Bitter Sweet Symphony: the impact of sugars on autoimmunity

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REVIEW

Checkpoints controlling the induction of B cell-mediated autoimmunity in human autoimmune diseases

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Abstract

B-cell targeting therapies are effective in various autoimmune diseases, amongst others Rheumatoid Arthritis, Pemphigus Vulgaris and Systemic Lupus Erythematosus. Given these successes, it is evident that B cells are central orchestrators in the processes leading to the signs and symptoms hallmarking many human autoimmune diseases. The pathways provoking the generation of such autoreactive B cells or mechanisms preventing their induction in health are, however, poorly explored. Nevertheless, such information is crucial for the development of preventative/curative interventions aiming to permanently deplete- or prohibit the emergence of autoreactive B cells. Hence, this review will focus on how B-cell tolerance might be breached, and which checkpoints are at play preventing the arousal of autoreactive B cells in human. Especially antigen presentation by follicular dendritic cells, somatic hypermutation and cross-reactivity to the microbiome/environment could operate as actors playing pivotal roles in the induction of B cell-mediated humoral autoimmunity. Moreover, we highlight the human autoimmune disease Rheumatoid Arthritis as a prototype where autoreactive B cells combine several mechanisms to overcome peripheral B-cell checkpoints.

Introduction

Clear evidence for a key role of B cells in human autoimmune diseases, such as Rheumatoid Arthritis (RA), Pemphigus Vulgaris (PV), Systemic Lupus Erythematosus (SLE), Myasthenia Graves or Anti-Neutrophil Cytoplasmic Antibody-Associated Vasculitis (AAV), is provided by the efficacy of B-cell targeted therapies in these diseases[1-4]. B cells might contribute in several ways to these disorders, including the production of pathogenic autoantibodies, the generation of inflammatory cytokines and the presentation of antigens to T cells combined with their excellent ability to activate these cells. The different B-cell functions in human autoimmune diseases have been extensively reviewed and will not be discussed in this review[5,6].

During B-cell development in the bone marrow (BM), B cells start to express B-cell receptors (BCRs) with diverse antigen-binding sites, created through a random combination of variable (V), joining (J) and diversity (D) gene segments[7]. This junctional diversity during V(D)J-recombination is further increased by the introduction of palindromic- and non-templated nucleotides[8,9]. Additionally, the association between heavy- and light chain creates further diversity in the BCR variable domains. Collectively these processes generate highly diverse BCR repertoires and B-cell populations able to recognize many different structures and molecules in a highly specific manner, including self-molecules expressed by healthy human tissues. It is estimated that approximately 50-75% of immature BM B cells carry self-reactive BCRs in both mice and humans[10-12]. A significant proportion of these B cells will undergo receptor-editing or clonal deletion both leading to the removal of self-reactivity as experimentally evidenced within Ig transgenic mouse models[13]. During receptor-editing, autoreactive B cells against multivalent antigens will change their light chain to prevent autoreactivity. If this mechanism fails or if the B cell shows a high affinity to self-antigens, it will most likely undergo clonal deletion. These negative selection processes are responsible for central B-cell tolerance and ensure that most B cells leaving the BM do not recognize self. However, as central B-cell tolerance is incomplete, self-reactive B cells will escape into the periphery. To further hinder the emergence of autoreactive B-cell responses, several complementary peripheral tolerance mechanisms and B-cell activation checkpoints are in place to prevent the induction of humoral autoimmunity. Immature self-reactive B cells coming from the BM are, for example, competing with other B cells to enter primary follicles and exclusion will result in cell death within 1-3 days. This follicular exclusion of (auto)antigen-reactive B cells, shown in different mouse systems, will depend on the diversity and abundance of competing cells and is not absolute. Nonetheless, it further minimizes the chance that autoreactive B cells enter the follicle and survive in the periphery[14,15]. After passaging the primary follicles, B cells can enter germinal centers (GC) where they interact with antigens presented by follicular dendritic cells (FDCs) and

