Anti-citrullinated protein antibody B cells in rheumatoid arthritis: from disease-driving suspects to therapeutic targets

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CHAPTER 1

General introduction

B-cell development and maturation

B cells, or B lymphocytes, are a key component of the adaptive immune system and are responsible for generating specific responses to pathogens and maintaining immunological memory. Antibody production, antigen presentation and cytokine production are examples of mechanisms exerted by B cells to contribute to humoral and cellular immunity. B cells recognize antigens through their B cell receptor (BCR), a transmembrane protein on the cell surface encompassing a signal transduction moiety (CD79A/Igα and CD79B/Igβ) and an antigen-binding immunoglobulin (Ig) molecule [1]. The latter is composed of two Ig heavy chains and two Ig light chains.

B cells arise from hematopoietic precursor cells in the bone marrow where they develop into immature B cells through various stages. First, pro-B cells rearrange the diversity (D) and joining (J) gene segments of the heavy chain, followed by a second rearrangement connecting the upstream variable (V) region to the already rearranged DJ segment, giving rise to the heavy chain [2, 3]. Additional variety is achieved by the addition of nucleotides at joints between gene segments during rearrangement [4]. Next, pre-B cells rearrange the V and J segments of the light chain κ and λ gene segments [2, 3]. This, in combination with the rearranged heavy chain, results in an IgM or IgD molecule expressed on the cell surface of the immature B cell. Altogether, these processes generate a highly variable B-cell repertoire capable of recognizing a multitude of different antigens. After BCR completion, immature B cells migrate from the bone marrow (BM) to the spleen where they undergo final steps of maturation and transition into naïve B cells. From here onwards, naïve B cells circulate between peripheral blood and secondary lymphoid organs [2].

B-cell activation

Upon antigen encounter, B-cell activation can occur through two main pathways: the extrafollicular response (EF) or the germinal center (GC) response, also described as the T cell independent or T cell dependent response, respectively. Toll-like receptors (TLRs) are described to play a role in the induction of EF responses by recognizing conserved components of pathogens such as liposaccharides (LPS) [5, 6]. Additionally, repeating epitopes present on antigens can mediate extensive BCR cross-linking and thereby activate B cells independently of T cells [7, 8]. Therefore, TLR- and BCR-triggering together may induce strong B-cell activation without requiring T-cell help. Generally, such EF responses are rapidly induced and short-lived, leading to the production of short-lived plasma cells primarily secreting low-affinity IgM antibodies [9]. However, EF responses can also induce Ig-class switching associated with somatic hypermutation (SHM) to increase affinity to the antigen [10, 11].

Beside the EF response, B cells can be activated through the GC response. After binding and internalization of an antigen presented by follicular dendritic cells (FDCs), B cells process the antigen into peptides which are loaded onto major histocompatibility complex class II (MHCII) for presentation to T-helper cells, specifically T follicular helper (Tfh) cells [12]. When Tfh cells recognize the presented peptides, they provide survival signals to the B cells through cytokine production

and CD40-CD40L interaction, stimulating Ig-class switching. Within the GC, the B cells shuttle to- and from the dark zone (DZ) and light zone (LZ) [13, 14]. In the DZ, the B cells proliferate and the variable region of the BCR undergoes SHM. In the LZ, the affinity of the BCR to its antigen is evaluated and the B cells are either sent back to the DZ to accumulate more SHM to increase affinity to its antigen, or the B cells exit the GCs either as long-lived plasma cells or memory B cells [15]. Long-lived plasma cells produce high affinity antibodies, whereas memory B cells will circulate the periphery and can be reactivated upon secondary antigenic stimulation, resulting in more rapid production of high-affinity long-lived plasma cells or short-lived plasma cells or re-enter the GC [15]. In general, primary B cell-immune responses following vaccination or infection induce rapid IgM antibody production by short-lived plasma cells, and later-onset production of high affinity, class-switched antibodies since the GC response takes more time. Following a second antigenic exposure, memory B cells are able to respond very fast and differentiate into plasma cells secreting high affine and class-switched antibodies.

B cell-receptor signaling

B cells can be activated by multivalent soluble antigens as well as by membrane-bound antigens [16]. However, membrane-bound antigen has a lower threshold for B cell activation, in accordance with the primary mode of *in vivo* antigen recognition through membrane-bound antigen presentation by antigen-presenting cells (APCs) [17]. Furthermore, monovalent membrane-bound antigen has been described to induce B-cell activation, whereas monovalent soluble antigen does not [16, 18, 19].

