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Beyond transitional selection: New roles for BLyS in peripheral tolerance

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Abstract

B cell targeted therapies have enjoyed recent success in the treatment of systemic autoimmune diseases. Among these, Belimumab, which blocks the B cell survival cytokine BLyS, was recently approved for the treatment of Systemic Lupus Erythematosus. It is therefore important to consider the roles BLyS plays in B cell tolerance. Herein, we review how BLyS contributes to the negative selection of autoreactive B cell clones from the preimmune repertoire as well as its role in regulating both germinal center and extrafollicular peripheral B cell responses. We further examine the complex role of Toll-like receptors (TLRs) in humoral autoimmunity, pointing out potential crosstalk between BLyS and TLR pathways.

Keywords

BLyS; BAFF; B lymphocyte; Autoimmunity; Tolerance; Toll like receptors; Belimumab

INTRODUCTION

Humoral autoimmune diseases, such as Systemic Lupus Erythematosus (SLE), Rheumatoid Arthritis (RA), and Sjögren's Syndrome (SJS), are characterized by immune cell infiltration, autoantibody formation, and subsequent damage to tissue caused by dysregulated inflammatory responses. Treatments have historically focused on relatively global immunosuppressive regimens that, while often effective, have numerous off-target side effects [Yildirim-Toruner and Diamond 2011]. Accordingly, therapies that specifically target cells and molecules directly contributing to disease onset and progression have been increasingly emphasized.

Given the association of autoantibodies with these diseases, it is not surprising that B cells contribute to their onset and pathology [Eggert et al. 2010; Yanaba et al. 2008]. Indeed, eliminating B cells ameliorates disease in several mouse models of autoimmunity [Hayakawa et al. 2007; Serreze et al. 1998; Shlomchik et al. 1994]. In addition to antibody secretion, the capacity of B cells to efficiently collect and process antigens via their BCR (AU Define) makes them particularly effective at autoantigen presentation [Chan et al. 1999]. Accordingly, several drugs have been developed that selectively ablate B cells. Rituximab, a CD20-specific monoclonal antibody originally developed for B cell lymphoma, has shown varying therapeutic potential. It has been approved for the treatment of RA patients who no longer respond to alternative therapies, and benefits some cohorts of

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SLE patients [Engel et al. 2011; Looney et al. 2004; Merrill et al. 2010]. A second monoclonal antibody, Belimumab, blocks the B cell survival cytokine B lymphocyte stimulator (BLyS, also known as BAFF) and was recently approved for the treatment of lupus [Sanz et al. 2011; Stohl et al. 2011].

BLyS is a key determinant in the tolerogenic selection and homeostatic control of primary B cells, and is elevated in several humoral autoimmune syndromes. These and other observations support the widely held views that excess BLyS contributes to autoimmunity by allowing newly formed autoreactive B cell clones to survive, and that BLyS blockade restores appropriate counter selection of cells entering pre-immune B cell pools [Cancro et al. 2009; Stohl et al. 2011]. Nevertheless, while this mechanism likely contributes to the outcomes of BLyS blockade, it may not be the sole point of action. Indeed, both activated and antigen-experienced B cells are involved in the etiology of humoral autoimmune diseases, making the appropriate selection of naïve B cell pools only one piece of the overall puzzle. For example, antibodies associated with autoimmune pathologies in both humans and mouse models display features characteristic of those derived from T-dependent (TD) humoral immune responses, including isotype switching and somatic hypermutation. Further, several Toll-like receptors (TLRs), which are key molecular components in T-independent (TI) humoral responses, are linked with autoimmune disease, thus implying that the failure to properly limit such responses might lead to the persistent generation of autoreactive antibodies.

These and other observations prompt the question of whether BLyS, in addition to its established roles in governing primary pools, also functions in processes that limit autoreactivity among activated and antigen-experienced B cells. Herein, we survey the range of B cell differentiation states where BLyS might influence processes associated with autoimmunity. We first briefly review current notions of how BLyS influences selection and survival in the pre-immune repertoire. We then consider the growing body of evidence suggesting that BLyS and its receptors are involved in the selection and survival of activated and antigen experienced B cell pools, with an emphasis on potential interplay between BLyS and Toll like receptors (TLRs).

