REVIEW

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Regulation of autoreactive B cells: checkpoints and activation

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Abstract

It has become clear that the autoreactive B cells are a part of the normal naïve B cell repertoire in the periphery, despite the fact that they undergo a series of checkpoints, which include receptor editing (revision), clonal deletion, and anergy. However, most of those B cells reactive against self antigen remain functionally naïve for autoantibody production by differential peripheral checkpoints. Therefore, the presence of autoreactive B cells does not always signify disease. Regulation of their activation and effector functions will determine the ultimate outcome. Although autoreactive B cell tolerance is well maintained in the healthy individual, the existence of pathogenic autoantibodies in autoimmune diseases indicates that these tolerogenic checkpoints are broken. Recent studies have demonstrated that autoreactive B cells are regulated by a composite of factors, such as genetic susceptibility and environmental triggers such as bacterial and viral infections as well as other immune cells. Interestingly, Toll-like receptors, previously considered as pattern-recognition receptors to detect and sense pathogens, may also have a potential to recognize self antigens and regulate autoreactive B cells for activation. Understanding the mechanisms of autoreactive B cell regulation and activation may help in identifying novel targets for the treatment of autoimmune diseases.

Key words: autoreactive B cells, tolerance, Toll-like receptors, infection.

Abbreviations: AutoAb – autoantibody, BCR – B cell antigen receptor, GC – germinal center, RA – rheumatoid arthritis, pDCs – plasmacytoid dendritic cells, RF – rheumatoid factor, SLE – systemic lupus erythematosus, snRNP – small nuclear ribonucleoprotein particle, TLRs – Toll-like receptors.

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INTRODUCTION

The production of autoantibodies (autoAbs) is a hallmark of systemic autoimmune diseases such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA). These autoAbs, produced by self antigen-experienced B cells, are involved in pathogenic immune complex formation and deposit in organs or tissues for disease progression. This also represents a breakdown of autoreactive B cell tolerance. Using different immunoglobulin transgenic mouse models, mechanisms by which autoreactive B cells are regulated have been proposed, including clonal deletion, receptor editing (revision), anergy, and ignorance. In addition, autoreactive B cells that escape these mechanisms are subject to regulation by recently identified peripheral checkpoints. However, many questions remain. For example, in which circumstance do autoreactive B cells activate for autoAb production? How do these autoreactive B cells escape checkpoints both in the developmental stage and peripheral stage? Moreover, it has not been determined where B cell tolerance is broken in disease development. It becomes apparent that the regulation of autoreactive B cells is influenced by a composite of factors such as genetic susceptibility and environmental triggers. Recent studies have also suggested that Toll-like receptors (TLRs) not only function to detect pathogens and activate innate immunity, but also have a potential to recognize self antigen and trigger autoreactive B cell activation. In this review we will discuss checkpoints to maintain autoreactive B cell tolerance and the regulation of autoreactive B cell activation.

CHECKPOINTS OF B CELL DEVELOPMENT AND B CELL TOLERANCE

Our immune system can react to a wide array of antigens. One of the features of the immune system is a diverse repertoire of antigen receptors that recognize non-self and self antigens. The majority of B cells in bone marrow are polyreactive and capable of binding self antigen, but only a small proportion enter the mature B cell pool; most of the potentially harmful autoreactive B cells are eliminated from the repertoire at a series of self-tolerance checkpoints on the pathway of B cell development from immaturity to plasma cell differentiation and germinal center (GC) formation in the bone marrow and periphery.

B cell tolerance checkpoints can be divided into central and peripheral tolerance. Central tolerance eliminates autoreactive B cells in the pre-immune B cell repertoire and can prevent the development of high-affinity autoreactive B cells by the mechanisms of deletion, receptor editing (revision), and anergy. Peripheral tolerance can prevent the activation and affinity maturation of the remaining, generally less autoreactive B cells during an immune response such as ignorant B cells. To develop autoAbs by autoreactive B cells and initiate systemic autoimmunity, either the central tolerance mechanism must be overcome or the mechanisms that regulate peripheral B cells must break down.