Upon sufficient triggering of the surface-Ig BCR, the immunoreceptor tyrosine-based activation motifs (ITAMs) of Igα and Igβ signal transducers are phosphorylated [20] by the Src family kinases LYN [21] and subsequently spleen tyrosine kinase (SYK) [22]. Once recruited and activated, SYK propagates the BCR signal by phosphorylating several adaptor proteins which initiate the assembly of the signalosome, encompassing a variety of signaling molecules activating multiple signaling pathways [23, 24]. Adaptor proteins such as CD19, B-cell linker (BLNK) and Bruton's tyrosine kinase (BTK) are important regulators of downstream signaling pathways. Inhibitory co-receptors can act to inhibit the BCR-triggered signalosome, for example CD22 which regulates BCR signaling by recruiting SHP-1 [25, 26].

PI3K/AKT/mTOR pathway

Together with ITAM phosphorylation, Lyn phosphorylates the cytoplasmic tail of CD19, resulting in phosphoinositide 3-kinase (PI3K) activation, which in turn generates PIP_3 from PIP_2. Protein kinase AKT is one of the most important downstream signaling molecules of PI3K. By inactivating pro-apoptotic proteins and transcription factors such as BCL-2, AKT can increase cell survival. Additionally, AKT can stimulate NF-κB to enhance expression of anti-apoptotic genes. Furthermore, by inhibiting GSK3 activation, AKT can regulate apoptosis and glucose metabolism. Through mTOR activation, AKT stimulates protein synthesis. In general, the PI3K/AKT/mTOR signaling pathway is crucial for regulating of cell growth, proliferation, metabolism and apoptosis [27].

