Dysregulation of autoreactive B cell responses in autoimmune diseases: from initial triggering to persistent activation

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CHAPTER 8

Summarizing discussion

Autoimmune diseases (AIDs) are a major burden to global health by affecting nearly 10% of the population. The clinical management is complicated by the chronic character of AIDs which is fueled by sustained activation of the immune system. Lifelong treatment with immunosuppressive drugs is often required to prevent damage to the function of organs and tissues, but disease flares still commonly occur. An important hallmark of many AIDs is the break in B cell tolerance, which leads to the induction of autoreactive B cell responses targeting self. In most cases, these autoreactive B cell responses closely associate with disease, disease onset and disease manifestations. These clinical associations suggest that autoreactive B cell responses act as crucial drivers of AIDs, which is underlined by the striking effects of B cell targeted therapies.

Given this importance of autoreactive B cells in AIDs, insights into mechanisms underlying their initiation, activation and role in pathogenesis could allow to improve clinical disease management, identify targets to treat or eventually cure disease and open possibilities for strategies that could prevent AIDs. This thesis focused on the dysregulation of autoreactive B cell responses in two prototypic human autoimmune diseases, studying B cells targeting topoisomerase 1 (TOP1) in systemic sclerosis (SSc) and citrullinated proteins in rheumatoid arthritis (RA), respectively. **Chapter 2** reviewed the role of autoreactive B cells in the pathogenesis of SSc. Analyses of the phenotypic and functional characteristics of TOP1-reactive B cells in **Chapter 3** revealed active and ongoing triggering of these cells. Antigenic triggers that could drive the activation of TOP1-reactive B cells were identified by studying patient-derived anti-TOP1 autoantibodies (ATAs) on the monoclonal and polyclonal level in **Chapters 4** and **5**. Finally, the dynamics of the autoreactive B cell response targeting citrullinated proteins were examined in RA patients treated with various immunosuppressive drugs to relate autoreactive B cell activation to clinical disease activity (**Chapter 6**). In addition, a dynamic population of B cells secreting anti-citrullinated protein antibodies was assessed (**Chapter 7**). In **Chapter 8**, the main findings of this thesis and their importance to understand autoreactive B cell responses in AIDs are discussed.

Origin of autoreactivity

Reactivity towards self-antigens is the defining feature of autoreactive B cells. In principle, B cells can either be autoreactive by origin if a self-reactive B cell receptor (BCR) is formed during B cell development in the bone marrow or become autoreactive in the periphery by modification of the BCR through somatic hypermutation in germinal centers (GCs)[1,2]. The relative contribution of these processes can be estimated from studying BCR sequences obtained from autoreactive B cells, followed by their expression as monoclonal antibodies in germline configuration. Reconstructing germline antibody configurations can be complex and are inference-based[3], especially for sequences with high mutational loads. Yet such studies have revealed that a subset of germline-reverted antibodies can, in principle, recognize self-antigens[4-9]. Somatic mutations may then further enhance the recognition of self by these BCRs or antibodies[4-9]. Autoreactivity in the germline repertoire is also suggested by the presence of autoreactive IgM, which typically contains few somatic mutations[10-12]. Nonetheless, other autoreactive B cell clones examined by germline reversion lost reactivity to self, suggesting that at least in certain cases,

autoreactivity depends on somatic hypermutation[4-9,13-17]. Thereby, these findings indicate that B cell autoreactivity in AIDs may depend on failure of either central tolerance required to remove autoreactive B cell clones in the bone marrow, or peripheral tolerance required to prevent B cells to gain reactivity towards self-antigens in the periphery, or both (Fig. 1).

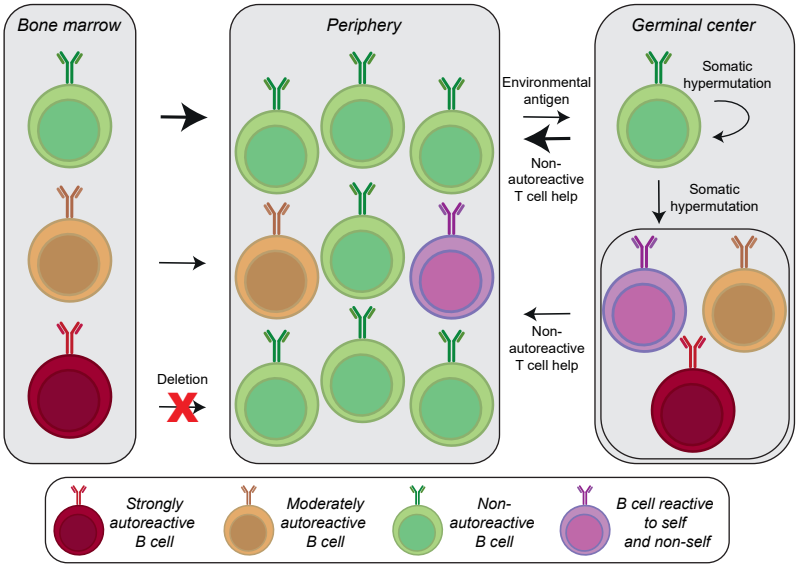


Figure 1. Origin of autoreactive B cells in autoimmune diseases. B cells can be autoreactive by origin and escape central tolerance mechanisms in the bone marrow to reach the periphery. In addition, non-autoreactive B cells can become autoreactive by the introduction of somatic mutations in germinal centers upon recruitment by environmental antigen. These B cells can be selected by non-autoreactive T cells if B cell retain reactivity towards non-self.

