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

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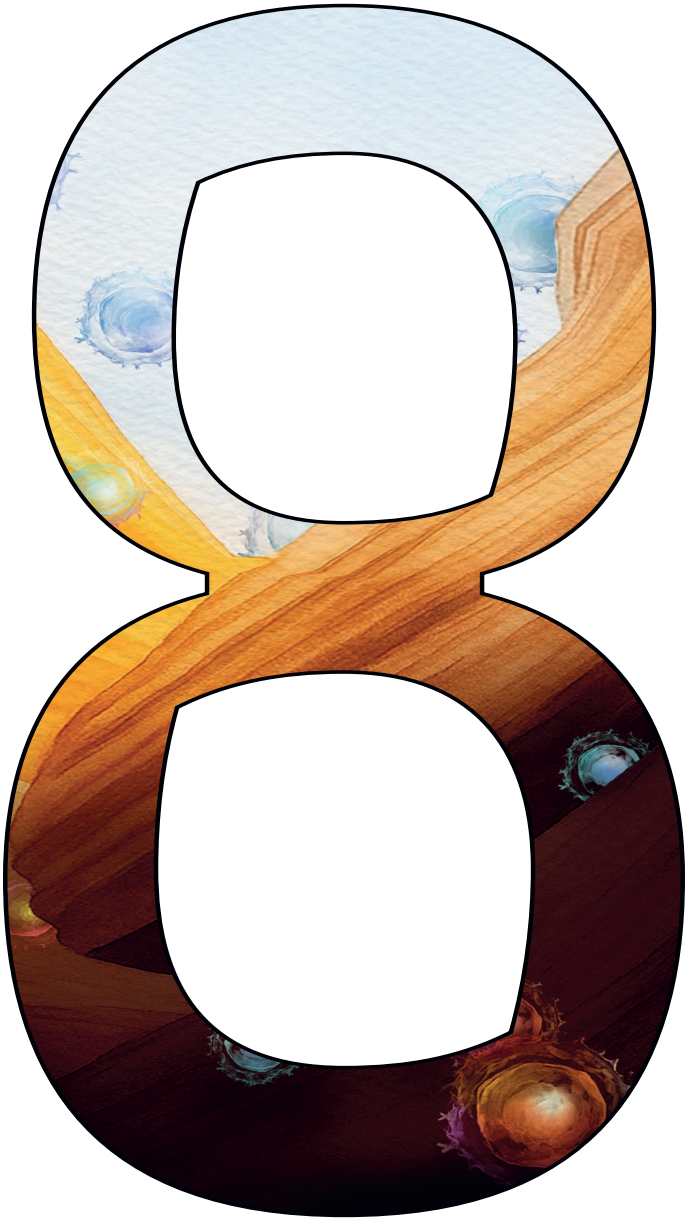

CHAPTER 8

Summarizing discussion

Many autoimmune diseases (AIDs) are characterized by the presence of autoantibodies targeting a plethora of autoantigens. For instance, autoantibodies targeting nuclear antigens are prevalent in systemic AIDs such as systemic lupus erythematosus (SLE), systemic sclerosis (SSc), vasculitis and Sjögren's syndrome (SjS). Likewise, autoantibodies recognizing post-translational modifications, including citrullination, carbamylation and acetylation, can be present in rheumatoid arthritis (RA). However, the mechanisms driving the development and persistence of the underlying autoreactive B-cell responses remain to be elucidated. Gaining insights on these mechanisms may advance strategies to target autoreactive B cells in an antigen-specific manner. Such approaches enable more precise treatments for AIDs, thereby reducing the need for systemic therapies.

Anti-Citrullinated Protein Antibodies in RA

In this thesis, we focus on anti-citrullinated protein antibodies (ACPA) and the ACPA-expressing B cells as they are disease specific and are associated with disease severity [1]. To explain the possible role of ACPA in RA pathogenesis, several mechanisms have been proposed, although primarily based on *in vitro* studies. First, ACPA-immune complexes have been described to trigger the classical and alternative complement pathways, which could lead to inflammation and tissue damage [2]. Second, ACPA-immune complexes can bind Fcγ receptors on monocytes and macrophages and thereby induce the release of pro-inflammatory cytokines [3]. Third, ACPA have been implicated in osteoclastogenesis. Osteoclasts express protein arginine deiminases (PADs), enzymes responsible for protein citrullination, which have been described to citrullinate vimentin during osteoclast differentiation. When adding autoantibodies against citrullinated vimentin to osteoclasts, the autoantibodies induced osteoclastogenesis and bone resorption [4]. Finally, ACPA have been reported to induce pain in mouse models, although caution is warranted when interpreting such findings, as the specificity of the used antibodies may not always be verified properly [5, 6]. Other important considerations when interpreting results from such studies include potential endotoxin (LPS) contamination in purified ACPA, antibody aggregation, and presence of rheumatoid factor, all of which could influence experimental outcomes. Moreover, monoclonal ACPA may not perfectly represent the polyclonal ACPA population observed in RA patients [7], which could have functional implications.

