heavy and light transcripts back to predicted germline in **Paper I** and **IV** for validation of SHM contribution, prediction of closest germline version of the ACPA mAb was constructed using the IMGT database with IgBLAST. All predicted SHM were reverted, included identified sites in the J-chain (FRW4). However, due to N-insertion, SHM in the junction cannot be predicted, leaving the CDR3 mostly intact.

4.3 GENERATION OF SINGLE B CELL DERIVED MONOCLONAL ANTIBODIES

To generate mAbs from single B cells, variable Ig heavy and light regions were amplified with RT-PCR. Ig-transcripts were ligated into expression vectors containing either Igγ1, Igλ or Igκ respective constant regions followed by DH5α bacteria transformation. Obtained heavy and light Ig-chain plasmids were co-transfect in Expi293 cells for recombinant monoclonal IgG1 mAb expression (Figure 9). Antibodies were purified on protein G affinity chromatography. A thorough quality control process was performed including SDS-PAGE validation, size exclusion for >90% monomeric protein purity by chromatography, polyreactivity assessment to exclude unspecific binding and endotoxin measurements as outlined in our method report [263]. One mAb was originally generated by genetic programming immortalization with Bcl-6 and Bcl-xL as previously described [238].

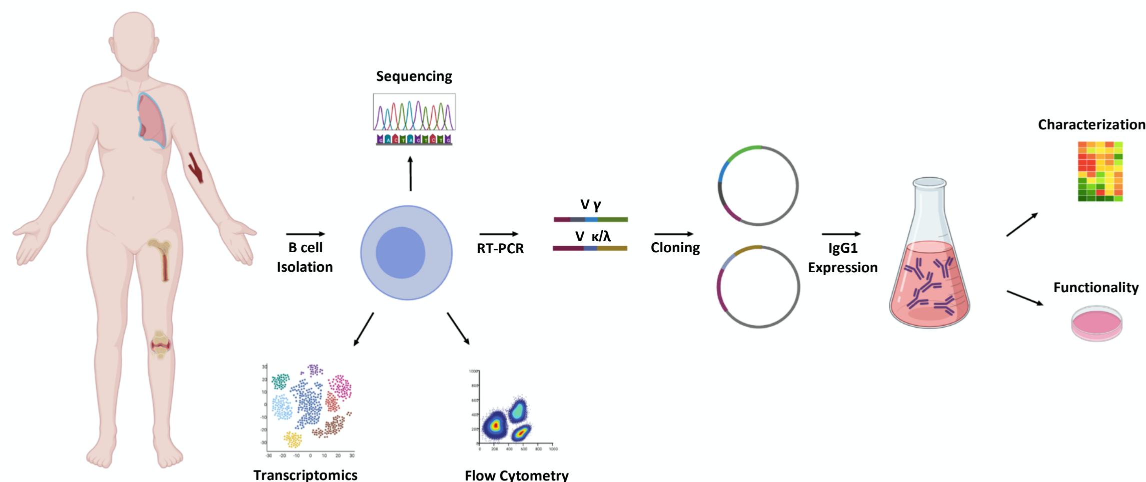

Figure 9. B cell isolation and mAb generation. RA patient B cells from blood, joint, bone marrow and lung were isolated or identified. Single cell sequencing, RT-PCR, vector cloning and subsequent IgG1 expression followed by characterization and functionality studies. Made with Biorender.com.

4.4 PROTEIN AND PEPTIDE MODIFICATIONS

Reactivities against PTM-proteins and peptides are extensively studied in **Paper I-V**. Protein were isolated or recombinantly expressed. Peptides were chemically synthesized with various length designed to contain selected PTM at the amino acid of choice. Antigens were enzymatically or chemically modified to induce the PTM of interest. Modification protocols were optimized throughout the thesis with emphasis on detection methods for validation together with suitable controls. For citrullination, commercially available PAD-enzymes were used. Chemical in house protocols were utilized for carbamylation, acetylation and MDA/MAA-modification. In addition, commercially available modified antigens were used.

4.5 IMMUNOASSAYS

A selection of immunoassays was used to study antibody-antigen interactions. The general immunoassay procedure involves a solid surface for antigen coupling. Antibody binding to target antigen coupled to solid surface followed by secondary fluorophore-coupled antibody and detection by absorbance methods are the main principles for validation of antibody characteristics.

ELISA

Enzyme-linked immunosorbent assay is perhaps the most commonly used method to detect proteins, peptides and other biomarkers in sera. ELISA was used in **Paper I-V** to investigate antibody-antigen interactions and to measure antibodies in patient sera. Protein or peptide antigens are coupled directly to the surface of a 96-well plate or alternatively, if biotinylated, coupled to streptavidin-coated plates. After blocking, investigated antibodies are added for incubation followed by washing and incubation with a secondary mouse, rat or rabbit anti-

human IgG Fc-specific antibody coupled to the enzyme horseradish peroxidase (HRP). The enzyme is activated with a substrate to produce light measured with a spectrophotometer. Antigen coating is usually in native conditions, meaning that if the antigen is a protein, it is likely to stay in native folding although folding may be altered in the procedure.

Western Blot

In **Paper I, II** and **V**, another commonly used immunoassay for detection of proteins was used. By running protein antigen samples on sodium dodecyl sulfate–polyacrylamide (SDS-PAGE) gel electrophoresis, reduced or non-reduced samples were denatured and separated by size and charge. After gel-separation, the protein is transferred to a membrane for antibody detection.

Peptide and Protein Arrays

Array technology was used for efficient evaluation of multiple antigens. Larger arrays with proteins or peptides coupled or printed on a solid surface followed by similar methodology as for ELISA. Incubation with the antibody of interest followed by washing and incubation with a secondary fluorophore-coupled antibody and measurement with spectrophotometer. The RA autoantigen peptide array in **Paper I-V** includes known RA autoantigen peptides. Different variations of similar multiplex peptide array have been used extensively for determine the presence of ACPA fine-specificities [62]. Modified peptides and their native counterparts including peptides from, fibrinogen, a-enolase, vimentin, collagen, histones, filaggrin are being used with a secondary antibody coupled to a fluorophore for detection. In **Paper I** and **III** we made use of a peptide array containing ca 50 000 citrulline and homocitrulline in situ-synthesized peptides and their native counterparts. Peptides were 16-mer with overlaps covering in total 1439 extracellular matrix and RA-related proteins. Fluorescence intensity (FI) was measured and the cut off for positivity was set to 5x the 98th percentile FI for non-ACPA control mAb. An array consisting of 20-mer histone peptides including a range of PTMs was used in **Paper III**. Cutoff was set to 3x the 98th percentile of FI values compared to non-ACPA control mAb. The protein array used in **Paper III** contain >20 000 *E. coli* on-array expressed protein fragments from >6000 genes from human fetal brain cDNA. Citrullination was performed with rabbitPAD and alkaline phosphatase conjugated secondary mAb together with substrate for FI detection followed by visual scoring. The large arrays with multiple antigens in **Paper I** and **III** enabled the illustration of consensus binding motifs by the flanking amino acids next to the PTM of interest.

4.6 MASS SPECTROMETRY

MS was used to validate and detect modified peptides in various tissues and cell suspension in **Paper II** and **V**. In **Paper I**, immune precipitation followed by MS was used to detect antibody-antigen interactions in solution. ACPA together with spiked citrullinated fibrinogen was immune precipitated and visualized on Coomassie stained SDS-PAGE gel for MS detection. In **Paper II** apoptotic cell antigens were immunoprecipitated with ACPA for MS detection.

4.7 CELL SYSTEMS

Binding to Neutrophils and NETs

Neutrophils from healthy donor whole blood were isolated using Ficoll separation. In **Paper II**, human neutrophils or murine β-estradiol differentiated ECoM-G cells were stimulated with ionomycin for 4h to induce NETosis. PAD enzyme inhibition was performed with Cl-amidine. Guide RNA vector targeting the *Padi4* gene was electroporated into ECoM-G cells for PAD4 enzyme removal. Cells were fixed and permeabilized for murine chimeric ACPA IgG2a mAb staining followed by secondary anti-mouse AlexaFluor488 for analyze with immunofluorescence microscopy. In **Paper IV**, stimulation with Ca^{2+} ionophore A23187 activated cells in culture for 45 min. Cells were fixed and permeabilized for intracellular staining with ACPA mAb and a secondary mouse anti-human IgG APC for flow cytometry analyze.

Binding to Thymus

In **Paper II**, human thymus tissues were embedded in pre-cooled isopentane were sectioned for staining with biotinylated mAbs followed by secondary streptavidin-AlexaFluor555 for fluorescence microscopy analyze. Alternatively, thymocyte single cell suspensions were prepared by enzymatic and mechanic digestion for staining with biotinylated mAbs followed by streptavidin-APC for flow cytometry analyze.

Binding to Apoptotic cells

In **Paper II**. Murine thymocytes were isolated and apoptosis was induced with dexamethasone followed by mAb staining with the addition of secondary biotin-coupled goat anti-human IgG for detection with streptavidin-PE. Antibody binding and apoptosis with Annexin 7 was analyzed with flow cytometry. Alternatively, apoptosis was induced in Jurkat cells by etoposide after pre-incubation with PAD or histone deacetylase (HDAC) inhibitors. Modified histone 2b was detected in the cell lysates with WB.

Osteoclast Assay

In **Paper I,** Ficoll separation and CD14 magnetic beads positive separation of monocytes from ACPA+ RA patient whole blood or buffy coats were differentiated into macrophages with macrophage colony-stimulation factor (M-CSF) for 3 days. Differentiation into osteoclasts with M-CSF, RANKL and ACPA mAb. Osteoclasts were analyzed with tartrate-resistant acid phosphatase (TRAP) staining and cells with minimum 3 nuclei were counted as osteoclasts using light microscopy. Erosions by osteoclasts grown on synthetic calcium-phosphate coated plates were quantified after 14-18 days by measuring resorption area using NIS elements software.

4.8 ADMINISTRATION OF APOPTOTIC CELLS *IN VIVO*

In **Paper II**, thymocytes from 4 to 5 weeks old sex-matched mice were cultured with apoptosis inducer dexamethasone for 6h. Generated apoptotic cells together with ACPA mAb were

transferred into mice. After 4 weeks, serum was collected and mouse antibodies against DNA, Ro-52, Ro-60, SmD1, SS-B and citrullinated vimentin, fibrinogen and histone 2b were measured with ELISA.

4.9 STATISTICAL ANALYSES

In **Paper I,** differences in the numbers of mutations and CDR3 were determined by Mann-Whitney nonparametric test. For the osteoclast *in vitro* studies, differences were considered statistically significant if $P \leq 0,05$ by using one-way Kruskal-Wallis test followed by Dunn's test for multiple comparisons. In **Paper II**, serological measurements had a non-Gaussian distribution and therefore t-test with Welch correction for unequal standard deviation for comparing continuous variables between groups. Spearman analysis was used for correlation analysis. For *in vitro* assays, $P \leq 0,05$ were considered significant using Student's t-test. In **Paper IV,** multiple comparisons of the Ig repertoire analysis were significant when $P \leq 0,05$ using Fisher's exact test or Dunn's joint rank method.