receive survival signals [16]. The activated B cells are migrating to the T-/B-cell border to present antigen and receive "help" from T helper cells. These B cells will then undergo proliferation and somatic hypermutation (SHM) in the dark zone following antigen-driven selection by FDCs in the light zone[17,18]. Murine studies have also shown that selected B cells can re-enter or exit the GC as memory B cells or plasmablasts/cells secreting high affinity isotype-switched antibodies. Thus, retained antigen presentation by FDCs and the recruitment of "help" from T helper cells are both essential peripheral checkpoints that are in place to permit the induction of B-cell responses by transmitting necessary survival and activation signals to B cells. We will further elaborate on the important role of T cells, antigen presentation and FDCs as peripheral checkpoints in the main part of the review. Additionally, B cells can also be activated in a T-cell independent manner by bacterial molecules such as Lipopolysaccharides (LPS). LPS, like other T-cell independent antigens, contains repetitive antigenic structures leading to an extensive BCR crosslinking and also provides additional co-stimulatory signals through toll-like receptor (TLR) activation. Although the activation of B cells by T-cell independent antigens can induce abundant IgM responses as evidenced by hyper-IgM syndrome, a primary human immune deficiency disorder characterized by defective CD40 signalling, chronic BCR crosslinking with high antigen density in the absence of T-cell help or co-stimulation, typically results in B-cell anergy. This silencing of B-cell clones has been shown to represent an additional peripheral checkpoint in mice as most self-molecules do not provide co-stimulation to B cells[19].

Despite all these checkpoints cooperating in controlling self-reactive B cells (Figure 1A), negative selection through central and peripheral checkpoints often fails as evidenced by the prevalence of auto-immune diseases in humans that are frequently characterized by the presence of disease-specific autoantibodies. How or when autoreactive B cells are induced in these diseases remains, however, often obscure. In this review we will highlight several mechanisms how B cells might bypass peripheral "tolerance" checkpoints including the important role of antigen presentation by FDCs, somatic hypermutation and T-/B-cell cross-reactivity between self and microbial/environmental antigens (Figure 1B). A special focus will be given to RA, highlighting different mechanisms used by the RA-specific autoreactive B cells producing anti-citrullinated protein antibodies (ACPAs) to overrun several B-cell checkpoints.