Although ACPA are specific to RA and highly abundant in the joints of RA patients [8], their role in disease pathogenicity remains uncertain. ACPA serve as a predictive marker for joint damage severity and are thought to engage in several possibly pathogenic pathways, as discussed above [1]. However, several factors argue against the pathogenicity of ACPA. For one, ACPA can be detected in asymptomatic individuals in a pre-disease autoimmune phase, suggesting they are not solely responsible for disease onset [9, 10]. Additionally, ACPA often persist in patients that have achieved clinical remission following successful treatment [11], indicating that their presence does not necessarily drives ongoing inflammation. Furthermore, passive transfer of ACPA into animal models does not consistently induce arthritis, arguing against a direct pathogenic role. Notably, multiple studies have even indicated a protective effect of ACPA in inducible mouse models for arthritis, highlighting the complexity of their involvement in RA. Already in 2013, a monoclonal ACPA was described to prevent the onset of inflammation in collagen-

antibody induced arthritis (CAIA) and collagen-induced arthritis (CIA) mouse models for RA [12]. Additionally, when administered therapeutically in the CAIA and CIA model, this ACPA monoclonal named CIT-013 halted a further increase of the inflammatory response. A citrullinated domain of histone-2A/4 (H2A/H4) was determined as the epitope recognized specifically by the protective CIT-013 antibody, not by the other non-protective antibodies [12]. Subsequently, CIT-013 was described to inhibit the release of neutrophil extracellular traps (NETs) and to stimulate NET clearance by macrophages through binding Fcγ receptors [13]. Moreover, binding of CIT-013 to RA synovium indicated the presence of citrullinated NETs which, upon binding by CIT-013, may be cleared [14]. Interestingly, E4, another antibody described to exert a protective effect in the CAIA model, does not bind H2A/H4, indicating different protective mechanisms may be at play. Indeed, E4 was shown to bind to α-enolase and its protective effect depends on immune complex formation between E4 bound to citrullinated α-enolase and FcγRIIb on macrophages, which resulted in an increased IL-10 production and attenuated osteoclastogenesis [15]. Therefore, E4 is thought to exert its protective effect by binding degraded collagen in CAIA mice and Fc-mediated binding to macrophages subsequently leading to secretion of anti-inflammatory IL-10. Other studies using the CAIA model have demonstrated similar anti-inflammatory effects of ACPA independent of H2A/H4 binding and additionally highlighted the importance of timing when administering ACPA for therapeutic effects [16, 17]. However, when interpreting results from studies such as the ones referenced here, it is important to consider that CAIA and CIA are induced in mice and that these models do not depend on citrullination and thus deviate considerably from RA.

Given the contradictory results regarding the role of ACPA in RA pathogenicity, one could argue that ACPA act as a proxy for pathogenic B- and/or T-cell responses, marking the ACPA-expressing B cells as pathogenic players in RA rather than their secreted autoantibodies. Given the convincing beneficial effects of B cell-targeting therapies in RA, which will be later touched upon, a key role of B cells in disease pathogenesis is undeniable. Understanding how autoreactive B cells develop and persist, enables the exploration of options for targeted therapies. In this thesis, we further elucidated the ACPA B-cell response and utilized it as a prototypic autoreactive B-cell response to investigate novel antigen-specific therapies.

Initiation of ACPA B cells

Previous studies on ACPA-expressing B cells primarily focused on the memory B-cell compartment, revealing a remarkably high degree of somatic hypermutation (SHM) in their BCRs [18]. SHM can play an important role in disease initiation as exemplified by pemphigus vulgaris (PV), a blistering AID driven by autoantibodies against desmoglein 3 (DSG3). In PV, B cells acquire *de novo* autoreactivity through SHM, as binding to DSG3 is lost when sequences are reverted to germline, indicating that autoreactivity arises from mutations originally generated in response to an unrelated antigen [19]. For the ACPA response, however, reactivity to citrullinated antigen is still observed in germline-reverted BCR sequences, indicating it does not necessarily rely on extensive SHM [20]. However, it is important to note that the more mutations an original sequence contains, the harder it becomes to accurately reconstruct its germline configuration. To further elucidate the role of SHM in the ACPA response, we studied naïve ACPA-expressing B cells,