Autoantigen recognition by B cells carrying unmutated BCRs is an important prerequisite for the removal of autoreactive B cell clones by central tolerance mechanisms in the bone marrow[18,19]. BCR triggering by autoantigen at sufficient strength subjects B cells to either undergo receptor editing or clonal deletion[20,21]. These mechanisms also affect B cells expressing BCRs that cross-react to antigens present on self and non-self[22-24]. Elimination of these cells, however, limits the breadth of the naive BCR repertoire, impairing B cell responses to certain microbes[25-28]. To balance this potentially dangerous limitation, a fraction of B cells generated in the bone marrow reaches the periphery despite a certain degree of autoreactivity[29]. Notably, central tolerance mechanisms might be more permissive in AIDs, including SSc and RA, because a higher fraction of naive B cells in the circulation of these patients has been described to be autoreactive[30-33]. In line with this notion, autoreactive B cells with a naive phenotype were detected at low frequencies in SSc and RA throughout experiments performed for this thesis (**Chapter 3** and **Chapter 6**). While the frequency of such reactivity towards self remains to be determined, defective removal of autoreactive B cells in AIDs might depend on low affinity towards self or the lack of exposure of B cells to the form of self-antigen that such cells might encounter in the periphery. In this respect, it is interesting to note that anti-post-translationally modified protein antibodies in RA recognize a plethora of antigens modified by citrullination,

acetylation and carbamylation[34]. In addition, nucleic acid binding can modulate the reactivity of autoantibodies reactive towards nuclear antigens, as we observed in this thesis for a subset of TOP1-reactive antibodies in SSc which particularly recognize TOP1 in complex with DNA (**Chapter 4**). Importantly, the presence of these recognition profiles in the IgM compartment favors the concept that these cells are incompletely removed in the bone marrow (**Chapter 4**)[11]. To explore how autoreactive B cells can escape from central tolerance mechanisms, in-depth analyses of autoreactivity (for example against post-translationally modified proteins or TOP1) in the naive B cell compartment and their reactivity towards antigens in the bone marrow are needed. In this regard, B cells using IGHV3-48 would be a particularly interesting subject to study in SSc, as increased usage of IGHV3-48 was observed for TOP1-reactive B cells (**Chapter 3**). This suggests that this V-gene, either by itself or when paired to other immunoglobulin genes, encodes for an antibody which is or is prone to become autoreactive. Interestingly, TOP1-reactive clone 7G6, which uses IGHV3-48, recognizes TOP1cc considerably stronger than TOP1 (**Chapter 4**). Possibly, low reactivity of IGHV3-48 to TOP1 without DNA and the lack of exposure to TOP1cc in the bone marrow might enable these cells to escape central tolerance mechanisms.

Next to the generation of autoreactive B cells in the bone marrow, B cells might also develop autoreactive BCRs in the periphery. Traditionally, somatic hypermutation in GCs was mainly considered a function of B cells to enhance reactivity to the encountered antigen[35]. However, somatic hypermutation can also modulate reactivity of B cells to other antigens[36]. For example, somatic mutations can redeem autoreactive B cells by decreasing the reactivity towards self, while increasing the reactivity to foreign antigen[37,38]. Contrastingly, GCs might also transform non-autoreactive B cells into autoreactive B cells by the introduction of mutations in immunoglobulin genes[4-9,13-17,39,40]. In the blistering skin disease pemphigus vulgaris, for example, germline reversion of anti-desmoglein 3 antibodies abolished their reactivity to self-antigen[13]. In such cases, the initial engagement of non-autoreactive B cells in GCs might depend on microbial antigens[2]. Random mutations in the immunoglobulin genes of initially non-autoreactive B cells might subsequently result in the generation of B cells that cross-react with self-antigen. A proof of concept for the involvement of microbes in this process was demonstrated by a study on antibodies recognizing the self-antigen GlialCAM, which is expressed by glial cells in the central nervous system in multiple sclerosis. These antibodies cross-react with Epstein-Barr virus antigen 1 (EBNA1)[40]. Interestingly, reactivity of the germline reverted variant of these cross-reactive antibodies was restricted to EBNA1. A similar mechanism might allow for the development of TOP1-autoreactive B cells in SSc, as a subset of TOP1-reactive antibodies can also recognize microbial TOP1 (**Chapter 5**). In addition, if such cross-reactive B cells retain their reactivity towards the environmental antigen during the process of SHM, such cells could recruit help from non-autoreactive T cells residing in GCs through the internalization and subsequent presentation of microbial antigen on MHC class II. This could facilitate their survival in GCs, despite being autoreactive. The possibility that autoreactive B cells in SSc originate from non-autoreactive B cells and the relative contribution of this pathway require further exploration, particularly by assessing the reactivity of cross-reactive antibodies towards microbial and human TOP1 upon germline reversion.

