Regulation of autoreactive antibodies

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Purpose of review

Significant progress has been made over recent years in uncovering the B-cell tolerance mechanisms that control development of autoreactive antibodies. This review examines current knowledge on the regulation and selection of autoreactive B cells in mouse models, and in healthy humans and patients with autoimmune disorders.

Recent findings

Autoreactive B cells undergo stringent selection either in the bone marrow or peripheral circulation by deletion, induction of anergy, or receptor editing. There is growing evidence that receptor editing represents the primary physiologic B-cell tolerance mechanism. Several checkpoints against autoreactive B cells have been established in bone marrow and peripheral blood of healthy humans. Recent studies demonstrate that some autoimmune disorders are associated with several alterations in B-cell tolerance checkpoints and often lead to a greater number of autoreactive B cells in the circulation.

Summary

Discovering the precise nature of B-cell tolerance alterations in patients with autoimmune diseases will lead to the identification of new targets for therapeutic interventions in patients with these disorders.

Keywords

antibody, autoimmunity, B cells

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Abbreviations

BCR	B-cell receptor
CDR	complementarity determining region
FcγR	Fcγ receptor
IgH	immunoglobulin heavy (chain)
IgL	immunoglobulin light (chain)
SLE	systemic lupus erythematosus

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Introduction

A vast repertoire of antibody specificities is somatically generated by a process of immunoglobulin gene rearrangement during early B cell lineage development [1,2]. Because the recombination reaction is random, it will inevitably generate antibodies that recognize self-antigens. We have shown that at least 50% of early immature B cells in bone marrow express autoreactive B-cell receptor (BCR) [3]. These autoreactive B cells undergo stringent selection either in the bone marrow or peripheral circulation by deletion, induction of anergy, or receptor editing (for review [4–7]). This multistep process shapes the final immunoglobulin gene repertoire and reactivity of peripheral B cells, so that the majority of healthy humans exhibit only low levels of autoreactive antibodies in serum. Of the three regulatory mechanisms that have been documented to have an impact on B-cell tolerance, there is growing evidence that receptor editing is of primary physiologic importance [8,9•].

Persistent recombination results in replacement of functional autoreactive antibody genes with new immunoglobulin gene rearrangements, which are then again tested for autoreactivity. B cells that successfully replace autoreactive with nonautoreactive BCR proceed through development, whereas those few that fail to edit autoreactivity are likely to be deleted. Several checkpoints against autoreactive B cells have been established in bone marrow and peripheral blood of healthy humans [3,10••,11••]. Recent studies have demonstrated that some of the autoimmune disorders are associated with several alterations in B-cell tolerance checkpoints and often lead to greater numbers of autoreactive naïve B cells in the circulation [12,13].

Regulation of autoreactive antibodies in mice

Receptor editing is a highly efficient process that occurs when secondary recombination is activated in B cells producing autoreactive antibodies [14,15]. There is some evidence that immunoglobulin heavy (IgH) chain can undergo replacement when secondary recombination occurs using a cryptic recombination signal sequence site embedded in the third framework region of the rearranged heavy chain V_H gene (for review [16]). Detection of V_H replacement is often complicated, however, because of the proximity of a cryptic heptamer to the 3′ end of the V_H gene, and the effects of exonuclease activity and P and N nucleotide additions on the final V_HDJ_H joint sequence. Despite the fact that the 12-base-pair recombination signal sequence within

the D segments are deleted during the primary rearrangements, V_H replacement was demonstrated in mice with nonfunctional V_HDJ_H rearrangements [17,18,19•], in 3H9 mice expressing an anti-DNA transgene [20], in normal human B cells carrying hybrid V_H genes [21], and in human acute pre-B leukemia cell lines [22]. It has been estimated that editing of the IgH chain may have occurred in as many as 12% of all human B cells [16].

The structure of the V and J gene segments within the Igκ locus allows secondary rearrangements to occur by joining of upstream V_κ and downstream J_κ gene segments. Pioneering work conducted in mice carrying an anti-DNA IgH chain transgene (3H9) [15] showed that tolerance can be achieved by multiple rounds of V_κ rearrangement that result in selection of an effective immunoglobulin light (IgL) chain editor. Igκ locus editing in mice results in enrichment of distal V_κ and $J_\kappa 5$ genes, and can involve the second allele, with an increase of B cells rearranging both κ alleles [15,23]. These IgL chain editors have V regions with low isoelectric points, and thus they have the capacity to prevent or reduce IgH chain binding to negatively charged DNA phosphate groups.