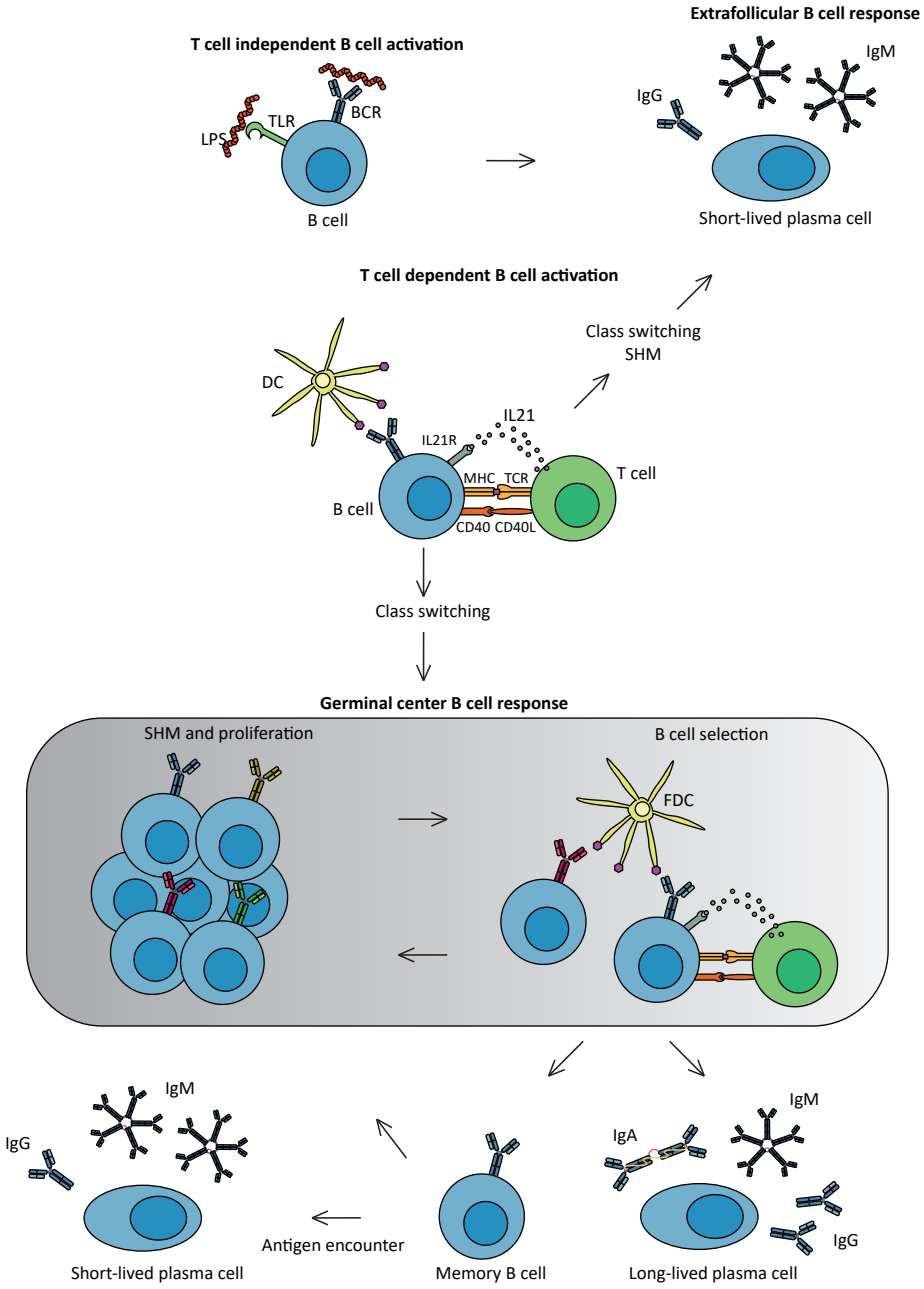

Figure 1. B-cell responses after activation. When the BCR and TLR are activated by antigen, B cells can be activated independent of T-cell help, leading to an extrafollicular B-cell response producing short-lived plasma cells. Alternatively, in presence of T-cell help, B cells migrate to the germinal center where they undergo SHM and affinity maturation, leading to long-lived plasma cells or memory B cells. Reactivation of memory B cells can make them re-enter the germinal center or can result in short-lived plasma cell production. Alternatively, T cell-dependent B-cell activation can result in class switching and SHM extrafollicularly, also resulting in short-lived plasma cells.

PLCγ2 pathway

Upon Lyn-mediated ITAM phosphorylation, SYK is recruited and phosphorylated and together with LYN, enables BTK phosphorylation. Additionally, activated SYK is responsible for BLNK phosphorylation. BTK and BLNK together recruit PLCγ2, which is then phosphorylated by BTK. Activation of PLCγ2 leads to activation of DAG and IP3. The latter initiates calcium mobilization, leading to activation of nuclear factor of activation T cell (NFAT). Activation of DAG generates protein kinase Cβ activation, which in turn activates ERK, MAPK and NFκB. NFκB functions in maturation and survival of the B cells [28].

ERK and MAPK pathways

The ERK pathway can also be induced via RAS. Upon BCR ligation, RAS is activated which in turn activates RAF. RAF is able to phosphorylate intermediate kinases which are responsible for activating ERK1 and ERK2. Dimer formation of ERK1 and ERK2 is necessary for nuclear translocation to initiate transcription of genes responsible for cell proliferation, survival and differentiation. Furthermore, the MAPK pathway is initiated by BLNK-mediated activation of VAV and subsequently RAC [29].

Ultimately, BCR triggering leads to the activation of several transcription factors controlling the expression of genes involved in B-cell activation, proliferation and differentiation. The exact outcome of the signaling cascade depends on multiple other factors such as the duration and strength of activation, nature of the antigen, the maturation state of the cell, and signaling coming from other receptors *e.g.* CD40, BAFF-R, CD22, FcγRIIB1 (CD32) [30, 31].

Antibodies

Antibodies are produced by terminally differentiated plasma cells and are the secreted form of BCRs, making their specificities identical. Monomeric antibodies are ~150kDa in size and are made up from two identical heavy chains and two identical light chains connected by disulfide bonds. Light chains consist of one constant domain (CL) and one variable domain (VL), whereas heavy chains contain three (in IgG or IgA) or four (IgM or IgE) constant domains (CH) and one variable domain (VH). Structurally, antibodies can also be divided in the antigen-binding fragments (Fab) and the crystallizable fragment (Fc). The Fab and Fc fragments are connected via the hinge region responsible for flexibility and therefore enhancing binding capacity of the antibody. The Fc portion of an antibody consists of the CH2 and CH3 domains of the heavy chains. The Fab fragment is made up from the CH1, CL, VH and VL domains. Two Fab arms and the hinge region together is known as F(ab')2 (Figure 3A). The variable domains of the Fab are responsible for antigen binding [32]. Each variable domain comprises three hypervariable regions, known as complementarity-determining regions (CDRs), because their structure complements that of an antigen. The CDRs from both the heavy chain and light chain variable regions form the antigen-binding site that form optimal shapes to fit its cognate antigen [33].