5 RESULTS

5.1 PAPER I

Citrulline reactive synovial plasma cell derived mAbs recognize amino acid motifs rather than specific proteins

In this study, we aimed to investigate the presence of ACPA-secreting B cells in the RA synovium. By isolating synovial fluid antibody secreting cells using the foci method [261], paired heavy- and light-chain variable Ig sequences from 182 antibody secreting cells were generated. 93 of those were recombinantly expressed as IgG1. Four out of 93 expressed mAbs were identified as ACPA with the CCP2 test. ELISA screenings of fibrinogen, histone and vimentin cit-peptides and PAD-citrullinated proteins revealed mAb binding to multiple cit-targets with unique binding patterns for each mAb without any reactivity to native antigens. We further analyzed the antibodies on a large peptide array containing >53 000 cit-peptides and >49 000 carb-peptides from extracellular matrix proteins and known RA autoantigens. By aligning the amino acids from the positive peptides with citrulline centralized, we could identify the autoantibody consensus binding motifs. The importance of glycine in +1 position to the citrulline and the homocitrulline was recognized. The B cell Ig sequence analysis showed that all ACPAs had high numbers of SHM that also introduced Fab variable N-glycosylation sites in comparison to non-ACPA, features that were in-depth studied in a separate report [249]. In addition, we noted replacement mutations in the CDRs and shorter heavy chain CDR3 compared to non-ACPA. One of the four ACPAs was clonally expanded by the identification of three clones with identical VDJ-gene usage and identical CDR3 nucleotide sequences. Next, we reverted the variable regions to closest predicted germline in the IMGT database for subsequent mAb analysis. When predicted germline reverted expressed mAbs were analyzed for binding to cit-peptides, reactivity was reduced or lost for two ACPA mAbs. However, one ACPA mAb gained reactivity when the variable heavy chain was left mutated and only the variable light chain was reverted back to predicted germline (Figure 10) (Table 2).

Differential effects of ACPA mAb on osteoclasts

Monocytes from ACPA+ patients were isolated and differentiated into osteoclasts together with ACPA mAb or patient derived control mAb. TRAP stained osteoclasts and bone erosion capacity was measured. One out of four ACPA mAbs had osteoclast promoting and bone erosion effects *in vitro* compared to patient derived control mAb (Figure 11).

Conclusions Paper I

We have identified ACPA producing plasma cells locally in the synovium of RA patients that may have direct functional effects by promoting osteoclastogenesis. By using monoclonal ACPA we could find that the immunoglobulins expressed by ACPA+ plasma cells in the RA joint displayed features of high SHM rates, affinity maturation introduced Fab variable N-glycosylation and multireactivity to cit-peptides and citrullinated proteins with unique

individual binding patterns. We recognize glycine in +1 position to the citrulline for ACPA mAb binding and that two out of four ACPA mAbs cross-reacted with carb-peptides.

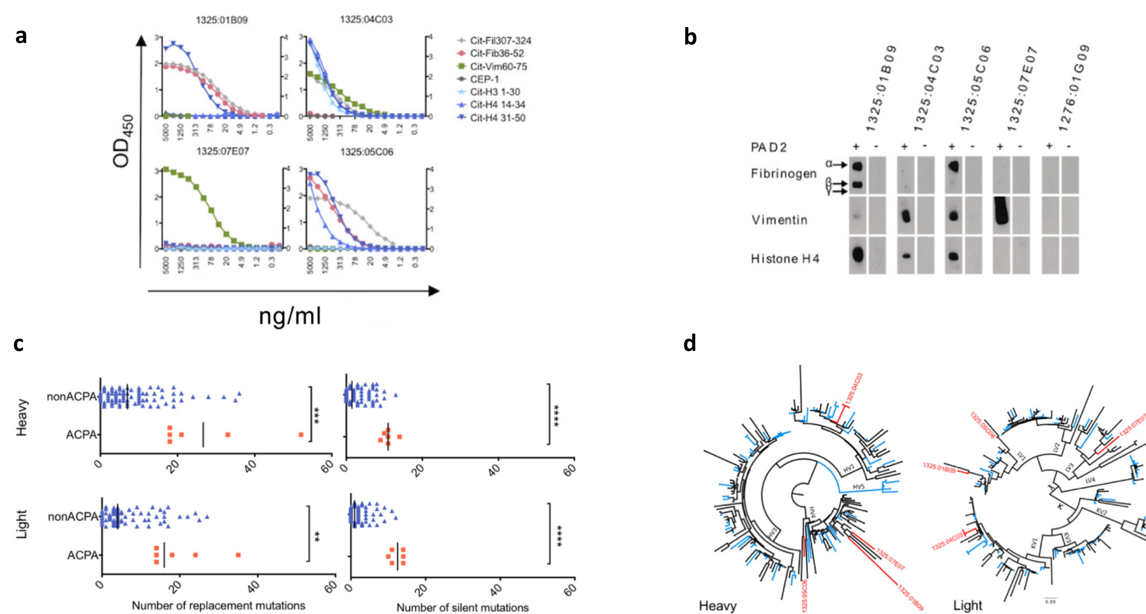

Figure 10. Synovial plasma cell ACPA. a, ACPA mAb binding to cit-peptides. ELISA 1:2 titration curves for each ACPA mAb starting at 5 µg/ml. Fil=filaggrin, Fib=fibrinogen, Vim=vimentin, CEP=alpha enolase, H=histone. **b**, Western blot binding of 1 µg/ml ACPA mAb or control mAb to 10 µg PAD2 citrullinated proteins. **c**, The number of replacement and silent mutations in the variable heavy- and light-chains for non-ACPAs (n = 78) and ACPAs (n = 6). One ACPA clone had in total three identical sequenced cloned. **d**, Phylogenetic trees for heavy-chain and light-chain sequences. Non-ACPA mAbs in blue, ACPA mAbs in red. Not expresses clones in black. The phylogenetic trees were generated using Phylogeny.fr with MUSCLE alignment and visualized by FigTree.

Figure X. ACPA reactivities and characteristics. a, ACPA mAb binding to cit-peptides, ELISA 1:2 titration curves for each ACPA mAb starting at 5 µg/ml, Fil=filaggrin, Fib=fibrinogen, Vim=vimentin, CEP=alpha enolase, H=histone. **b**, western blot binding of 1 µg/ml ACPA mAb or control mAb to 10 µg PAD2 citrullinated proteins. **c**, The number of replacement and silent mutations in the variable heavy- and light-chains for non-ACPAs (n = 78) and ACPAs (n = 6). One ACPA clone had in total three identical sequenced cloned. **d**, Phylogenetic trees for heavy-chain and light-chain sequences. Non-ACPA mAbs in blue, ACPA mAbs in red. Not expresses clones in black. The phylogenetic trees were generated using Phylogeny.fr with MUSCLE alignment and visualized by FigTree.

and **b**, citrullinated proteins.

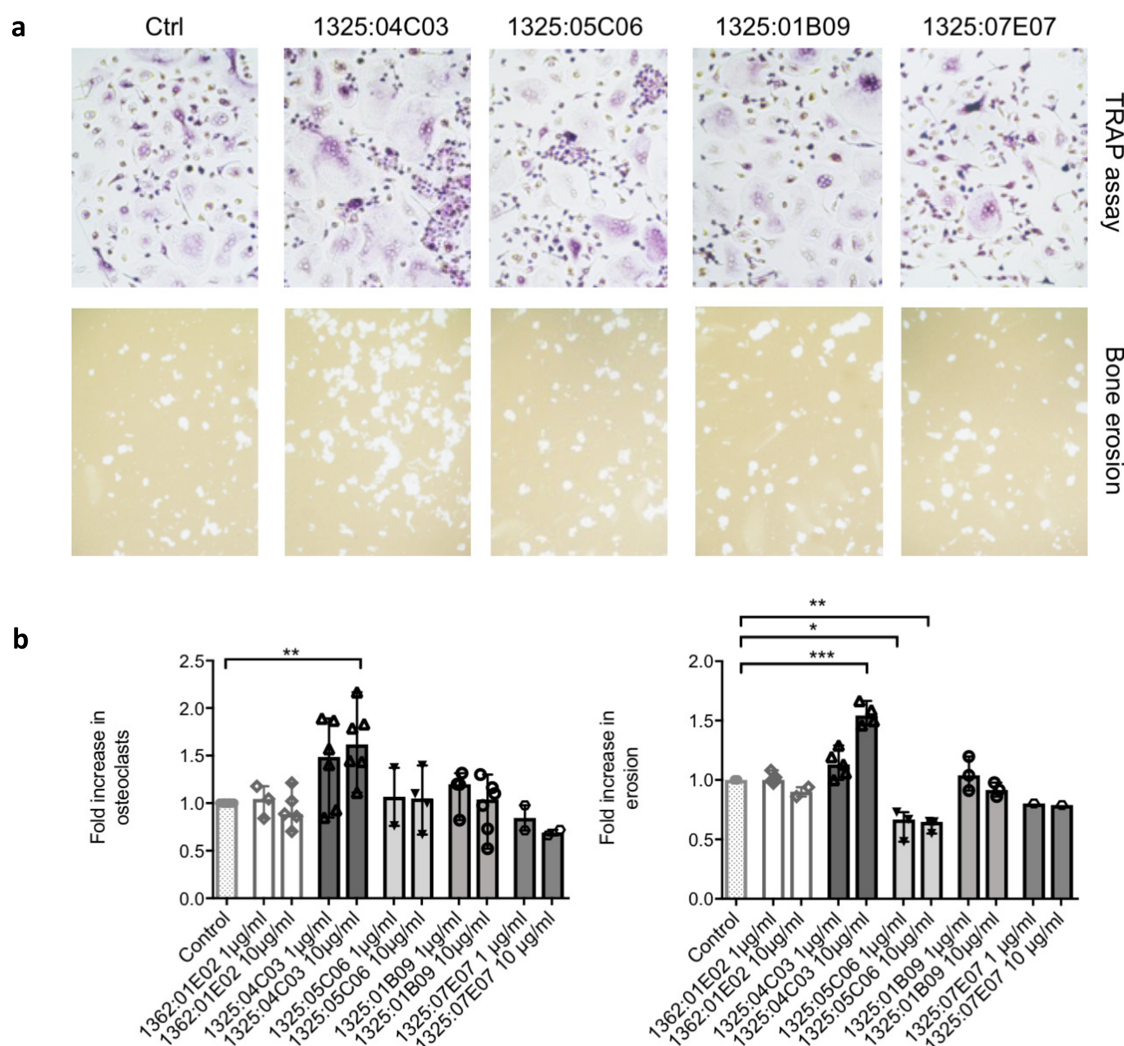

Figure 11. **Effects of ACPA mAbs on osteoclast differentiation and bone erosion**. **a**. Top, osteoclast differentiation in the presence of ACPA mAb or control mAb. TRAP stained cells with >3 nuclei were defined as osteoclasts. Bottom, ACPA mAb or control mAb effect on osteoclast bone erosion. **b**, Fold increase in osteoclast numbers and bone erosion. Results are the mean ± SEM of 1–6 samples per group. * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$, by Kruskal-Wallis test followed by Dunn's test for multiple comparisons.