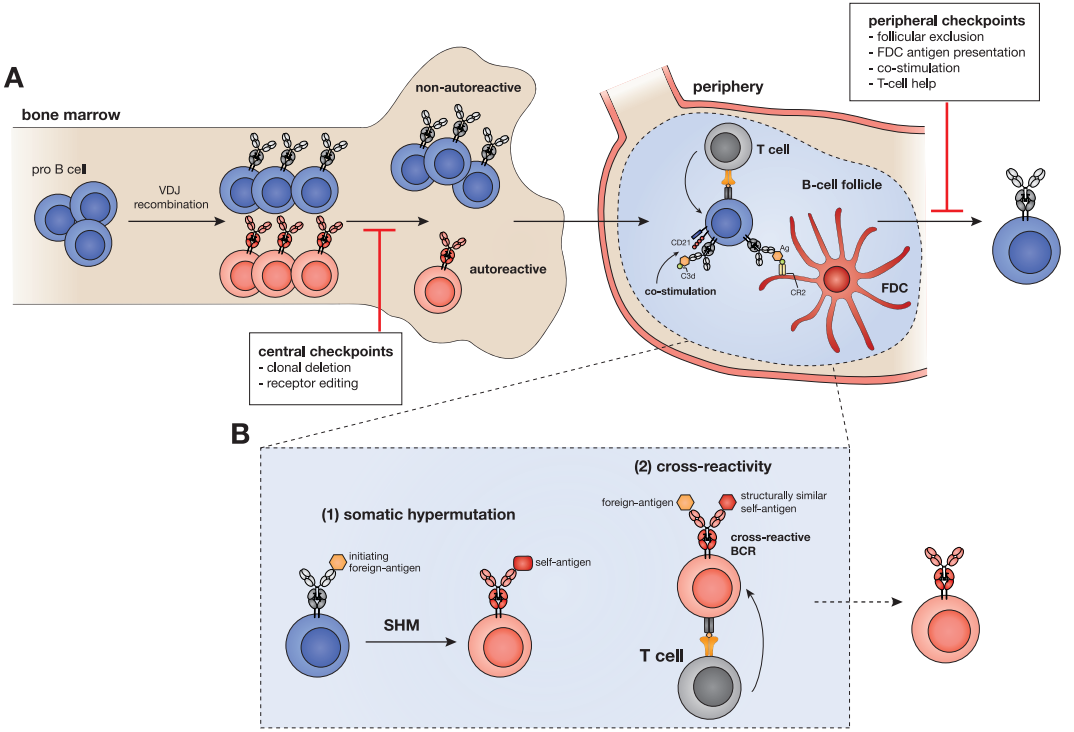

Figure 1. B-cell checkpoints and mechanisms to escape. (**A**) Schematic representation of the B-cell development in the BM and the periphery including the respective checkpoints. Random V(D)J-recombination of the B-cell receptor in the BM can induce autoreactive B cells. Clonal deletion and receptor editing as main central checkpoints eliminate most of these autoreactive B-cell clones. B cells that emerge into the periphery have to overcome additional checkpoints including B-cell follicular exclusion, antigen presentation by FDCs, co-stimulation by e.g. complement-C3d coated antigens and T-cell help. (**B**) Proposed main mechanisms of B cells to overrun peripheral checkpoints. The generation of self-reactive B cells via somatic hypermutation is one possible mechanism. B cells will alter their receptor reactivity towards self-antigens. Secondly, cross-reactive BCRs, able to recognize foreign- and structurally similar self-antigens, can overrun important gatekeepers as they receive "help" from non-autoreactive T cells.

Antigen-presentation and co-stimulation as checkpoint to control autoreactive B-cell activation

As noted above, many B-cell responses typically undergo an antigen-driven selection controlled by FDCs and follicular T helper cells in the GC. FDCs are essential for efficient GC formation and the generation of high-affinity antibodies as they are a major source of CXCL13 involved in attracting B and specific T cells to the follicles[20]. Likewise, FDCs also produce factors promoting B-cell survival by the supply of IL-6 and the B-cell activating factor BAFF[21]. FDCs are stromal derived cells characterized by a dendritic morphology and the expression of various markers such as CD21 (CR2), CD35 (CR1), MFGE8, and Fcγ-receptors[22]. Furthermore, FDCs express multiple TLRs enabling them to respond to viral- or bacterial products in their vicinity. TLR2 and TLR4 stimulation of FDCs in the gut induces, for example, the production of TGF-β and BAFF involved in IgA class switching of B cells[23]. Importantly, the expression of high levels of CR1 and CR2 are essential for FDC-mediated antigen retention in a complement dependent manner[24]. FDCs retain internalized antigens, complement coated immune complexes (ICs), within cycling non-degradative endosomal compartments and periodically present them on the cell surface to B cells via CR2 during affinity maturation[22]. In this way, microbes and other particles or molecules that have activated the complement system become preferentially presented to B cells by FDCs. Moreover, such complement-opsonized antigens can also deliver co-stimulatory signals to responding B cells via CD21/CD35 in conjunction with BCR activation. If this complement dependent co-stimulation is lacking, B cells will not be activated properly and undergo anergy induction[25]. Because FDCs are crucially involved in the regulation of humoral immune responses, they are also an important checkpoint controlling autoreactive B-cell responses. In general, most self-antigens will not activate the complement system and hence will not be presented efficiently by FDCs and/or recognized by B cells in a way allowing concurrent co-stimulation and BCR-engagement. Indeed, antigens conditionally expressed by FDCs in a Fc- and complement-receptor independent manner, induce B-cell tolerance in a mouse model, indicating that FDCs can play an important role in B-cell tolerance induction to sequestered self-antigens[26]. Nonetheless, some self-constituents, such as apoptotic cells or DNA-complexes, can induce complement activation under certain pathological conditions, potentially leading to the retainment of these antigens by FDCs and induction of B-cell autoimmunity[27,28]. The latter could be strengthened further through the ability of FDCs to attract follicular T helper cells, required for the efficient formation of T-B-cell interactions and productive GC reactions[29]. Indeed, the important role of FDCs is also suggested by the frequent presence of ectopic lymphoid, GC-like, structures in chronically inflamed tissues of several human autoimmune diseases such as the inflamed synovium of patients with RA[30,31]. Collectively, these findings identify FDCs as important gatekeepers governing the induction of (unwanted) B-cell responses through the control of antigen-presentation and provision of activation- and survival-signals.