Initiation of autoreactive B cell responses

The activation of B cells depends on triggering of the BCR by antigen. However, sole triggering of the BCR induces cell death[41]. The induction of durable B cell responses requires a coordinated sequence of events in which BCR triggering coincides with stimulation of additional receptors (such as toll-like and complement receptors) which is followed by cognate interaction with T cells[42,43]. For autoreactive B cells, it is often not possible to reconstruct the nature and context in which these cells have initially been triggered. Particularly, because autoreactive B cell responses are typically induced years before the onset of clinical disease[44-47]. Nonetheless, circulating autoantibodies and molecular features of the interaction between autoantibodies and autoantigens can provide important clues as to the context and mechanisms involved in the initiation of autoreactive B cell responses.

Chapter 4 exploited this possibility, identifying TOP1 in complex with DNA as potential trigger for the TOP1-reactive B cell response hallmarking SSc (**Chapter 4**). TOP1 binds DNA in a covalent manner to form TOP1-DNA cleavage complexes (TOP1cc). We observed that TOP1cc was recognized by all TOP1-reactive monoclonal antibodies studied. Interestingly, some of these monoclonal antibodies bound TOP1cc with a higher relative affinity in comparison to TOP1. TOP1-reactive antibodies with enhanced reactivity towards TOP1cc were also abundantly present in the circulation of patients (**Chapter 4**). These findings suggest that TOP1cc could enhance signals necessary to induce the potent activation of TOP1-reactive B cells by, for example, co-engagement of the BCR and DNA-binding Toll-like receptors[48]. This effect may also apply to B cells expressing a BCR that recognizes TOP1 and TOP1cc to a similar extent. In addition, the recognition of TOP1cc by circulating IgM antibodies suggests that naive TOP1-reactive B cells are susceptible to triggering with TOP1cc. While these findings favor the hypothesis that TOP1cc could facilitate a break in B cell tolerance, the context in which the nuclear enzyme TOP1cc becomes available to B cells remains to be elucidated. Like other nuclear proteins, TOP1cc could become available if cellular debris of dying cells is not properly cleared[49]. In fact, TOP1cc accumulates in dying cells under inflammatory conditions, a consequence of TOP1 binding to DNA containing lesions caused by reactive oxygen species[50]. Hence, an inflammatory context could generate the triggers required to initiate this autoreactive B cells response.

Next to nucleic acids, also microbes have been considered as potential inducers of autoreactive B cell responses. Microbes can trigger autoreactive B cells by expressing antigens that structurally mimic the autoantigen[51]. Guillain-Barré Syndrome, for example, is caused by autoantibodies targeting gangliosides, which are expressed by cells of the nervous system. These are induced by an infection with *Campylobacter jejuni*, which has cell wall oligosaccharides that mimic the structure of gangliosides[52]. Such cross-reactivity of autoantibodies has been observed for several AIDs[15,39,40,52,53]. In this thesis, we identified cross-reactivity of anti-human TOP1 autoantibodies with TOP1 from *S. cerevisiae* (**Chapter 5**). In addition, *S. cerevisiae* TOP1 was able to induce active BCR signaling in a B cell line expressing a cross-reactive ATA. Besides triggering the BCR, microbial antigen or microbial molecules associated with the antigen commonly contain ligands of innate immune receptors. For example, microbial TOP1 can bind microbial DNA, a potent stimulus of TLR9 (more potent in comparison to human DNA)[54]. In addition, and as described above,

autoreactive B cells recognizing microbial TOP1 could recruit help from microbial-directed T cells. Such T cell help is crucial for the generation of durable B cell responses[43]. The induction of autoreactive B cell responses is also likely to be dependent on T cell help, as the presence of particular autoantibodies is strongly associated with specific HLA class II alleles. Interestingly, help of non-autoreactive T cells would bypass the need to break the tolerance of T cells to human TOP1. The concept that B cells engage T cells with a different specificity is well-known and commonly observed in the context of protective B cell responses[55,56]. This also applies to AIDs as, for example, shown in celiac disease where autoreactive B cells recognizing transglutaminase enzymes can interact with gluten-specific T cells though engagement of gluten-transglutaminase complexes[57,58]. Based on these considerations, microbes could form an ideal context to induce autoreactive B cells responses in AIDs (Fig. 2).

