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Review

Sites and Stages of Autoreactive B Cell Activation and Regulation

Mark J. Shlomchik[1,*]
[1]Department of Laboratory Medicine and Department of Immunobiology, Yale University School of Medicine, New Haven, CT 06520, USA
*Correspondence: mark.shlomchik@yale.edu
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B cells are essential for the development and pathogenesis of both systemic and organ-specific autoimmune diseases. Autoreactive B cells are typically thought of as sources of autoantibody, but their most important pathogenetic roles may be to present autoantigens to T cells and to secrete proinflammatory cytokines. A rate-limiting step in the genesis of autoimmunity then is the activation of autoreactive B cells. Here, mechanisms are discussed that normally prevent such activation and how they break down during disease. Integrating classic work with recent insights, emphasis is placed on efforts to pinpoint the precursor cells for autoantibody-secreting cells and the unique stimuli and pathways by which they are activated.

Introduction

B cells are critical for promoting autoimmunity in a spectrum of diseases, ranging from classical B cell-mediated autoimmune diseases such as systemic lupus erythematosus (SLE) (Chan et al., 1999b; Leandro et al., 2002; Sfikakis et al., 2005; Shlomchik et al., 1994) to rheumatoid arthritis (RA) (Edwards et al., 2004) to diseases that until recently were thought to have no contribution by B cells, including diabetes (Hu et al., 2007; Serreze et al., 1996) and multiple sclerosis (Antel and Bar-Or, 2006). B cells facilitate autoimmunity not just by secreting autoantibodies, but also by presenting autoantigens to T cells and most likely by secreting proinflammatory cytokines (Chan and Shlomchik, 1998; Chan et al., 1999a, 1999b; Harris et al., 2000; Wong et al., 2004). As a consequence, targeting B cells has become one of the most effective treatments of autoimmune disorders to emerge in recent years, with the promise to modify disease course (Sfikakis et al., 2005). This raises interest in understanding how autoreactive B cells develop, how they are normally controlled in health, and how they are activated in disease. A variety of new insights have provided a clearer picture of these complex processes, although there remain a number of unanswered questions about regulation, the origins of autoreactive B cells, and how they promote disease once activated in the course of autoimmunity.

Self-Tolerance Mechanisms

B cell receptors are assembled via a stochastic process of joining V, (D), and J region segments in developing B cell precursors. Thus, the resulting receptor comprised of randomly selected H and L chains has an unpredictable specificity that could include ability to bind "self." Indeed, it has been suggested that on the order of 50% of B cell receptors (BCRs) carry unacceptably high degrees of autoreactivity (Merrell et al., 2006; Nemazee, 1995; Novobrantseva et al., 2005; Souroujon et al., 1988; Wardemann et al., 2003). The concept of self-reactivity is complicated by the fact that there is no discrete affinity cutoff, nor even a catalog of relevant "self" molecules and epitopes. Rather, self-reactivity can be defined either via in vitro binding assays with arbitrary definition of "positive" or in vivo by whether a B cell carrying a putative self-reactive receptor is affected by interaction with the self-target. In vivo assessment is more physiologically relevant but harder to accomplish.

Initial studies suggested that the immune system dealt with self-reactive B cells through either clonal deletion (abortion) or inactivation (anergy) (Nossal and Pike, 1978, 1980). Subsequent experiments have generally relied on BCR transgenic (Tg) or site-directed transgenic mice, in which a preformed receptor or receptor chain is introduced into the germline. In such mice all, or a fraction of, developing B cells express the self-reactive BCR and, depending on the system design, the effects of autoantigen recognition can be studied (Goodnow, 1992). A summary of the phenotypes observed in some of these systems is given in Table 1. Classical studies of Nemazee and of Goodnow confirmed that self-recognition could lead to clonal deletion and clonal anergy, respectively (Goodnow et al., 1988; Nemazee and Burki, 1989). It was later recognized that deletion occurs when the cell has exhausted possibilities of further V gene rearrangement (Gay et al., 1993; Tiegs et al., 1993). This continuing rearrangement process is termed receptor editing. After these initial systems, a variety of analogous self-reactive BCR Tg and site-directed transgenic models have been generated, oftentimes via self-antigens of greater disease relevance (see below). These have contributed to our understanding of regulation of self-reactive cells and particularly to how this breaks down in disease states.

The parameters that govern whether a cell becomes anergic, undergoes receptor editing, or is deleted are not fully worked out; however, avidity plays a role, with stronger signals promoting the editing-deletion pathway and weaker signals permitting anergic cell development (Fulcher et al., 1996; Hartley et al., 1991; Hippen et al., 2005). B cells expressing receptors that are weakly crosslinked by self will emerge from the bone marrow (BM) and appear in the periphery (provided that their ligand remains unavailable) as normal non-self-reactive B cells (Aplin et al., 2003; Hannum et al., 1996; Liu et al., 2007a; Nemazee et al., 1991; Shlomchik et al., 1993). This has been termed "clonal ignorance."

Although anergy is typically discussed as a discrete phenomenon, there are degrees of self-reactivity and hence degrees of "anergy" that may partially limit the responsiveness of a given