De novo autoreactivity gained through somatic hypermutation

When a mature B cell recognizes its antigen, it becomes activated and can undergo SHM. During this process, random mutations might result in an enhanced antigen recognition, leading to avidity maturation of the antibody response through the selection of B cells with the highest antigen affinity. This random mutation process also bears the risk that it might lead to a shift in antigen recognition towards other (structurally similar) self-antigens. However, many such B cells will undergo apoptosis as they compete with other responding B cells for survival signals provided by FDCs and follicular T helper cells. As a consequence, many B cells will mutate away from self-reactivity as it provides a means to overcome anergy[32].

Nonetheless, the development of autoimmunity in humans as a consequence of SHM has been described in several studies. For example, in Pemphigus Vulgaris, an autoimmune disease characterized by blistering and autoantibodies directed against desmoglein (DSG) proteins 1 and 3[33-35], DSG3-specific antibodies were reverted to their respective germline sequence. As no reactivity to DSG3 could be detected anymore when these antibodies were expressed in germline configuration, the data suggest that the DSG3 reactive B cell was initially activated by another, DSG3-unrelated antigen. Interestingly, one monoclonal antibody (mAb) expressed only 4 amino acid replacements, implying that no more than a few mutations are needed to gain autoreactivity[36]. Likewise, in pulmonary alveolar proteinosis (PAP), a rare autoimmune disease characterized by an impaired alveolar macrophage function and a shortness of breath, similar observations have been made. The disease is characterized by the presence of autoantibodies against granulocyte-macrophage colony-stimulating factor (GM-CSF), in which the reduced bioavailability of GM-CSF is causing an impaired alveolar macrophage development and function[37]. Analyses of several patient-derived autoantibodies reverted to germline, revealed that binding to GM-CSF was strongly diminished or undetectable, indicating that somatic mutations critically determined the specificity to the autoantigen[38]. Likewise, and in concordance with the autoimmune diseases described above, this diminished/loss of antigen recognition after germline reversion has also been described for ACPAs, the specific autoantibodies present in patients with RA [39-41]. However, IgG ACPA-expressing B cells are unique as they harbor, on average, an extraordinary amount of mutations compared to other (auto)antigen-specific B cells such as anti-Tetanus Toxoid B cells, anti-DSG3 or anti-DNA antibodies[36,42,43]. The high mutation load is, possibly, a consequence of the propensity of ACPAs to cross-react with several post-translational modifications and modified antigens[40,44-47], the putative chronic presence of these antigens and repetitive GC-reactions in the absence of affinity maturation. The high mutation rate complicates the determination of the correct germline sequence, increasing the chance that not the original germline sequence is investigated. In this case, investigating ACPA IgM might be more suitable as these are likely to harbor less mutations. The loss of autoreactivity after germline conversion of autoreactive IgG has also been noted in other rheumatic autoimmune diseases. For example, the analyses of a large set of mAbs, generated from BCR sequences of IgG

memory B cells from Sjögren's syndrome or SLE patients, revealed that a substantial proportion was reactive towards extractable nuclear (auto)antigens (ENA) Ro52 and La. However, when reverted to their unmutated ancestor, the ENA antibodies showed poly-reactivity with a low, non-specific, binding to Ro52 supporting the idea that SHM contributed to antibody specificity and introduction of autoreactivity[48,49]. Similar phenomena have been described in SLE in studies where two dsDNA-directed autoantibodies did not show self-reactivity anymore when expressed in their germline configuration[43,50]. Nevertheless, SHM is certainly not exclusively responsible for self-reactivity in these diseases, as it has also been shown that specific Ro52 reactivity is maintained after reverting somatic mutations of IgG autoantibodies to germline configuration[51].

Thus, several lines of evidence indicate that autoreactivity in certain B cell-mediated diseases might be gained by SHM, although additional checkpoints at the postmutational state are likely to be present. However at this stage it is unknown, why SHM, that results in an increased avidity of most pathogen-specific responses, could also result in autoimmunity and a putative drift away from the initial T-cell help involved in the response. A possible explanation could be cross-reactivity of BCRs, as discussed in the next paragraph.