5.2 PAPER II

ACPA mAbs bind nuclear antigens

To further dissect the direct involvement of ACPA in the RA joint pathogenesis we investigated ACPA binding to nuclear antigens and apoptotic cells. Within the inflamed joints of RA patients, neutrophils have been suggested for involvement in the pathogenesis by their unique form of apoptosis known as NETosis. NET products have been suggested as source of autoantigens in RA but not much is known about autoreactivities to nuclear antigens and apoptotic cells. Histones in particular have been suggested as autoantibody targets in this context [118]. The RA associated citrullinating enzymes PAD2 and PAD4 are involved in inflammatory processes and are expressed in the RA joint [264]. Enhanced NET-production in

the RA joint could provide an important source of ACPA autoantigens such as histones [208]. We screened RA patient and healthy control serum samples for antibody reactivity against full-length citrullinated histone 2B (His2B) and its native counterparts. The screening revealed that ACPA+ patients had higher levels of autoantibodies against citrullinated histones compared with ACPA- patients (p<0,0001) and controls (p=0,0005). In total 29% of ACPA+ patients had anti-cit His2B reactivities after normalization for His2B. Next, we investigated the reactivity of 10 RA patient derived ACPA mAbs for reactivity against citrullinated histones and different citrullinated heterogeneous ribonucleoproteins (hnRNPs), which are RNA binding proteins. Four of the 10 ACPA mAbs are the synovial plasma cell derived mAbs used in **Paper I.** The remaining six are previously described cit-tetramer sorted peripheral blood memory B cell derived ACPA mAbs (Table 2) [234]. All clones but two bound citrullinated nuclear histone and hnRNP proteins in ELISA settings. We investigated the ACPA mAbs using a nuclear antigen antibody (ANA) Hep-2 staining test showing that some but not all of the citrulline-histone positive ACPA had high nuclear binding in ANA.

ACPA mAbs bind acetylated histones in apoptotic cells

We investigated binding to apoptotic cells. By treating the human Jurkat T cell line with anti-Fas and by treating murine thymocytes with dexamethasone, we could investigate binding to early and late apoptotic cells using flow cytometry. A subset of the citrullinated nuclear antigen reactive ACPA mAbs also bound apoptotic cells. The later apoptotic stage was predominantly recognized. Surprisingly, co-incubation with PAD-inhibitor did not affect ACPA binding to the apoptotic cells. The finding suggests PAD-independent binding. Immunoblotting of the murine thymocyte lysate revealed that the ACPA-binding proteins with sizes around 14 kDa and 17 kDa turned out to be histones by MS analyze. Interestingly, the IP-MS analysis revealed that the histones were acetylated instead of citrullinated. In addition, acetylated histone reactivity was confirmed in deacetylation inhibited Jurkat cells analyzed with Western blot and by analyzing reactivity against acetyl-histone peptides with ELISA (Figure 12). Furthermore, the acetylated histone binding ACPAs were associated with positivity by the ANA test, suggesting that the nuclear antigens detected are acetylated.

ACPA mAbs target histones in NETs

Next, we used two different systems to investigate nuclear ACPA binding to activated neutrophils and NETs. We used human primary neutrophils and the murine myeloid stem cell line ECoM-G for stimulation with PMA and ionomycin to induce NETosis *in vitro*. A subset of ACPAs bound NETosis induced neutrophils as analyzed with fluorescent microscopy. The non-nuclear binding ACPA bound cytoplasmic targets. The PAD4 inhibitor Cl-amidine was added to the Ca^{2+} dependent ionomycin induced NETosis which reduced the binding of the cytoplasmic binding ACPA whereas NETosis binding of the nuclear binding ACPAs were attained. This suggests PAD-independent ACPA reactivity. By applying a CRISPR-Cas 9 approach to generate ECoM-G PAD4 knock out, NETosis was decreased but, intriguingly, for two of the ACPA some binding remained to PAD4 knock-outed NET-induced cells.

Conclusions Paper II

Results reveal a subset of ACPA mAbs that bind to acetylated histones which can mediate binding to nuclear structures in apoptotic cells and activated neutrophils in a PAD-independent way. RA patient B cell derived ACPA mAbs can have ANA reactivity and target apoptotic cells and NETs. Hence, while considerable NET-binding of ACPA can be explained by targeting citrullinated protein, a subset of ACPA may also target NETs in a PAD-independent manner through binding to acetylated histones.

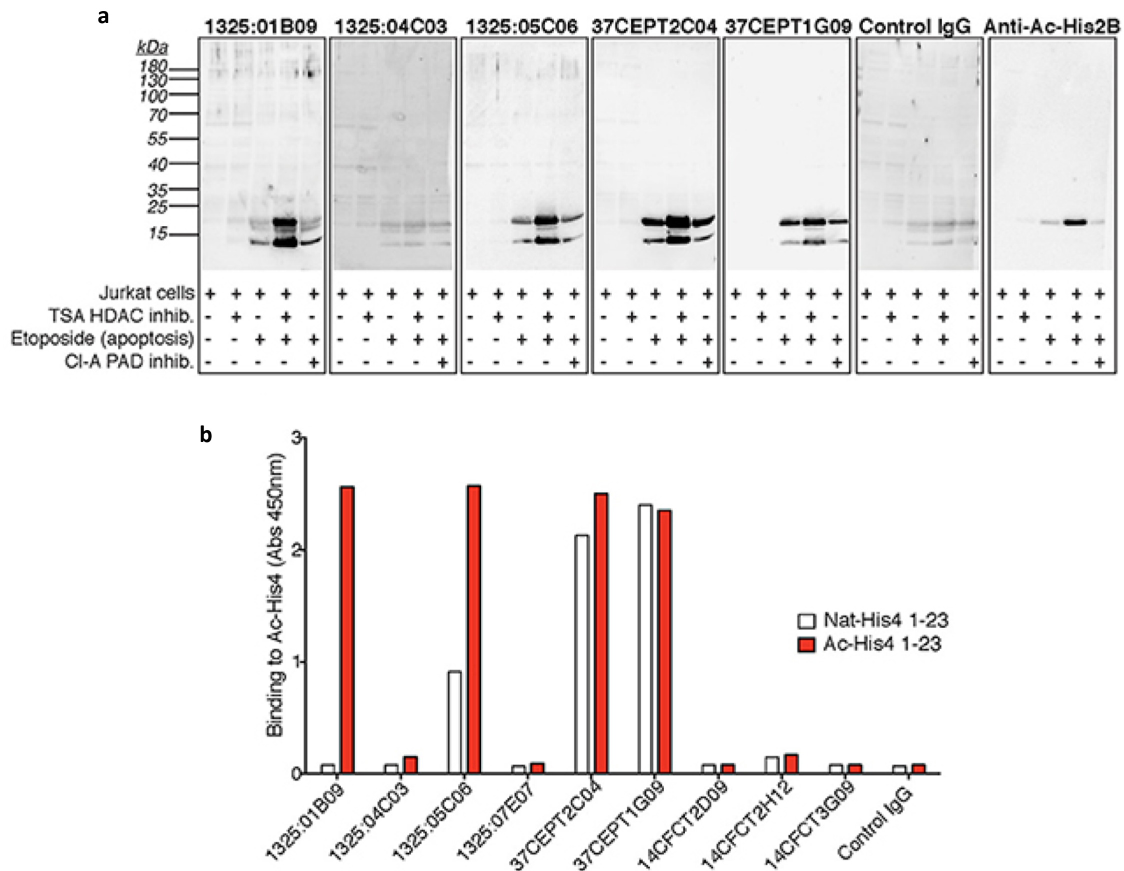

Figure 12. ACPA mAb reactivity to acetylated histones. a, Western blot binding of 5 µg/ml ACPA mAb to acetylated histones in apoptosis induced and histone deacetylation inhibited Jurkat cell lysates. Anti-acetylated histone 2B antibody was used as control. **b**, ELISA of 5 µg/ml ACPA mAb reactivity to biotinylated acetyl-histone 4 peptide compared to native control peptide.

5.3 PAPER III

Differences in ACPA mAb cit-peptide multireactivity is explained by consensus binding motif

In this study we substantially extend the previous efforts in **Paper I** and **Paper II** showing that ACPA mAbs are multireactive to cit-peptides with unique binding patterns and that ACPA can bind to carbamylated and acetylated peptides In **Paper III,** we aimed to in depth understand the biochemical interaction of ACPA and explain the extent of multireactivity and cross-reactivity of individual ACPA positive autoreactive B cells in RA. As tools for exploring

ACPA reactivity we used a panel of RA single B cell derived ACPA mAbs from different patients, B cell compartments, and tissues. Large peptide and protein arrays covering in total 7898 modified antigens with different PTMs. 12 RA patient single B cell derived ACPA mAbs were analyzed. Four ACPA mAbs from synovial plasma cells identified in **Paper I**, six ACPA mAbs from cit-tetramer sorted circulating memory B cells [234], one ACPA mAb from a synovial memory B cell [238] and one from circulating memory B cell [249]. With the extensive analysis, we learned that all 12 ACPA mAbs shared features of high levels of SHM and contained Fab variable N-glycosylation sites introduced by SHM during germinal center responses (Table 2). All 12 ACPA mAbs were CCP2 positive and reacted to multiple RA associated cit-peptides, although with distinct unique binding patterns (Figure 13). In addition, by using the same large cit- and carb-peptide array as in **Paper I,** we learned that the investigated ACPA mAbs bound multiple cit-peptides and that some ACPA mAbs, but not all, bound carb-peptides. The glycine in +1 position to the citrulline was present in several ACPA mAbs but also other consensus binding motifs were identified. The head to head comparison enabled recognition of additional consensus binding motifs as well as motifs outside of the +1 and -1 position to the citrulline. In addition, we learned that the consensus binding motif is influenced by neighboring amino acid charge and polarity. The motifs were not dependent on B cell subtype or tissue origin.

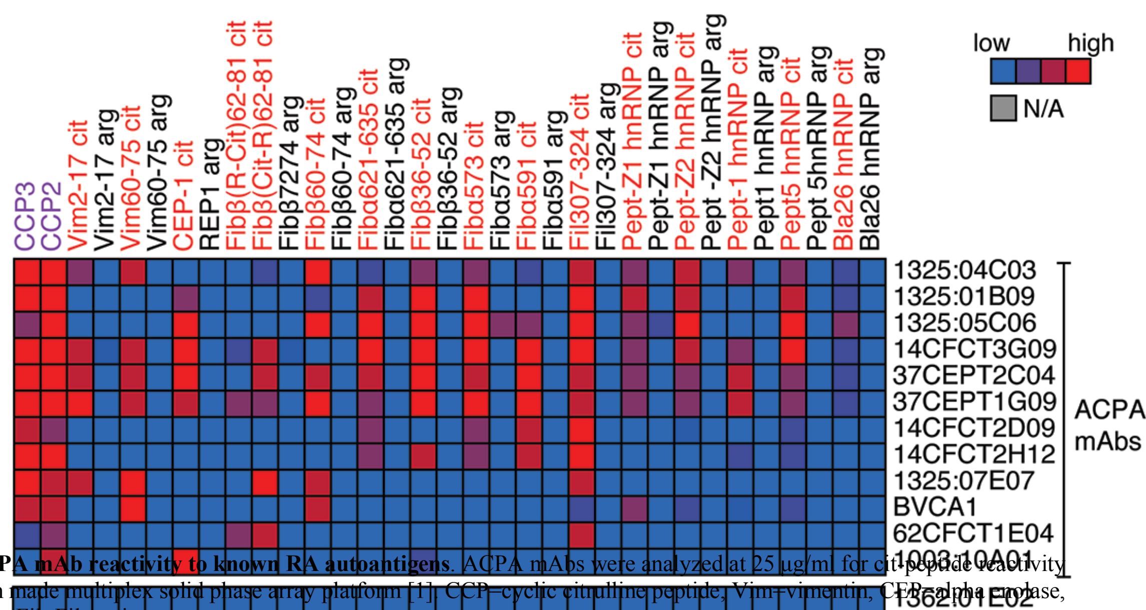

Figure X. **ACPA mAb reactivity to known RA autoantigens**. ACPA mAbs were analyzed at 25 µg/ml for cit-peptide reactivity using a custom made multiplex solid phase array platform [1]. CCP=cyclic citrulline peptide, Vim=vimentin, CEP=alpha enolase, Fib=fibrinogen, Fil=Filaggrin.

