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Molecular pattern recognition in peripheral B cell tolerance: lessons from age-associated B cells

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Although central tolerance mechanisms purge self-reactive B cells during development based on BCR signal strength, mechanisms that block the differentiation of autoreactive effector and memory B cells from mature pools remain poorly understood. Prior observations implicate nucleic acid sensing TLRs in autoimmunity, and more recent findings show that TLR9 is also involved in maintaining peripheral tolerance. Studies of the immunological changes that occur during aging revealed a subset of B cells denoted Age-associated B cells which expands in settings of aging and in autoimmunity. Further studies demonstrated that TLR9 signals poise activated B cells to adopt an Age-associated B cell phenotype, but BCR-delivered TLR9 signals cause programmed cell death that, if circumvented by costimulation, allows continued differentiation to the ABC fate. Together, these observations suggest molecular pattern recognition, rather than BCR epitope specificity *per se*, is a fundamental mediator of tolerogenic outcomes in the peripheral B cell activation.

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Introduction

Herein we consider implications for peripheral B cell tolerance that arise from studies of the Age-associated B cell (ABC) subset. A growing literature shows that ABCs, also known as Tbet-positive B cells, are antigen-experienced B cells that not only contribute to pathogen-specific humoral immunity and memory, but are also a prominent feature in autoimmune diseases. Although the exact basis for this connection remains unclear, the signals that prompt the ABC differentiative fate provide an ideal setting for a breach in peripheral tolerance, as they center on antigens complexed with natural nucleic acid adjuvants that can be derived from either microbial or self components. This, in conjunction with observations indicating that innate nucleic acid sensors like TLR9 inhibit autoimmunity, prompts reassessment of the tolerance mechanisms operating in mature B cells. Thus, we posit that in contrast to deletional checkpoints active during B cell development, the burden of maintaining tolerance in mature B cell subsets involves classifying activating antigens based on associated molecular patterns, rather than on BCR epitope specificity *per se*. Accordingly, we briefly review conventional notions of B cell negative selection and findings implicating nucleic acid sensing TLRs in autoimmunity and tolerance, followed by a discussion of ABCs and how the signals required for their formation places them at the nexus of peripheral tolerance to antigens that are complexed with nucleic acid ligands.

Prevailing notions of B cell tolerance rely on BCR epitope specificity

The clonal selection hypothesis accommodates acquired tolerance by positing clonally distributed antigen receptors, thereby allowing monospecific clones to be interrogated and either preserved or eliminated, based on their likelihood of breaching 'horror autotoxicus' [1–4]. The theory's proposed receptor clonality was established through single cell analyses [reviewed in Ref. [5]] or study of B lineage neoplasms [6,7], and subsequent observations showed that deletional mechanisms are driven by B cell antigen receptor (BCR) signals [8,9]. These processes purge frankly self-reactive BCRs during the final stages B cell maturation; more than 90% of immature bone marrow B cells generated die, and only about 1/3 of the remaining 10% survive the transitional B cell stages to join the mature follicular or marginal zone pools [10].

Although well established, deletional mechanisms based on BCR signal strength alone are unavoidably imperfect. First, because interactions between BCRs and their ligands are governed by reversible chemical bonds, autospecificity is necessarily a relative property dictated by receptor density and ligand concentrations, which may vary based on locale and circumstances. Second, the requirement for tonic BCR signaling in the selection and survival of mature B cells implies that cells in all mature preimmune pools

likely have some degree of intrinsic self-reactivity ([11–15] discussed in Ref. [16]). Consistent with these arguments, mature preimmune and antigen-experienced B cell pools include clonotypes with measurable autoreactive or polyreactive capacity (reviewed in Ref. [17]). These and other considerations have prompted 'second signal' models for circumventing the generation of autoreactive effector populations—usually acting during initial stages of antigen-driven B cell activation [18]. For example, requiring cognate T cell help for the survival and differentiation of activated B cells ensures that antigens internalized through the BCR yield peptide/MHC II epitopes that can engage extant TCR specificities, relying on thymic negative selection as a secondary gatekeeper. Nonetheless, all of these models remain closely allied with a *de facto* assumption that humoral autoimmunity reflects the escape of developing B cells from deletional mechanisms based on BCR epitope specificity, followed by fortuitous events enabling their activation and differentiation to effector function. However, recent findings question this belief, and suggest that mechanisms enforcing tolerance among mature and activated B cells may rely on antigen-associated molecular patterns, rather than on BCR epitope specificity *per se*.