BLyS MODULATES COUNTERSELECTION AMONG DEVELOPING PRIMARY B CELLS

After immunoglobulin heavy- and light-chain gene rearrangement during the pro- and pre-B cell stages in the bone marrow (BM), newly formed B cells undergo specificity-based selection before joining mature, pre-immune pools (reviewed in [Monroe and Dorshkind 2007]). This selection begins at the BM immature (IMM) B cell stage, where avid BCR ligation leads to receptor editing and/or death [Gay et al. 1993; Hartley et al. 1991; Luning Prak et al. 2011; Nemazee and Weigert 2000]. Roughly 90% of all newly formed IMM B cells are lost to these central tolerance processes [Allman et al. 1993; Forster and Rajewsky 1990].

Surviving IMM B cells leave the BM and continue to develop as transitional (TR) B cells, where they undergo additional selection based on BCR specificity [Allman et al. 1993; 2001; Fulcher and Basten 1994; Goodnow et al. 1988; Loder et al. 1999; Rolink et al. 1998]. Selection at this step spares only those cells whose BCR signal strengths fall below the threshold for negative selection, yet above levels required for positive selection via so-called “tonic” signaling [Monroe 2006]. Importantly, aberrant TR selection has been identified in patients with humoral autoimmunity, highlighting the importance of understanding the molecular mechanisms involved [Meffre and Wardemann 2008; Menard et al. 2010; Wardemann et al. 2003; Yurasov and Nussenzweig 2007; Yurasov et al. 2005]. A key

feature of this checkpoint is that, in contrast to central tolerance mechanisms, the stringency of TR selection is plastic, and can be varied based on interclonal competition [Cyster et al. 1994; Thien et al. 2004]. Moreover, mature follicular (FO) and marginal zone (MZ) B cells continue to require an appropriate range of BCR signaling to survive, whose breadth is similarly dictated by interclonal competition [Freitas et al. 1995; Gaudin et al. 2004; Gu et al. 1991; Lam et al. 1997; Lam and Rajewsky 1998; McLean et al. 1997]. Accordingly, the nature of this competition, as well as how competitive survival processes are integrated with BCR signal strength, have proven crucial to understanding the basis for TR B cell selection and homeostasis.

Evidence accumulated over the last decade firmly establishes BLyS as the limiting factor for which TR and mature primary B cells compete to survive. The BLyS family of ligands and receptors consists of two cytokines, BLyS (BAFF) and A proliferation-inducing ligand (APRIL); and three receptors, BLyS receptor 3 (BR3), transmembrane activator and cyclophilin ligand interactor (TACI), and B cell maturation antigen (BCMA) [Hahne et al. 1998; Kelly et al. 2000; Mackay et al. 1999; Madry et al. 1998; Moore et al. 1999; Shu and Johnson 2000; von Bulow and Bram 1997]. BLyS binds with the greatest affinity to BR3, less strongly to TACI, and with the lowest affinity to BCMA [Bossen and Schneider 2006; Day et al. 2005]. In contrast, APRIL binds with high affinity to both TACI and BCMA, but negligibly to BR3.

The three BLyS family receptors are differentially expressed among B cell subsets. Within the pre-immune pools TR, FO, and MZ B cells express BR3, and require signals via this receptor for survival. Accordingly, either BLyS or BR3 deficiency results in profound TR and mature B cell losses [Harless et al. 2001; Lentz et al. 1996; Lentz et al. 1998; Miller et al. 1992; Miller and Hayes 1991; Yan et al. 2001a]. Moreover, BCR signal strength dictates relative BLyS reliance, explaining how excess BLyS might promote autoimmunity via the rescue of autoreactive B cells normally deleted during TR selection [Hondowicz et al. 2007; Lesley et al. 2004; Thien et al. 2004]. Consistent with this idea, artificially increasing BLyS levels leads to systemic autoimmunity in mouse models [Groom et al. 2002; Hondowicz et al. 2007; Khare et al. 2000; Mackay et al. 1999] and elevated BLyS levels are correlated with the incidence and severity of SLE, RA, and SJS [Cheema et al. 2001; Mariette et al. 2003; Stohl et al. 2003; Zhang et al. 2001]. Together, these observations have led to the idea that BLyS depletion therapies restore the stringency of TR selection by interfering with BLyS-BR3 interactions, thus decreasing the likelihood that autoreactive B cell specificities continue to enter the mature peripheral pools. While correcting TR selection likely plays a role in the efficacy of BLyS blockade, it is unlikely the sole mechanism through which BLyS depletion impacts disease.