Environmental or microbial cross-reactivity as an inducer of autoimmunity

The observations described above clearly point to the possibility that autoreactive B-cell responses emerge from SHM and aberrant subsequent selection of memory B cells. Although observed in several disorders, the prevalence of this mechanism is unclear as this selection process is controlled by other mechanisms as well. For example, B cells typically require the provision of "help" from follicular T helper cells directed against the same antigenic moiety as the responding B cell. In the case of autoantigen recognition as a consequence of SHM to another (unrelated) antigen, the B cells will be severed from T-cell help due to the lack of accompanying autoreactive T cells. Therefore, such B-cell responses will not be sustained and likely disappear[52,53]. In this respect, it is remarkable to note that many autoreactive humoral responses are directed against antigens that are tightly linked to RNA or DNA. One of these autoreactive responses is SLE, which is characterized, amongst others, by autoantibodies directed against ribonucleoproteins (RNP), such as anti-Ro (or anti SS-A), anti-La (or anti-SS-B), or anti-Sm[54]. Similarly, anti-RNP and/or anti-nuclear autoantibodies are found in a variety of other autoimmune diseases such as scleroderma (anti-topoisomerase I; anti-centromere), Sjögren's syndrome (anti-SS-A/B), or dermatomyositis (anti-Jo1 recognizing histidine-tRNA ligase)[55-57]. Thus, all these autoantibodies recognize proteins that are intimately linked to DNA/RNA, both able to trigger Toll-like receptors (TLRs). Antigen recognition by such autoreactive B cells will lead to the concurrent provision of TLR7 or TLR9-mediated co-stimulatory signals, hence allowing effective initial activation of these B cells. Intriguingly, highly similar proteins to which these autoreactive B cells react to, are also expressed by microbes. As many DNA- and RNA-binding proteins are highly conserved across species, it is plausible that autoreactive B cells

attract "help" from T cells directed against homologous DNA-/RNA-binding proteins expressed by microbes. Noteworthy, orthologs of Ro60 with a high sequence similarity to human Ro60 can be found in multiple bacteria, including species of *Corynebacterium*, *Propionibacterium* and *Bacteroides,* which are present in the human skin, oral and gut microbiota[58]. Thus, individuals chronically colonized by commensal bacteria's expressing Ro60 orthologs, might develop antibodies against both the bacterial and human Ro60 leading to B cell-mediated autoimmunity via cross-reactivity[59]. In this way, the responding B cell could secure the provision of T-cell "help" by attracting microbe-specific T cells. Indeed, it has been shown that Ro60 ortholog-containing bacteria are commonly present in the human microbiome and that lupus patients with anti-Ro60 autoantibodies harbor B-cell responses to bacterial Ro60 orthologs in vitro[59]. Moreover, it has been shown that the persistent presence of Ro60 orthologs repetitively stimulates short-lived Ro60-specific B cells leading to a sustained autoantibody production and a chronic disease course[60]. These experimental evidences point to the notion that B-cell cross-reactivity to conserved epitopes expressed by bacterial and human DNA-/RNA-binding proteins might be central in the breach of peripheral "tolerance" checkpoints in lupus[61].

Similarly, convincing evidence for a "microbe-autoreactive B-cell axis" has been presented recently in the case of the systemic autoimmune disorder antiphospholipid syndrome (APS)[62]. APS is characterized by a well-defined B-cell autoantigen, β_2-glycoprotein I (β_2GPI)[63]. It has been shown in vitro and in vivo that anti- β_2GPI B cells cross-react with mimotopes from the human gut commensal *Roseburia intestinalis*, expressed by the bacterial DNA methyltransferase (DNMT). APS patients express high levels of anti-*Roseburia intestinalis* DNMT IgG antibodies, which correlate with the anti-β_2GPI antibody levels[62]. Additionally, immunizing mice with *Roseburia intestinalis* resulted in the generation of autoantibodies directed against human β_2GPI, showing that bacterial proteins, conserved between man and microbe, can provide continued T-cell help and hence induce autoimmunity[62]. In these cases, the translocation of microbes to lymph nodes or systemic organs as a result of mucosal barrier breakdown[64], might be at the start of autoimmunity. Translocating bacteria are not only likely presenting antigens to the adaptive immune system but will also activate complement and other innate immune triggers, allowing efficient antigen-presentation to B cells by FDCs as well as co-stimulatory signals for the efficient initiation of B-cell responses.