as reported in **chapter 2** of this thesis. Beside highlighting important technical issues leading to the detection of false-positive cells due to streptavidin reactivity, we in fact report one naïve ACPA-expressing B cell. This BCR clone contains no somatic hypermutations, but shows clear binding to cyclic citrullinated peptide and exhibits cross-reactivity to cyclic homocitrullinated peptide. This finding indicates that the formation of an ACPA-response does not necessarily rely on SHM and can, in fact, result from germline-encoded autoreactive B cells. However, it is tempting to speculate that ACPA-expressing B cells do rely on SHM in terms of maturation and acquiring pathogenic features. As such, the process of SHM enables the introduction of N-linked glycosylation sites, leading to the presence of N-linked glycans on the variable domains of the ACPA BCR which are shown to be predictive for RA development [21]. The presence of these glycans enhances BCR signaling and affects antigen uptake, potentially promoting the expansion of ACPA-expressing B cells and increasing autoantibody production towards disease onset [22].

As ACPA can be present in serum of patients with RA and healthy individuals, it raises the question of whether naïve B cells expressing germline-encoded ACPA are part of the repertoire of healthy individuals as well [23-26]. Germline autoreactive sequences within the naïve BCR repertoire are crucial for maintaining a baseline level of autoreactivity, which is thought to contribute to immune surveillance in all individuals [27-29]. This inherent autoreactivity arises because germline-encoded BCRs must exhibit a broad recognition profile to protect against a wide variety of pathogens [27]. However, this broad recognition profile carries the risk that BCRs recognizing foreign antigens may inadvertently cross-react with self-antigens that share similar sequences or structural features, a phenomenon known as molecular mimicry [30, 31]. Molecular mimicry may be particularly relevant in the context of PTMs such as citrullination, which widely occur in bacterial- as well as human proteins. Such similarities increase the likelihood that B cells primed against foreign citrullinated antigen could also react with self-antigens, potentially contributing to the generation of autoreactive B cells [32, 33]. In fact, molecular similarities between Epstein-Barr virus (EBV) and self-antigens relevant in RA have been observed and ACPA-IgG has been shown to recognize citrullinated EBV peptides [34, 35]. Additionally, EBV reactivation is linked to disease onset and flares in RA, SLE, pSS and multiple sclerosis (MS) [36].

Another hypothesis of how EBV may contribute to the initiation of ACPA-B cells, is by directly contributing to the survival of autoreactive B cells, *e.g.* through mimicking B cell-receptor activation and T cell-help signals. This could theoretically enable a proliferative phenotype while evading elimination during B-cell selection. We challenged this hypothesis by the studies described in **chapter 3**. Despite clear clinical associations between RA and EBV infection, we found no evidence of EBV viral copies within ACPA-expressing B cells, suggesting a more indirect role for EBV in RA pathogenesis. It is plausible that instead of causing RA, uncontrolled EBV activity, characterized by elevated antibody titers and increased numbers of EBV-infected B cells, reflects the immune dysregulation already present in RA patients. In contrast, recent longitudinal data from a US military cohort showed a 32-fold increased risk of developing MS following EBV infection, implicating EBV as a primary cause of MS [37]. This raises intriguing questions about whether autoreactive B-cell populations in MS patients, such as anti-myelin basic protein-expressing B cells, harbor EBV copies. Investigating this relationship could provide

valuable insights into the role of EBV in MS and other autoimmune conditions. Although also in MS an important role for molecular mimicry has been indicated, as a monoclonal antibody derived from cerebrospinal fluid of an MS patient revealed binding to both EBV nuclear antigen 1 (EBNA1) and the central nervous system protein glial cell adhesion molecule (GlialCAM) [38].

Persistence of ACPA B cells

Advances in antigen-specific phenotyping techniques have enabled extensive characterization of the ACPA B-cell response. The majority of ACPA-B cells present in circulation of RA patients belong to the IgG-memory compartment and exhibit a highly proliferative phenotype in comparison to anti-tetanus toxoid (TT) B cells, which are used as antigen-specific comparators [39]. As such, ACPA-B cells in established RA patients highly express CD19, CD80, CD86, Ki67 along with a reduced expression of CD21, CD24 and FcγRIIb [39-42]. High expression of CD80 and CD86 molecules indicate that ACPA-B cells can provide T cells with costimulatory signals necessary for T-cell activation and survival, and downregulation of CD21 and CD24 indicate recent B-cell activation and germinal center (GC) emigration [43]. Additionally, in some patients, the ACPA B-cell population consists of up to 60% plasmablasts (PBs) and both these cells as well as ACPA-expressing memory B cells (MBCs) express elevated levels of CXCR3 [41]. This chemokine receptor for CXCL9, CXCL10 and CXCL11 allows the ACPA-expressing MBCs and ACPA PBs to locate to synovial tissue, as increased concentrations of these chemokines are observed in synovial tissue [44]. Moreover, increased amounts of ACPA and ACPA-B cells have been demonstrated in synovial fluid of RA patients [39, 45]. Overall, the phenotype of the ACPA B-cell response fits with anti-vaccine B cells within the first two weeks of vaccine boosting. However, unlike vaccine-induced antigen-specific B-cell responses which rapidly transition to a resting memory state, ACPA-B cells remain persistently activated throughout the disease course [40]. To date, it is unclear whether persistence of ACPA-B cells is maintained merely by the continuous presence of (auto)antigens, or if continuous T-cell help is also required. If so, identifying which antigens are recognized by these T cells is vital as their effect on the ACPA-B cells may be the driver of disease chronicity.