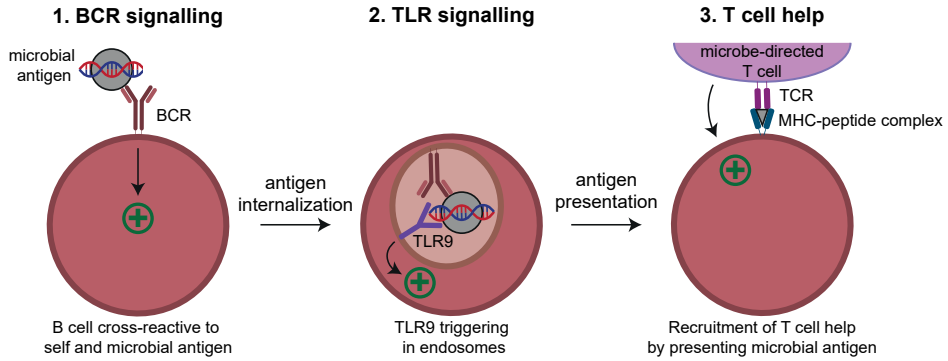


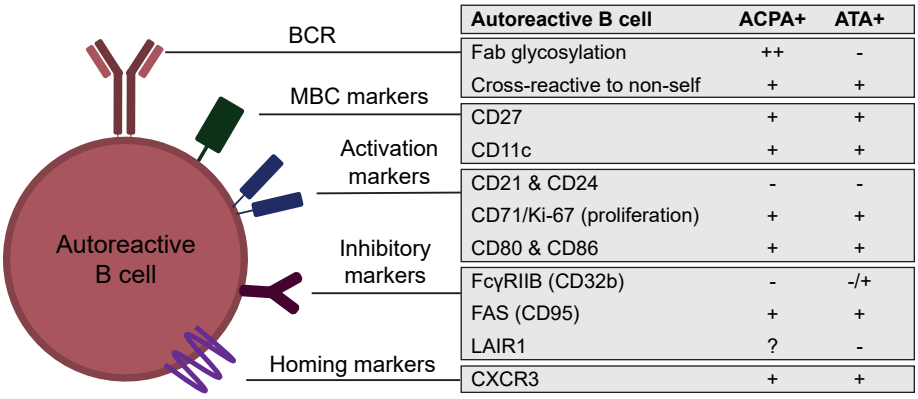
Figure 2. Microbes can provide potent signals to stimulate autoreactive B cells responses. Antigen derived from microbes can trigger BCR signaling of autoreactive B cells that express cross-reactive BCR that can recognize both self and non-self. Microbial antigen could also trigger innate immune receptors, for example toll-like receptor 9 (TLR9) if microbial antigen containing DNA is internalized. Moreover, presentation of microbial peptides could enable autoreactive B cells to recruit help from non-autoreactive T cells.

Insights into how autoreactive B cells are induced are important for the development of strategies that prevent or delay the development of AIDs. The mechanisms proposed above are hypothetical extrapolations from available data. Further insights are therefore needed to understand how autoreactive B cell responses are induced. In this context, studies on the reactivity, repertoire and properties of autoantibodies and BCRs before disease onset can help to reconstruct early steps in the development of autoreactive B cell responses. In RA, such studies uncovered that the development of autoreactive B cells targeting citrullinated proteins occurs in at least two stages: an initial step in which ACPAs are generated, and a second step in which the ACPA B cell response matures under the influence of T cells[59]. While the first stage has been observed in individuals that do not develop RA, the second stage more closely relates to the development and onset of RA. In SSc, similar studies on the development of autoreactive B cell responses are currently lacking. There, defining the epitope targeted by germline-reverted autoantibodies or autoantibodies detected early in the disease may help to identify inducing antigens and to reveal whether initial recognition depends on the binding of TOP1 to DNA. Moreover, identification of the epitope could help to identify the microbe involved in inducing the

response. Many homologues of human TOP1 were identified, including fungi, bacteria and viruses (**Chapter 5**). In this thesis, we used *S. cerevisiae* TOP1 as proxy for microbial TOP1 based on availability of this antigen. However, other microbes known to colonize or infect affected tissues could be more likely candidates responsible for the induction of the TOP1-reactive B cell response.

Persistent activation of autoreactive B cells

Cellular analyses of autoreactive B cells have provided direct insights into the phenotypic and functional dysregulation of autoreactive B cell responses in AIDs. The direct assessment of autoreactive B cells depends on antigen-specific staining approaches, which are challenging to develop and require extensive validation (**Chapter 3**). In SSc and RA, such staining approaches have enabled the identification of autoreactive B cells directed against TOP1 and citrullinated proteins, respectively. The autoreactive B cell compartment in these diseases mainly consists of conventional class-switched memory B cells (MBCs) expressing CD27 (**Chapter 3, Chapter 7**). In comparison to non-autoreactive counterparts, these autoreactive MBCs display a proliferative and activated phenotype as evidenced by the increased expression of Ki-67, CD71, CD80 and CD86 and lower expression of CD21 and CD24 (**Chapter 3, Chapter 7**)[60]. Moreover, autoreactive plasmablasts are commonly observed in patients, either by direct staining or culturing (**Chapter 7**)[61]. Interestingly, the activated phenotype of TOP1-reactive B cells in SSc and citrullinated protein-reactive B cells in RA are highly comparable (Fig. 3). This could indicate that similar mechanisms could underly the dysregulation of autoreactive B cells in either AIDs.