In contrast to the variable regions of an antibody, the constant regions only occur

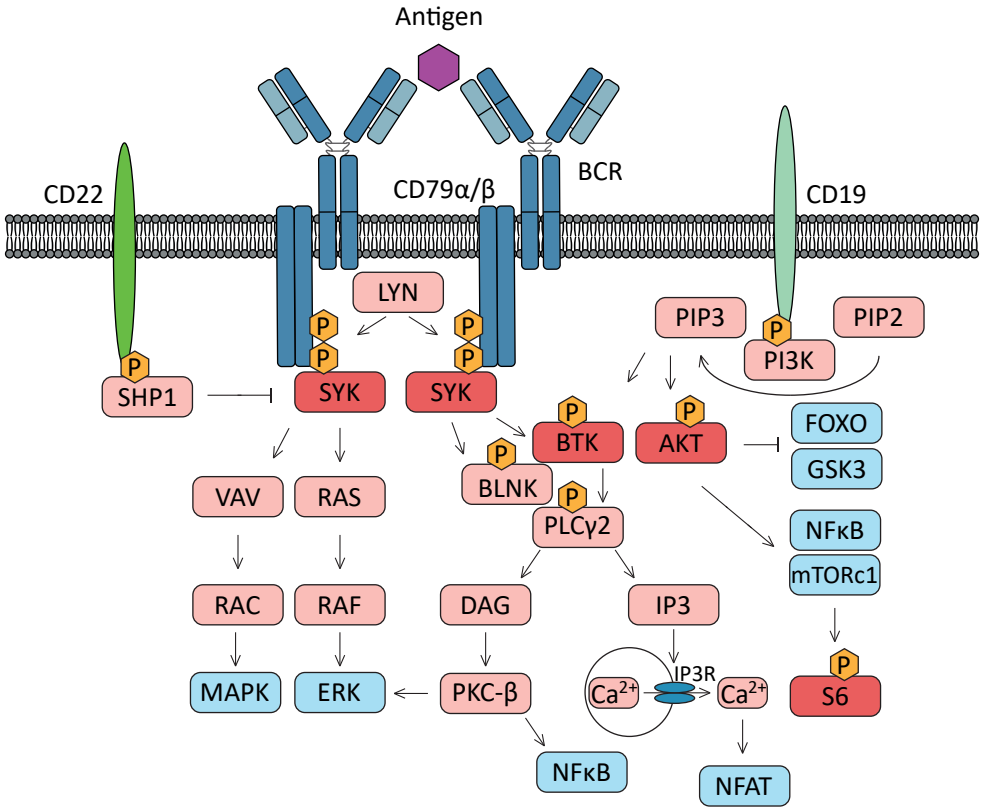

Figure 2. Signaling cascades initiated by BCR activation. Upon antigen-mediated BCR activation, a plethora of signaling pathways are initiated, some of which are depicted in this figure.

in a few variants (Figure 3B). In humans, there are five heavy chain isotypes present (μ, γ, α, ε and δ) corresponding to five antibody isotypes: IgM, IgG, IgA, IgE and IgD. IgM is expressed as a monomeric BCR, but upon activation, plasma cells secrete IgM in a pentameric form linked via disulfide bonds and a J-chain which facilitates secretion at mucosal sites [34, 35]. IgM antibodies are expressed early in B-cell responses and are rarely mutated, thereby eliciting a quick response to a broad range of antigens without requiring T-cell help. IgG, IgA and IgE are generated by class switching and IgG is the most abundant antibody isotype in serum. IgG antibodies are divided in four subclasses based on structural differences in the constant domains and their associated effector functions: IgG1, IgG2, IgG3 and IgG4. Antibodies of the IgG1 subclass are the most abundant subclass and dominate in the response against protein antigens, whereas IgG4 is the least abundant isotype and often observed after repeated antigenic exposure. IgG2 antibodies are potent anti-carbohydrate antibodies, and antibodies of the IgG3 subclass are particularly effective in the induction of effector functions [36]. IgA antibodies are secreted at respiratory and intestinal sites, making them the main mediators of mucosal immunity. IgA displays a monomeric form in serum but forms a dimer at mucosal sites. Dimeric IgA is linked via the J-chain and also exhibits a polypeptide chain called the secretory component [34, 35]. IgA antibodies are divided in two