Highlighting the role of ACPA B-cell activity in RA pathogenesis, ACPA-expressing MBCs are less active in long-term clinically suspect arthralgia (CSA) patients who do not convert to RA for over two years, compared to those with active disease or CSA patients who eventually convert to RA [39, 46]. Also in patients in sustained disease-modifying antirheumatic drug (DMARD)-free remission (SDFR), ACPA MBCs demonstrated lower CD80 expression than in active RA patients. Additionally, in CSA non-converters, less ACPA PBs were present in circulation, ACPA-B cells expressed lower Ki67 and FcγRIIb was not reduced [46]. Altogether, these findings indicate that the activation level of ACPA$^+$ MBCs may predict RA progression or SDFR.

To date, it remains unclear how ACPA-expressing B cells can continuously exhibit such a proliferative phenotype without going into apoptosis or becoming anergic or exhausted. To further elucidate this observation, we sought to characterize intracellular signaling molecules in ACPA-expressing B cells, as outlined in **chapter 4**. We developed a flow cytometry staining approach enabling the

simultaneous measurement of protein phosphorylation and antigen specificity. This adapted method was essential, as antigen-specific staining itself triggers the phosphorylation of protein kinases downstream of the BCR, potentially affecting the readout. We measured protein phosphorylation of kinases spleen tyrosine kinase (SYK), Bruton's tyrosine kinase (BTK), the serine/threonine-specific protein kinase AKT (AKT) and ribosomal protein S6 (S6) in ACPA-expressing MBCs and anti-TT-expressing MBCs from RA patients, directly after isolation and without any *in vitro* BCR stimulation. This antigen-specific phosphoflow staining revealed that circulating ACPA-expressing MBCs, compared to anti-TT-expressing MBCs, exhibit elevated kinase phosphorylation. This observation possibly reflects recent antigen encounter and/or impaired clonal deletion of these autoreactive B cells. Understanding the molecular mechanisms underlying chronic ACPA B-cell activation is essential for developing more precise and effective therapies for AIDs. As such, the observations described in **chapter 4** indicate protein kinases as possible targets for therapy. For instance, BTK inhibitors may offer potential for modulating the activity of ACPA-B cells.

Targeting B cells in RA

Autoreactive B cells contribute to disease pathogenesis of AIDs, including RA, through multiple mechanisms. While the direct pathogenic role of produced autoantibodies remains a topic of debate, B cells are known to secrete pro-inflammatory cytokines promoting inflammation and immune cell filtration. Moreover, B cells are highly effective antigen-presenting cells, capable of activating HLA class II-restricted T cells, thereby potentially sustaining and amplifying autoreactive T-cell responses. Due to their central role in RA pathogenesis, B cells have become a major therapeutic target. Several classes of targeted therapies have been developed to modulate B-cell activity at various levels of differentiation and function.

BTK inhibitors

While BTK inhibitors were initially developed to treat B-cell malignancies, they are currently being investigated as therapeutic candidates in the treatment of RA. As described in **chapter 4**, we observed elevated BTK phosphorylation in ACPA-expressing MBCs. Additionally, levels of BTK and phosphorylated BTK are increased in the total B-cell compartment of ACPA-positive compared to ACPA-negative RA patient [47]. These findings support the rationale for targeting BTK in ACPA-positive RA and indeed, several (pre)clinical studies investigate(d) BTK inhibitors for treating RA. For example, evobrutinib, a second-generation irreversible BTK inhibitor, demonstrated inhibition of arthritis progression in the CIA mouse model despite failure to decrease autoantibodies [48]. In subsequent clinical trials, evobrutinib showed to be well-tolerated but regarding efficacy, primary endpoints were not met [49-51]. However, evobrutinib did reveal a trend towards improved clinical responses when given at higher doses. Additionally, fenebrutinib, a second-generation reversible BTK inhibitor, also demonstrated efficacy in the CIA mouse model [52]. In following clinical studies, fenebrutinib was well-tolerated and demonstrated efficacy when combined with methotrexate, similar to adalimumab combined with methotrexate [53]. However, when administered as monotherapy, fenebrutinib failed to induce significant improvement in disease activity scores [53]. Altogether, several BTK inhibitors are being evaluated for

the treatment of RA and outcomes so far have been variable with some studies observing modest therapeutic effects. Increasing these effects may be possible by extending treatment duration, optimizing dosing, or combining with other immunomodulatory agents.