Autoreactive B cell	ACPA+	ATA+
Fab glycosylation	++	-
Cross-reactive to non-self	+	+
CD27	+	+
CD11c	+	+
CD21 & CD24	-	-
CD71/Ki-67 (proliferation)	+	+
CD80 & CD86	+	+
FcγRIIB (CD32b)	-	-/+
FAS (CD95)	+	+
LAIR1	?	-
CXCR3	+	+

Figure 3. Phenotypic characteristics of citrullinated protein-reactive (ACPA$^+$) and topoisomerase 1-reactive (ATA$^+$) B cells in rheumatoid arthritis and systemic sclerosis, respectively. Comparison of the BCR and expression of memory B cell (MBC), activation, inhibitory and homing markers by ACPA$^+$ and ATA$^+$ MBCs. Indicated expression levels are relative to non-autoreactive MBCs.

The observation that autoreactive B cell responses are strongly activated in practically all RA and SSc patients assessed is remarkable. In the context of recall responses to vaccination and infection, protective B cells with an activated CD27$^+$ MBC or plasmablast phenotype typically appear in the circulation within a week upon exposure to microbial

antigen[62-64]. Following this initial burst, these activated B cell populations contract in the following weeks and months. Such a contraction does not seem to take place for TOP1-reactive and citrullinated protein-reactive B cell responses in SSc and RA, respectively, as activated autoreactive B cells are consistently present in the circulation. Even in patients followed over time, frequencies of activated autoreactive B cells remained continuously increased (**Chapter 6**). This persistent activation indicates that these autoreactive B cells are continuously triggered. The increased phosphorylation of molecules that signal downstream of the BCR in citrullinated protein-reactive B cells in RA further supports this hypothesis[65]. Triggers driving continuous autoreactive B cell activation likely overlap with but do not need to be limited to the signals that initiate autoreactive B cell responses. The ubiquitous expression of TOP1 and abundant presence of post-translationally modified proteins in human cells could provide a systemic pool of antigen. Besides endogenous antigen, and based on the common cross-reactivity of autoreactive B cells to microbial antigen (as described above), antigen could also be of microbial origin. Commensal or opportunistic microbes residing in mucosal tissues could constantly deliver antigen to B cells in a tissue-specific manner. Enrichment of antigen in mucosal tissue could explain the presence of IgA autoantibodies and expansions of autoreactive IgA-expressing B cells observed in AIDs (**Chapter 3, Chapter 5**)[60]. Moreover, local enrichment of antigen provides a potential link between local B cell activation and the involvement of mucosal tissues in AIDs.

In addition to antigen, T cells are known drivers of B cell responses through cellular interactions and secretion of various cytokines, including IL-4 and IL-21[66]. T cells orchestrate the maturation of B cell responses in GCs by providing survival signals selectively to B cells that improved their affinity to antigen by somatic hypermutation[35]. Autoreactive B cell responses in RA and SSc probably also originate from GCs, as TOP1-reactive and citrullinated protein-reactive B cells are commonly class-switched and express BCRs with frequent somatic mutations (**Chapter 3**)[67]. Moreover, strong clonal expansions of TOP1-reactive B cells in SSc patients provide further evidence for a GC origin. As described above, T cell help to autoreactive B cells might be provided by non-autoreactive T cells if B cells cross-react to microbial antigen. Identification of T cells that help autoreactive B cells and definitions of their recognition profiles, as well as assessing the dynamics of autoreactive GCs (e.g. by fine-needle aspirates of lymph nodes), would be instrumental to understand mechanisms underlying the activation and maturation of autoreactive B cells. Of note, the effects of T cells on autoreactive B cells are not limited to GCs, but likely also take place in inflamed tissues. In fact, T cells expressing various molecules that can promote B cell activation are expanded in tissues affected by AIDs[68]. Such T cells, termed peripheral helper T cells, could sustain autoreactive B cell activation locally.

The persistent activation of autoreactive B cells could also be reflective of impaired regulatory mechanisms. Negative regulation of B cells prevents excessive activation and mediates the contraction of B cell responses. Signaling of various regulatory receptors, including FcγRIIB (CD32b) and LAIR1, inhibits signaling pathways induced by BCR triggering[69,70]. The expression of FcγRIIB and LAIR1 by TOP1-reactive and citrullinated protein-reactive MBCs was found to be decreased in comparison to non-autoreactive B cells (**Chapter 3, Chapter 6**). It is likely that the differential expression of these molecules

desensitizes autoreactive B cells to negative feedback provided by immune-complexed IgG (the ligand of FcγRIIB) or the ligands of LAIR1, collagen, surfactant protein D, adiponectin and complement component 1q (C1q)[69,70]. An impaired effect of collagen on LAIR1 could be of particular interest on TOP1-reactive B cells, as SSc is characterized by excessive deposition of extracellular matrix components in affected tissues[71]. In contrast to FcγRIIB and LAIR1, autoreactive B cells express high levels of the death receptor Fas (CD95; **Chapter 3**). T cells express Fas-ligand upon activation and induce apoptosis of B cells by triggering the death receptor Fas[72]. The increased expression of Fas could facilitate the removal of autoreactive B cells, while effective crosstalk between T and B cells prevents Fas-mediated cell death[73]. Therefore, the effect of these inhibitory receptors to counteract activation does not only depend on their expression, but also on the availability of ligands and the context in which these ligands are presented. In addition to regulatory receptors, recent studies have revealed inhibitory effects of secreted autoantibodies on B cell responses[74,75]. This effect particularly influences recall responses and is based on the competition of secreted antibodies, particularly those of high affinity, with B cells for antigen binding. It is unclear whether autoantibodies affect the activation of autoreactive B cells. In principle, the abundant and systemic presence of autoantigen might overrule this antibody feedback mechanism in AIDs.