Several B cell developmental checkpoint mechanisms have been identified. The early checkpoints of B cell development include (1) receptor editing (revision): the receptor is arranged to change the specificity of the B cell antigen receptor (BCR) that is less self reactive by V(D)J recombination [17, 44]. Receptor editing involves RAG gene activation and leads to a new BCR with altered antigen specificity [24]; (2) clonal deletion: some of the immature B cells expressing a BCR with high affinity for the self antigen might be deleted by apoptosis in the bone marrow when they encounter high-avidity forms of self antigen [42, 43, 45]; and (3) clonal anergy: converts autoreactive B cells to a state that is no longer response to BCR engagement and less able to compete for survival factors [16]. Maintenance of B cell anergy requires constant antigen receptor occupancy and signaling [15]. Many features of anergic cells are rapidly reversed after dissociation of self antigen using hapten competition and these cells regain antigen responsiveness. In addition to these mechanisms, several novel checkpoints to maintain autoreactive B cell tolerance in the periphery have been identified, such as GC arrest. Recent study has shown that a subset of autoreactive B cells in V(H)3H9 anti-dsDNA Tg mice that escape the central and peripheral checkpoints can be activated and enter into GCs. However, these B cells fail to develop further into antibody-secreting plasma cells [48]. Further study confirmed this mechanism and also identified another novel checkpoint for autoreactive B cell regulation using an anti-rheumatoid factor (RF) immunoglobulin transgenic mouse [70]. RF-expressing B cells in normal BALB/c background mice do not secrete autoAb but can be activated by self antigens. These activated autoreactive B cells actively participate in GCs. However, these B cells fail to develop further into antibody-producing cells. Moreover, analysis of the somatic hypermutation pattern of V regions from RF B cells demonstrates that these B cells fail to efficiently select and expand autoreactive B cell clones. These studies have proposed the interesting point that some of the antigen-specific autoreactive B cells are not quiescent or ignorant, but can be activated in the periphery and that autoimmunity can proceed much further than originally thought, even in non-autoimmune-prone mice.

To further support this notion, previous studies demonstrated that up to 20% of mature naive B cells from healthy donors produce low-affinity self-reactive antibodies and 5% secrete antibodies with low levels of polyreactivity [69]. Therefore, autoreactive B cells are a part of the normal naive B cell compartment. However, these potentially autotoxic autoAbs are not likely to make a significant impact on the circulating B cell repertoire. On the contrary, these B cells with self activity from the naive B cell pool failed to develop IgM^+ memory B cells due to a failure of somatic mutation [65]. This may represent another novel checkpoint for B cell tolerance between naïve and memory B cell development.

DEFECTIVE B CELL TOLERANCE CHECKPOINTS IN AUTOIMMUNE DISEASES

Despite these checkpoints, not all autoreactive B cells are eliminated from the preimmune repertoire. Many studies have demonstrated that these checkpoints which maintain autoreactive B cell tolerance are broken in autoimmune diseases. Previous study indicated that autoreactive B cells from SLE patients are associated with a failure to establish self tolerance during early B cell development, leading to increased numbers of autoreactive B cells in the mature naive B cell compartment [75]. Similarly impaired early B cell tolerance also occurred in patients with RA [56]. In addition, autoreactive B cells display impaired transitional B cell production and selection in the non-obese diabetic mouse [51].

Recent studies have significantly advanced our understanding of the mechanisms by which autoreactive B cells escape tolerance. These factors include genetic susceptibility and environmental triggers. A large number of loci and candidate genes have been identified to be associated with susceptibility to the development of autoimmune diseases [29, 40, 41, 68]. Among these, defects of inhibitory signaling pathways such as inhibitory Fc receptor (FcγRIIB) have been shown to be genetically associated with the development of autoimmunity. Partial restoration of FcγRIIB levels on B cells from lupus-prone mouse strains is sufficient to restore B cell toler-

ance and prevent autoimmunity [36]. Recent study has also indicated that autoreactive B cells from lupus-prone mice are regulated by the lupus susceptibility gene Ly108 [29]. The Ly108.1 isoform of the Ly108 gene is found to be highly expressed in immature B cells from lupus-prone mice. Compared with wild-type Ly108.2-expressing normal B cells, immature B cell lines transfected with Ly108.1 gene display reduced calcium flux, reduced RAG expression, and decreased cell death. Autoreactive B cells with an elevated level of Ly108.1 expression escape multiple B cell tolerance mechanisms such as deletion, receptor revision, and anergy induction by tuning down BCR signaling at the immature stage of B cell development. These studies underscore the pivotal role of Ly108 gene in the regulation of autoreactive B cell checkpoints.