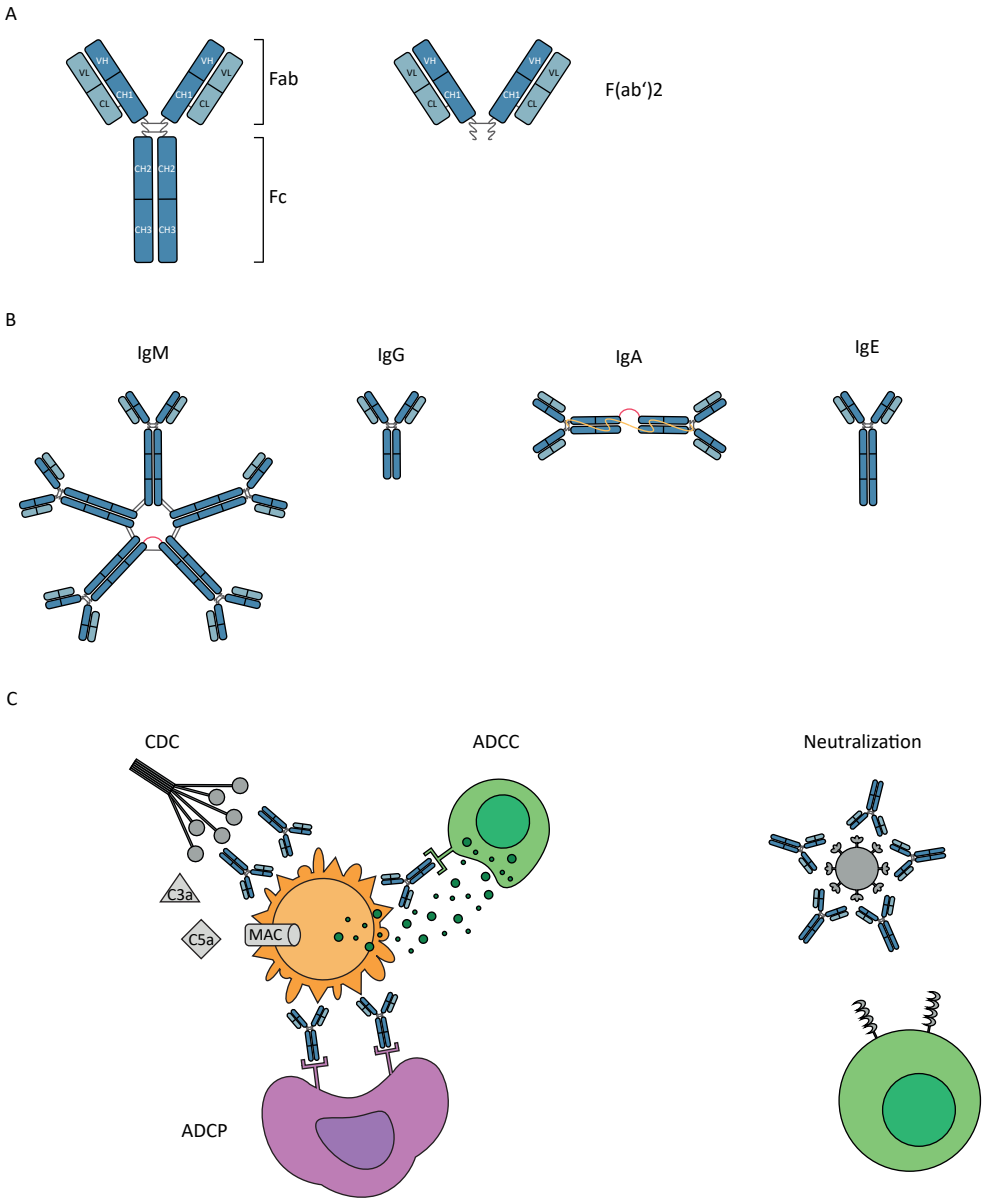

Figure 3. Antibody structure and effector functions. A. Structural architecture of an antibody. Here, an IgG1 antibody was used as an example. **B.** Structural architecture of the different antibody isotypes in their secreted forms. The J-chain in IgM and IgA antibodies is indicated in red. IgA's secretory component is depicted in yellow. **C.** Effector functions mediated by antibodies. Antibodies can mediate several effector functions by binding to a pathogen via its variable domain, and binding of its Fc domain to 1) Fc receptors of NK cells or T cells, which will release cytotoxic granules and thereby kill the target cell via ADCC, 2) Fc receptors of macrophages which will phagocytose the target cell (ADCP), 3) C1q which will recruit the complement system leading to MAC formation and subsequent cell lysis. Furthermore, by antibody binding to antigens, the antibodies can block pathogens from entering target cells and thereby neutralize the pathogen.

subclasses based on the size of their hinge region. IgA1 is the dominant subclass in serum and it has a longer hinge region, making it more vulnerable to bacterial proteases, whereas IgA2 with its shorter hinge region is found more at mucosal sites. IgE antibodies, the least abundant class in the human peripheral blood, are the main antibody class mediating responses to allergens. IgD is expressed on naïve, antigen-unexperienced B cells and its expression has been associated with differences in activation status. However, its role in serum is poorly understood. There are also two variants of light chains: κ and λ. However, since they do not exhibit distinct functions, the antibody isotype is only determined by the isotype of the heavy chain constant regions.

The Fc portion of an antibody is responsible for exerting several effector functions [37]. Firstly, upon formation of immune complexes (antibody bound to antigen), various immune cells can be recruited which bind the Fc tails of the antibodies via their Fc receptor. This initiates antibody-dependent cytotoxic phagocytosis (ADCP) by *e.g.* macrophages or antibody-dependent cellular cytotoxicity (ADCC) by *e.g.* NK cells. Secondly, the complement system can be activated by antibodies, especially by IgG3 and IgM. Upon binding of C1q to the Fc portions of antibodies, cleavage of complement components C3 and C5 yields C3a and C5a. These mediate the recruitment of inflammatory cells, pathogen uptake by macrophages and the formation of the membrane attack complex (MAC) [38]. Third, IgG or IgA binding to antigen-coated pathogens can block entry into host cells (Figure 3C) [37].

B-cell tolerance