The continuous activation of autoreactive B cell responses persists without signs of exhaustion. For T cells, exhaustion is commonly described in the context of chronic infections and cancer where T cells are persistently triggered with antigen[76]. Exhausted T cells are less responsive to antigen and have reduced effector functions. Such a decay in activation has not been observed for TOP1-reactive and citrullinated protein-reactive B cell responses in SSc and RA, respectively, even in patients with long-lasting disease (**Chapter 3, Chapter 6**). Also, frequencies of activated autoreactive MBCs are continuously high in RA patients followed over time (**Chapter 6**). Therefore, defining the clonal dynamics of B cells forming autoreactive B cell responses could provide insights into the cellular entity underlying this seemingly unlimited activation of autoreactive B cell responses. In principle, a limited population of long-lived B cells could form durable autoreactive B cell responses and be subject to repetitive activation. Longevity of MBCs can exceed decades in specialized niches that minimize their metabolic activity, but it is unclear whether this also occurs in settings of repetitive antigenic triggering[77]. Alternatively, autoreactive B cell responses could consist of a dynamic pool of autoreactive B cells which continuously evolves through the maturation of existing clones, deletion of other clones, and generation of new clones. Whether such new clones arise during disease is an important question, as it would indicate that the break in B cell tolerance to self occurs continuously rather than within a restricted timeframe early in the disease.

Relation between activation of autoreactive B cells and clinical disease

The dynamics of autoreactive B cells throughout disease stages and their link to clinical disease are often unclear. Therefore, in **Chapter 6**, autoreactive B cells were studied over time and in patients treated with various therapeutics. A remarkable finding was that the activated phenotype of autoreactive B cells in RA mostly persists despite treatment with immunosuppressive drugs, including conventional disease-modifying antirheumatic

drugs (csDMARDs), TNF-α inhibitors and Janus kinase (JAK) inhibitors (**Chapter 6**). Notably, no correlation was found between the degree of activation of citrullinated protein-reactive B cells and disease activity. This persistence of activated, autoreactive B cells may be interpreted as sign of immunological activity underlying the disease, and indicate that such activity persists even if inflammation as a sign of clinical disease is in remission under treatment. Consequently, such immunological disease activity might reflect disease chronicity and could be responsible for the frequent occurrence of flares if anti-inflammatory treatment is stopped. Indeed, sustained-drug free remission in patients affected by AIDs such as RA is rare.

Nonetheless, the phenotype of autoreactive B cells is not fixed but dynamic throughout disease stages (Fig. 4). A previous study showed that citrullinated protein-reactive B cells can already be detected in patients who are at risk to develop RA (individuals with arthralgia, without signs of arthritis)[78,79]. In such individuals, the autoreactive B cell phenotype was found to be less activated in comparison to patients diagnosed with RA[78]. Interestingly, a prospective analysis revealed that autoreactive B cell-activation, as assessed by the expression of elevated levels of CD80 and Ki-67, was present in patients who developed RA within one year, in comparison to patients who did not develop RA[79]. In addition, more autoreactive B cells with a plasmablast phenotype were observed in RA converters, while barely detectable in non-converters. These data support the hypothesis that the activity of the citrullinated protein-reactive B cell response acts as a marker of immunological disease activity by predicting disease onset and characterizing chronic disease. This concept is further supported by the phenotype of citrullinated protein-reactive B cells observed in a rare population of RA patients which are in sustained drug-free remission, which closely resembled the phenotype in individuals with arthralgia that did not develop RA. Hence, a more quiescent phenotype of citrullinated protein-reactive B cells could reflect less immunological activity of disease. While this finding could enable the identification of patients who are eligible to taper medication[79], more markers that accurately reflect quiescent autoreactive B cells are required beyond the limited set of currently used markers. Of note, such comparative studies could also provide clues as to the differential presence of activating triggers across disease stages. Presumably, such activating triggers could entail fluctuations in the presence and quality of T cell help and, possibly, the availability of microbial antigens. As such triggers might differ between diseases and between particular disease stages, a comparative study on the dynamics

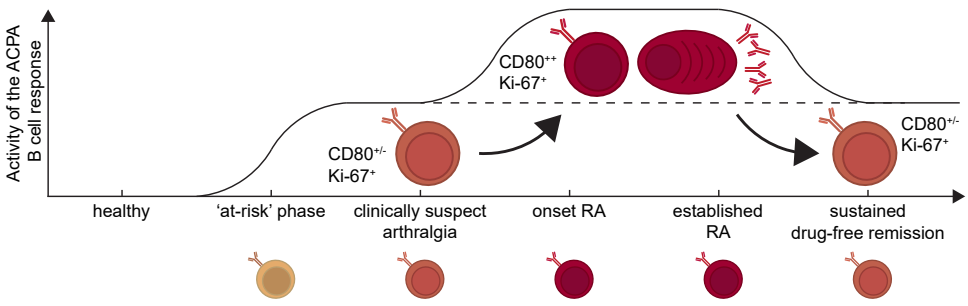


Figure 4. Dynamics of the ACPA B cell response throughout disease stages in rheumatoid arthritis (RA).

between TOP1-reactive B cells in SSc and citrullinated protein-reactive B cells in RA throughout disease could be highly informative.