3H9 mice on a Balb/c background have a limited IgL chain repertoire in the periphery and do not develop autoimmune disease, although they do express IgM anti-DNA antibody [15,23]. Placing the same transgene in a MRL/lpr background or a C57Bl/6 background greatly accelerates the development of anti-DNA IgG, suggesting that genetic background alters the mechanisms of receptor editing and the ability to sequester autoreactive specificities [24,25]. Introduction of additional positive charges by replacement of aspartate for arginine at position 56 in complementarity determining region (CDR)2 (3H9/56R) and replacement of serine with arginine at position 76 in frame work region 3 of the 3H9 heavy chain (3H9/56R/76R) results in an increased affinity for DNA, and makes it more difficult to find an appropriate editor IgL chain [26]. Although a small number of IgL chains can veto DNA binding in 3H9/56R mice, these light chains fail to inhibit DNA binding in 3H9/56R/76R mice. Additionally, negatively charged aspartates of editor IgL chains that are not completely opposed by positively charged arginines can introduce new reactivity against positively charged proteins such as myelin basic protein and lead to polyreactivity caused by residual DNA affinity and acquired protein binding [27•,28•].

Cells that fail to find an adequate Igκ editor can proceed to the Igλ locus, where editing can present a bigger challenge because of the locus structure [28•]. Activation of rearrangement in Igλ locus of Igκ-expressing cells can result in co-expression of both light chains and relative silencing of autoreactivity by dilution [29,30]. The co-expression of anti-DNA receptor and non-anti-DNA receptor dilutes either receptor to the point that cross-linking of autoreactive BCR is not sufficient for activation, but provides enough tonic stimulation to allow B cells to survive in the marginal zone area of the spleen [29]. Additionally, B cells have been found to co-express two λ chains (allelic inclusion), as described in 3H9/56R mice with κ locus deletion, which can further change antibody reactivity and lead to polyreactivity [28•]. It is believed that double expressor B cells with attenuated autoreactivity or polyreactivity can also contribute to the production of so called 'natural' antibodies, which play an important role in innate immune defense, as well as clearance of apoptotic debris, thus having a protective effect against autoimmune inflammation (for review see [31]).

A series of studies have demonstrated that receptor editing plays a crucial role in wild-type mice, in which physiologic BCR selection is preserved. By creating an allelic polymorphism of the κ constant region in mice, Casellas and colleagues [32] were able to estimate that as many as 25% of all B cells carrying both mouse and human Igκ constant locus undergo light chain receptor editing. Using a different approach limited to B cells that express Igλ because of Igκ deletion, Retter and Nemazee [33] found that as many as 50% of such cells were products of receptor editing. The phenomenon of both κ and λ chain being expressed in B cells carrying anti-DNA reactive BCRs has been confirmed by using a fluorochrome-labeled tetrameric form of a peptide that behaves as a double-strand DNA mimetope [34]. Receptor editing becomes the dominant mechanism of autoreactivity prevention when competition is introduced in mice that carry both rearranged V regions of autoreactive hybridoma and the intact IgH and IgL loci [8]. In these mice, the efficiency of vetoing the autoreactive BCR approached 100% with a minimal loss of B cells. Any significant cell deletion was observed only when the degree of receptor editing was artificially limited by the deletion of downstream J_L genes. Additionally, these animals had a significant number of allelically and isotypically included B cells that simultaneously expressed the original autoreactive and new nonautoreactive BCR [35].