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Large extent of ACPA mAb multireactivity to citrullinated proteins

The peptides and proteins present on arrays are usually preselected by RA association or with specific tissue or compartment origin. In **Paper III**, we aimed at performing an as unbiased large protein array validation as possible. The protein array of choice consisted of >6800 genes expressed on a PVDF membrane. Three of the ACPA mAbs described in **Paper I and II** were selected for analysis. The three ACPA mAbs bound multiple citrullinated protein fragments

with distinct individual binding patterns. The sub analysis displayed a binding bias for nuclear RNA-binding and protein-binding proteins.

Histone PTM peptide array reveals ACPA mAb cross-reactivity restriction to carbamylated and acetylated antigens

A histone peptide array containing a range of PTMs show that a subset of carb-peptide reactive ACPA also bound acetyl-histone peptides. No other PTM than citrulline, carbamyl or acetyl were detected by any of the 12 analyzed ACPA mAbs. Consensus binding motifs for the 20 strongest binding peptides for each ACPA mAb were constructed. Interestingly, by analyzing the 20 strongest hits on the array, we learned that acetyl-peptide cross-reactive ACPA mAbs preferred binding to acetyl-peptides over cit-peptides (Figure 14). This finding was confirmed with competition ELISA using cit- and acetyl-histone peptides.

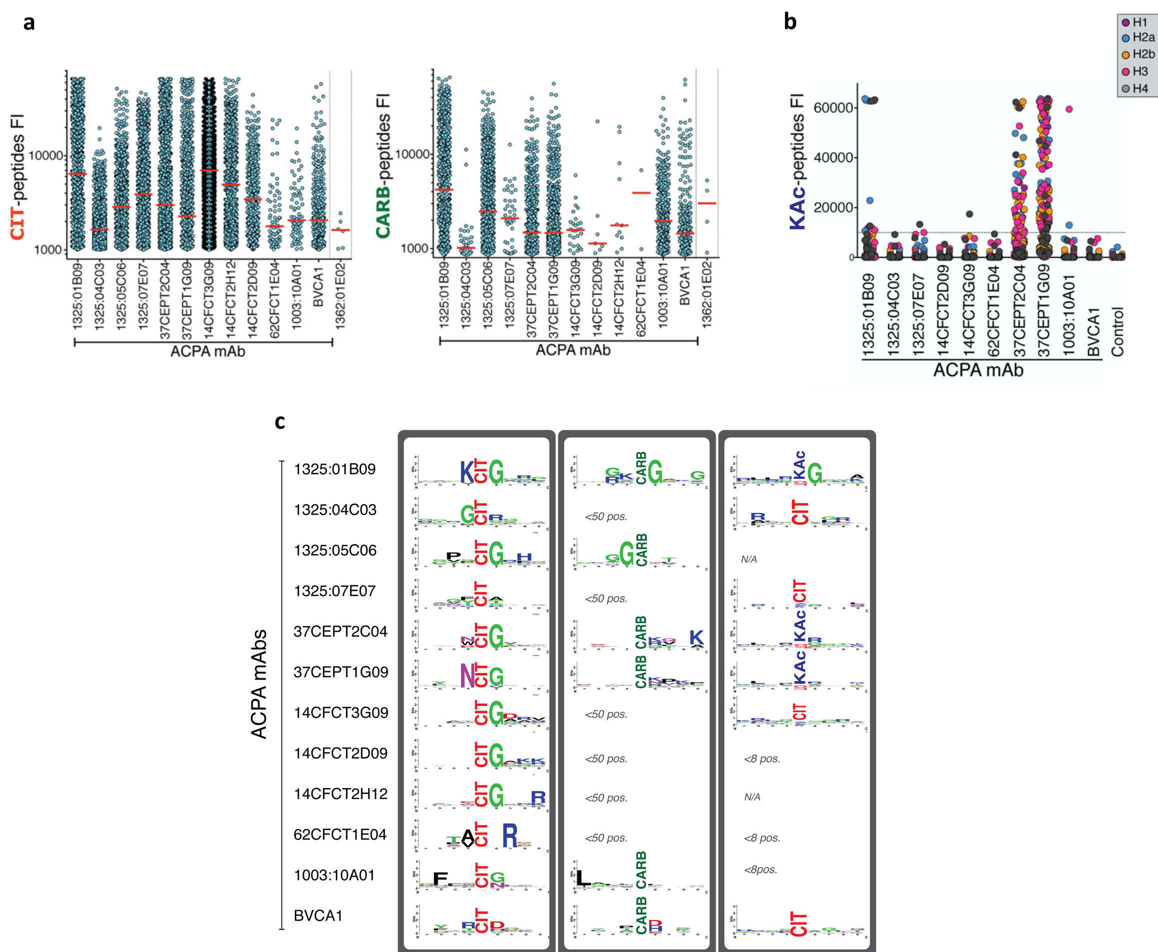

Figure X. ACPA mAb multireactivity to cit-, carb- and kac-peptides enables consensus binding motif comparison across platforms. **a,** ACPA mAb binding to array containing 53,019 cit-peptides and 49,211 carb-peptides from extracellular matrix proteins and known RA autoantigens. **b,** ACPA mAb binding array containing 314 kac-peptides from histones. The array also contained 132 cit-peptides and other PTM-peptides (data not shown). FI=fluorescence intensity. **c,** Consensus binding motif was generated for the 20 peptides with the highest FI values using the Weblogo server.

Figure 14. ACPA mAb multireactivity to cit-, carb- and kac-peptides enables consensus binding motif comparison across platforms. a, ACPA mAb binding to array containing 53,019 cit-peptides and 49,211 carb-peptides from extracellular matrix proteins and known RA autoantigens. **b,** ACPA mAb binding array containing 314 kac-peptides from histones. The array also contained 132 cit-peptides and other PTM-peptides (data not shown). FI=fluorescence intensity. **c,** Consensus binding motif was generated for the 20 peptides with the highest FI values using the Weblogo server.

Conclusions Paper III

ACPA mAbs bind multiple cit-peptides and citrullinated proteins to a very large extent. The ACPA mAb consensus binding motif range over neighboring amino acids additional to glycine in +1 with the influence of charge and polarity. The ACPA mAb cross-reactivity is restricted to carbamylated and acetylated antigens. Interestingly, one ACPA mAb displayed apparent higher affinity for acetylated antigens compared to citrullinated antigens.

5.4 PAPER IV

RA patient bone marrow plasma cell repertoire

In **Paper I,** ACPA+ antibody producing plasma cells were identified in the RA synovium. ACPA memory B cells and plasmablast have been described in the circulation by other groups [193, 234, 265]. Interestingly ACPA are still detectable at relatively stable levels over time after CD20 B cell depletion treatment which suggest ACPA production by long lived plasma cells in the bone marrow. In **Paper IV,** we used two single cell approaches, flow cytometry sorting and transcriptomics to investigate bone marrow plasma cell repertoire in four ACPA+ and one ACPA- RA patients. More than 900 paired heavy and light Ig chains from BM plasma cells defined either by surface CD138 expression or RNA expression of plasma cell genes including MZB1, JCHAIN and XBP1 (Figure 15). As expected, the bone marrow plasma cells were dominated by class switched affinity maturated IgA and IgG expressing cells with high SHM. The transcriptomics analysis revealed that the frequencies of variable N-glycosylation sites were 35% for the ACPA+ patient compared to 17% for the ACPA- patient. Both 10X investigated patients had higher frequency of IgA1 compared to IgG1 positive BM plasma cells. Interestingly, the ACPA+ patient had detectable IgG4+ plasma cells that were not detectable in the ACPA- patient. Furthermore, the ACPA+ patient had lower frequencies of IgA2+ cells compared to the ACPA- patient (11% vs 20%). Expanded BM plasma cell clonotypes defined by identical paired HCDR3 and LCDR3 amino acid sequences were identified in four out of five patients. Furthermore, the finding of identical heavy chains but different light chains in bone marrow plasma cells in four of the patients may indicate receptor revision.

Discovery of ACPA plasma cells in RA patient bone marrow

In **Paper I-III,** we described ACPA mAb features of high SHM and Fab variable N-glycosylation sites (Table 2). In **Paper IV,** we used these features as criteria for selecting bone marrow plasma cell clones for IgG expression. We expressed in total 44 plasma cell mAbs. All but one originally IgGs and one IgA. We expressed 38 bone marrow plasma cell mAbs from ACPA+ patients and 6 from the ACPA- patients. Out of those 38 ACPA+ patient plasma cell mAbs, 11 were identified by transcriptomics using 10X droplet barcode sequencing and the remaining were flow sorted. We discovered two flow sorted, and one 10X identified CCP2-reactive mAbs with individual binding-patterns to cit-peptides without binding to the native counterparts. None of the ACPA clones were expanded and no ACPA-related clonotypes could be identified based on VH-VL gene usage. Two of these ACPA mAbs displayed high

CCP2 reactivity and recognized multiple citrullinated peptides including cit-histone 4. The third ACPA mAb, with low CCP2 reactivity, was reactive to nuclear RNA-binding protein cit-hnRNP A1. When all of the SHMs were reverted back to predicted germline, the citrulline reactivity was lost for the two multi-reactive ACPA mAbs with strong CCP2 reactivity but attained for the cit-hnRNP A1 binding ACPA mAb with low CCP2 reactivity. The two multi-reactive and cit-histone 4 binding ACPA mAbs bound Ca^{2+} ionophore activated neutrophils *in vitro* as analyzed with flow cytometry (Figure 16).