Table 1. Mechanisms of Self-Tolerance Illustrated by BCR Transgenic Mice

Phenotype	Example	Description	References
Deletion	anti-MHCII Tg, anti-RBC, high-affinity anti-IgG (RF) Tg (20.8.3)	lack of cells beyond the pre-B cell stage in BM and in peripheral lymphoid tissue	Murakami et al., 1992; Nemazee and Burki, 1989; Wang and Shlomchik, 1998
Receptor editing	anti-MHCII Tg, 3H9/Vκ4 anti-dsDNA Tg, 3H9 or 56R site-directed Tg, HEL site-directed Tg with mHEL	cells express an endogenous V_L rather than the Tg-encoded one, or cells express a limited repertoire of L chains that veto autoreactivity	Gay et al., 1993; Hippen et al., 2005; Li et al., 2001; Tiegs et al., 1993
Allelic inclusion	anti-MHC II site-directed Tg, 3H9 56R	cells coexpress an endogenous V_L with the Tg-encoded one, or cells express two L chains, including one that vetoes autoreactivity	Li et al., 2002b; Liu et al., 2005
Anergy	anti-HEL Tg with sHEL, anti-DNA (3H9/Vλ$_1$), anti-Sm Tg (2-12), ArsA1	cells have variably reduced sIgM expression, shortened life span, and localize to the T-B interface; variably impaired response to LPS and reduced response to BCR ligation	Erikson et al., 1991; Goodnow et al., 1988; Merrell et al., 2006; Santulli-Marotto et al., 1998
Clonal ignorance	anti-IgG (RF) AM14 Tg, anti-La	cells assume a normal follicular B cell phenotype	Aplin et al., 2003; Hannum et al., 1996; Shlomchik et al., 1993

cell according to the degree to which its receptor is constitutively engaged (Liu et al., 2007a; Merrell et al., 2006) and possibly the degree of B cell activating factor of the TNF family (BAFF)-mediated signals (Thien et al., 2004). A defining feature of anergy is the reduced or absent ability to stimulate B cells via their Ag receptor (Cooke et al., 1994; Goodnow et al., 1988). There may be other defects that vary by system, including downregulation of sIgM, resistance to LPS-mediated activation, shortened half-life in vivo, and expression of an "immature-like" phenotype (Erikson et al., 1991; Fulcher and Basten, 1994; Goodnow et al., 1988; Liu et al., 2007a; Mandik-Nayak et al., 2000; Qian et al., 2001; Roark et al., 1997; Santulli-Marotto et al., 1998). These individual BCR Tg mice may reflect the spectrum of anergic B cells found among a polyclonal repertoire in normal mice. This notion has recently been supported elegantly in studies that recognized parallels between anergic cells with a BCR that crossreacts with a nominal hapten, azophenylarsonate, and ssDNA, and a subpopulation of B cells in normal mice termed "An1" (Merrell et al., 2006).

In addition to anergic and ignorant cells, the products of receptor editing can also appear in the periphery (Casellas et al., 2001; Gay et al., 1993; Li et al., 2002b; Prak and Weigert, 1995; Tiegs et al., 1993). In some cases, editing will destroy the autoreactive receptor, for example via a secondary rearrangement that replaces an in-frame Vκ-Jκ join with another via an upstream Vκ and a downstream Jκ. However, in some cases the second rearrangement could come on the other kappa chromosome or at any one of the lambda loci, in which case a cell with two productive rearrangements will result (Li et al., 2001, 2002b; Liu et al., 2005). If the second receptor sufficiently dilutes the autoreactivity of the initial HL pair, the cell will develop, as either an anergic or an ignorant cell. L chains that can negate the autoreactivity of pre-existing H chains are termed "editors." Cells carrying dual receptors may have unique properties and it has been speculated that they could be important precursors for autoantibodies generated during autoimmune disease (Li et al., 2001; Liu et al., 2005). Thus, a variety of mechanisms ranging from elimination to functional inactivation of autoreactive B cells serves to shape the developing B cell repertoire.

Phenotypes and Locations of Autoreactive B Cells in the Periphery

Although B cells that survive BM self-tolerance mechanisms do populate secondary lymphoid tissue, their phenotype, location, and function can depend on the nature of their BCR. At least some types of anergic B cells are found at the T cell-B cell border, consistent with their partially activated phenotype. Interestingly, this location allows anergic B cells to interact with Ag-specific T cells (Cook et al., 1998). The fate of dual-receptor cells may depend on the residual degree of self-reactivity. One type of edited anti-DNA B cell differentiates into a marginal zone (MZ) phenotype (Li et al., 2002b); this is likely due to residual self-reactivity, consistent with data indicating that many MZ B cells are positively selected by receptor crosslinking (Martin and Kearney, 2002). Indeed, some autoreactive B cells with specificity for the Smith (Sm) antigenic complex of ribonucleoproteins involved in mRNA splicing are positively selected into the MZ and the related B-1 pool of B cells (Qian et al., 2001). Finally, ignorant B cells are found in follicles and possibly in the MZ (Wang and Shlomchik, 1999).

What Is the State of Tolerance of Autoreactive Precursor B Cells?

A major challenge has been to determine how the tolerance mechanisms that shape the B cell repertoire break down during disease. The precursor-product relationships that lead to autoreactive B cells in autoimmunity are only partially elucidated. Natural leakiness or genetically mediated failures in receptor editing and/or anergy induction could lead to increased frequencies of partially tolerized or nontolerized B cells in peripheral lymphoid tissue. These could be precursors for activated autoreactive B cells in systemic autoimmunity. Ignorant cells with latent autoreactivity could also be precursors for such cells.

Regardless of the origins of autoreactive cells, they must be stimulated to divide and differentiate in order to generate

Table 2. Comparison of Self-Reactive B Cell Behavior in Normal and Autoimmune-Prone Genetic Backgrounds

BCR Tg Model	Normal Phenotype	Autoimmunity Model; Phenotype	References
Anti-HEL, sHEL	anergy	MRL.Fas^{lpr}; anergy	Rathmell and Goodnow, 1994
Anti-MHCI	deletion, receptor editing	MRL.Fas^{lpr}; deletion, receptor editing	Rubio et al., 1996
Anti-ss and dsDNA (3H9 Tg and 3H9 56R site-directed Tg)	anergy	MRL.Fas^{lpr}; follicular entry and anti-DNA production	Mandik-Nayak et al., 1999
	receptor editing	MRL.Fas^{lpr} and chronic GVHD; escape of partially edited cells and altered editing	Chen et al., 2006; Li et al., 2002a; Sekiguchi et al., 2002
Anti-IgG (RF)	clonal ignorance	MRL.Fas^{lpr} and B6/lpr; extrafollicular B cell AFC response	Wang and Shlomchik, 1999; William et al., 2002, 2005a
Anti-Sm	developmental arrest and anergy	MRL.Fas^{lpr}; anti-Sm secretion	Santulli-Marotto et al., 2001