More recently, the degree of receptor editing in mice heterozygous for human Cκ and mouse Cκ was studied in great detail using a combination of flow cytometry, single cell reverse transcription (RT)-PCR, confocal microscopy, and hybridoma analysis [9•]. The investigators concluded that 11% of all B lymphocytes in heterozygous animals express in-frame IgL chain transcripts from both κ alleles. Kinetics studies showed that these allelically included B cells were delayed at the pre-B cell stage for

several hours, suggesting that dual receptor expression is not the result of simultaneous biallelic Igκ recombination or failure in allelic exclusion, but rather results from secondary IgL chain gene rearrangements. Additionally, many of these double expressor B cells were found in the marginal zone and exhibited autoreactivity, conferred by either the human or mouse light chain [9•]. Co-expression of autoreactive and nonautoreactive receptors might dampen signaling from autoreactive receptor, thus allowing further B-cell development. It is believed that the high-avidity autoreactive BCR is continually internalized in these double expressor cells, wheeas only nonautoreactive BCR is present on the surface. Alternatively, IgL chain editors may tolerize autoreactive B cell by outcompeting autoreactive IgL chains for pairing with IgH chain [9•,29,35].

Regulation of autoreactive antibodies in healthy humans

Our group has developed an approach that allows single-cell analysis of human immunoglobulin gene repertoire and BCR reactivity [3]. IgH and IgL chain gene rearrangements are amplified by RT-PCR from single B cells and expressed *in vitro* as a recombinant IgG antibody. The purified antibodies are tested for reactivity in a number of assays: polyreactivity against a variety of unrelated antigens (double-stranded DNA, insulin, lipopolysaccharide); autoreactivity in an enzyme-linked immunosorbent assay with HEp-2 human cell line nuclear extract; indirect immunofluorescence assay for nuclear and cytoplasmic staining; Western blotting against defined autoantigens; and enzyme-linked immunosorbent assay against bacterial extracts.

In our first report, we analyzed B cells isolated from bone marrow and peripheral blood of healthy volunteers [3]. Precursor B cells with the surface phenotype of pre-B cells that expressed functional IgH and IgL chain transcripts were designated early immature B cells. These were distinguished from pre-B cells that did not express functional IgL chains and from $CD10^+$ immature B cells that expressed cell surface IgM. Peripheral blood $CD10^+IgM^+CD27^-$ B cells carried no somatic mutations and were designated new emigrant B cells, as opposed to unmutated peripheral blood $CD10^-IgM^+CD27^-$ B cells, which were defined as mature naïve B cells. We found that up to 75% of early immature B cells expressed antibodies that were reactive with HEp-2 cell lysates, and most of these were removed from the repertoire at two discrete checkpoints. The first checkpoint occurs in the bone marrow between the early immature and immature B-cell stage. The second selection step takes place in the periphery in the transition from new emigrant to mature naïve B cells. The majority of polyreactive B cells were removed at the first checkpoint, with less than 7% of bone marrow immature B cells and peripheral new emigrant and mature naïve B cells retaining the ability to react with several unrelated antigens. In addition, antibodies with nuclear or nuclear/cytoplasmic staining pattern were efficiently removed from the repertoire at the first checkpoint in the bone marrow, and the vast majority of mature naïve B cells that retained HEp-2 reactivity exhibited low levels of anticytoplasmic reactivity. Long IgH CDR3s with high numbers of cationic amino acids were enriched in pre-B and early immature B cells. These features of autoreactivity [36] were selectively lost from the repertoire as B cells progressed through development. In approximately 5–10% of peripheral blood B cells, both light chain transcripts were present, with either one or both being productively rearranged. Comparison of downstream J_κ usage as a measure of receptor editing between early immature and mature naïve B cells did not reveal downstream gene usage bias. This analysis was based on a limited number of clones, however, and it did not take into account the prominent role of Igλ chain in the human repertoire.

During immune responses mature naïve B cells undergo clonal expansion, affinity maturation, and selection before they differentiate into either antibody-secreting plasma cells or memory B cells [2]. In humans, CD27 expression marks B cells that have mutated antigen receptors. Circulating $CD27^+$ cells can be divided into those that express surface IgM, which participate in T cell independent immune responses (for review [37]), and class-switched memory B cells, which are produced in germinal centers (for review [38]). Based on their cell surface phenotype and gene expression profile, it has been proposed that $CD27^+IgM^+$ B cells are circulating splenic marginal zone B cells, which play an important role in defense against polysaccharide antigens and bacterial infections [39]. Single cell reactivity analysis showed that the transition from naïve B cells into the circulating IgM^+CD27^+ compartment is accompanied by efficient counter-selection against autoreactive naïve B cells before the onset of somatic hypermutation, with only 2–1% of cells remaining autoreactive or polyreactive in the IgM^+ memory compartment [10••]. IgM^+CD27^+ B cells exhibited significant overrepresentation of the V_H3 gene family, which has been associated with ability to bind *Staphylococcus* protein A (reviewed [40]). V_H3^+ antibodies cloned from IgM^+CD27^+ B cells were no more reactive to *Staphylococcus* protein A than were V_H3^+ antibodies from mature naïve B cells, however, and they had often lost *Staphylococcus* protein A reactivity by somatic hypermutation [10••]. In addition, broadly bacterially reactive antibodies were selected against during IgM^+ memory B-cell development.