JAK inhibitors
In contrast to BTK inhibitors, Janus Kinase (JAK) inhibitors have shown convincing favorable clinical benefits and are used to treat RA [54]. JAK inhibitors target the JAK-signal transducer and activator of transcription proteins (STAT) signaling pathway. Normally, the JAK-STAT pathway is initiated by a ligand (*e.g.* cytokine) binding its receptor, inducing the phosphorylation of JAK, which subsequently phosphorylates the receptor. This creates docking sites for STAT proteins, which are then also phosphorylated by JAKs. Phosphorylated STATs dimerize and translocate to the nucleus to regulate gene expression [55]. As such, cytokines and growth factors can regulate cell function, growth and differentiation. JAK inhibitors such as tofacitinib and baricitinib inhibit the activity of JAK proteins. This way, JAK inhibitors do not directly target a single cell type such as B cells. However, they do contribute to silencing hyperactive B cells by blocking the activity of various cytokines and growth factors [54]. Despite clinical efficacy, JAK inhibitors fail to silence the activated phenotype of ACPA-expressing MBCs [40]. Whether JAK-STAT signaling is enhanced in ACPA-expressing MBCs, and whether this is effected by JAK inhibitors, remains to be investigated. In the studies described in **chapter 4**, phosphorylated STAT was not included since its staining requires a different permeabilization method containing buffers incompatible with our current antigen-specific staining panel.

Rituximab
Rituximab is a chimeric IgG1-monoclonal antibody targeting CD20 used as a treatment for RA, in particular patients refractory to TFN inhibitors. It depletes CD20-expressing B cells through multiple mechanisms, including complement-dependent cytotoxicity, Fcγ receptor-mediated antibody-dependent cellular cytotoxicity, Fcγ receptor-mediated antibody-dependent cellular phagocytosis, and direct induction of apoptosis [56]. CD20 is expressed by the majority of B cells, except for pro-B cells, PBs and terminally differentiated plasma cells (PCs), which are therefore not depleted by rituximab. Despite this, rituximab has demonstrated clinical efficacy in RA, suggesting that the unaffected B-cell subsets may not be the primary drivers of disease [57]. However, in other AIDs such as SLE, the clinical efficacy of rituximab has been controversial. Clinical trials failed to meet their primary endpoints, although concerns were raised regarding background immunosuppression potentially masking the benefits of rituximab. Additionally, numerous observational studies did demonstrate relevant clinical improvements in patients with refractory SLE, particularly in lupus nephritis and neuropsychiatric lupus [58-61]. In these cases, rituximab is often used off-label when conventional therapies fail.

Importantly, the preservation of terminally differentiated PCs allows for the maintenance of vaccine-induced immunity acquired prior to rituximab treatment, although patients are advised to get booster vaccinations prior to rituximab treatment to maximize responses [62]. However, responses to vaccinations administered after rituximab treatment are often diminished [62, 63]. Additionally, treatment cessation often induces relapse of disease, entailing

repeated rituximab administration. Moreover, patients receiving rituximab can experience infusion-related side effects, particularly during the first infusion, due to the rapid destruction of B cells and release of cytokines. Furthermore, upon receiving rituximab, patients have an increased risk for infections, particularly in the context of hypogammaglobulinemia or repeated dosing [64, 65]. Despite these disadvantages, the success of rituximab in RA was pivotal in establishing B cells as key contributors to RA pathogenesis and catalyzed the development of B cell-targeted therapies in AIDs.

Anti-CD19 CAR-T cells

A more recent and highly promising B cell-targeted therapy involves autologous anti-CD19 chimeric antigen receptor (CAR) T cells, for which autologous T cells are isolated and engineered to express anti-CD19 single-chain variable fragments (scFv). CD19 is expressed throughout a broad spectrum of B-cell subsets, including pro-B cells, PBs and some subsets of PCs, making it a broader target than CD20. Unlike rituximab, which relies on passive diffusion for reaching target B cells, CAR-T cells can actively migrate to inflammatory environments containing activated B cells [66]. This migratory capacity, combined with their long-term persistence, enables more effective infiltration and depletion of B cells in tissue compartments such as the synovium. These features are particularly advantageous in AIDs characterized by tissue involvement, where conventional monoclonal antibody therapies may show limited efficacy.