The concept that persistent immunological activity is reflected by the continuous activation of autoreactive B cells aligns well with the striking effects of therapeutic B cell depletion. In contrast to the drugs described above that primarily inhibit inflammation, resetting B cell immunity through B cell-depleting therapies has shown sustained clinical effects[80]. In fact, emerging data of patients treated with CD19-targeting CAR T cells show that most treated patients reach the state of sustained drug-free remission[81]. These striking effects are thought to depend on the ability of CAR T cells to deeply deplete B cells, including those residing in tissues residing in tissues. This effect is not observed upon treatment with the monoclonal antibody rituximab, which targets CD20-expressing B cells[82]. To date, it is unclear whether this reset in B cell immunity is permanent, whether B cell autoreactivity returns in the naïve repertoire upon B cell repopulation, or whether autoreactive B cells can develop again in a multi-year process. However, such processes can now be closely monitored by assessing B cell autoreactivity upon repopulation of the B cell compartment, using the technology presented in this thesis.

Role of autoreactive B cell responses in autoimmune diseases

The active involvement of autoreactive B cells in AIDs implies that these cells might be capable of orchestrating destructive effector responses. In principle, B cells can exert exerting multiple effector functions, including the production of antibodies, the stimulation of T cells and the secretion of cytokines[83]. While autoantibodies have direct pathogenic effects in some AIDs, the exact contribution of autoreactive B cells to most AIDs remains to be determined[84-86].

In RA, there is no clear evidence for the pathogenicity of anti-citrullinated protein antibodies. In fact, recent studies have suggested that some ACPAs could have anti-inflammatory properties, but mechanisms underlying these effects remain elusive[87,88]. In line with these findings, B cell depleting therapies are effective in RA, but seroconversion of autoantibody levels is not a prerequisite for reaching sustained drug-free remission[89-91]. Therefore, the contribution of ACPA-expressing B cells to RA likely depends on other effector mechanisms. In this perspective, it is interesting to note that B cells directed against citrullinated proteins are capable of producing pro-inflammatory cytokines, such as IL-8, IL-6 and TNF-α[78]. Moreover, these B cells abundantly express various co-stimulatory molecules for the interaction with helper T cells, like CD80, CD86 and HLA-DR. These helper T cells could amplify the pro-inflammatory cascade induced by B cells through the recruitment and activation of other innate immune cells and the production of various cytokines. *Vice versa*, T cells could further activate autoreactive B cells, possibly initiating a positive feedback loop.

In SSc, autoantibodies directed against TOP1 strongly associate with disease onset, clinical disease phenotype and disease manifestations[92]. Several studies have suggested a direct link between autoantibodies and fibrosis in SSc[93-95]. These studies reported that TOP1-reactive autoantibodies could bind TOP1 displayed on the surface of fibroblasts to induce

their activation. The mechanisms by which this binding could lead to the activation of fibroblast, however, as well as the clinical relevance of this activation, has not been fully elucidated. Also, not all ATA$^+$ patients have progressive disease (**Chapter 5**), despite the presence of abundant ATA in serum. In fact, like in RA, the clinical effects of rituximab or CD19-targeted CAR T cell therapy precedes the decline in autoantibody levels in SSc, suggesting that ATA are either not pathogenic, or that the total pool of ATA-IgG as assessed in clinical practice may not adequately reflect its pathogenic components[96-98]. Based on these considerations, it is likely that TOP1-reactive B cells contribute to disease by production of cytokines and the activation of T cells. Indeed, TOP1-reactive B cells express high levels of CD80, CD86 and HLA-DR, as observed for ACPA-expressing B cells in RA, that could facilitate T cell activation. T cell activation might, in turn, amplify inflammation in SSc, but could also induce the secretion of pro-fibrotic cytokines TGF-β and IL-1β[99]. Furthermore, B cells might directly contribute to tissue fibrosis by stimulating collagen production by fibroblasts and secretion of the pro-fibrotic molecule PDFG-β and extracellular matrix remodeling enzyme LOXL2[100,101]. TOP1-reactive B cells are eligible candidates to secrete such fibrotic mediators, particularly because they express lower levels of LAIR1, a regulatory receptor which binds to extracellular matrix collagens[102,103]. Thereby, autoreactive B cells could fulfill an important function in mediating inflammation and fibrosis in SSc.