These observations appear to be at odds with the transgenic mouse experiments that showed that B cells expressing polyreactive antibodies are selected into the

marginal zone B-cell compartment (for review [41]). The reactivity of marginal zone B cells has never been measured in normal mice or humans, however, and experiments using transgenic mice can alter the development, half-life, and anatomic distribution of autoreactive B cells. Thus, the repertoire of authentic marginal zone B cells developing in the presence of a complete B-cell repertoire may differ from those that arise in transgenic mice in the absence of competition.

Development of most class-switched memory B cells depends on the germinal center reaction and T-cell help, and is accompanied by extensive somatic hypermutation, which may alter antibody affinity or reactivity (for review [42]). Single-cell IgG^+ memory B-cell analysis [11••] showed that the frequency of HEp-2 autoreactive antibodies was higher in this compartment than among mature naïve and IgM^+ memory B cells. In addition, a fraction of these autoreactive antibodies in three healthy donors exhibited a true antinuclear antibody staining pattern by indirect immunofluorescence, including nuclear and nucleolar patterns typically associated with autoimmune disorders. IgG^+ memory B cells also exhibited a significant increase in polyreactivity, including bacterial antigens. Only a small number of these antibodies recognized several strains of bacteria but did not react with self-antigens. When the immunoglobulin genes of these reactive IgG^+ memory B-cell antibodies were reverted to their germline unmutated counterparts, the majority of antibodies lost their reactivity, indicating that reactivity was acquired during somatic hypermutation. Finding autoreactive and polyreactive B cells in the IgG^+ memory B-cell compartment is surprising because most polyreactivity in serum has been attributed to IgM antibodies (for review see [43]). Any direct comparison of monomeric IgG would naturally favor IgM, however, because of the greater avidity of the latter. Comparison of monomeric serum IgM with IgG showed that it is actually the IgG fraction that exhibits greater reactivity [11••].

Regulation of class-switched IgG^+ B cells is associated with acquisition of new signaling properties that distinguish them from IgM^+ cells in that IgG cytoplasmic tail contributes to signaling by the Igα/β heterodimer. This leads to a very different downstream gene expression pattern and possibly explains differences in the levels of autoreactivity in IgM versus IgG antibodies [44•,45•]. Thus, it is clear that autoreactive antibodies are tightly controlled in healthy humans by several checkpoints, which lead to a gradual deletion of autoreactive specificities among naïve B cells, whereas de-novo autoreactivity is often found in the IgG^+ memory B-cell compartment. Currently work is under way to determine whether these autoreactive antibodies are retained in the human plasma cell compartment.

Autoreactive antibody selection in patients with autoimmune diseases

Autoreactive antibody production is often recognized as a hallmark of a variety of autoimmune diseases, and the presence of such antibodies is often a characteristic feature of a particular autoimmune condition [46]. Consistent with our findings in IgG^+ memory B cells [11••], autoreactive antibodies are often found in healthy human volunteers. It is generally agreed that the persistence of isotype-switched and somatically mutated autoreactive antibodies is the result of a break in B-cell tolerance, which allows these autoreactive B cells to escape the selection process that shapes the spectrum of peripheral B-cell antibody reactivity (for review see [47]). Sequence analysis of productive IgV gene rearrangements showed that the peripheral B-cell repertoire in patients with autoimmune disease is diverse and often comparable to that in healthy humans, varies between patients, and, finally, does not predict the antibody reactivity (for review see [12,48,49]).