Innate pattern recognition systems mediate both activation and tolerance in the B lineage

Over the last several decades, growing awareness of innate pattern recognition and danger sensing systems have yielded a framework within which adaptive immune responses are shaped by signals from innate receptors that recognize broad pathogen-related or danger-related molecular patterns [discussed in Refs. [19,20]. A key conceptual feature of this framework is that innate receptor systems parse stimuli based on characteristic molecular *categories*, rather than epitopes unique to a particular antigen. Among these, the Toll-like receptors (TLRs) play central roles in directing the outcome of adaptive immune responses.

Until recently, these innate pattern recognition sensors have been viewed primarily as a means to tailor effector activities of adaptive immune responses activated by microbial pathogens or commensals. However, an increasing body of literature shows that some, particularly endosomal nucleic-acid sensing TLRs, play key roles in autoreactive antibody responses as well. Foundational work showed that the endosomal nucleic acid sensors TLR7 or TLR9 are essential for maximal activation of rheumatoid factor expressing B cells stimulated by chromatin-containing immune complexes [21,22]. These and subsequent findings have forged a link between nucleic acid sensing TLRs and the long-recognized fact that many autoantibodies bind components of RNA-containing and DNA-containing complexes, and that inefficient clearance of apoptotic debris is a major source of such antigens, even in the absence of infection [23]. Critical roles for nucleic acid sensing TLRs and their downstream signaling machinery in promoting humoral autoimmunity are now widely confirmed both in mouse models and in human disease (reviewed in Refs. [24,25]). For example, as would be predicted for molecular drivers of autoimmunity, TLR7 or MyD88 deficiencies ameliorate disease, whereas TLR7 duplications worsen disease or increase risk [26].

However, despite general acceptance that nucleic acid sensing TLRs are positive drivers of autoimmunity, recent studies have yielded an opposing finding; TLR9 deficiency accelerates disease onset and exacerbates disease severity in all mouse models of humoral autoimmunity tested [27••]. Moreover, deficiencies in TLR9 signaling components yield increased frequencies of polyreactive B cells surviving transitional differentiation [28,29], and signaling deficiencies in TLR9, but not TLR7, are observed in B cells from SLE patients [30,31]. Interestingly – and consistent with a dominant role for DNA sensors in modulating humoral immunity – earlier studies had shown that antibodies to histone proteins can be generated when the histones are conjugated to RNA, but immunogenicity is lost when further complexed with DNA [32,33].

Together, these observations indicate that TLR9 can also *sustain* B cell tolerance, a conclusion that has provocative and potentially fundamental implications. First, in contrast to the epitope-centric deletion mechanisms active during B cell development, sensors that instead classify BCR-engaged and internalized antigens by molecular category may be key mediators of tolerance among mature B cells. Second, understanding the collective signaling networks that parse these categories to dictate effector generation versus tolerance may prove key to understanding the etiology of humoral autoimmune disease. The recent discovery of an effector memory B cell subset whose generation is regulated by endosomal TLR signals and that is associated with humoral autoimmunity provides support for these ideas, as well as a framework for their study.