autoantibodies. Clues to the nature of this process derive from autoantibody-secreting clones recovered from diseased mice and humans. These often contain somatic mutations in their V regions and have switched isotype (Marion et al., 1989; Randen et al., 1993; Shlomchik et al., 1987a, 1987b, 1990; Winkler et al., 1992). In many cases, these mutations are distributed nonrandomly, consistent with selection by autoantigen. Some of these mutations increase affinity for self-antigens, particularly in the case of anti-DNA, in which mutations to Arg and Asn often result in higher affinity for DNA (Radic and Weigert, 1994). Finally, autoantibodies in diseased animals frequently belong to large clones that presumably result from chronic autoantigen-driven proliferation (Marion et al., 1989; Shan et al., 1994; Shlomchik et al., 1987a, 1987b, 1990). Together, these findings suggest that autoreactive B cells are generated from responses to naturally derived self-antigens that are either identical to or contain the same epitopes as nominal self-antigens upon which the in vitro detection assays (such as for DNA binding) were based.

Thus, there is reasonable understanding of how autoreactive B cells are normally regulated as well as the nature of autoantibody-secreting cells found in disease. The major remaining questions relate to how regulation fails in disease. What are the autoantigens that break tolerance? At what microanantomical location(s) does the response occur? What are the precursor cells and do the requirements for stimulation and/or the outcomes depend on the nature of the precursor cells? What other cell types are required to promote and/or regulate the initial activation and differentiation of autoreactive B cells? What cytokines and surface costimulatory receptors are critical for the development of autoantibody-secreting cells?

Crossing BCR Tg Tolerance Models to Autoimmune-Prone Strains

A primary approach to these questions has been to cross the Ig-Tg systems that helped define B cell tolerance to strains of mice genetically predisposed to develop systemic autoimmunity. The concept behind these experiments is to observe autoreactive B cell recruitment into the autoimmune response from the starting point of a cell with a defined state of self-tolerance, as determined by the study of such cells on "normal" genetic backgrounds. Table 2 is a summary of several of the systems that were crossed onto autoimmune-prone backgrounds and a comparison of the phenotype in wild-type versus autoimmune-prone strains.

Initial studies used the HEL plus anti-sHEL and the anti-MHCI models (Rathmell and Goodnow, 1994; Rubio et al., 1996). It was important to study the Tg B cells in both the presence and absence of the autoantigen, which was possible in both of these models. Because autoimmunity is accompanied by apparently nonspecific B cell activation (Klinman and Steinberg, 1987), this comparison allowed specific activation to be distinguished. However, in both cases, there was little if any evidence that self-tolerance was broken in the murine lupus model strain MRL.Fas^{lpr}. Both clonal anergy and clonal deletion/editing seemed to be intact, and there was little recruitment of these self-reactive B cells into the antibody-secreting pool.

It is interesting to consider why these models did not reveal loss of tolerance, and hence were not particularly useful in elucidating this aspect of autoimmunity. One possible explanation is that they were not specific for the autoantigens typically targeted in systemic autoimmunity. Indeed, it is remarkable that, despite the polygenic and variable nature of lupus in humans and mice, three categories of autoantigens are immunodominant: DNA-associated, RNA-associated, and IgG (von Muhlen and Tan, 1995; Witte et al., 2000). Recognizing this point, Weigert and colleagues (Erikson et al., 1991; Gay et al., 1993; Shlomchik et al., 1993), along with a number of other groups, subsequently generated Tg and site-directed transgenic BCRs specific for disease-related autoantigens, such as DNA, IgG (the RF specificity), and Sm (Pewzner-Jung et al., 1998; Santulli-Marotto et al., 1998). Like the HEL and MHCI-specific models, in some cases B cells specific for disease-related autoantigens demonstrated altered development on normal genetic backgrounds, including anergy, receptor editing, and deletion. The spectrum and phenotype of anergy was variable, including developmental arrest and degrees of resistance to LPS activation and exclusion from follicular entry. To some extent, this seemed related to affinity for self. At the other end of the spectrum, IgG-specific RF B cells developed into normal follicular B cells and hence were considered clonally ignorant on a BALB/c genetic background (Hannum et al., 1996).

In contrast to the HEL and MHCI systems, B cells carrying Tg and site-directed transgenic BCRs specific for disease-related autoantigens, such as DNA, Sm, and IgG (the RF specificity), were in some cases dysregulated in the context of autoimmune-prone genetic backgrounds (Hoyer et al., 2004; Li et al., 2002a; Mandik-Nayak et al., 1999; Roark et al., 1995; Santulli-Marotto et al., 2001; Wang and Shlomchik, 1999; William et al.,

2002, 2005a). This was ascertained by the appearance of autoantibodies in the serum and antibody-forming cells (AFCs) in spleen. Thus, although BCR Tg mice specific for arbitrary antigens remained tolerant on lupus-prone genetic backgrounds, BCR Tg mice with a variety of disease-related specificities demonstrated activation of these cells specifically in lupus-prone mice. That autoantibody-secreting cells are generated when these models are crossed onto lupus-prone genetic backgrounds highlights two questions: what are the precursor cells that initially are activated, and how has self-tolerance been broken?

Are Central Tolerance Checkpoints Overcome in Systemic Autoimmunity?

Genetic or acquired defects could lead to failure of receptor editing or anergy induction. In some lupus-prone models, gross defects in anergy induction have not been seen, in that cell populations found anergic in normal mice still retain similar phenotypes in autoimmune-prone mice (Rathmell and Goodnow, 1994). Yet, subtle defects have been observed (Mandik-Nayak et al., 2000). In normal mice, expression of a Vh region that confers anti-DNA specificity with most L chains leads to extensive receptor editing with resultant rescue only of cells that express a narrow spectrum of V_κ regions that veto DNA binding (Li et al., 2001; Radic et al., 1993). In MRL.Fas^{lpr} mice and also in C57Bl/6 mice (which harbor some genes that enable autoimmunity), there is a broader spectrum of L chains found either in single sorted cells or hybridomas compared to those seen in normal BALB/c mice (Chen et al., 2006; Li et al., 2002a; Sekiguchi et al., 2006). These L chains are more permissive for DNA binding. These studies raise the possibility of primary defects in the stringency of self-tolerance in mice genetically predisposed to SLE.