Multiple steps during B-cell development in the BM yield highly diverse BCR repertoires and B cells able to recognize a diversity of antigenic structures, including self-antigens expressed in healthy human tissues. In fact, around 50-75% of B cells in the BM are estimated to express BCRs reactive to self-antigens, both in mice and in humans [39-41]. Several checkpoints are present to ensure deletion of B cells reactive to self-antigen, also referred to as autoreactive B cells [42]. During B-cell development in the BM, central checkpoints significantly decrease the number of autoreactive B cells by receptor editing and, if necessary, subsequent clonal deletion. During receptor editing, autoreactive B cells rearrange their light chain to avoid autoreactivity. If self-affinity is too high or if receptor editing does not result in a loss of autoreactivity, the B cell is clonally deleted [43]. However, these central processes are not optimal and so, upon escape of autoreactive B cells into the periphery, peripheral checkpoints are present to add a second layer of controlling autoreactivity. First, multiple mouse models have revealed the principle of follicular exclusion where naïve autoreactive B cells compete with other B cells to enter a follicle. Upon exclusion from entering primary follicles, these autoreactive B cells will die within 1-3 days [44, 45]. Although the exact mechanism behind follicular competition of autoreactive B cells is not known, this poses a checkpoint to control peripheral B cell autoreactivity. Second, after exiting a follicle, B cells can enter GCs where they are presented with antigen by FDCs and subsequently receive survival signals in the form of IL-6 and BAFF [46, 47]. In case no antigen is presented to the B cell, it is unlikely to mediate a B cell response. Additionally, once activated, B cells migrate to the T/B cell border of the GC to present antigen and subsequently receive help signals from the T cells

(CD40-CD40L interaction), which poses a third peripheral B-cell checkpoint. Finally, FDCs deliver co-stimulation signals by presenting antigen opsonized by complement. These co-stimulatory complement signals reach the B cells via complement receptors CD21 and CD35 [48]. Since most self-antigens do not activate the complement system and are therefore not presented efficiently, they fail to activate B cells. Altogether, the central and peripheral B cell tolerance checkpoints prevent the majority of autoreactive B cells to mature and become activated. However, these checkpoint mechanisms are not absolute as evidenced by autoimmune diseases characterized by the presence of autoreactive B cells.

Rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by inflammation of synovial tissue and subsequent bone erosions, causing swollen and painful joints. In 2019, 18 million people were living with RA, of which 70% are women [49]. The risk of developing RA increases with age and although the exact cause is unknown, research indicates that a combination of environmental factors and genetics can trigger the development of RA. One established environmental risk factor is cigarette smoking, which has been described to increase the risk of RA development by 2-fold [50]. Additionally, infection with Epstein-Barr virus (EBV) is suspected to play a role in RA development, despite the lack of a known pathogenic mechanism. Moreover, the composition of intestinal bacteria has been suggested to play a role in RA pathogenesis but this hypothesis also lacks mechanistic evidence. The most significant genetic risk factor for RA is carrying human leukocyte antigen (HLA)-DRB1 alleles encoding shared epitope motifs, a sequence motif of five amino acids which is associated with susceptibility to RA and predicts poor disease prognosis [51, 52].

Currently available anti-rheumatic therapies alleviate pain and symptoms but do not cure the disease. Disease-modifying antirheumatic drugs (DMARDs) such as methotrexate or sulfasalazine are used to dampen the overactive immune system, and biologicals such as etanercept, abatacept or rituximab are used to inhibit specific inflammatory molecule such as tumor necrosis factor (TNF), co-stimulatory signals CD80 and CD86 or B-cell marker CD20, respectively. Glucocorticoids such as prednisolone may be administered to manage disease flares. Although the development of these anti-rheumatic therapies has greatly expanded RA-treatment options, the disadvantage of these treatments are unwanted side-effects as they affect the whole immune system, as well as, depending on the drug, other organs (*e.g.* liver) and sustained drug-free remission is rarely achieved. Therefore, specific targeted therapies could, potentially, maximize treatment efficacy while minimizing side effects. The presence of disease specific antibodies and the efficacy of B cell-depleting therapy in RA together highlight the importance of (antigen-specific) B cells in disease pathogenesis. Therefore, this thesis focuses on these autoreactive B cells and on strategies to more specifically dampen or even eradicate disease.

Autoantibodies in RA

The first type of autoantibodies detected in RA was rheumatoid factor (RF), which are polyclonal antibodies directed against the Fc-tail of IgG. Although different

isotypes and titer levels are observed in healthy individuals and in multiple diseases [53], high titers of IgM-RF and IgA-RF are considered to be indicative of RA [54]. However, the biological function of RF involvement in disease pathogenesis is still unclear [55, 56]. Under healthy conditions, RF may promote immune responses by forming immune complexes with IgG bound to its antigen [57, 58]. Since RF displays characteristics of affinity maturation in RA, it is thought that high titers of high-affinity RF may lead to a derailed inflammatory reaction and overproduction of proinflammatory cytokines [59, 60]. Other autoantibodies found in RA, but, like RF, not disease specific are anti-collagen antibodies, anti-nuclear antibodies (ANA), anti-malondialdehyde-antibodies (anti-MDA) and anti-malondialdehyde-acetaldehyde antibodies (anti-MAA).

In contrast, antibodies directed against citrullinated proteins or peptides, or anti-citrullinated protein antibodies (ACPA), are specifically found in patients with RA. ACPA are detected in approximately 60-90% of established RA patients and in 50-60% of early RA patients [61, 62]. In healthy individuals, ACPA are detected in only 1-3%, usually at low levels [63]. This makes the presence of ACPA a valuable diagnostic tool [64]. ACPA were first discovered in 1964 as antiperinuclear factor [65], yet their citrulline specificity was described years later [66]. ACPA are known to bind many different citrullinated epitopes including epitopes derived from vimentin, fibrin, alpha-enolase, fibrinogen, and myelin basic protein [67-70]. Some are cross-reactive, thus able to bind multiple citrullinated epitopes [70]. Additionally, ACPA display broad cross-reactivity to the other PTMs carbamylation and acetylation [71].

Based on the presence or absence of the disease specific ACPA, RA can be divided in two major subsets with different disease courses. Development of ACPA-positive RA is strongly associated with HLA-DR alleles encoding shared epitope, and is associated with worse disease prognosis hallmarked by more severe bone and joint destruction [72, 73]. Additionally, ACPA-positive RA patients typically respond well to $CD20^+$-B cell-depleting therapy [74]. In contrast, the etiology and development of ACPA-negative RA is less well understood and seemingly more heterogeneous [55]. For these reasons, this thesis focuses on ACPA-positive RA.