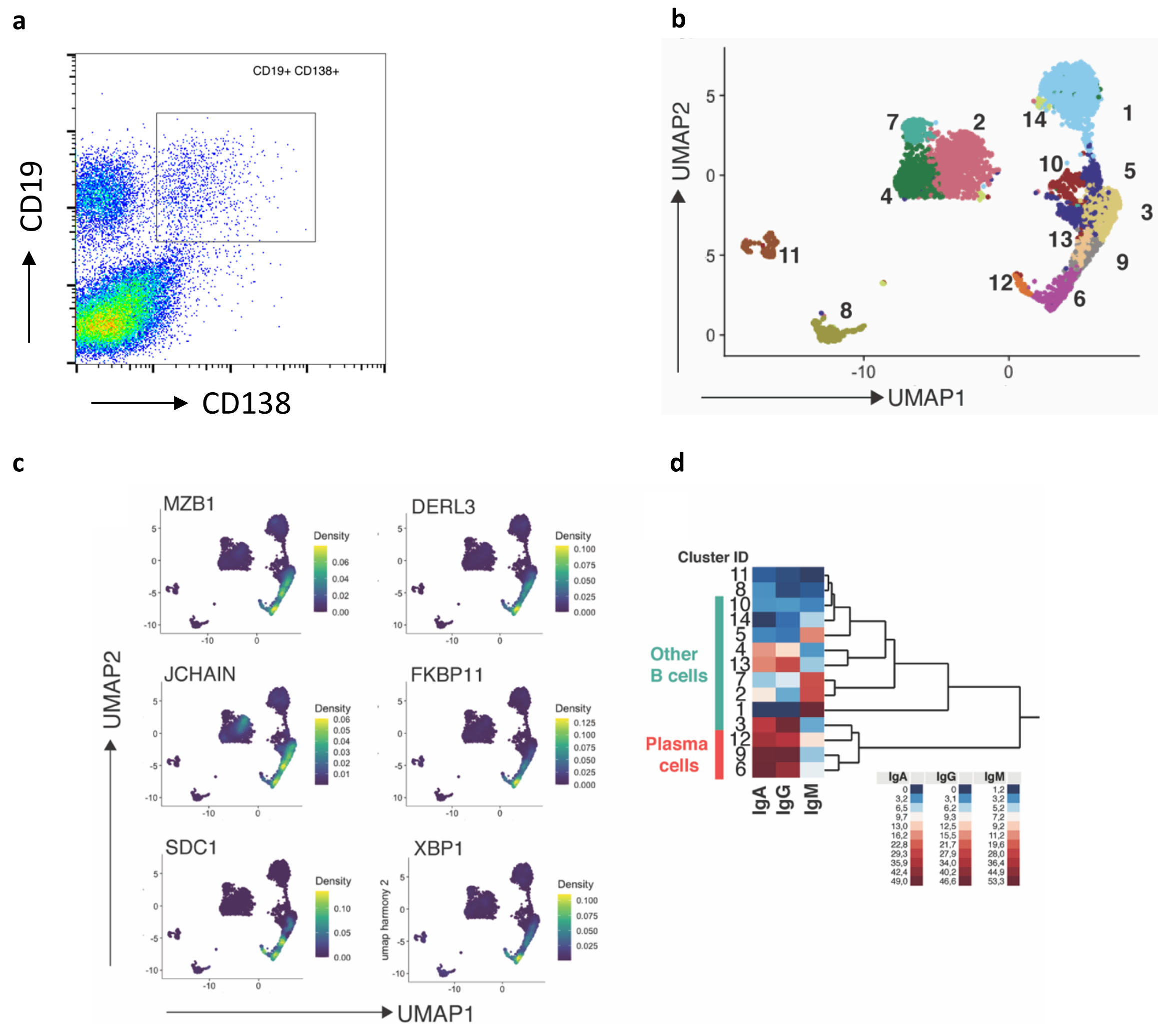

Figure 15. Bone marrow plasma cell identification. a, Gating of CD19+ CD138+ display bone marrow plasma cell distribution. **b**, Cluster analysis of transcriptomics data from bone marrow mononuclear cells of two RA patients integrated with Harmony and visualized with UMAP. Expression profiles identified cell clusters as plasma cells (clusters 6, 9, 12, 13), other B cells (cluster 1-5, 7, 10, 14) monocytes (cluster 8) or T cells (cluster 11). **c**, UMAP density plot showing expression of selected plasma cell markers. **d**, Frequency of events positive for paired heavy-light chain immunoglobulins in the different clusters by isotype.

Figure X. **Bone marrow plasma cell identification**. **a**, Gating of CD19+ CD138+ bone marrow plasma cells for flow cytometry sorting. **b**, Cluster analysis of transcriptomics data from bone marrow mononuclear cells of two RA patients integrated with Harmony and visualized with UMAP. Expression profiles identified cell clusters as plasma cells (clusters 6, 9, 12, 13), B cells (cluster 1-5, 7, 10, 14) monocytes (cluster 8) or T cells (cluster 11). **c**, UMAP density plot showing expression of selected plasma cell markers. **d**, Frequency of events positive for paired heavy-light chain immunoglobulins in the different clusters by isotype.

Conclusions Paper IV

By analyzing the RA patient bone marrow plasma cell repertoire, we observed higher frequencies of Fab variable N- ACPA- RA patient and we ga marrow plasma cell ACPA m

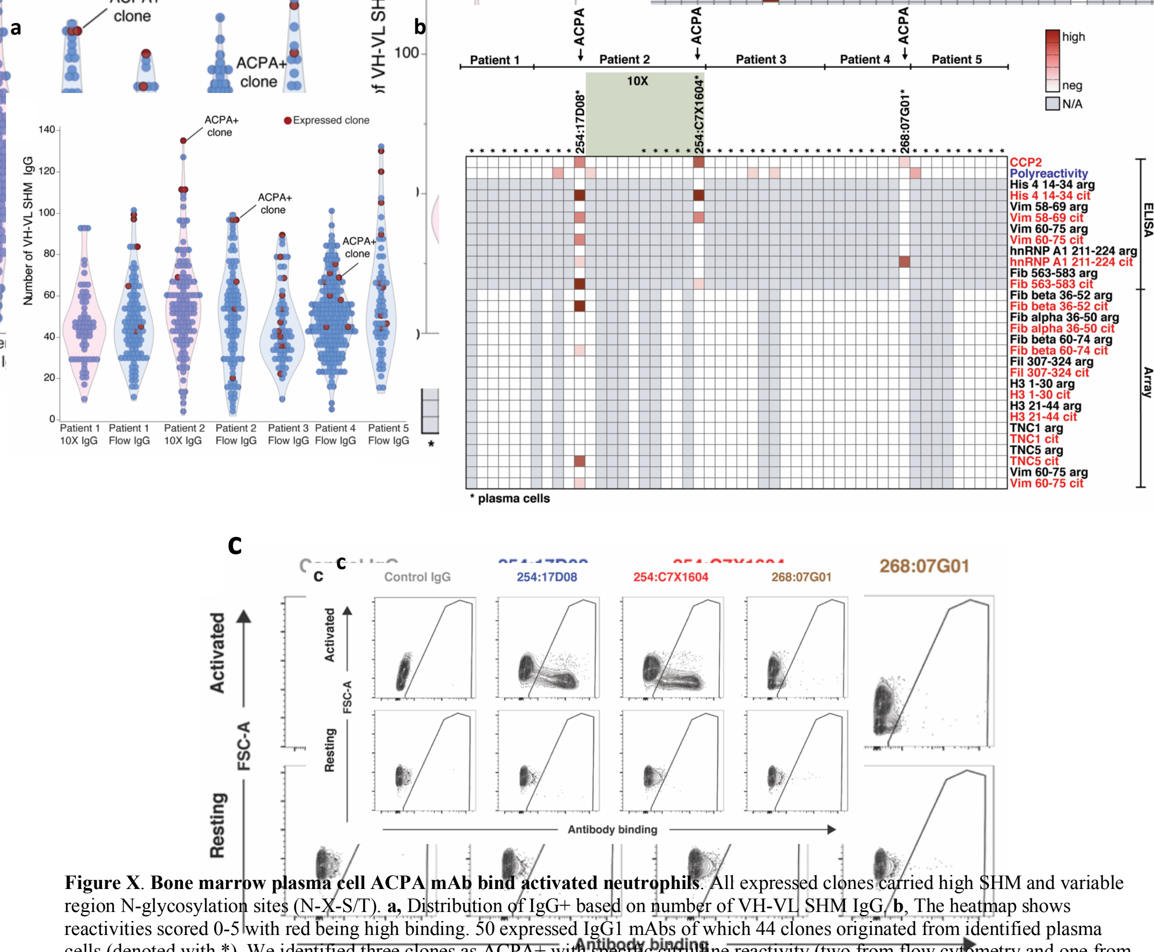

Figure X. **Bone marrow plasma cell ACPA mAb bind activated neutrophils**. All expressed clones carried high SHM and variable region N-glycosylation sites (N-X-S/T). **a,** Distribution of IgG+ based on number of VH-VL SHM IgG. **b**, The heatmap shows reactivities scored 0-5 with red being high binding. 50 expressed IgG1 mAbs of which 44 clones originated from identified plasma cells (denoted with *). We identified three clones as ACPA+ with specific citrulline reactivity (two from flow cytometry and one from 10X transcriptomics) from two different ACPA-positive RA patients. **c**, ACPA and predicted germline mAb binding to activated ionophore neutrophils evaluated by flow cytometry. The figure shows data from a representative experiment.

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5.5 PAPER V

Autoreactive Malondialdehyde-protein B cells in the bone marrow and lung of ACPA+ RA patients

MDA and MAA are protein modifications that lately are gaining more attention in the field of RA. MDA is a byproduct from oxidation induced lipid peroxidation that preferentially form adducts on lysines. The addition of acetaldehyde, possibly from smoking or infection, give rise to the fluorescent MAA-adduct. Furthermore, MDA and MAA form intra-protein cross-links that changes protein structure and possibly creates neo-epitopes targeted by antibodies. Germline encoded anti-MDA/MAA IgM antibodies are likely beneficial by clearing modified proteins and apoptotic material. Interestingly, both IgM and IgG isotypes are elevated in autoimmune conditions such as RA. We have previously generated MDA-reactive osteoclast promoting mAbs from RA patient synovial B cells. These MDA-mAbs are not cross-reactive with citrullinated antigens. However, their preferential binding to different MDA/MAA-modifications are unresolved [147, 203]. In **Paper V**, we aimed at investigate the presence of MDA/MAA-reactive B cells in the bone marrow and lung of ACPA+ RA patients and ACPA+ individuals at risk of developing RA for evaluation of target specificity.

We analyzed bone marrow plasma cell mAbs from **Paper IV** together with patient derived lung mAbs for reactivity against MDA-BSA. We found MDA-BSA reactivity in two out of 44 BM mAbs and three out of 98 lung mAbs, two from ACPA+ individuals at risk of developing RA from an untreated ACPA+ RA patient [266]. The MDA-reactive mAbs were further analyzed together with previously described synovial derived anti-MDA/MAA mAbs [147, 203]. The sequence analysis revealed a variety in Ig-subclass, gene usage, Fab N-glycosylation sites, SHM and CDR3. The bone marrow plasma cells had high SHM and notably, the lung mAbs had less SHM compared to the bone marrow plasma and synovial mAbs. One previously described synovial memory IgG1+ B cell mAb had germline encoded variable regions.