Although anti-CD19 CAR-T-cell therapy has not yet been evaluated in RA, it has been investigated in patients with SLE, SSc and idiopathic inflammatory myositis (IM) [67]. In both SLE and IM patients, disease symptoms fully resolved, while patients with SSc experienced a decrease in the severity of skin and lung involvement. Remarkably, a single administration of autologous anti-CD19 CAR-T cells enabled all treated patients to discontinue their immunosuppressive therapy without experiencing relapses or disease progression, as observed at the most recent follow-up (maximally two years at the time of writing this). Furthermore, B cell were reconstituted while serum autoantibody levels declined, and vaccine-induced antibody levels remained stable, suggesting that CD19-negative long-lived PCs are not depleted by the therapy [67]. Importantly, observed side effects of the treatment were minimal, with no cases of severe cytokine release syndrome which is commonly observed in oncology settings, possibly due to the lower antigenic load in patients with AIDs compared to those with cancer. While only 15 patients have been included so far, this study demonstrates the ability of anti-CD19 CAR-T cell therapy to re-establish immunological tolerance and even induce sustained, drug-free remission. The sustained absence of disease may be a result from the disruption of the germinal center reaction. Upon B-cell depletion, follicular dendritic cells (FDCs), which present the antigen to the B cells and rely on the B cells for survival, also die. As a result, (auto)antigen presentation ceases, halting the germinal center response and essentially resetting the system to a naïve-like state. Nevertheless, further studies are required to determine the long-term durability of the responses.

Despite its promise, rare reports of T-cell lymphoma following anti-CD19 CAR-T cell therapy raise concerns that may limit its use in relatively mild or non-life threatening AIDs such as RA, although further studied are needed to clarify any causal relationship [68]. In addition, the overall clinical application of anti-CD19 CAR-

T-cell therapy faces significant challenges related to manufacturing logistics and high costs, particularly due to the need for intensive hospital-based care during- and after treatment [69]. An intriguing strategy to overcome these limitations involves *in vivo* CAR-T-cell generation, as demonstrated in a recent study using lipid nanoparticles to deliver CAR constructs selectively to $CD8^+$ T cells [70].

Blinatumomab
Blinatumomab is a bispecific T-cell engager (BiTE) containing anti-CD3 scFv and anti-CD19 scFv linked through a short peptide linker. By redirecting autologous T cells to CD19-expressing B cells, blinatumomab facilitates T cell-mediated killing of target cells.

A recent study reported the treatment of six patients with multidrug-resistant RA using blinatumomab. The treatment led to B-cell depletion in the periphery as well as in synovial tissue [71]. Notably, a reduction in disease activity was observed after 12 weeks, which persisted for up to 6 months. Similar to anti-CD19 CAR-T cells in SLE, SSc and IM patients, treatment with blinatumomab did not induce severe cytokine release syndrome, although the dosage used in this context was significantly lower than typically used in hematological malignancies [71]. In addition, a case report describes clinical improvement of an SSc patient treated with blinatumomab [72]. While these preliminary findings are promising, larger and more controlled studies are necessary to assess the treatment's long-term efficacy and its potential advantages over other (B cell-targeting) therapies.

Targeting ACPA-B cells in RA

While multiple B cell-targeted therapies hold promise in treating AIDs, it remains a challenge to treat these diseases in an antigen-specific manner. In AIDs such as RA, where the overall disease burden experienced by patients is lower than in *e.g.* SLE or SSc, the need for more precise therapies is particularly important to minimize treatment-related side effects. For example, broad B cell-targeting therapies such as anti-CD19 CAR-T cells or blinatumomab can effectively reduce disease activity but also leave patients vulnerable to serious infections. Moreover, anti-CD19 CAR-T cells come with substantial drawbacks, including high costs and the requirement for preconditioning with chemotherapy, which is both toxic and potentially harmful to fertility. Altogether, this highlights the ongoing need for more refined therapeutic approaches, particularly strategies that enable antigen-specific B-cell targeting. Such therapies have the potential to improve efficacy while reducing toxicity and preserving healthy immunity.

In **chapter 5**, we reviewed currently explored antigen-specific B cell-targeting approaches as treatments for AIDs. We highlight a variety of treatment modalities, ranging from synthetic polymers to cellular therapies and describe their advantages, challenges and status in research and development. Among the more advanced and promising strategies discussed in **chapter 5** are chimeric autoantigen receptor (CAAR)-T cells. These are T cells engineered to express autoantigen on their surface, enabling them to selectively bind and deplete autoreactive B cells expressing BCRs specific to that autoantigen. While CAAR-T cells have not yet been described in the context of RA, significant progress has been made in other AIDs. For instance, CAAR-T cells expressing DSG3 and CAAR-T cells expressing

muscle-specific kinase (MuSK) have shown promise in preclinical studies of PV and MuSK myasthenia gravis, respectively [73, 74]. These encouraging results have led to the initiation of phase I clinical trials (NCT04422912, NCT05451212). Although not discussed in this thesis, CAAR-expressing natural killer (NK) cells also represent a noteworthy development. These cells offer advantages over CAAR-T cells, most notably their potential for off-the-shelf use due to their compatibility with allogeneic donors [75]. However, a key limitation of CAAR-NK cells is their reduced persistence, which may compromise their long-term therapeutic efficacy [75]. Notably, CAAR-NK cells targeting anti-La/SSB-expressing B cells have shown specific cytotoxicity *in vitro* in the context of SLE and SjS, although no subsequent *in vivo* studies have been reported to date [76].