A caveat in the current understanding of autoreactive B cells concerns the role of B cells in affected tissues. Most studies, including the studies presented in this thesis, focus on autoreactive B cells in circulation. However, B cells infiltrate tissues to exert important effector functions during immune reactions[104]. In the context of influenza infection, for example, antigen-specific B cells infiltrate the lungs to mediate protective immunity[105-107]. In AIDs, autoreactive B cells infiltrating tissues could fuel local pathology. B cells are commonly present in tissues affected by AIDs, while rarely encountered in the same tissues in the healthy state[108,109]. Homing of autoreactive B cells to tissues may be mediated by the chemokine receptor CXCR3, which is abundantly expressed by autoreactive B cells (**Chapter 3**)[60]. Indeed, citrullinated protein-reactive B cells were enriched in synovial fluid of RA patients[78]. In SSc, autoreactive B cells in tissues have not yet been investigated. Studies on the phenotypic characteristics of tissue-infiltrating autoreactive B cells and their interaction with other cells are needed to understand the role of autoreactive B cells in affected tissues.

Targeting autoreactive B cells

B cell-depleting therapies currently target molecules widely expressed by B cells during various phases of differentiation. Hence, broad depletion of B cells also interferes with the generation of protective B cell responses and, consequently, mediates risk for infections[110,111]. To prevent the depletion of protective B cells, targeting methods that specifically eradicate autoreactive B cells are needed. Chimeric autoantibody-receptor (CAAR) T cells may represent an elegant method to specifically eradicate autoreactive B cells[80,112]. CAAR T cells express autoantigen and kill B cells upon binding the antigen with the autoreactive BCR. By doing so, CAAR T cells can efficiently target autoreactive B cells *in vitro* and in murine models *in vivo*[112-116]. Despite these promising results, CAAR

T cells are not yet widely applied in clinical settings. Possibly, dose optimizations and immunoadsorption prior to administration of CAAR T cells are necessary, as circulating autoantibodies might block the effect of CAAR T cells by binding to the expressed antigen. Moreover, CAAR T cells could fail to target B cells expressing low levels of surface BCR, such as antibody-secreting cells[117]. Therefore, the clinical potential of CAAR T cells to effectively silence autoreactive B cell responses in AIDs remains to be demonstrated.

Other strategies to target autoreactive B cells more specifically could be based on distinctive traits of autoreactive B cells. As discussed above, autoreactive B cells display a differential phenotype in comparison to non-autoreactive counterparts, as for example observed for autoreactive B cells recognizing TOP1 and citrullinated proteins in SSc and RA, respectively (**Chapter 3, Chapter 6**). These autoreactive B cells overexpress numerous activation markers, like CD11c, CD71, CD80, CD86 and CD95. Such markers are attractive targets, particularly due to the level and persistence of expression on autoreactive B cells. Nevertheless, protective B cells and other immune cells also express these markers upon activation. Avoiding depletion of these cells is complicated and likely requires expression level-dependent targeting in a cell-specific manner (e.g. by bispecific antibodies). Alternatively, specific properties of BCRs expressed by autoreactive B cells could be targeted. Specific IGHV genes represent interesting targets, as increased usage of selected IGHV genes is commonly described for particular autoreactive B cell responses[118]. In SSc, we observed that TOP1-reactive B cells commonly used IGHV3-48 (**Chapter 3**). Targeting such IGHV genes and other targetable markers described above will still affect part of the non-autoreactive B cell population, but would be more specific in comparison to currently employed B cell targeting strategies.

An alternative approach to silence autoreactive B cells would be to interfere with stimulating triggers or to provide regulatory signals. Depletion or targeting of autoantigen is probably complicated due to the physiological functions of autoantigens[119,120]. However, regulatory signals can be provided to autoreactive B cells in an antigen-specific manner using regulatory CAAR T cells or tolerogenic dendritic cells loaded with autoantigen. Regulatory CAAR T cells express, in addition to autoantigen, regulatory cytokines and molecules (e.g. IL-10, CD25 and CTLA-4) which are induced by transduction of transcription factor FoxP3[121-123]. Thereby, these regulatory CAAR T cells can inhibit the activation of B cells, at least *in vitro* and in mice[121-123]. Autoreactive B cell activation can also be limited by tolerogenic dendritic cells loaded with autoantigen which primarily inhibit the ability of T cells to provide help to B cells[124-126]. Besides providing regulatory signals, microbes expressing antigens that stimulate autoreactive B cells can also be targeted. As proof of principle, vaccination against the pathobiont *E. gallinarum* prevented the induction of autoantibodies by this microbe in mice[127,128]. Thus, if microbes trigger autoreactive B cells, controlling these microbes through vaccination, antibiotics, antimycotics, antiviral drugs or bacteriophages could limit the activation of autoreactive B cell responses in AIDs.

Conclusion

The dysregulation of B cell immunity remains an enigmatic hallmark of many AIDs. Autoreactive B cell responses likely represent a chronic driver of AIDs, as autoreactive B cells are persistently activated independent of disease activity and even despite treatment

with immunosuppressive drugs. The activation of autoreactive B cells is probably driven by chronic exposure to antigen, like microbial-derived antigen containing structures resembling self-antigen and nucleic acid-binding antigens, that potently stimulate these autoreactive B cells. Silencing or depleting these activated autoreactive B cell responses may be required to effectively treat and, potentially, cure human AIDs.

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