Malondialdehyde-induced cross-links as targets for autoreactive B cells

When analyzing reactivity against a selection of MDA- and MAA-modified antigens, we demonstrate that all mAbs bound both MDA- and MAA-modified proteins with the germline encoded mAb displaying the strongest binding to both MDA- and MAA-modified proteins. Interestingly, MDA can spontaneously break down to acetaldehyde *in vitro* to form MAA-adducts on proteins. By blocking the MAA-formation, we show that only the germline encoded antibody bound MDA-restricted protein modifications. Furthermore, the germline encoded mAb was the only investigated mAb that bound linear MDA/MAA-modified peptides. Consequently, we hypothesized that the other investigated clones needed cross-linked MAA-modification for binding. To further evaluate this hypothesis, we produced a MDA/MAA-hapten that we coupled to BSA and investigated mAb reactivity. Only the germline encoded mAb recognized the MDA/MAA-hapten BSA (Figure 18). Lastly, we investigated if the cross-linked MAA reactive clones are dependent of antigen amino acid composition for recognition. Interestingly, all mAbs bound MDA/MAA-modified cross-

linked Poly-L-Lysine which suggest antigen independent recognition of the MAA-residues by anti-MDA/MAA autoantibodies.

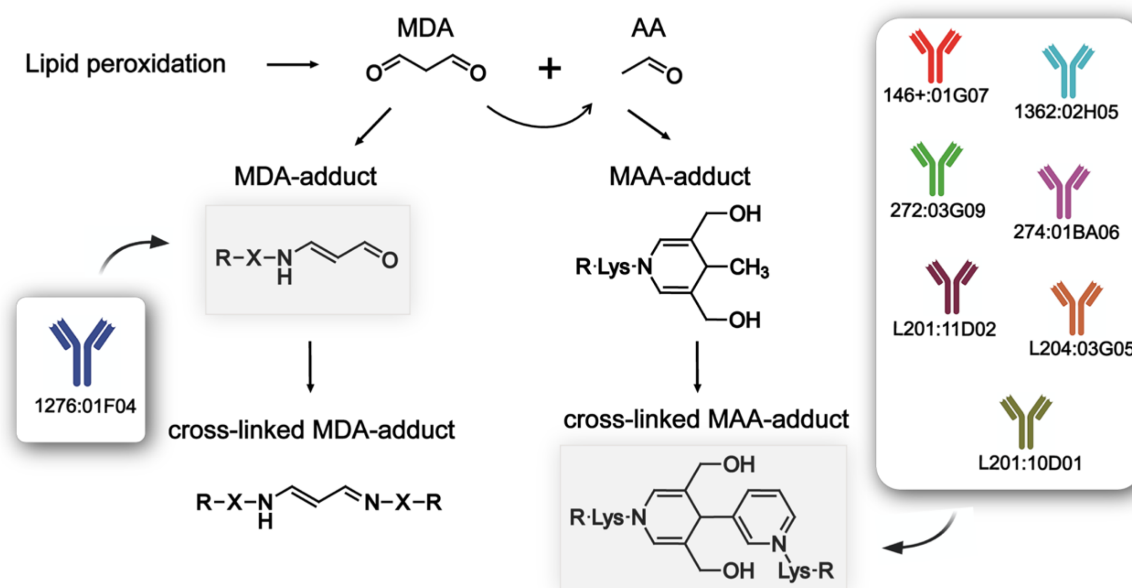

Figure 17. Schematic overview of RA mAb binding to MDA- and MAA-adducts. Lipid peroxidation produces MDA that forms MDA-adducts (N-Propenallysine) on amino acid (X= lysine, arginine, glutamine and histidine) in proteins. MDA can spontaneously break down to AA *in vitro* but is likely distributed exogenously *in vivo*. MDA and AA form together MAA-adduct (DHP-lysine) on amino acid lysine in proteins. Both MDA-adducts and MAA-adducts can further react to form intra-protein cross-links (1-Amino-3-Iminopropene and pyridium DHP). Our investigations show that the germline encoded 1276:01F04 mAb without osteoclast promoting function binds linear MDA-adducts. On the contrary, the remaining mAbs bind preferentially to cross-linked MAA-adducts.

Figure X: Schematic overview of RA mAb binding to MDA- and MAA-adducts
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Conclusions Paper V

MDA/MAA-reactivity is present in ACPA+ established and early untreated RA patients as well as in ACPA+ individuals at risk of developing RA. Furthermore, MDA/MAA-reactivity is common in different RA compartments and tissues such as the synovium, lung and bone marrow. Out of eight discovered B cell derived anti-MDA/MAA mAbs, one mAb bound linear MDA-antigens and the remaining seven, including the osteoclast promoting mAbs, need cross-linked MAA-protein for recognition independent of protein backbone (Figure 17). The origin of these autoreactive B cells and their potential role in the RA pathogenesis remains to be elucidated.

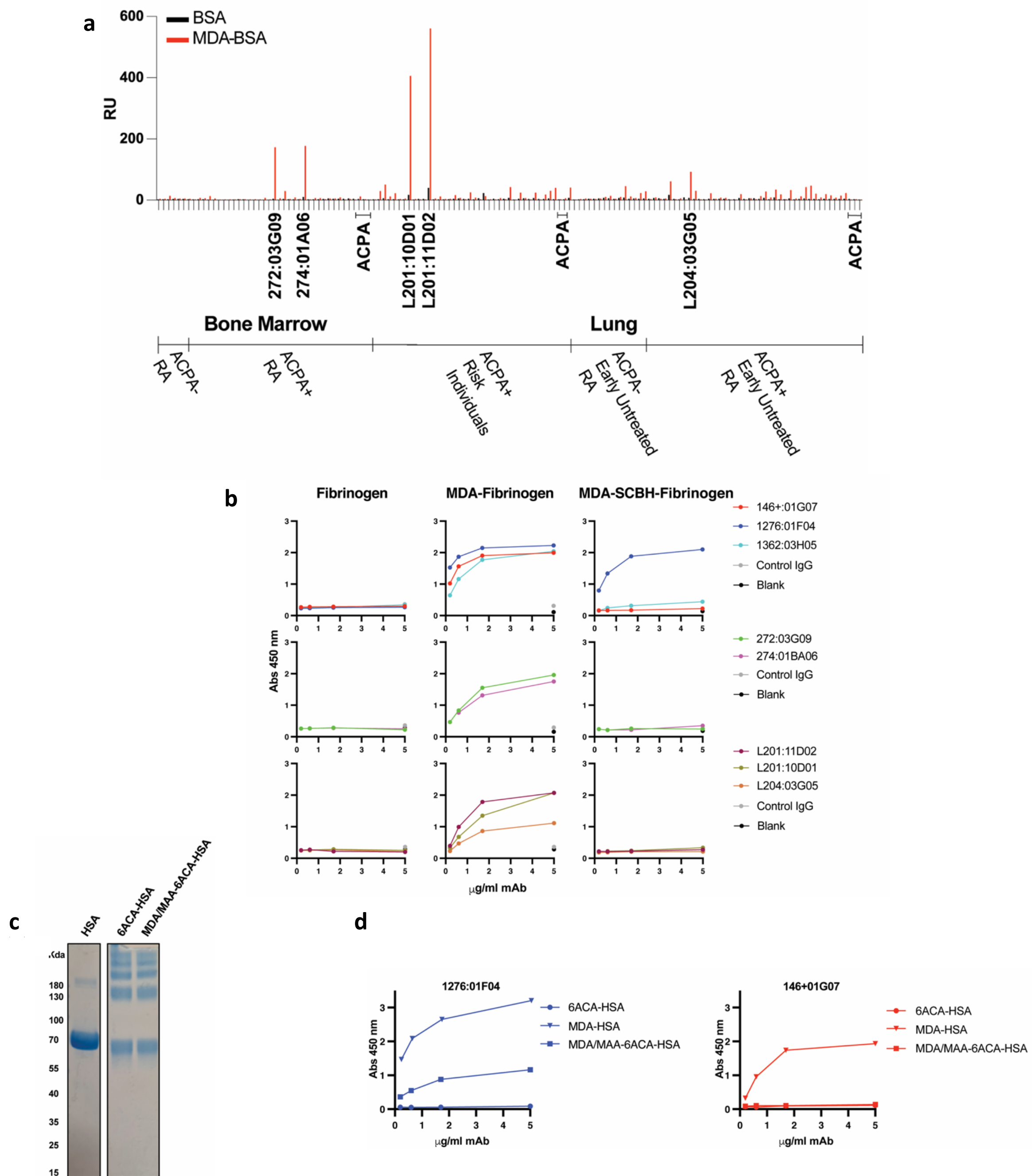

Figure X. Identification of bone marrow and lung anti-MDA mAbs for characterization of linear MDA-recognition or alternatively, cross-linked MAA-recognition. a. Recombinant human IgG1 mAbs expressed from single B cells. Bone marrow plasma cell and lung B cell mAbs without unspecific polyreactivity were screened at 5 µg/ml for binding to BSA or MDA-BSA with ELISA. Quantification by relative units (RU) was calculated based on reactivity to a reference curve from the positive MDA-reactive mAb 1276:01F04. **b.** Serial dilutions of mAbs to MDA-, MDA-SCBH-modified fibrinogen and mock treated fibrinogen coated on the same ELISA plate respectively. OD values of mAb reactivities. c, MDA/MAA-modified haptens were coupled to HSA. Protein Coomassie stained SDS-PAGE gel electrophoresis confirms MDA/MAA-hapten coupling to HSA without cross-linking. **d**, mAb serial dilutions against modified antigens coated at normalized concentrations based on fluorescence to ensure similar number of MAA-adducts on each antigen. Since the measured fluorescence for MDA-HSA was four times higher compared to the MDA/MAA-modified hapten, 3 µg/ml MDA-HSA and alternatively, 12 µg/ml MDA/MAA-6ACA-HSA was coupled on the 96-well plate. ELISA measurements (Abs at 450nm) reveal binding of the non-mutated germline mAb 1276:01F04 to MDA-HSA and MDA/MAA-6ACA-HSA whereas the mutated clone 146+:01G07 only bind MDA-HSA which indicates cross-linkage dependence for binding.

Figure 18. Identification of bone marrow and lung anti-MDA mAbs for characterization of linear MDA-recognition or cross-linked MAA-recognition. a. Bone marrow plasma cell and lung B cell mAbs were screened at 5 µg/ml for binding to BSA or MDA-BSA with ELISA. Quantification by RU was calculated based on reactivity to a reference curve from the positive MDA-reactive mAb 1276:01F04. **b**, Serial dilutions of mAbs to MDA-, MDA-SCBH-modified fibrinogen and mock treated fibrinogen coated on the same ELISA plate respectively. OD values of mAb reactivities. c, MDA/MAA-modified haptens were coupled to HSA. Protein Coomassie stained SDS-PAGE gel electrophoresis confirms MDA/MAA-hapten coupling to HSA without cross-linking. d, mAb serial dilutions against modified antigens coated at normalized concentrations based on fluorescence to ensure similar number of MAA-adducts on each antigen. Since the measured fluorescence for MDA-HSA was four times higher compared to the MDA/MAA-modified hapten, 3 µg/ml MDA-HSA and alternatively, 12 µg/ml MDA/MAA-6ACA-HSA was coupled on the 96-well plate. ELISA measurements (Abs at 450nm) reveal binding of the non-mutated germline mAb 1276:01F04 to MDA-HSA and MDA/MAA-6ACA-HSA whereas the mutated clone 146+:01G07 only bind MDA-HSA which indicates cross-linkage dependence for binding.