There is a growing appreciation that BLyS and its receptors also influence activated and antigen-experienced pools. In contrast to preimmune B cells that require BLyS signaling through BR3, activated B cells alter their BLyS receptor profiles. For example, B cells stimulated with TLR ligands (discussed below) dramatically upregulate TACI expression [Groom et al. 2007; Trembl et al. 2007], and long-lived plasma cells express BCMA. These changes in receptor expression establish novel survival requisites based on their interacting ligands, thus defining distinct survival niches for activated B cells and antibody-secreting effectors [Crowley et al. 2005; Trembl et al. 2006]. This idea, combined with evidence that humoral autoreactivity often arises from antigen-driven B cells, highlights the importance of understanding how BLyS and its receptors are involved during B cell activation per se, as well as how dysregulated interplay between various B cell stimuli and BLyS receptor signaling might potentiate autoimmunity.

Different modes of B cell activation pose distinct peripheral tolerance risks

Two general pathways of B cell activation – congruent with the major modes of stimulation during humoral responses to conventional antigens – have been associated with autoimmunity. During thymus-dependent (TD) activation, T cells supply co-stimulatory signals leading to germinal center (GC) formation, somatic hypermutation, affinity maturation, Ig class switching, and the eventual generation of long-lived plasma cells (LLPCs) [Gatto and Brink 2010]. Thus a major challenge to self-tolerance in the GC response is the elimination of newly emerging self-reactive clones, to prevent their entrance into long-lived effector pools.

In contrast, thymus-independent-type 1 (TI-1) activation does not require T cell help, and instead involves signaling through the TLR family of receptors. Typically, strict TI-1 responses do not form GCs but yield a wave of short-lived antibody secreting effectors with minimal class switching. The risks for autoimmunity posed by TI-1 driven activation therefore differ, inasmuch as these responses exhibit negligible somatic hypermutation or affinity maturation, and tend to form only short-lived PCs. As a result, instead of regulating insipient autoreactive clones, the primary risk is allowing activated autoreactive cells derived from the FO or MZ pools to persist and evolve as effectors. These distinct features – based on mode of activation – present fundamentally different challenges to avoiding pathogenic autoreactivity.

REGULATION OF THE GC RESPONSE AND THE GENERATION OF LONG-LIVED PLASMA CELLS

Controlling autoreactive specificities arising within GCs and preventing their differentiation to LLPC are crucial to preventing autoimmunity in the context of TD activation. Indeed, failed regulation of the GC reaction is correlated with the emergence of B cell autoimmunity [Shlomchik et al. 1987; Zaheen and Martin 2011], and pathogenic antibodies are typically class-switched to IgG. Because GC B cells alter their BCRs through somatic hypermutation, TD forms of activation pose similar risks to those of VDJ recombination [Gatto and Brink 2010], as these newly modified specificities may prove self-reactive. Therefore, forms of specificity-based selection, analogous to those acting during TR B cell selection, presumably also act during the GC reaction. Although Fas/FasL-mediated cell death has been implicated in this process, the mechanistic details whereby self and non-self reactive GC cells are distinguished remain obscure [Hao et al. 2008; van Eijk et al. 2001]. Considering its role in B cell survival and TR selection, BLyS likely also regulates the selection of newly mutated autoreactive B cells within the GC. Indeed, BLyS transgenic mice have increased numbers of GCs, whereas loss of BLyS/BR3 leads to aberrant GC formation [Mackay et al. 1999; Rahman et al. 2003]. This strongly indicates a role for BLyS in GC maintenance and evolution. Interestingly, TACI knockout mice have an increased number of GCs, suggesting that TACI plays a role in limiting the GC reaction [Yan et al. 2001b]. The exact relationship between BLyS family ligands and receptors and normal GC regulation are yet undetermined, as neither the expression levels of BLyS receptors on GC B cells, nor the availability of BLyS within the GC are known. Exploration of these factors should provide insight into their relative roles in GC negative and positive selection, and how these roles might be related to autoimmune etiology.