In addition to Ly108 gene, a Y chromosome-linked "autoimmune accelerator" locus (Yaa) has been identified as another lupus susceptible locus. Yaa in combination with FcγRIIB gene significantly increases the onset and severity of lupus development in normal non-autoimmune-prone C57Bl/6 mice [3]. Interestingly, B cells containing the Yaa locus exhibit increased TLR7 expression, which responds to RNA-related antigen stimulation. The TLR7 gene is duplicated in Yaa mice due to a 4-megabase expansion of the pseudoautosomal region [50]. This effectively doubles the TLR7 gene dosage and significantly increases the responsiveness of B cells to TLR7 ligand. In addition, TLR7 translocation has shown to accelerate systemic lupus autoimmunity [63].

REGULATION OF AUTOREACTIVE B CELLS BY TLRs

TLRs are pattern-recognition receptors that serve the important function of detecting pathogens, activating innate immunity, and eliciting inflammatory responses [38, 64]. However, TLRs may also have a potential to recognize self antigens and trigger autoimmune diseases such as lupus and RA. Notably, SLE patients have elevated levels of circulating plasma DNA which is enriched in hypomethylated CpGs [27, 35]. Recent studies have demonstrated that DNA-containing immune complexes from the serum of patients with active lupus stimulated plasmacytoid dendritic cells (pDCs) to produce cytokines and chemokines in a TLR9- and CD32-dependent manner [37]. In addition, the U1 small nuclear ribonucleoprotein particle (snRNP) immune complexes found in SLE patients trigger interleukin 6 and interferon (IFN)-α production in pDCs in a TLR7-dependent manner [57]. These studies further demonstrate the pathogenic role of TLRs in human lupus disease development.

TLR ligands, such as bacterial DNA and stimulatory CpG-oligodeoxyribonucleotides, are potent B cell activators, inducing proliferation, Ig isotype switching, costimulatory molecule expression, and cytokine secretion [28, 74]. A possible role for TLRs in autoreactive B cell activation has also been examined. For example, endogenous DNA containing CpG motifs has been demonstrated to function as a more ubiquitous and powerful adjuvant for autoAb secretion via the TLR9 pathway [27, 31]. Low-affinity RF B cells can be activated by self antigen IgG when it forms immune complexes with self CpG DNA via a TLR9-dependent pathway or by RNA and RNA-associated antigens such as snRNP via a TLR7-dependent pathway [30, 31]. U1 RNA, the endogenous ligand for U1-70 Kd ribonucleoprotein particle, has been demonstrated to induce a TLR3-mediated innate immune signaling pathway for proinflammatory cytokine secretion [19]. The demonstration that message RNA (mRNA) is an endogenous ligand for TLR3 also supports this possibility [25]. Indeed, double-stranded RNA (dsRNA) treatment on MRL *lpr* mice aggravates lupus nephritis although serum autoAb levels are unaltered [47]. In addition, a recent study showing that TLR 3 is not necessary for the generation of anti-Sm autoAb may also implicate the involvement of other innate receptors such as TLR7 or protein kinase R for anti-RNA Ab production in SLE [6, 10, 18]. Indeed, RNA-containing immune complexes have demonstrated a stimulatory effect on low-affinity RF B cells for activation through TLR7 [30]. Furthermore, a BCR/TLR co-engagement hypothesis has been proposed to explain how silent autoreactive B cells are activated in certain circumstances [67]. The dual-engagement hypothesis is confirmed in the regulation of low-affinity RF autoreactive B cells, hapten antigen B cells, as well as 3H9 anti-dsDNA B cells *in vitro* [31, 66]. Our studies using an anti-snRNP Tg mouse model also demonstrate that TLR engagement (TLR3 and TLR9) can stimulate previously developmentally arrested autoreactive anti-snRNP B cells for activation both *in vitro* and *in vivo*. The autoAb production elicited by TLR agonists appears to be CD4 independent [11]. It is likely that self antigens could act in conjunction with "danger triggers", such as endogenous hypomethylated CpG motifs or self RNAs, to generate stimuli for low-affinity autoreactive B cell activation via TLR9, TLR3, and TLR7 pathways.