Clone name	Tissue	Reactivity	B cell	subclass	VH	Vλ/κ	γ-chain CDR3	λ/κ-chain CDR3	VH mutations	VL mutations	total glyco	total SHM
1325:01B09	Synovium	ACPA	Plasma	IgG1	4-39	LV1-44	ATDGEGVLFDE	AVWDDDLSGVV	58	37	2	95
1325:04C03	Synovium	ACPA	Plasma	IgG2	1-2	KV1-5	ARTNFSPFRH	QQYNGPSET	36	25	2	61
1325:05C06	Synovium	ACPA	Plasma	IgG1	4-39	LV1-51	AKLGCSGGGCYFDFDY	GTWDSSLSAGL	42	28	2	70
1325:07E07	Synovium	ACPA	Plasma	IgG1	4-39	LV3-21	ARLDPFDY	QVYDRKTDHQV	35	46	3	81
1003:10A01	Synovium	ACPA	Memory	IgG1	3-49	LV2-14	TRDLAPRAPRSFDY	SSYTNNSTLFYV	16	19	1	35
BVCA	Blood	ACPA	Memory	IgG1	3-49	KV1-6	VGPWFGDLLM	LQDDGFPFT	56	38	1	92
37CEPT1G09	Blood	ACPA	CEP+MB	IgG1	4-59	LV1-47	ARTNASDSSEQLAS	AAWDVRLWL	51	23	1	87
37CEPT2C04	Blood	ACPA	CEP+MB	IgA	4-59	LV1-47	AATKISDSGPQLGH	AAWGARFWL	48	44	4	92
14CFCT2D09	Blood	ACPA	CFC+MB	IgG1	4-59	KV4-1	ARQTGDREYMLAYYLDH	QQCSSTISLT	41	22	1	63
14CFCT2H12	Blood	ACPA	CFC+MB	IgG1	4-59	KV4-1	ARQVGSWEYLLGYYFDH	QQYYSTSSIT	35	21	1	56
14CFCT3G09	Blood	ACPA	CFC+MB	IgG1	4-59	KV4-1	ARQVGRWDYILADYFDH	QQYRSTSSIT	51	23	3	69
62CFCT1E04	Blood	ACPA	CFC+MB	IgA	4-59	KV4-1	YASGSHDTFDI	QQNSVLPFT	69	60	3	129
254:17D08	BM	ACPA	Plasma	IgG4	3-30	LV1-51	AKEHGYWKTATIDL	GTWDSSLDADDVI	49	47	4	96
268:07G01	BM	ACPA	Plasma	IgG1	3-33	LV2-23	ARGQGGVALNKYFDY	CSYANNSWV	26	34	2	60
254:C7X1604	BM	ACPA	Plasma	IgG1	4-61	KV1-33	VRFDYGPSYYFDN	QKYAGRPYA	76	52	3	128
1362:03H05	Synovium	MDA/MAA	Plasma	IgG1	3-53	KV4-1	ARDRRGWSGYYSLRYGMDV	QQYYSTPYT	13	12	0	25
146+:01G07	Synovium	MDA/MAA	Memory	IgA	3-33	KVD3-15	ARARRGDGYNQARYYYFDY	QQYIKWPPEYT	1	18	0	19
1276:01F04	Synovium	MDA/MAA	Memory	IgG1	4-39	LV1-5	VRVRGYFDY	GTWDSSLSVWV	0	1	0	1
272:03G09	BM	MDA/MAA	Plasma	IgG1	4-34	KV1-5	ARGLYLMTLRTVNRRTSSPRPVWYFDL	QQHQTYFPT	49	31	1	80
274:01BA06	BM	MDA/MAA	Plasma	IgG1	3-30	LV2-11	AKMTGEHS	CSYTGTYSWV	26	19	1	45
L201:10D01	Lung	MDA/MAA	Double neg#	IgG1	1-3	KV1-27	ARSIFFSGWGYYYYGMDV	QKYNSAPLT	13	6	0	19
L201:11D02	Lung	MDA/MAA	Switch mem§	IgG4	3-49	KV1-5	TRIPFASYYYFYGMDV	QQFHSFWT	14	14	1	28
L204:03G05	Lung	MDA/MAA	Double neg#	IgG1	4-59	KV1-5	ARGITMVRGVKFPLFDY	QQYNSYPLT	3	2	0	5
1362:01E02	Synovium	Control	Memory	-	3-21	LV2-28	AREGRNLGYCRGSACYPGWDYHAMDV	GFYTGSSPYV	10	16	0	26

Table 2. **Human mAbs generated from RA patient B cells.** BM = Bone marrow. CEP+MB = CEP1 tetramer sorted memory B cell. CFC+MB = CFC1 tetramer sorted memory B cell.
$CD27^-$ IgD^-
§ $CD27^+$ IgD^-

6 DISCUSSION

Our studies have considerably contributed to the understanding of autoreactivity to posttranslational modifications in RA. By generating mAbs from identified single B cells, we were able to dissect their unique binding properties and learned that ACPA fine-specificities are highly related due to the multireactivity of ACPA mAbs and that different ACPA seem to have diverse functional properties. Different ACPA mAbs promoted bone destruction, bound activated neutrophils, targeted nuclear antigens in apoptotic cells. Furthermore, we have learned that ACPA mAbs have unique variable Ig sequence characteristics although they share features of high SHM and mutation introduced Fab variable N-glycosylation sites. In addition, our investigations reveal that a subset of ACPA mAbs reacted with carbamylated and acetylated antigens. Another type of AMPA, the anti-MDA/MAA antibodies were identified in the RA bone marrow plasma cell niche and in the lung. Our results indicate that mAbs binding to MAA-modified cross-linked proteins, independent of protein amino acid backbone, can induce osteoclast promoting functions [147, 203].

We generated mAbs by isolation of single patient B cells by different means, followed by RT-PCR amplification of the variable Ig genes, cloning into expression vectors and recombinant IgG1 expression in a mammalian cell line. The original strategy was derived from the Nussenweig's laboratory that implemented the method for investigations of B cell development [35, 36]. Several important aspects need to be considered for the generation of large amounts of reproducible high quality autoantibodies. A significant challenge is to avoid false positive reactivity to PTM-antigens often seen in autoimmune patients and to instead identify true strong antigen-specific binders. Control steps during purification, storage and specificity-validation, ensures mAb quality. Our optimized strategy for generating citrulline-specific B cell derived mAbs are available in a peer-reviewed protocol [263].

An alternative approach for investigate mAb functionality *in vitro* and *in vivo* could be performed by generating high affinity mAbs against interesting PTM-antigens. There are other applications for developing human derived high affinity mAbs. Phage display is method that has generated mAbs directed against citrullinated antigens from RA patient antibody repertoires [267]. However, since our main interest is to study individual autoreactive B cells, the RT-PCR amplification of paired variable Ig genes was our method of choice. One investigated single B cell mAb investigated in **Paper III** was originally discovered using an immortalization technique but the success rate of such method was low and the procedure labor intensive [238].

One main aim of this thesis was to study unique characteristics of AMPA in RA. In **Paper I**, we utilized the foci method [261] to isolate synovial RA plasmablasts for mAb expression without biased pre-selection. We identified four ACPA mAbs out of total 93 expressed mAbs. That is less than 4% ACPA mAb which is a rather low yield which makes the method rather insufficient. Another research group used flow cytometry to isolate CD19+ synovial B cells for subsequent mAb generation and assessment of citrulline reactivity. In total 59 mAbs were

produced in which around 40% were citrulline reactive, although the criteria for positivity may have been less strict than in our current investigations [20]. For our remaining studies, selection procedures were introduced to increase our yield of identified autoreactive B cells for mAb expression. One such method is the cit-tetramer flow sorting procedure which enabled isolation of cit-reactive single B cells to a much higher frequency [234]. Cit-alpha enolase and cit-filaggrin peptides were used to produce the tetramers and even though it generated the identification of citrulline reactive B cells, the procedure introduce a bias in antigen recognition. In **Paper IV** and **V,** we made use of other methods to identify AMPA+ B cells. Flow cytometry was used but since plasma cells downregulate their BCR, it is not possible to tetramer-sort these cells [15]. To select clones for mAb generation, we utilized known variable Ig sequence ACPA characteristics such as high SHM, Fab N-glycosylation sites, gene usage or related clonality [249]. In addition to flow, we used droplet barcode single-cell sequencing technologies to screen the transcriptome of paired heavy and light chain Ig repertoires. Since most bone marrow plasma cells had high rates of SHM, the variable Fab N-glycosylation sites were directive for our selection of clones for mAb generation. Three out of 38 plasma cells were indeed citrulline reactive which makes us conclude the selection strategy was successful. Even if we might have missed ACPA+ B cells with the selection method used, it would have been difficult for us to express all 657 identified plasma cells.

In **Paper I**, we recognized ACPA mAb features of high SHM, Fab N-glycosylation sites and reactivity to multiple cit-peptides and citrullinated proteins as well as the ability of some ACPA mAbs to cross-react with carb-peptides. These features are ACPA mAb characteristics that have been confirmed elsewhere [20, 193, 228, 234-236, 240, 249]. The extremely high SHM rate demonstrated by the B cell variable Ig sequence repertoire suggest re-current rounds of germinal center induced affinity maturation. The apparent increased antigen affinity by SHM was appreciated for some of the ACPA mAbs by the production of predicted germline reverted versions of the ACPA mAbs. However, one ACPA mAb increased antigen reactivity when the light chain was reverted back to predicted germline and the heavy chain was left mutated which suggest a different scenario to SHM driven affinity maturation. More extensive studies elsewhere have shown that the SHM induced affinity maturation is indeed a feature of ACPA mAbs [193, 235]. The ability of ACPA mAbs to cross-react with carbamylated antigens has led to the now commonly used term AMPA for description of such cross-reactive ACPA mAbs. Both the high numbers of SHM and the Fab variable N-glycosylation sites that we and others report on are likely introduced during affinity maturation in T cell dependent manner [76]. One could thereby speculate that the early ACPA-responses seen in individuals without RA is T cell independent. Furthermore, the lost reactivity displayed when variable mutations are reverted back to predicted germline are in favor of the importance of SHM for ACPA specificity gained during affinity maturation [193, 235].

In **Paper II**, we analyzed our multireactive and cross-reactive AMPA described in **Paper I** together with previously described AMPA [234]. Results in **Paper II** reveal unique binding patterns to nuclear antigens and apoptotic cells. One AMPA mAb with PAD-dependent NET binding bound cytoplasmic antigens whereas a subset of PAD-independent NETs binding

AMPA bound nuclear acetylated histones. These characteristics, among others, suggest different effector functions and possible B cell origins that represent AMPA subsets. ACPA mAb NET binding have been described before but not binding to apoptotic cells [20]. In addition hypercitrullination is suggested a feature of NETs in RA [208, 214].