Although DNA-/RNA-binding proteins are involved in the examples mentioned above, similar principles are also implicated for other autoimmune responses directed against different self-antigens. Celiac disease, although not primarily a B cell-mediated autoimmune disease, is characterized by disease-specific autoantibodies against the enzyme tissue transglutaminase (tTG)[65]. It is now evident that the T cells underlying the autoreactive tTG-specific B-cell response are directed against gliadin, a foreign-antigen present in food. tTG can catalyse a specific deamidation of certain glutamine residues in gliadin, leading to the creation of modified gliadin

peptides able to bind to the Human Leukocyte Antigen (HLA)-DQ-molecules predisposing to disease and that are recognized by the disease-causing gliadin-reactive T cells. tTG can form complexes with gliadin and through the uptake of tTG-gliadin complexes, autoreactive tTG-specific B cells can recruit T-cell help through an HLA-DQ-restricted presentation of modified gliadin epitopes[66]. Recently, several bacterial mimics of gliadin-epitopes have been identified that were recognized by gluten-reactive T cells from celiac disease patients. Intriguingly, the analyses of crystal structures of T-cell receptors derived from gliadin-reactive T cells in complex with HLA-DQ bound to two distinct bacterial peptides, derived from *P. fluorescens*, demonstrated that molecular mimicry drives cross-reactivity toward the gliadin epitopes[67]. Thus, also in the case of celiac disease, it is indicated that T-cell reactivity towards microbial antigens displaying cross-reactivity to an antigen present in food, is at play in disease development and the formation of autoreactive B-cell responses to an ubiquitously expressed self-antigen.

However, next to microbial antigens, also other environmental triggers might provide T-cell help to support autoreactive B-cell responses. This is best exemplified for endemic Pemphigus Foliaceus in Brazil[68]. Pemphigus Foliaceus is known worldwide, but an endemic variety, Fogo Selvagem, characterized by the presence of autoantibodies against DSG1, is only present in certain areas of Brazil[69]. In these areas the unique combination of predisposing HLA-molecules expressed by the population[70] and a well-defined environmental trigger, the bite of a sand fly, has been shown to lead to the formation of anti-DSG1 antibodies[68]. This is explained by the observation that the salivary protein LJM11 from the sand fly is recognized by Fogo Selvagem antibodies as well as anti-DSG1 mAbs derived from Fogo Selvagem patients. Although, the underlying T-cell response has not yet been defined, it is highly conceivable that also in this case the autoreactive B-cell response arises through cross-reactivity via the "help" of T cells directed against environmental antigens.

Thus, together, the picture emerges that B-cell autoimmunity often arises in the absence of autoreactive T cells. Instead, these B cells contain BCRs showing cross-reactivity between self-antigens and environmental/ microbiome triggers and thereby recruit T-cell help.

Mechanisms of ACPA-expressing B cells in RA to overrun B-cell checkpoints

As described above, the prominent human autoimmune disease RA is characterized by disease-specific autoantibodies and responsive to B-cell targeted therapies. It is manifested by synovial inflammation and progressive joint damage if left untreated. The autoantibody response in RA has been characterized in great detail in the past decade, revealing important insights that harmonize with the findings into the emergence of the autoreactive B-cell response uncovered in other autoimmune diseases. However, these studies also unveiled additional processes which are likely involved in violating checkpoints controlling B-cell activation in humans. The most disease-specific antibodies in RA are directed against citrullinated proteins, and hence,

are named anti-citrullinated protein antibodies. Remarkably, ACPAs are reactive towards a broad spectrum of citrullinated antigens, including self-proteins as well as proteins present in microbes[71-75]. Moreover, recent studies have shown that they are not only restricted to citrulline recognition, but that they can also cross-react to other post-translational modifications (PTMs), more specifically acetylated- and carbamylated-lysine residues. This has not only been shown at the polyclonal and monoclonal autoantibody level, but also on the level of BCR signaling[40,44-46,76-78]. Thus, in this case, autoreactive B cells expressing a BCR directed against one particular PTM antigen can not only be activated by this specific modification, but also by (other) antigens expressing different modifications[45]. Hence, various antigens and PTMs could potentially drive the expansion of autoreactive B cells in RA, possibly including microbe-derived antigens. Experimental studies have reflected on a role for the oral, gut or lung microbiota in the development of RA, such as mediated by *Porphyromonas gingivalis* (*Pg*), *Aggregatibacter actinomycetemcomitans (Aa)* and *Proteus mirabilis* (*Pm*)[74,79,80]. For example, a high similarity between *Pg* enolase and the human α-enolase has been shown[74]. This similarity between foreign and self allows cross-reactive antibodies to bind to self-antigens, as evidenced by the correlation between ACPA levels directed against citrullinated human α-enolase and the levels of antibodies directed against citrullinated α-enolase from *P.gingivalis*[74]. Additionally, it has been reported that ACPAs might evolve from immune responses to *Pg*[81]. For *Aggregatibacter actinomycetemcomitans* it has been suggested that a pore-forming toxin, leukotoxin A (LtxA), causes hypercitrullination and thus generates multiple citrullinated epitopes that can potentially be targeted by the cross-reactive autoantibodies[80]. Nevertheless, ACPAs are not only present in individuals exposed to *Aa*[82]. Thus, also in this autoimmune disease, the prevailing humoral autoimmune response is recognizing both (modified) self- and non-self-proteins expressed by microbes. Moreover, these autoantibodies also recognize DNA-binding molecules such as histones as citrullination and acetylation represent prominent epigenomic modifications[83]. Therefore, it is conceivable that ACPA-expressing B cells can recruit co-stimulatory signals from both (opsonized) microbes as well as (modified) protein-DNA-complexes.