Among the other described modalities in **chapter 5** are polymers and antigen-drug conjugates. In **chapter 6**, we describe the synthesis and evaluation of antigen-drug conjugates consisting of dimeric CCP4 linked to the toxin monomethyl auristatin E (MMAE), as well as the synthesis and evaluation of antigen-drug polymers carrying CCP4 and MMAE molecules. While the CCP4-MMAE dimers were successfully synthesized and demonstrated efficient internalization into target cells and cleavage by cathepsins to release active MMAE, they failed to selectively kill the target cells. In contrast, CCP4-MMAE polymers did achieve antigen-specific cell killing. The substantial differences in structure and antigen valency between dimers and polymers likely influence BCR engagement, internalization efficiency, and trafficking to cathepsin-rich endolysosomal compartments, possibly causing the observed differences in killing efficiency. Additionally, the higher MMAE payload on the polymers likely enhances toxin delivery, contributing to their superior killing efficiency. Thus, dimers may not deliver their cargo as effectively as polymers, emphasizing synthetic polymers as a suitable toxin delivery platform to eradicate autoreactive B cells.

In the studies described in **chapter 6**, the polymers carried CCP4 in combination with MMAE, a potent toxin that inhibits mitosis by preventing tubulin polymerization [77]. Due to its high toxicity, MMAE cannot be administered on its own and therefore requires conjugation to a carrier, in this case a polymer with CCP4, to allow for targeted delivery. However, despite this advantage of targeted delivery, clinical approval of such a therapeutic for diseases like RA remains uncertain, given the possibility of severe side effects associated with MMAE. Thus, while the CCP4-MMAE polymer strategy serves as a clear proof of principle, it may be more desirable to link a less toxic agent to the CCP4-carrying polymers. As discussed in **chapter 4**, several protein kinases are upregulated in ACPA-expressing MBCs compared to other MBCs, highlighting them as promising therapeutic targets. For example, conjugating a selective BTK inhibitor to the polymers could potentially silence hyperactive ACPA-expressing MBCs by inhibiting BCR signaling upon in response to (autoantigenic) stimulation.

Another antigen-specific treatment strategy described in this thesis involves bispecific complement engagers (BiCE). The studies presented in **chapter 7**, demonstrate the synthesis and proof of principle of BiCEs composed of CCP4 linked to the anti-C1q nanobody Nb75. We show that the C1qNb75-ta-CCP4 BiCE is able to selectively target multiple ACPA-expressing Ramos cell lines, without affecting anti-TT-expressing Ramos cells. This effect is dependent on the presence of normal human serum containing complement components and could be inhibited by the

C5 inhibitor eculizumab, confirming a complement-dependent mode of action. Additional experiments on primary, patient-derived ACPA-expressing B cells are still ongoing, but current results suggest that this approach holds promise as an off-the-shelf targeted therapy for ACPA-positive RA patients.

Both therapeutic approaches described in **chapters 6 and 7**, namely CCP4-MMAE polymers and C1qNb75-ta-CCP4 BiCEs, show strong potential for the selective targeting of ACPA-expressing B cells while sparing non-autoreactive B cells. However, their efficacy may be compromised by the presence of circulating autoantibodies, as ACPA can bind to CCP4 on the therapeutic, thereby blocking its activity. This limitation could potentially be overcome through strategies such as drug dosage increase or plasmapheresis. Future steps should include testing these modalities on patient-derived ACPA-expressing B cells, followed by evaluation in relevant *in vivo* models. Given the difficulty of eliciting an ACPA B-cell response in mice through immunization [78], the use of BCR-transgenic mice may offer the possibility to test these therapeutics *in vivo*.

One additional benefit of such antigen-specific approaches is their modularity. By exchanging the antigen on the polymer or BiCE, these platforms could be adapted for use in other AIDs. For BiCEs, however, intact endogenous complement activity is a prerequisite for efficacy, meaning that their application would be limited in *e.g.* SLE patients with C1q deficiency [79]. Moreover, the modularity of the approaches opens the possibility to combine several antigens. In the context of RA, emerging evidence suggests that disease pathogenesis may not be driven exclusively by ACPA-expressing B cells, but also by B cells recognizing other post-translational modification such as acetylation and homocitrullination [41]. Adding acetylated and/or homocitrullinated peptides to polymers and/or BiCEs can therefore be of added value.