There are two limitations to this interpretation. First, it is not clear whether the emergence of partially edited cells is a primary or a secondary defect. Evidence suggests that preexisting autoantibodies—which block exposure of developing B cells to autoantigens that, if not impeded by the autoantibodies, would promote tolerance—could lead to secondary defects in central tolerance (Fulcher et al., 1996; Wang and Shlomchik, 1998). Inflammation can also influence early B cell development (Nagaoka et al., 2000; Ueda et al., 2004), and this in turn could promote survival of autoreactive B cells. Second, the maturation of partially edited B cells does not establish that these cells are the precursors of autoantibody-secreting B cells, although this is possible. In one model, many of the autoantibody-secreting B cells no longer used the Vh site-directed transgenic, thus obscuring the relationship (Li et al., 2002a). In a Vh site-directed transgenic with very high affinity for DNA, spontaneous hybridomas were recovered that used the Vh along with permissive L chains; however, in these mice, there were very few B cells and a very limited repertoire (Chen et al., 2006).

In the case of lupus-associated activation of anergic B cells specific for DNA, it is easier to establish a precursor-product relationship. In 3H9 H Tg mice, B cells carrying a λ_1 L chain react with dsDNA. Such B cells generate serum autoantibody and splenic AFCs on the MRL.Fas^{lpr} background (Mandik-Nayak et al., 1999). However, DNA-reactive B cells in MRL.Fas^{lpr} mice are no longer developmentally arrested or anergic, nor are they excluded from follicles. Thus, the immune and genetic defects in this background seem to short-circuit the self-tolerance mechanisms themselves. Hence, the DNA-reactive B cells that are recruited to become AFCs may derive from these B cells that are no longer anergic rather than directly from anergic or developmentally arrested cells. Nonetheless, both in vitro (Noorchashm et al., 1999) and in vivo (Seo et al., 2002) experiments demonstrate that if anergic anti-DNA B cells are presented with T helper-related signals, they will proliferate and differentiate, arguing that, particularly in the context of strong T cell help, anergic B cells can be directly drawn into autoimmune responses.

These results beg the question of the genetic basis for central tolerance defects. Recently, insights have come from studying particular central tolerance checkpoints in the context of genetic loci that were isolated from a lupus-prone strain, NZM2410. The genes and allelic variants that comprise these loci are being isolated while at the same time the phenotypes they confer are being studied. Mice carrying the Sle1^z allele have altered isoform expression of Ly108 that is correlated with impaired induction of anergy, possibly because of effects on BCR signaling (Kumar et al., 2006). A very interesting recent finding in the NZM2410 model is that mice carrying the disease-associated *Sle2*^{z} allele have reduced receptor editing and altered differentiation of autoreactive DNA-specific B cells, associated with increased serum anti-DNA titers (Liu et al., 2007b).

Studies in humans have addressed this question by interrogating the B cell repertoire via single-cell sorting. In a series of impressive studies, such cells were sorted from various stages of B cell development, from immature through to memory cell, and from normal and diseased (lupus and RA) subjects (Samuels et al., 2005; Tiller et al., 2007; Wardemann et al., 2003; Yurasov et al., 2005). The H and L chains were cloned and re-expressed to reconstitute mAbs corresponding to the originally sorted cell. Extensive panels assembled in this way were tested in a series of binding assays that served as surrogates for either cell-constituent-, nuclear-, or poly-reactivity. In normal individuals, it was found that such self-reactivity was progressively filtered out of the B cell repertoire with cell maturation. However, this was not the case in most lupus and RA patients, who incompletely purged cells at both critical stages. In some cases, young lupus patients were studied both prior to and after initial treatment (Yurasov et al., 2005), eliminating the confounding effect of treatment. The finding that, at least in some effectively treated patients, the defects persisted suggested that these defects were primary rather than secondary to the disease. Overall, although multiple lines of evidence in mice and humans support defects in central tolerance in lupus and other autoimmune syndromes, more work is needed to determine which of these is a primary versus a secondary defect and what the genetic basis is for these defects.

Clonally Ignorant B Cells Generate Autoantibodies

Many studies have noted that the normal preimmune repertoire includes B cells that react with self-constituents (McHeyzer-Williams and Nossal, 1988; Souroujon et al., 1988; Wardemann et al., 2003). Although the tolerance status of these self-reactive cells has not always been clear, at least some of them can respond to LPS. Self-reactivity of these cells has been determined by in vitro assays, but such studies were not able to establish

that these B cells could be triggered in vivo by such antigens during autoimmune disease.

The AM14 BCR Tg mouse model, with B cells specific for IgG2a (the RF specificity), has helped to elucidate role of clonally ignorant cells. AM14 B cells can only recognize IgG2a of the "a" allotype. By crossing the BCR Tg onto Ig allotype congenic mice of either "a" or "b" allotype (IgH^a or IgH^b), we created animals in which a disease-related autoreactive B cell either could or could not recognize a self-Ag. These B cells are not tolerized in a normal BALB/c mouse (IgH^a) and instead develop into phenotypically and functionally typical follicular-type B cells that are indistinguishable in the BALB/c and IgH^b allotype congenic CB.17 strains (Hannum et al., 1996; Shlomchik et al., 1993).