Additionally, a unique feature of ACPAs is the presence of N-linked glycans in the variable (V) domain as it is found that over 90% of ACPA molecules in serum of RA patients contain V-domain glycans[84,85]. These glycans displayed on ACPAs are primarily complex-type carbohydrates containing a high degree of sialic acids[84] and are acquired through the introduction of N-linked glycosylation sites following SHM[86]. As the introduction of glycosylation sites in the BCR by SHM is a random process, and as ACPA V-domain glycans are found in the vast majority of ACPA molecules in almost all RA patients, it is likely that the expression of such glycans by anti-citrullinated protein BCRs, provides a selective advantage to ACPA-expressing B cells. Interestingly, the presence of V-domain glycans in ACPA-positive healthy subjects is associated with the transition towards disease[87]. These observations, together with the recent discovery that the HLA-molecules predisposing to RA are associated with the introduction of ACPA

V-domain glycans[88], further emphasizes that the introduction of glycans into the BCR V-domain likely represents an additional mechanism involved in the "breach of B-cell tolerance" in RA and possibly other human autoimmune diseases including Anti-Nuclear Antibody (ANA)-associated vasculitis and Sjögren's syndrome[89,90]. How V-domain glycans expressed by autoreactive anti-citrullinated protein-directed B cells contribute to the development and/or expansion of the ACPA response is presently unclear, although V-domain glycans have been shown to modulate antigen recognition and to interact with lectins in vicinity to the BCR or on neighboring cells and thereby could deliver survival signals[91-95].

Thus, collectively, autoreactive B-cell responses that hallmark RA are of a highly cross-reactive nature, recognizing different PTMs that are expressed on foreign- and self-proteins. Their development encompasses the introduction of V-domain glycans through the formation of N-linked glycosylation sites by SHM. Both characteristics are likely to be involved in overrunning B-cell checkpoints as cross-reactivity could trigger T-cell help as a "first hit"[96], whereas the introduction of V-domain glycans might be an additional "second hit" mechanism to gain a selective activation/survival advantage despite autoreactivity. Autoreactive B-cell responses in RA might therefore emerge from "multiple hits", including the high cross-reactive nature of ACPAs and their abundant presence of V-domain glycans (Figure 2).