ACPA-expressing B cells

ACPA-expressing B cells can be isolated from peripheral blood and synovial fluid of RA patients using fluorescently-labeled streptavidin tetramers carrying biotinylated cyclic citrullinated peptide (CCP) [75]. This approach allows for characterization of the ACPA-expressing B response. ACPA-expressing B cells exist in relatively low frequencies in the peripheral blood of RA patients, comparable to anti-tetanus toxoid (TT)-expressing B cells [75, 76]. The latter is often used as antigen-specific comparator. However, in contrast to anti-TT-expressing B cells, ACPA-expressing B cells show elevated levels of CD19 and proliferation marker Ki67 [76]. Additionally, ACPA-expressing B cells highly express T cell-stimulating ligands CD80 and CD86 [76], and BCR-encoding genes from ACPA-expressing B cells demonstrate an extensive degree of SHM [77]. Moreover, the majority of detected ACPA-expressing B cells resemble a $CD27^+$ memory B-cell phenotype [76], although recently updated staining procedures including an intracellular staining step now also reveal an active plasmablast compartment [78]. Altogether,

these characteristics suggest that ACPA-expressing B cells may undergo continuous (re)activation in GCs. Glycans present in the variable domain of the ACPA-BCR may play an important role in this, as these glycans have been shown to affect antigen binding and BCR-mediated B-cell activation [79].

Thesis outline

The overall aim of this thesis is to gain insight into the origin and persistence of the ACPA B-cell response in RA and to explore novel strategies to antigen-specifically target these cells. Unraveling the mechanisms underlying the development and maintenance of ACPA-B cells is of importance as it could lead to the identification of vulnerabilities which may be exploited therapeutically. Developing antigen-specific targeting approaches holds promise for more precise and effective treatments, potentially minimizing side effects and improving patient quality of life.

The majority of ACPA-expressing B cells are highly mutated, $CD27^+$ memory B cells. However, as described in **chapter 2**, $CD27^-$ cells can also be detected. BCR sequencing demonstrated mutated and germline sequences. By producing the (variable domain) sequences as monoclonal antibodies, we found that the majority of germline sequences were actually streptavidin reactive. We identified one germline-encoded ACPA sequence, indicating that ACPA-expressing B cells can in fact be germline encoded and present in the naïve compartment.

For many years, EBV infection has been implicated in RA pathogenesis, despite lacking mechanistical evidence. **Chapter 3** describes the investigations on the hypothesis that EBV infection directly facilitates the development and persistence of autoreactive B cells. To this end, we isolated ACPA-expressing B cells and found that these are not enriched for EBV-DNA-containing clones.

Recent investigations have described the activated state of ACPA-expressing B cells. **Chapter 4** reports an antigen-specific staining adapted to simultaneously measure the phosphorylation status of kinases downstream of the BCR, directly *ex vivo*. This staining approach demonstrated increased phosphorylation levels of signaling molecules downstream of the BCR, implying recent BCR triggering of these ACPA-expressing B cells.

Identification of disease-specific, possibly pathogenic, B cells allows for the exploration of antigen-specific targeting of these B cells. **Chapter 5** outlines recently investigated approaches to establish antigen-specific B-cell targeting in the context of autoimmune diseases.

Chapter 6 outlines the application of antigen-drug conjugates to specifically target ACPA-expressing B cells. Conjugates composed of CCP4 and the toxin MMAE were tested on B-cell lines expressing ACPA BCRs. This revealed that dimeric CCP4-MMAE conjugates were unable to kill ACPA-expressing cell lines, despite confirmed functionality of the conjugates. However, polymeric scaffolds carrying CCP4 and MMAE could antigen-specifically eradicate several ACPA-expressing cell lines.

Chapter 7 describes the usage of a bispecific complement engager (BiCE) composed of the anti-C1q nanobody Nb75 conjugated to CCP4. We demonstrated the efficient, antigen-specific killing induced by this BiCE on ACPA-expressing cell

lines. Additionally, we investigated the effects on patient-derived, primary ACPA-B cells. Collectively, we demonstrated the suitability of this modality for antigen-specific elimination of autoreactive B cells.

In **chapter 8**, the results described in this thesis are summarized and discussed.

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