The LLPC generated during GC reactions migrate to the BM, where they can reside and produce antibodies for years [Shapiro-Shelef and Calame 2005; Slifka et al. 1998]. These cells are of particular concern in systemic autoimmune disease [Hiepe et al. 2011], inasmuch as they no longer express many of the surface receptors targeted by current B ablation therapies. Moreover, BCMA expression allows LLPCs to utilize either BLyS or APRIL for

survival [Belnoue et al. 2008; O'Connor et al. 2004]. Consequently, it will be important to consider how the BCMA survival pathway contributes to sustaining ongoing autoimmunity, and ways in which BCMA/APRIL production might be locally modified to ameliorate disease.

TI RESPONSES POSE UNIQUE CHALLENGES FOR PERIPHERAL B CELL TOLERANCE

Despite numerous checkpoints in primary B cell development, the mature B cell repertoire in healthy individuals includes low-avidity self-reactive B cells [Wardemann et al. 2003]. It follows that BCR engagement by autoantigen, though necessary, is insufficient to yield activation or disease, and that additional signals are required to initiate B cell expansion and differentiation. The two-signal hypothesis [Bretscher and Cohn 1970] implies a tertiary tolerance checkpoint in TD responses, whereby an autoreactive B cell that escapes tolerance must also receive help from an activated autoreactive T cell. However, under normal conditions, autoreactive T cell activity is tightly controlled, and naïve B cells are poor antigen presenting cells (APCs) for naïve T cells [Sprent and Kishimoto 2002]. Thus the initial event in the activation of autoreactive B cells is unlikely to be entirely T cell-dependent.

An alternative means of activating autoreactive B cells involves combined BCR and TLR signaling. TLRs were originally identified as a family of receptors that recognize conserved microbe-associated molecular patterns [Medzhitov et al. 1997]. Importantly, B cells express most of the known TLRs and their ligation augments BCR signaling, thereby promoting rapid T cell-independent activation [Booth et al. 2011]. In addition to surveillance for exogenous microbial incursions, TLRs can also bind endogenous ligands, especially in the context of sterile inflammation and/or injury [Rifkin et al. 2005]. Thus, both experimental and endogenous TLR9 ligands promote wound repair [Gregorio et al. 2010; Sato et al. 2010], and TLR-mediated detection of endogenous nucleic acids may facilitate autoreactive B cell deletion [Isnardi et al. 2008]. Considered in concert with the skewed antibody repertoires associated with humoral autoimmunity, these findings raise the prospect that certain combinations of BCR specificity, endogenous TLR ligands, and elevated BLyS could yield dire consequences.

Most autoantibodies in systemic autoimmune diseases recognize DNA, RNA, or parts of nucleic acid-associated macromolecules [Tan 1988]. Moreover, TLR3, 7, and 8 detect RNA, whereas TLR 9 detects DNA [Alexopoulou et al. 2001; Diebold et al. 2004; Heil et al. 2004; Hemmi et al. 2000]. These four TLRs are found within endolysosomes [Brinkmann et al. 2007; Kim et al. 2008], where they play a critical role in detecting viral infections. Nevertheless, they can also bind mammalian DNA- or RNA-associated complexes, so long as these gain access to the endolysosomal compartment [Green and Marshak-Rothstein 2011]. Since BCR-bound antigens are transported to the endosome even in the absence of BCR cross-linking, low affinity autoreactive B cells might efficiently deliver autoantigen to endosomal TLRs, yielding sustained activation. This BCR/TLR co-engagement paradigm was first demonstrated *in vitro* using BCR transgenic B cells expressing an IgG2a-specific rheumatoid factor (RF) [Wolfowicz et al. 1989]. These cells proliferated robustly to immune complexes (ICs) in which IgG2a-bound antigens contained mammalian DNA or RNA, and the responses were TLR9- and TLR7-dependent, respectively [Leadbetter et al. 2002; Lau et al. 2005; Uccellini et al. 2008]. *In vivo* models of autoimmunity have further strengthened these connections. For example, autoimmune-prone mice that express twice the normal level of TLR7 develop more severe autoimmunity [Pisitkun et al. 2006; Subramanian et al. 2006]. Conversely, inactivation of the nucleic acid-sensing TLR results in dramatically reduced autoantibody titers, remarkably attenuated clinical disease, and improved survival