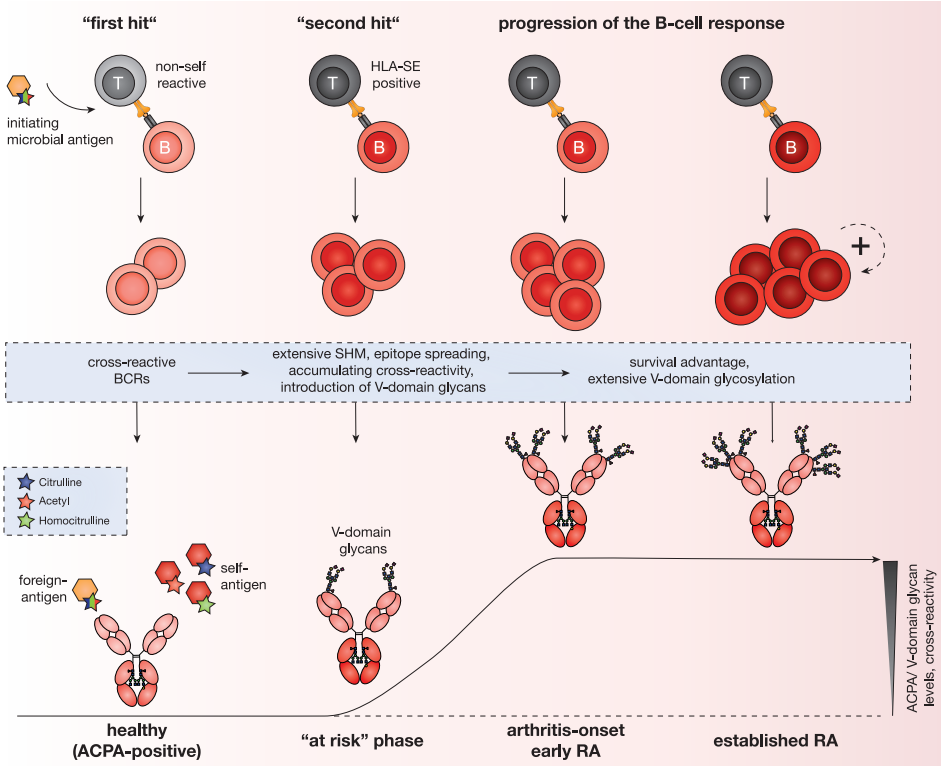

Figure 2. Escape mechanisms of ACPA-expressing B cells and RA development. "Multiple hit" theory of the ACPA-expressing B-cell response in RA and changes in ACPA characteristics towards disease-onset. The autoreactive B-cell response in RA is initiated by microbial triggers allowing T-cell help and the generation of cross-reactive, citrullinated protein-directed B cells and autoantibodies (ACPAs). These B cells are able to recognize post-translationally modified (citrullinated, acetylated, homocitrullinated) foreign- and self-antigens. This "first hit" is followed by a "second hit", including T cells restricted to HLA-molecules predisposing to RA, and results in extensive SHM leading to epitope spreading and an increase in cross-reactivity of the B-cell response. This stage is also characterized by the introduction of glycans into the variable domain of the BCRs. Current epidemiological evidence indicates that these V-domain glycans provide a selective advantage to ACPA-expressing B cells explaining the increase of V-domain glycosylated ACPAs pre-disease and the abundant presence of V-domain glycosylated ACPAs in RA. B cells presenting cross-reactive, highly V-domain glycosylated BCRs are probably able to escape important gatekeepers, which will lead to a progression of the B-cell response, a rise in autoantibody/ ACPA levels and thus in the onset of RA.

Concluding remarks

The mechanisms described in this review shed light on how autoreactive B cells might breach "tolerance" in humans. It is probable that autoreactivity arises in the periphery where B cells have to escape fewer "tolerance" checkpoints than autoreactive B cells emerging in the BM. As the induction of B-cell responses in the periphery is stringently controlled by co-stimulation from innate triggers, FDCs and T helper cells and as most autoantibodies have undergone class switch recombination and SHM, it is likely that autoreactive B-cell responses arise by misleading these gatekeepers.

Accumulating evidence suggests, although not consistent throughout diseases, that autoreactivity might be gained during SHM allowing mutated BCRs to obtain reactivity to self-antigens. SHM might also introduce cross-reactivity, which allows the BCRs to react towards both self- and foreign-antigens. Cross-reactivity to non-self as an inducer of autoimmunity is highlighted in several studies demonstrating an important role for the microbiome or environmental triggers in the induction of B cell-mediated autoimmune responses. A common denominator of these findings is the notion that autoreactive B cells cross-react between self and the "environment"/microbiome, whereas the accommodating T cells supporting these B cells do not necessarily recognize self. In this way, the need to depose both B-cell and T-cell checkpoints to induce B cell-mediated autoimmunity is overturned, conceivably explaining the relative high prevalence of B cell-mediated autoimmune responses in the human population. Additionally, glycans present in the variable domain of autoreactive BCRs might provide an additional, still poorly studied, mechanism involved in the induction of autoimmunity in humans. Further understanding of these, and other pathways controlling the induction of autoreactive B-cell responses in humans, is crucial for the development of preventative and/or curative strategies in B cell-mediated autoimmune diseases; diseases that are still incurable to date.

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Conflict of interest

R.E.M.T. is named as co-inventor on a patent on ACPA IgG V-domain glycosylation. Other authors declare no competing interests.

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