It is still controversial how TLRs play a role in autoreactive B cell activation and autoAb secretion *in vivo*. For example, the expression levels of anti-dsDNA and anti-chromatin autoAbs are inhibited in TLR9-deficient lupus-prone mice. In contrast, ablation of TLR3, a receptor for dsRNA, did not inhibit the formation of autoAbs to either RNA- or DNA-containing antigens [6]. However, in fully back-crossed TLR9-deficient lupus-prone MRL mice, TLR-9 signaling plays a protective role, perhaps by modulating the activity of regulatory T cells [71]. These results highlight the need for caution in the assessment of disease paradigms based on *in vitro* study as well as *in vivo* studies of knockout animals involving non-ideal genetic models. More studies need to be done to further dissect the role of TLRs *in vivo* for the autoreactive B cell activation.

It is interesting to examine the cross-talk between

the TLR and the BCR signaling pathways, since some TLRs, such as TLR7 and TLR9, are not expressed on the B cell surface but inside the cells. The expression levels of TLR9 and TLR10 are low in human naive B cells, but can be rapidly induced following BCR ligation, suggesting synergistic effect between BCR and TLR signaling [1, 2]. However, the relationship between BCR and TLRs on autoreactive B cells is not very well defined. Mammalian DNA must enter discrete endocytic compartments within the cell, the only way being through engagement of IgG-nucleoprotein complexes with the BCR on B cells [31] or Fcγ receptors on dendritic cells [33]. In addition, TLR engagement may not necessarily lead to autoreactive B cell activation depending upon the status of the autoreactive B cells. For example, anergic anti-HEL B cells which constantly bind self antigen are resistant to CpG DNA stimulation for Ab secretion [53]. This may reflect two different aspects of the regulation of autoreactive B cells by TLRs. High-affinity autoreactive B cells such as anti-HEL B cells are resistant to CpG DNA stimulation. This mechanism may be also responsible for the self-antigen-induced tolerance in the context of immunogenic stimuli acting through divergent TLRs. On the other hand, low-affinity autoreactive B cells such as RF B cells can be activated by TLR engagement to secrete pathogenic autoAb.

INFECTION AND ACTIVATION OF AUTOREACTIVE B CELLS

Susceptibility to autoimmune diseases is determined by a combination of genetic and environmental factors. Among the environmental factors, the role of infectious agents in the pathogenesis of autoimmune diseases is still a matter of debate. Although some studies show that infections may have a protective effect against autoimmune disease, clinical observations and experimental models have demonstrated that autoimmune disease may be initiated or worsened by infections. The infectious organisms may contribute to the onset of several autoimmune diseases, such as rheumatic fever and animal models of multiple sclerosis. There are several major pathways through which viruses and other microbes can initiate or modulate autoimmunity [5]. For example, molecular mimicry (structural cross-reactivity between the microbial antigens and self antigens) or bystander activation of the immune system can lead to humoral autoimmunity [7, 26, 46].

Helicobacter pylori infection may be involved in autoimmune disorders such as RA or idiopathic thrombocytopenic purpura in addition to various gastroduodenal diseases. Previous study showed that purified *Helicobacter pylori* urease predominantly stimulates the B-1 cell population to secrete IgM-type RF, anti-ssDNA autoAb, and anti-phosphatidyl autoAbs [72]. Studies by Soulas et al. [62] demonstrated that chronic infection by *Borrelia burgdorferi* in RF immunoglobulin Tg mice (of low or intermediate affinities) breaks autoreactive B cell immunological ignorance, leading to the production of RFs. The production of autoAb is mediated by immune complexes and involves collaborative signaling pathways between the BCR and TLRs as well as help from T cells. These findings indicate that chronic infection can activate autoreactive B cells for pathogenic autoAb production and establish conditions that can drive them to further differentiate into memory B cells.

In addition to bacteria infection, virus infection has been long considered as one of the environmental triggers for the initiation of autoimmune diseases. Epstein-Barr virus (EBV) has been implicated by association in both human multiple sclerosis and SLE [20–23]. Many hypotheses have been proposed to explain autoreactive B cell activation and EBV infection. For example, Pender proposes that autoreactive B cells may be infected by EBV and become latently infected memory B cells. These infected autoreactive B cells lodge in organs where their target antigen is expressed and act there as antigen-presenting cells. When $CD4^+$ T cells are activated in lymphoid organs by cross-reactivity with infectious agents, they migrate to the target organ, proliferate, and produce cytokines, which recruit other inflammatory cells and cause target organ damage and chronic autoimmune disease [49]. Recent elegant studies from Drs. Harley and James's group demonstrate that some humoral autoimmunity in human lupus patients arises through molecular mimicry between the latent viral protein EBV nuclear antigen-1 and lupus autoantigens [34]. By tracing back the autoAb response in SLE patients, they identified the initial autoantigenic epitope for some lupus patients with positive autoAb for 60-kDa Ro autoantigen. These findings, along with others, provide strong evidence to support the hypothetical etiologic role of EBV in the development of autoimmune diseases.