However, when the AM14 H or H+L Tg were crossed onto the autoimmune-prone B6.Fas^{lpr} or MRL.Fas^{lpr} backgrounds, mice developed large numbers of RF-secreting cells in the spleen and elevated RF titers in serum (Wang and Shlomchik, 1999; William et al., 2002, 2005b). Importantly, this response occurred only on the IgH^a autoimmune-prone strains and not the IgH^b ones, formally establishing that the response was driven by autoantigen. Thus, clonally ignorant cells do have the capacity to be stimulated in vivo, despite the fact that the self-Ag signal is not normally strong enough to enforce tolerance. The onset of this spontaneous autoreactive B cell response was stochastic, with an increasing fraction of mice with high degrees of RF B cell activation with age (William et al., 2005a). This pattern of onset indicated specific triggers—whether environmental, developmental, or relating to other events in the body such as Ag availability—that initiate the response were operating, opening the possibility of identifying them by means of this system. In any case, these results establish directly that clonally ignorant cells can be the precursors for disease-related autoantibodies.

Contributions of Extrafollicular and Germinal Center Pathways to Autoreactive B Cell Responses

Studies of autoreactive B cell activation in lupus-prone animals raised several questions. First, could the stage and site of tolerance breakdown be isolated? Second, what are the triggers for this breakdown and why was it observed more readily in the DNA-, RNA-, and IgG-specific (i.e., disease-related) models than in the synthetic HEL- and MCHI-specific models?

Several themes have emerged from work in all of these systems. The first is that in some contexts, autoreactive responses bypass the GC reaction. Seminal work with non-Tg systems had already identified the T cell zone as an area of B cell activation and AFC localization for autoreactive B cells in MRL.Fas^{lpr} mice (Jacobson et al., 1995). In the RF system, we showed that the spontaneous response in the spleen does not involve the GC and instead proceeds at extrafollicular sites that are similar to the location of T-independent responses and the AFC phase of T-dependent responses (William et al., 2002). RF B cells were observed to proliferate and differentiate into plasmablasts at the border of the T zone and red pulp, in the marginal sinus-bridging channels of the spleen. Figure 1 diagrams the cellular interactions of the extrafollicular reaction, highlighting the cell-interaction molecules that might govern this process. The anti-DNA response in lupus-prone mice has not been as extensively studied at the histologic level, but available data indicate that it too does not proceed via the GC. Instead, DNA-specific (λ_1^+) B cells in 3H9 MRL.Fas^{lpr} mice are found in the outer T zone as well as the T zone-red pulp border (Mandik-Nayak et al., 1999; Seo et al., 2002).

The dominant role of the extrafollicular response for autoreactive specificities such as RF and anti-DNA is somewhat surprising, because the AFCs secreting these autoantibodies have many somatic mutations and have undergone affinity maturation, clonal expansion, and isotype switch. Classically, somatic mutation in particular has been thought restricted to the GC. By microdissection of small clusters of B cells at extrafollicular sites, we found that V region mutation does occur outside the GC in this response (William et al., 2002). Furthermore, histologic and FACS studies demonstrated two populations of B cells participating in the RF response, both of which were rapidly dividing. $CD22^{hi}CD44^{hi}$ cells were not secreting Ab, but appeared to be the precursors of $CD22^{lo}CD44^{bright}$ cells that did secrete Ab (William et al., 2005b). The latter cells had the appearance of plasmablasts and were also rapidly undergoing apoptosis, indicative of a short-lived compartment. Both $CD22^{hi}$ and $CD22^{lo}$ cells contained somatically mutated V regions.

There is evidence for extrafollicular responses and short-lived plasmablasts in other models of lupus. A majority of AFCs in spleens of NZB/W mice are short lived; these include anti-DNA AFCs (Hoyer et al., 2004). Similarly, the histologic appearance of the anti-DNA response generated by a parent → F1 "GVHD" manipulation that generates a lupus-like response is also consistent with an extrafollicular response (Sekiguchi et al., 2002). Mice overexpressing BAFF via a Tg have autoimmunity accompanied by large numbers of AFCs in splenic red pulp and mesenteric LN medullary cords (Mackay et al., 1999). Lupus patients, particularly when undergoing a flare, demonstrate elevated frequencies of plasmablast-like cells in PBL (Arce et al., 2001; Jacobi et al., 2003).

Despite the predominance of the extrafollicular reaction in anti-DNA and RF B responses in spleens of MRL.Fas^{lpr} mice, this does not exclude a role for GCs for other specificities and in other genetic contexts. GCs have been reported to be numerous in several autoimmune-prone strains of mice, though whether they contain autoreactive B cells and whether they are responsible for autoantibodies was not determined, nor was a complete survey of "normal" strains performed (Luzina et al., 2001). Disregulation of entry of autoreactive B cells into GCs has been observed in lupus patients as well (Cappione et al., 2005). In BAFF-Tg mice, plasmablasts are accompanied by GCs as well (Mackay et al., 1999); recent evidence suggests that the extra-GC pathway may be most important (Groom et al., 2007). Though the extrafollicular plasmablast pathway is emerging as a primary site and mechanisms by which disease-related autoantibodies are generated (Weisel et al., 2007), more needs to be known about the precise sites and pathways used to generate various specific autoantibodies in lupus and other autoimmune diseases.

What Are the Rules that Select and Direct Autoimmune Responses?