ABCs arise in both microbe-specific and autoimmune humoral responses

A splenic B cell subset that enlarges continuously with age, correspondingly dubbed 'age-associated B cells' (ABCs), was reported by companion papers in 2011 [34•,35•]. Subsequent studies have confirmed and extended these observations, revealing that the ABC pool continues to enlarge in mice 24 months of age and beyond, eventually comprising as much as half of all splenic B cells [34•,36]. Distinguishing phenotypic criteria for ABCs include low to nil expression of CD23 and CD21, expression of the transcription factor Tbet, and expression of CD11c. Although heterogeneity exists among cells meeting these criteria (discussed in Ref. [37]), several characteristic functional features are shared. ABCs are unresponsive to BCR ligation alone, but

proliferate briskly to either TLR7 or TLR9 stimulation [34•,35•]. They are well represented in the spleen, variably found in the blood, but largely absent from lymphatics and most lymph nodes [34•].

In both mice and humans, there is strong evidence that most ABCs are antigen-experienced B cells. For example, ABCs can be generated from follicular B cells through both *in vitro* stimulation and *in vivo* adoptive transfer [34•,38]. In addition, their BCRs are somatically mutated and they are poorly generated or absent in CD154-deficient and CD40-deficient mice [38], suggesting they are the result of prior activation and likely have a germinal center origin. Recent publications from multiple groups have shown directly that Tbet+ ABCs emerge and comprise a portion of antigen-specific memory B cells following a variety of viral, bacterial, and parasitic infections. These include influenza, Mouse Hepatitis Virus, Lymphocytic Choriomeningitis Virus, *Erlichia muris*, and *plasmodium* infections in mice, as well as influenza, Human Immunodeficiency Virus, Hepatitis C virus, and malaria infection in humans (see Ref. [39] for collected reviews).

In addition to their roles in microbe-specific antibody responses, ABCs are associated with autoimmunity. This association was noted by Rubtsov *et al.*; the numbers of cells with an ABC phenotype expanded inordinately early in mouse models of humoral autoimmunity and in several human autoimmune diseases [35•]. Notably, earlier studies had also identified an atypical and unresponsive memory B cell subset closely resembling ABCs in immunodeficient and autoimmune patients [40,41], and in HIV infected individuals [42]. In accordance with these initial observations, accumulating reports reveal that cells with the Tbet+ ABC phenotype are significantly elevated in both human autoimmune disease and mouse models of autoimmunity [43,44••,45•]. Moreover, the degree of ABC elevation has been correlated with clinical disease severity, and ABCs from SLE patients are enriched for lupus-associated autoantibody specificities [44••].

In toto, these observations suggest commonality in the cellular and molecular origins of both pathogen-specific and autoreactive ABCs. Moreover, they imply that ABC generation likely includes a tolerogenic versus effector differentiation checkpoint, that when breached allows autoreactive ABC pools to be established. This notion is supported by studies that show the signaling requirements for ABC generation are the same as those involved in fostering programmed cell death in activated B cells.

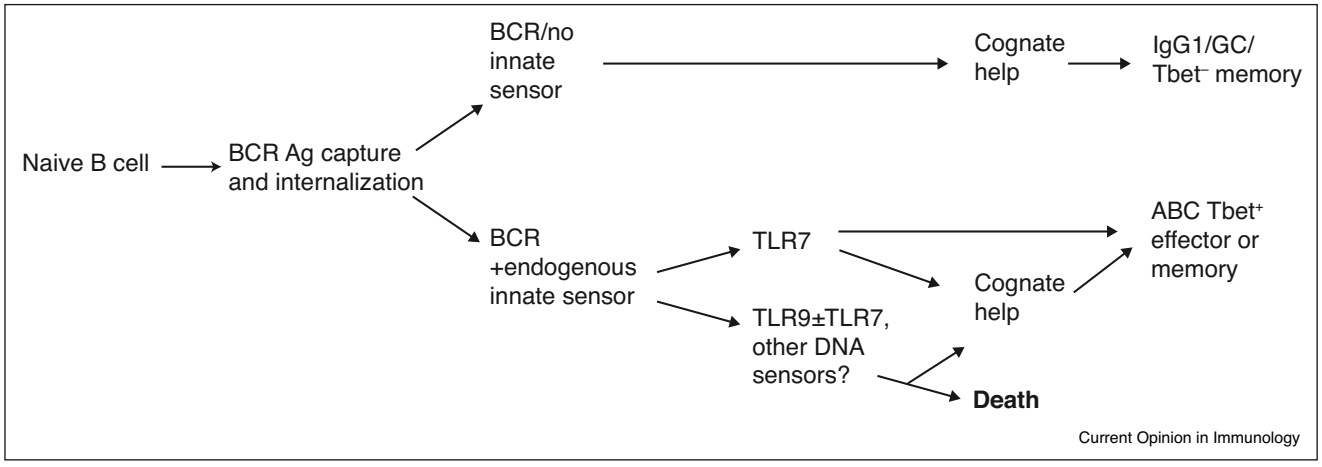
TLR9 signals poise activated B cells for ABC fate but initiate a cell death program