[Brinkmann et al. 2007; Herlands et al. 2008; Kawai et al. 1999]. In addition, TLR9-deficient autoimmune prone mice do not make anti-chromatin autoantibodies and TLR7-deficient autoimmune prone mice do not make antibodies against RNA-associated autoantigens [Christensen et al. 2005; Christensen et al. 2006; Nickerson et al. 2010]. Finally, situations associated with increased endogenous TLR ligand levels can hasten or exacerbate disease, further supporting a role for BCR-mediated trafficking of endogenous TLR ligands in humoral autoimmunity. Thus, either higher rates of lymphocyte apoptosis or decreased ability of macrophages to clear apoptotic debris are features of SLE [Amanna et al. 2003; Emlen et al. 1994]. Moreover, nucleosomes are displayed on apoptotic blebs and can serve as the endogenous TLR ligands [Radic et al. 2004], and mice that fail to clear apoptotic cells develop anti-nuclear antibodies (ANAs) [Rodriguez-Manzanet et al. 2010].

INTERPLAY BETWEEN TLR-DRIVEN SYSTEMS AND BLYS-MEDIATED SURVIVAL

Mounting evidence linking BCR/TLR co-engagement, endogenous TLR ligands, and BLYS availability with autoimmunity suggests that cross-talk between this receptor triumvirate may normally prevent autoimmunity arising from activated self-reactive specificities in the primary repertoire. It is tempting to speculate that such mechanisms serve as a “fourth checkpoint” to quench innate B cell responses that, while absolutely essential to clear microbial infections or endogenous apoptotic debris, must cease before isotype switching, somatic hypermutation, or differentiation into long-lived memory and effector pools might occur. In this scenario, responses resulting from BCR/TLR co-engagement are intrinsically programmed for brevity, and events promoting their persistence raise the likelihood of secondary events leading to autoimmunity.

Multiple observations suggest TLRs and BLYS receptors may engage in cross talk to regulate these types of responses. For example, BLYS overexpression engenders B cell autoimmunity via T-independent, but TLR-dependent, mechanisms [Groom et al. 2007]. Thus, excess BLYS might inappropriately prolong responses initiated by BCR-TLR co-engagement, affording sustained autoantibody production or fortuitous interactions leading to long-lived effectors. Consistent with this idea, chromatin immune complex stimulation of autoreactive B cells in normal mice is generally of limited duration [Mevorach et al. 1998], but will eventually yield frank disease on autoimmune-prone backgrounds [William et al. 2006]. Similarly, activated T cells may migrate to extrafollicular compartments formed following combined BCR/TLR9 stimulation, driving the further expansion, mutation, isotype switching, and differentiation among persistent autoreactive plasmablasts [Odegard et al. 2008; Sweet et al. 2011].

Previously unappreciated cross-talk between these three systems might also underlie seemingly intractable phenomena. For example, in contrast to TLR7-deficient mice, TLR9-deficient autoimmune-prone mice, despite lacking DNA and chromatin-reactive autoantibodies, develop more severe clinical disease than their TLR9-sufficient counterparts. This phenomenon, verified in numerous autoimmune models, still lacks explanation.

CONCLUSIONS

The roles for BLYS family members in systemic humoral autoimmunity are manifold, and likely impact both the counterselection of autoreactive specificities, as well as the persistence of autoreactive B cells following their activation. Increased BLYS decreases the stringency of TR negative selection, thereby increasing the chance that autoreactive clonotypes will enter the naïve B cell pool. Additionally, excess BLYS influences GC reactions, likely contributing to the selection against autoreactive B cells that arise through

somatic hypermutation. Finally, BLyS may function beyond clonotypic selection *per se*, by regulating the persistence of B cells activated via combined BCR and TLR co-engagement. Thus increased BLyS availability might extend the otherwise short lifespan of B cells activated in this fashion, thereby providing sufficient time for secondary events leading to persistent pathogenic autoantibody secretion. Accordingly, an understanding of the interplay between the BCR, TLRs, and the BLyS family requires further investigation, and could facilitate novel targeted therapies for humoral autoimmunity.

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