That there are at the least two pathways for the activation of autoreactive B cells in vivo, and that there are most likely at least two types of precursor B cell (anergic and ignorant), raises further questions: what controls the locus of activation, what

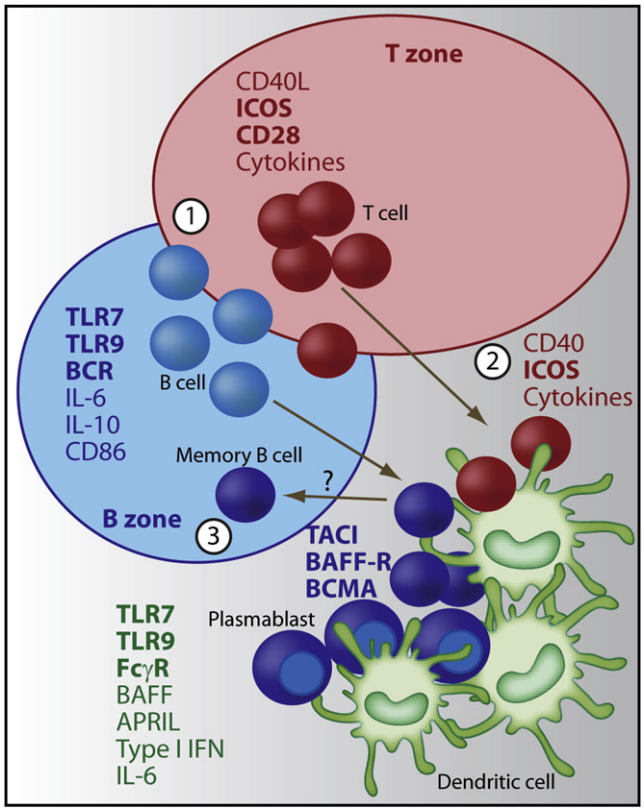

Figure 1. The Extrafollicular Pathway of Autoreactive B Cell Activation

Shown is a putative pathway by which autoreactive plasmablasts develop in the marginal sinus-bridging channels at the T zone-red pulp border. Most of the details of this process have not yet been worked out, so this scheme should be considered speculative. B cells are in blue, T cells in red, and DCs in green. Molecules expressed by each cell type that are likely to play key roles in activation and differentiation at each site are listed, with molecules that receive signals on the cell in bold. (1) Most likely the response initiates with antigen recognition and possibly T cell-B cell interaction at the T-B border, as occurs in a variety of B cell immune responses. Notably, $CD22^{+}$ activated RF B cells are seen at this site during the spontaneous RF response in autoimmune MRL.Fas^{lpr} mice. Activation requires BCR recognition of Ag as well as TLR7 or TLR9 signaling in the case of anti-RNA, anti-DNA, or RF B cells. T-B crossactivation via various costimulatory molecules may also take place. (2) Activated B cells presumably migrate to the T zone-red pulp border where they continue to proliferate and differentiate into plasmablasts (eccentric cells with nuclei shown). Some of these cells contact T cells and thus there may be continued T cell stimulation or regulation of the process. Others are in contact with DCs, which may provide rate-limiting help for plasmablast survival and also secrete factors such as BAFF and APRIL that promote plasmablast development and survival. (3) Whether memory B cells, which can recirculate into the B zone, are generated via this pathway is unknown, as suggested by the question mark.

are the differential roles of T cells and other B cell costimuli at the different sites, and what are the consequences of extrafollicular B cell activation—do memory cells form via this pathway? More proximally, are there different outcomes or activation requirements depending on whether the precursor cell is anergic or ignorant? To a large degree, the answers to these questions are not in, but it is worthwhile to integrate some recent studies and to propose some ideas about how the responses are regulated.

Roles of Toll-like Receptors, T Cells, and Other Stimuli in Accounting for the Specificities and Nature of Autoantibody Responses

Although MRL.Fas^{lpr} mice, particularly when aged, have few GCs in spleen (Luzina et al., 2001; Masuda and Kasajima, 1999), RF B cells in MRL.Fas^{lpr} or BALB/c mice can readily be recruited to GC responses by immunization with T-dependent antigens (William et al., 2002; Herlands et al., 2007). Thus, RF B cells are not inherently incapable of a GC response, nor are MRL.Fas^{lpr} mice inherently unable to support one. These results instead suggest that the stimulus itself influences the outcome. The T-dependent RF antigens were comprised of IgG2a complexed with foreign proteins such as KLH or CGG, which presumably recruited T help. In contrast, the physiologic antigen for the spontaneous RF B cell immune response in MRL.Fas^{lpr} mice has been unclear. In vitro experiments showed that RF B cells are potently stimulated to proliferate by naturally occurring immune complexes (ICs) in the serum of MRL.Fas^{lpr} mice. Moreover, IgG2a chromatin Abs, which complex with abundant chromatin found in culture supernatants (Rifkin et al., 2000), are potent stimuli. Because chromatin autoantibodies are abundant in MRL.Fas^{lpr} and many other lupus-prone mice and patients, we hypothesized that these may be an important or dominant trigger for in vivo RF B cell activation. Indeed, the stochastic onset of RF B cell activation in AM14 Tg MRL.Fas^{lpr} mice correlated with the presence of increased concentration of IgG2a chromatin Abs in the same mouse (William et al., 2005a). Recently, we tested this in vivo by infusing IgG2a chromatin mAbs in AM14 Tg mice. Indeed, this resulted in a robust extrafollicular response but no RF GC response (Herlands et al., 2007).

These data have implications for both how RF responses are triggered and also the control and initiation of the extrafollicular pathway. One connection is with Toll-like receptor costimulation. In vitro stimulation of RF B cells with chromatin-containing ICs absolutely requires MyD88 signaling (Leadbetter et al., 2002). Subsequently, it was shown that TLR9 and/or TLR7 were transducing this signal in the B cell, along with signals via the BCR (Lau et al., 2005; Viglianti et al., 2003). Notably, although first identified for RFs, this same paradigm should apply to activation of anti-DNA B cells, and evidence in vitro supports this (Viglianti et al., 2003). Similarly, RNA-specific B cells are activated by their autoantigen in a TLR7-dependent fashion in vitro (Lau et al., 2005). These in vitro studies highlighted specific TLRs with potential specificity for lupus-related self-antigens as major determinants of the characteristic profile of lupus autoantibodies, thus addressing a long-standing mystery of why certain autoantigens are preferred targets in SLE.