Concluding remarks

An increasing number of studies suggest that the ACPA-B cells, rather than their secreted antibodies, play a central role in disease pathogenesis of ACPA-positive RA. Collectively, the work described in this thesis advances our understanding of the initiation and persistence of the ACPA B-cell response and introduces novel, targeted therapeutic strategies aimed at the selective depletion of ACPA-expressing B cells in RA patients.

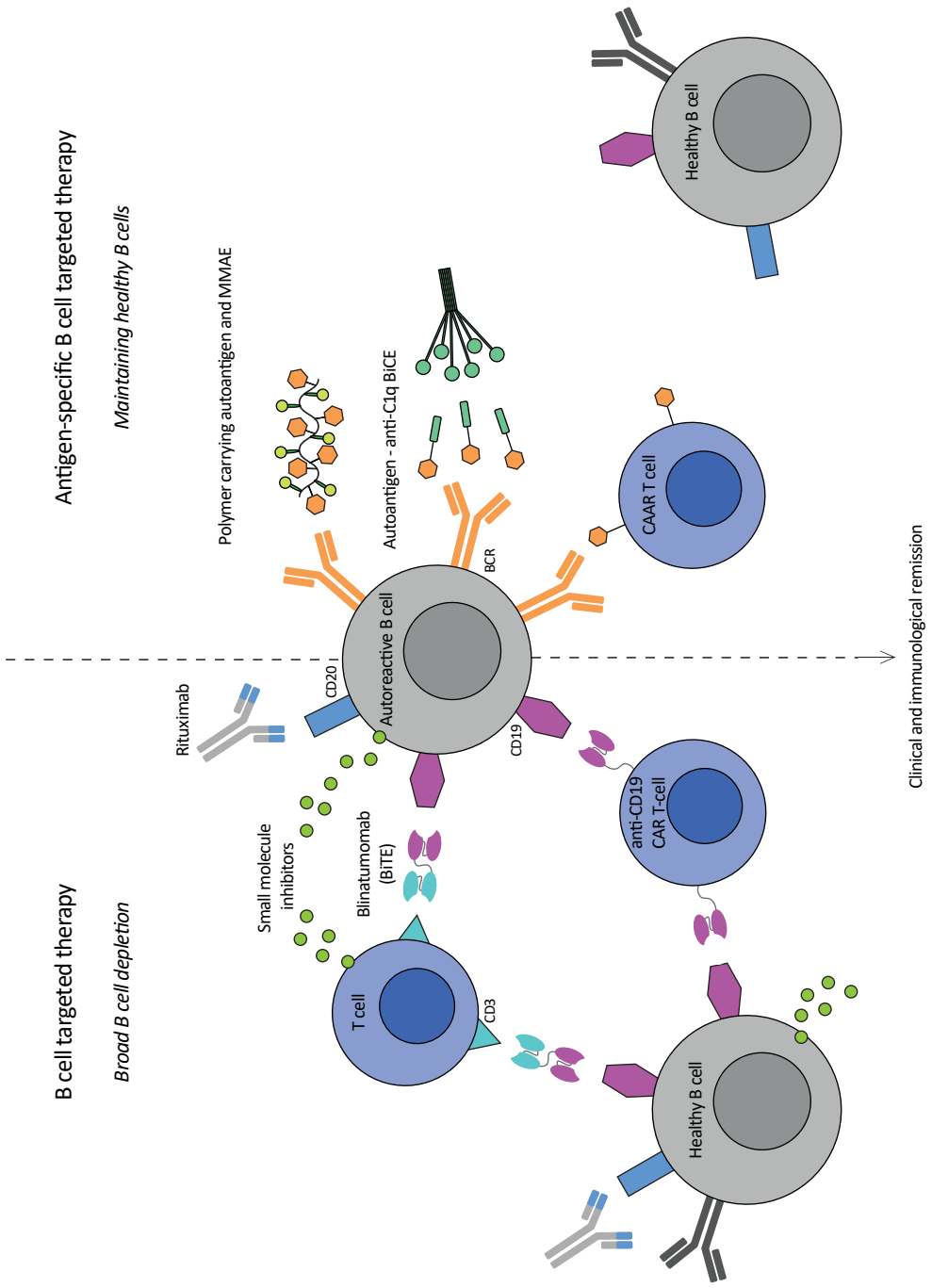

Figure 1. Potential treatment modalities enabling B cell targeted therapy (left) or antigen-specific B cell-targeted therapy (right) for achieving clinical and immunological remission.

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