Single-cell antibody cloning has made it possible to define the checkpoints that control autoantibody selection in patients. An analysis of three untreated pediatric patients with systemic lupus erythematosus (SLE) [12] showed that autoreactive antibodies were not removed from the naïve repertoire, which led to accumulation of large numbers of autoreactive/polyreactive B cells in the mature naïve B-cell compartment. Although these were presumably low-affinity and unmutated antibodies, we speculate that large numbers of autoreactive mature naïve B cells might overwhelm the later antibody tolerance checkpoints and contribute to the development of pathogenic antibodies.

Early checkpoints remained defective in SLE patients in remission after treatment, when high levels of autoreactive mature naïve B cells were present in the peripheral circulation [49]. This persistence of autoreactive antibodies in SLE patients was demonstrated despite the changes in immunoglobulin gene repertoire before and after treatment. SLE patients before treatment exhibited a bias for certain V gene families or individual members (V_H3, $V_κ4–1$), but no consistent pattern of receptor editing alterations was established (for review [48,49]).

The first B-cell tolerance checkpoint that normally removes most polyreactive B cells in the bone marrow is also defective in rheumatoid arthritis patients, as reflected by the increased percentage of polyreactive B cells in their new emigrant B-cell compartment [13]. Many of these patients exhibited IgL chain features associated with altered selection processes, such as an increased proportion of new emigrant B cells expressing 11-amino-acid long CDR3 immunoglobulin chains. Most polyreactive antibodies enriched in peripheral B cells in

rheumatoid arthritis were also reactive with peripheral B cells specific antigens and exhibited low rheumatoid factor and anti-cyclic citrullinated peptide reactivity. The second peripheral checkpoint that accounts for additional removal of autoreactive B cells in the periphery between new emigrant and mature naïve B-cell stage is also defective, and allows accumulation of a large number of self-reactive B cells in the mature naïve B-cell compartment in SLE patients and those with rheumatoid arthritis. Interestingly, many patients with rheumatoid arthritis appear to exhibit one of two patterns of V_κ and J_κ gene family usage. Some showed an increase in downstream V_κ genes associated with the most upstream J_κ, suggesting inefficient secondary recombination and therefore a potential defect in receptor editing. In contrast, the others exhibited an increase in upstream V genes associated with downstream J genes, suggesting extensive secondary recombination in those cells [13] (Meffre E, personal communication, 2007).

These recent findings demonstrate that some of the autoimmune disorders are associated with several alterations in early B-cell tolerance, which are often divergent in their nature as indicated by specific immunoglobulin gene usage, and often lead to a greater number of autoreactive B cells in the naïve B-cell compartment. The low-affinity autoreactive B cells, which are normally excluded during the early stages of germinal center reaction, might escape this checkpoint in SLE patients and participate in germinal center reaction, with subsequent expansion in class-switched memory and plasma cell compartments [50]. These potentially autoreactive B cells are further regulated by a variety of mechanisms, including activation receptor Fcγ receptor (FcγR)IV and its inhibitory receptor counterpart FcγRIIb [25,51]. FcγRIIb is routinely upregulated on memory B cells in the peripheral blood of healthy volunteers, whereas this is considerably less evident in SLE patients [52•]. Deficiency or reduced signaling via FcγRIIb on long-lived bone marrow plasma cells is associated with decreased levels of apoptosis and leads to a significant expansion of these cells [53••]. Lack of negative regulation can contribute to inappropriate activation and increased survival of autoreactive memory and plasma cells in these patients.

Conclusion

We are beginning to develop an understanding of the checkpoints that regulate autoreactive antibodies in normal humans, but this understanding is still incomplete in that a number of important B-cell types, including plasma cells, have yet to be investigated. In addition, numerous questions remain about the development and regulation of autoantibodies in autoimmune disease. Preliminary studies in patients with autoimmune diseases indicate that early B-cell checkpoints are defective in SLE and rheumatoid arthritis. Although the increase in autoreactive naïve B cells may ultimately contribute to pathogenesis by increasing the size of the precursor pool for high-affinity class-switched autoantibody producing cells, the validity of this idea has yet to be demonstrated. Finally, the B-cell compartment that contains highly specific pathogenic autoreactive antibodies and the frequency of such cells relative to the normal population remain to be determined.

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