The idea that TLR signals are important for activation of classical lupus autoantibody responses has been tested directly in vivo by crossing TLR7, TLR9, and MyD88 null alleles onto autoimmune-prone backgrounds (Christensen et al., 2005, 2006; Lau et al., 2005; Sadanaga et al., 2007). Indeed, anti-chromatin responses depended on TLR9 (Christensen et al., 2005, 2006; Ehlers et al., 2006), and anti-Sm responses were found to be TLR7 dependent (Christensen et al., 2006). A spontaneous mutation that duplicated TLR7 onto the Y chromosome also promoted anti-RNA and disease when crossed onto a permissive genetic background (Pisitkun et al., 2006; Subramanian et al., 2006). On a mixed genetic background that included MRL genes,

anti-nuclear Abs were absent in the absence of MyD88, as were anti-Sm (Lau et al., 2005). The dependence of ANA on MyD88 was recently confirmed on a more fully backcrossed MRL.Fas^{lpr} background (Sadanaga et al., 2007; our unpublished data).

There are many cell types, including DCs, that can both respond to TLR signals delivered via chromatin-containing ICs (Boule et al., 2004) and influence autoantibody generation (see below). Thus, it will be important to determine the B cell-intrinsic versus -extrinsic requirements for TLR gene expression. In any case, the fact that anti-chromatin Abs stimulate extrafollicular responses by AM14 RF B cells in vivo and that AM14 B cell stimulation in vitro requires MyD88 suggests that TLR-dependent signals could direct extrafollicular responses in vivo. This bears further in vivo investigation in part because the in vitro cultures resulted only in proliferation and not differentiation.

Conversely, the reliance on TLR signals, along with the fact that T cell-independent responses occur at extrafollicular sites (MacLennan et al., 2003), raises the question of how T cells influence this type of autoantibody response. There is reason to think T cells should be critical: the importance of bidirectional T-B interactions in lupus and other autoimmune diseases is well documented (Chan et al., 1999b); B cells undergo mutation, which is thought to depend on T cells; and T cells can be seen in contact with activated and dividing RF B cells in AM14 MRL.Fas^{lpr} mice (Wang and Shlomchik, 1999; William et al., 2002). However, there are reasons to question the role of T cells: there is no obvious T cell epitope on IgG2a anti-chromatin Abs, yet even in BALB/c mice, chromatin Abs elicit a robust extrafollicular RF response within 7 days, suggesting that an Ag-specific T cell response may be dispensable (Herlands et al., 2007); TLR signals can induce activation-induced cytidine deaminase directly in B cells, leading to isotype switch and possibly somatic hypermutation (Jegerlehner et al., 2007), which would substitute for signals previously thought to require T cells; and many RF B cells are not in contact with T cells in the extrafollicular reaction (William et al., 2002, 2005b).

In the case of clonally ignorant B cells, the extrafollicular response could proceed independently of T cells, as long as TLR and BCR signals are provided by the autoantigen. In the case of anergic B cells, T cell help may be more important. The responsiveness of anergic B cells to TLR signals such as LPS is controversial (Goodnow, 1992; Noorchashm et al., 1999); notably, anti-DNA anergic B cells do not respond well to LPS. Given this, and that anergic B cells do not respond to BCR stimuli, it is reasonable to suspect that the combination of BCR and TLR signals may not be sufficient to break anergic B cell tolerance. Indeed, anergic HEL-specific B cells display a split tolerance upon BCR and TLR ligation, proliferating but failing to produce AFCs (Rui et al., 2003). However, signals that simulate T cell help, in particular CD40 ligation and IL-4, do activate anergic B cells (Cooke et al., 1994). Indeed, if provided strong T cell help, anti-DNA B cells are induced to proliferate and differentiate along both the GC and the extrafollicular pathways (Seo et al., 2002). Similarly, anergic B cells can be activated in vivo by strong T cell help when the constitutive low-level tolerizing BCR signal is replaced with a more immunogenic form of Ag (Cooke et al., 1994; Goodnow et al., 1991).

In addition to T cell and TLR signals, there are other factors and cell types that influence the generation of autoantibodies. These include: BAFF and a proliferation-inducing ligand (APRIL) (Schneider, 2005); type I IFN, which has direct and indirect impacts on B cells (Coro et al., 2006; Le Bon et al., 2001); and DCs, which capture ICs and secrete cytokines including BAFF, APRIL, and IFN-I (Boule et al., 2004; Schneider, 2005). It is highly likely that both T cells and TLR signals contribute to and direct autoantibody responses; however, more work is needed to understand how these and other signals precisely direct the onset and nature of autoreactive B cell responses.

Implications for Understanding Disease Pathogenesis and Designing Therapy

Figure 2 depicts the contrasting stimulatory requirements, molecular and cellular interactions, and outcomes of the extrafollicular versus the GC pathway of stimulating autoreactive B cells. Different signals and cell types may be required at each site. For example, conventional DCs are prominent at extrafollicular sites whereas stromal follicular dendritic cells are exclusively found in the GC. Interventions that affect key molecules like BAFF or IFN-I could have differential effects on the two types of response. Similarly, if it is true that strong TLR signals promote the extrafollicular response, then TLR inhibitors may selectively affect this pathway. It has been suggested that because somatic mutations could create de novo self-reactivity, self-tolerance mechanisms should exist in the GC, and there is some evidence for this (Han et al., 1995; Hande et al., 1998; Pulendran et al., 1995); we have speculated that ICs trapped in the GC contribute to tolerance rather than activation (Haberman and Shlomchik, 2003). We have also suggested that B cell activation outside of GCs may not be subject to self-tolerance mechanisms that would filter out high-affinity self-reactive cells generated in GCs (William et al., 2002).

Although it seems likely that the GC pathway could generate autoreactive memory cells, whether autoreactive B cells stimulated via the extrafollicular pathway can generate memory is unknown. Memory cells could serve as a reservoir of autoreactive cells that are easier to reactivate (Tangye et al., 2003), which in turn could contribute to chronicity and refractoriness to therapy (Jacobi et al., 2003; Manz et al., 2006).