A consequence of the ACPA mAb cross-reactivity detected in **Paper I** could also be a sign of undefined mAb autoantigen. It motivated us to investigate a large amount of autoantigens beyond those already associated with RA or joint pathology. In **Paper III**, the extent of ACPA mAb multireactivity and cross-reactivity was evaluated using a selection of array platforms. We concluded that ACPA are to a much greater extent, than previously acknowledged, multireactive to cit-peptides and citrullinated proteins. Furthermore, we extended the consensus binding motifs by including neighboring amino acids in addition to glycine in +1 which highlighted the importance of charge and polarity. However, there are difficulties comparing consensus binding motifs for the cross-reactivity patterns since the lysine modifications are different to that of arginine. The AMPA nomenclature is now widely used and several independent research groups are recognizing the ACPA cross-reactivity and have been studied that using different methodologies [193, 236, 240, 257, 258]. Efforts in elucidating how different reactivities may be involved in the initial breakage of tolerance have been performed by tetramer B cell stainings using cit-, carb- and acet-tetramers in ACPA+ individuals without RA [134]. Furthermore, the finding that immunization in mice with one PTM can lead to the development of another type of anti-PTM autoantibodies suggest that citrulline is not necessarily responsible for the initial breakage of tolerance [257]. In addition, a B cell line was activated with different PTMs [236] and tetramer-sorted circulating IgM have been shown to cross-react with citrullinated, carbamylated and acetylated antigens [258]. Lastly, ACPA mAbs have been extensively used to study the feature of introduced Fab variable N-glycosylation sites during SHM [246-249, 252].

In **Paper I-III**, we identify peripheral citrulline reactive B cells. Such cells are likely targeted by CD20 B cell depletion therapy. On the other hand, long lived plasma cells in the bone marrow are CD20- and would not be affected by such treatment. Although ACPA levels significantly decrease after rituximab CD20 treatment, and by other DMARDs, baseline serum ACPA level remains stable which suggest ACPA production in the bone marrow [55, 268]. In addition, ACPA+ memory B cells have a proliferative and activated phenotype which suggest turn-over of short-lived plasmablasts in the circulation [269]. In **Paper IV**, we studied RA patient bone marrow plasma cell repertoires using flow cytometry and droplet 10X transcriptomics followed by recombinant mAb generation for identification of ACPA. The transcriptomics analysis reveal differences in IgA2 frequencies between the patients. We have previously described ACPA IgA+ B cells in the circulation [234] and other reports show clonal relationships between gut and serum IgA, indicating that mucosal immune responses can be found in the bone marrow [270]. The class-switched high SHM bone marrow plasma cells with identical heavy chains, based on CDR3, but with different light chains may represent receptor revision [271]. Furthermore, we observe higher frequencies of IgG4+ plasma cells in the ACPA+ patient compared to the ACPA- patient. This is in line with our previous finding that

IgG4+ B cell frequencies are increased in the circulation of ACPA+ RA patients compared to healthy individuals [272]. However, by comparing two individuals only, it is not possible to draw conclusions. In addition, frozen samples were used for the transcriptomics analysis which may affect the result. Still, no reports of paired bone marrow plasma cell repertoires from RA patients have been conducted for comparison and validation. Excitingly, **In Paper IV**, we could for the first time identify citrulline reactive RA patient bone marrow plasma cells. The finding highlights the successful use of single cell flow isolation and transcriptomics for identification of ACPAs by pre-selecting sequenced clones based on high SHM and Fab variable N-glycosylation sites. Not surprisingly, most identified plasma cells had high SHM. However, the frequency of plasma cells with Fab variable N-glycosylation sites were higher in ACPA+ patients compared to the ACPA- patient. It has previously been suggested based on transcriptional profiles that ACPA B cells may have a higher probability to develop into long lived plasma cells whereas RF B cells may develop into plasmablasts. This is explained by the more innate like transcription profile of RF B cells and the antigen-experienced transcription profile in ACPA B cells [92]. In **Paper IV**, two ACPA plasma cells were identified by flow and one by 10X transcriptomics. They displayed various multireactivity and binding strength to analyzed cit-peptides. Interestingly, the ACPA mAb with the most restricted cit-reactivity bound only to a cit-peptide from the RNA-binding hnRNP A1 protein. The peptide contains the gly-cit-gly motif including cit-gly which is a motif suggested in **Paper I** and **III** to be the dominant ACPA binding motif. Surprisingly, the reactivity to cit-hnRNP A1 was attained when the variable heavy and light regions were reverted to predicted germline. This is a rare feature for ACPA not seen in **Paper I** nor by others [235]. In addition, nuclear small RNA-binding antigens are in **Paper III** suggested to be over-represented autoantigens. The other two ACPA mAbs with cit-histone reactivity bound activated neutrophils. ACPA targeting citrullinated histones in NETs is reported in **Paper II** and also by others [20].

In **Paper V**, we investigate another type of AMPA associated with inflammation in RA, namely IgG anti-MDA/MAA modified protein. MDA-adducts are present in the RA synovium [147, 148] and MAA-adducts are produced with the addition of acetaldehyde from suggested exogenous sources such as smoking, alcohol and infection [152-157]. Anti-MDA/MAA IgG antibodies are elevated in RA and SLE [147, 148, 159, 160]. Our mAb investigations reveal that the MDA/MAA-reactivity is different from the investigated cross-reactive AMPA described in **Paper I-IV** by not binding to citrulline-, carbamyl- or acetyl-antigens. By screening mAbs from various RA tissue-compartments we identified MDA-reactive B cell derived mAbs from synovia, bone marrow and lung [147]. MDA/MAA-reactive bone marrow plasma cells provide continuous amounts of IgG to the circulation and the presence of MDA/MAA-reactive B cells at mucosal sites in the lung emphasize the concept of mucosal origin for autoimmunity [273]. It is thereby of particular interest that we find MDA/MAA-reactive B cells in the lung of ACPA+ individuals at risk of developing RA. This may suggest that the smoking- and infection-induced formation of MAA-adducts at mucosal sites is linked with the breakage of tolerance. This is in line with reports that airway induced inflammation in the lung give rise to MDA-derived modifications [274] and that MDA/MAA-antibodies appear

just before RA disease onset [161]. Furthermore, MDA/MAA-modification gave been shown to co-localize with citrullinated autoantigens [158]. It is tempting to speculate that the MDA/MAA-autoreactivity fuels the inflammation together with ACPAs by the possible formation of immune complexes. Indeed, our previous *in vitro* studies suggest FcγRI mediated MDA/MAA-mAb induced bone destructive events by osteoclasts [147, 203]. In addition, our ACPA mAbs have been shown to target modified IgG peptides, which emphasize the involvement of RF in the formed immune complexes [275].

As for the other AMPAs, the functional target autoantigen or autoantigens for the anti-MDA/MAA mAbs are unknown. In order to examine anti-MDA/MAA mAb antigen specificity, we investigated binding to a range of MDA- and MAA-modified proteins. In addition, we investigated the various protein cross-linking MDA- and MAA-modifications can induce. By blocking the formation of MAA-adducts from MDA followed by investigations of binding to linear modified peptides and modified proteins with and without cross-linking, we could conclude that all but one anti-MDA/MAA mAbs recognized MAA-induced cross-linked proteins, including the osteoclast promoting mAbs. One germline encoded mAb recognized linear MDA-modified antigens. Except for MS analysis of the modified proteins and peptides, we used immune assays to determine the modifications and mAb modified antigen recognition. Since MDA spontaneously breaks down to acetaldehyde and forms MAA-adducts *in vitro*, which are not distinguished by antibody recognition, difficulties lies in the detection of the modifications and determination of mAb antigen recognition [150, 151, 276]. The use of more robust measurements such as MS and HPLC on routine basis would increase efficiency. Such methods would enable the generating of MAA-exclusive antigens.

Lastly, the AMPA mAbs described in this thesis have been used in several additional functional and characteristics studies. Functional *in vitro* investigations reveal that ACPA mAbs and anti-MDA/MAA mAbs promote bone degrading osteoclasts [147, 200, 203, 234] and that ACPA mAb induce differentiation of fibroblasts [205]. Furthermore, *in vivo* studies showed that ACPA mAbs can cause pain in mice [226]. In addition, we analyzed the variable Fab N-glycosylation of ACPA mAbs [249] and other have shown that our ACPA mAbs can target modified IgG-peptides [275].

The remarkable diversity and function of AMPA demonstrated in this thesis sets a foundation for future studies that possibly will unravel the autoreactive origin and involvement in RA pathogenesis.

7 CONCLUSIONS AND POINTS OF PERSPECTIVE

In this thesis, autoreactive B cells in various RA tissue compartments were identified by the generation of single cell derived mAbs. We investigated mAbs from the synovium, circulation, bone marrow and lung of RA patients. Remarkable insights that ACPA mAbs from different tissue compartments and B cell subsets are able to bind multiple cit-peptides and citrullinated proteins with unique binding patterns were discovered. Furthermore, some ACPA mAbs were cross-reactive with carbamylated and acetylated antigens. Our investigations show that the multireactivity and cross-reactivity is possible since ACPA recognize short peptides with consensus binding motifs. The consensus binding motif and cross-reactivity can be used to determine what cellular compartments the mAbs recognize since our investigations show that a subset of ACPA mAb preferentially target nuclear antigens whereas others prefer binding to the cytoplasm. The generation of mAbs enabled functional studies and since the ACPA mAbs have unique antigen specificities, they exhibit individual distinct functions. Our investigations show osteoclast promoting effects and the ability to bind activated neutrophils.

The identified mAb features may also be true for other autoimmune diseases where potentially pathogenic autoantibodies develop for years. It is intriguing that the investigated autoantibodies may recognize different targets in different organs and may exhibit different functions in different cellular systems and thereby provide new areas of research and understanding of if and how B cells contribute to the triggering of inflammatory diseases like RA.

The multireactivity and cross-reactivity patterns essentially expands the understanding of AMPA and unravel how different PTMs are connected. This raises important questions on which protein modifications are primarily involved in the initiation of autoimmunity and if these are continuous pathogenic responses in RA.

These striking properties of AMPA and their reactivity profiles can possibly provide information about how human autoimmune responses evolve and may help clarify the etiology behind the break-of-tolerance to modified antigens in autoimmune disease.

For the first time, we describe ACPA producing plasma cells in the bone marrow of RA patients. The finding is important for current and future B-cell targeted therapeutic strategies. In addition, by exploring the RA bone marrow plasma cell niche, insights into human bone marrow plasma cell clonalities and diversity were gained. The RA bone marrow work in this thesis sets the foundation. By including more patients and expanding the plasma cell repertoire investigations, significant valuable confirmations of the observed clonal expansions, class switching and receptor revision can be obtained for RA patient bone marrow plasma cells.

We also widen the field of AMPA by gaining increased knowledge in another set of autoreactive B cells in RA, namely anti-MDA and anti-MAA autoantibodies. We show that autoreactivities specific for malondialdehyde protein modifications can be found in the lungs of RA risk individuals and early RA as well as in the bone marrow plasma cell compartment and in the inflamed synovium of RA patients. Furthermore, we show that although there are B

cell clones that bind the linear MDA modification, the majority of clones in our study preferentially recognize MAA-cross-linked amino acid adducts independent on the protein backbone. We are only now starting to unravel the specific target autoantigens for these anti-MDA/MAA mAbs and their potential role in the RA pathogenesis. With our approach to detailed protein modification studies, a foundation for future investigations is served.

Taken together, the findings of this thesis are achieved by the generation of single B cell derived mAbs. The diversity and function of the AMPA response was demonstrated by our head-to-head comparison of several AMPA mAbs from different tissue compartments and B cell subsets identified with a range of methods. The gained knowledge is important to consider when investigating possible pathogenic or therapeutic roles of AMPA. Investigations that eventually will lead to better understanding of the RA pathogenesis and consequently to improvement of disease interventions. Hopefully to that of cure.

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