$CD4^+$ T CELLS AND AUTOREACTIVE B CELL ACTIVATION

Naive B cell activation is dependent on the sequential integration of two signals: signal one from the antigen binding to BCR, signal two from T-helper (Th) cells. Recent studies have proposed TLR stimulation as a third signal required for the activation of human naive B cells [54]. Early studies by Shores et al. demonstrate the importance of $CD4^+$ T cells in driving the *in vitro* anti-Sm response of MRL-derived B cells [60]. Furthermore, T cell lines from MRL *lpr* mice have been isolated and characterized [9, 12]. Datta and colleagues further demonstrate the presence of T cells that could help B cells secrete autoAbs and identify peptides that are recognized by these T cells [39, 55]. More strikingly, these T cells could often recognize these peptides in the context of multiple different MHC allotypes [59]. Thus the recognition of autoAg by lupus T cells is MHC dependent, but unrestricted. These studies provide a rationale to develop "universally tolerogenic" epitopes for antigen-specific lupus treatment [8]. Recent studies from Erikson and

Singh's laboratories also showed that the anergy of anti--dsDNA B cells in non-autoimmune mice could be reversed by the provision of T cell help [58, 61], although the induction of autoAb synthesis and renal pathology appears to be mild and transitory. Our previous studies have indicated that T cells from lupus-prone MRL *lpr/lpr* 2-12 Tg mice can help B cells of non-autoimmune origin to initiate an anti-snRNP response [73]. Work from Hahn's laboratory has identified $SmD1^{83\text{-}119}$-reactive T cells that could provide T cell help for pathogenic anti-dsDNA autoAb production [52]. In addition, studies using the chronic graft-versus-host disease model suggest that a diverse $CD4^+$ T cell repertoire is required to produce sustained levels of autoAb responses capable of mediating systemic autoimmunity. The diverse repertoires of $CD4^+$ T cells are also important for the generation of autoAb isotype switching [4]. Moreover, Th2 cells can promote anti-chromatin B cell survival and autoAb production *in vivo*. Th1 cells induce anti-chromatin B cell GC formation, and their ability to stimulate anti-chromatin Ab production correlates with their level of IFN-γ production [14]. Recent studies also demonstrate that even naive $CD4^+$ T cells can promote autoreactive B cell survival via a CD40-CD40L pathway [32]. To directly confirm the possibility that T-B collaboration could cause autoimmune disease, mice doubly transgenic for an Id^+ Ig light chain and an Id-specific TCR are generated. These studies clearly demonstrate that Id-specific T cells can provide chronic help for Id^+ B cells and lead to a lethal systemic autoimmune disease [13].

The $CD4^+CD25^+$ T-regulatory (Treg) cells have been shown to block several organ-specific autoimmune diseases in which T cells are the primary effector lymphocytes, but how Treg cells inhibit autoimmune diseases in which B cells and autoantibody production are prominent mediators of pathology is not very clear. Previous studies demonstrated that anergic anti-dsDNA B cells can be activated simply by the provision of T cell help. However, the autoreactive B cell activation is aborted in the presence of $CD4^+CD25^+$ Treg cells [58]. Recent studies demonstrate that anti-chromatin B cells secrete Abs *in vivo* upon provision of undeviated, Th1- or Th2-type $CD4^+$ T cell help, but this secretion is blocked by the co-injection of $CD4^+CD25^+$ Treg cells. However, Treg cells must be administered during the initial phases of the Ab response to exert full suppression of autoAb production. These findings imply that Treg cells may act to inhibit the maturation, rather than the initiation, of autoAb responses [13]. Altogether, these results suggest that the spontaneous development of autoimmunity may be a 2-fold process involving both the production of activating factors, such as T cell help, as well as the surmounting of inhibitory factors, such as Treg cells.

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