Insights about the nature of the extrafollicular response can also help to explain results of clinical trials of B cell depletion with anti-CD20 in RA and SLE. Certain autoantibodies, notably anti-DNA and RF, decayed rapidly after treatment (Cambridge et al., 2003; Sfikakis et al., 2005), although AFCs lack CD20 and are not directly depleted (Ahuja et al., 2007; Jacobi et al., 2003). Therefore, these autoantibodies were being generated from rapidly renewing CD20^{+} precursors, most consistent with the short-lived plasmablast pathway. On the other hand, anti-Sm Abs were generally found to decay slowly, if at all, more consistent with their generation via the GC pathway (Cambridge et al., 2006). This is one illustration of how the concepts and information derived from murine studies can help in understanding therapeutic approaches and studies.

Conclusion

There are many facets to B cell tolerance and how this breaks down in autoimmune diseases. Central and peripheral mechanisms like deletion, receptor editing, and anergy all shape the responsive B cell repertoire. Moreover, it is clear that some

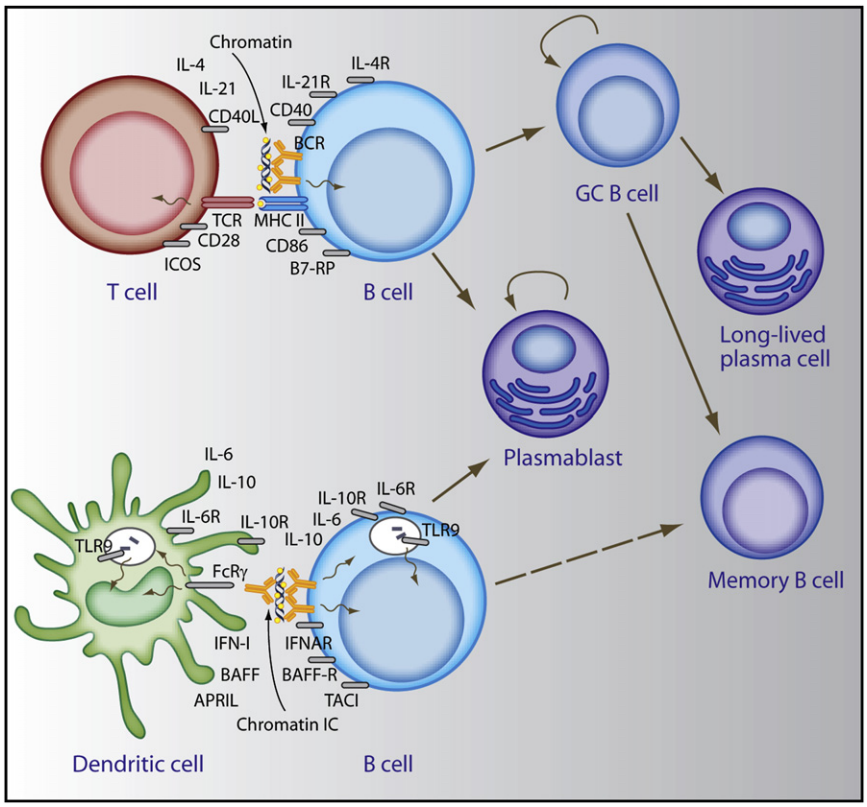

Figure 2. Different Outcomes of Activating Anti-Chromatin B Cells via T Cell or DC Interactions

This figure emphasizes some of the different molecular interactions that are likely to occur at the T-B versus DC-B cell interface as well as the divergent pathways of differentiation that ensue. When anti-chromatin B cells recognize chromatin either with (top) or without (bottom) T cell help, they receive a BCR signal and a TLR signal (shown in the bottom panel only for simplicity; signals denoted by arrows within the cell). B cells will also take up and process the protein components of chromatin, such as histones, into peptides for display in the context of MHCII (shown top only). If T cells that recognize chromatin peptides are present, they will be activated and presumably a classical T-B interaction will ensue, involving bidirectional costimulation via CD40L-CD40, CD86-CD28, and B7-RP-ICOS; all of these have been implicated in the development of lupus autoantibodies in mice. Cytokines such as IL-4 and IL-21 may also be activated. This would be expected to lead to a GC response that generates memory and long-lived plasma cells. Although there is good evidence for both in lupus patients and for long-lived plasma cells in some lupus models, there has been little direct evidence for anti-chromatin B cells in GCs as yet. In model systems, when all anti-chromatin B cells could receive help from T cells that had been activated by Ag, a prominent plasmablast response ensued (Seo et al., 2002), as depicted via the arrow, suggesting that when T cell signals combine with TLR signals and/or when the target B cell is anergic, T help can promote the extrafollicular pathway. In addition, a wholly T cell-independent pathway is proposed that may depend on DC-B interactions, as inferred from work on model T-independent responses. Here the anti-chromatin B cells would be activated by BCR and TLR signals, potentially along with IFN-I, BAFF, and APRIL—all secreted by DCs that in turn were stimulated via activating FcRs by chromatin-containing ICs. There could be bidirectional cytokine-mediated interaction as well, for example via IL-6 and IL-10. The result would be development of plasmablasts that at some stage induce AID and undergo isotype switching as well as somatic hypermutation. Whether this pathway can also generate autoreactive memory B cells is uncertain, as indicated by the dashed arrow.

self-reactive B cells are not subject to self-tolerance, yet remain quiescent in normal individuals. Environmental and genetic factors in certain individuals can lead to the aberrant activation of some or all of these types of autoreactive B cells, and if this is not controlled, B cell-dependent autoimmune disease will ensue. This activation, which is likely influenced by T cells and controlled by TLRs, can take place at extrafollicular sites in the spleen, leading to robust generation of short-lived plasmablasts. Thus, the autoantibody response, though chronic in terms of the individual, is actually a dynamic and ongoing process. This dynamic response could explain why a number of autoimmune diseases are rapidly responsive to therapies that target B cells. Further elucidation of how autoreactive B cells are regulated and activated and why certain autoantigens uniquely direct the nature of the response will advance basic knowledge of the disregulated immune response as well as enhance design of therapies for diseases in patients suffering from SLE and related disorders.

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