Early studies connected the appearance of ABCs with an inflammatory cytokine milieu, particularly IFNγ. More recently, detailed analyses of the signals driving ABC fate were reported by Naradikian *et al.* [46••]. A key finding in these studies was that ABC fate choice is independent of BCR and CD40 signals, but instead relies on B cell intrinsic TLR7 or TLR9 engagement. Importantly, while these endosomal TLR signals are not sufficient to drive cells to the Tbet+ ABC phenotype, they are necessary to poise B cells for this fate upon subsequent exposure to either IFNγ or IL-21.

The requisite for endosomal TLR signaling and association with both normal and autoimmune humoral responses suggested possible correlates between ABCs and the prior observations implicating TLR9 and TLR7 in both immune activation and tolerance. The nature of these relationships was explored in reports from Nündel *et al.* and Sindhava *et al.* [47••,48••]. Both of these studies reveal a B cell-intrinsic role for TLR9 in terminating incipient B cell responses that, when skirted by additional signals, leads to survival and the ABC differentiative fate. A central finding in both studies was that when CpG DNA-containing molecular complexes are internalized via the BCR, the activated B cells proliferate briefly but then undergo cell cycle arrest and mitochondrial death within 60 hours. Significantly, this programmed death response relies upon TLR9 ligands complexed and internalized with the antigen bound by the BCR, rather than on BCR specificity *per se*. In addition, internalization and trafficking by the BCR is critical, since simultaneous but separate BCR and TLR ligation do not yield this programmed death response. In the Nündel *et al.* studies, DNA-containing immune complexes were internalized via an IgG2a-specific BCR, whereas Sindhava *et al.* were able to generalize this response to all follicular, transitional, and marginal zone B cells by using an anti-BCR reagent complexed with DNA. Importantly, B cells can be rescued from this programmed cell death response either by the survival cytokine BAFF or by CD40 ligation. Moreover, consistent with the notion that once B cells receive TLR9 signals they are positioned to assume ABC fate if subsequent activation steps proceed, cells rescued in this manner adopt a Tbet+ ABC phenotype in the presence of IFN-γ or IL-21.

In aggregate, these observations prompt us to propose a stepwise decision model whereby activated B cells are triaged based on molecular category, rather than BCR epitope specificity or signal strength. A model depicting this decision tree is shown in Figure 1. Antigens engaged by the BCR are internalized and assessed by innate sensors. Antigens lacking associated nucleic acid moieties bypass further interrogation, whereas those that include endosomal TLR ligands are parsed further. TLR7 signals alone foster activation and a direct inflammatory response engendered by poising cells to a Tbet+ ABC effector fate. In contrast, antigen-associated DNA yields TLR9 signals that initiate a dominant default non-inflammatory response, unless circumvented by pro-survival signals or costimulation via cognate T cell help.

Figure 1



Step-wise decision tree for B cell responses governed by molecular patterns. Antigen captured by the B cell receptor is internalized and engages with innate molecular pattern sensors. If no innate sensors are engaged, but cognate T cell help is provided, a typical B cell response develops that is characterized by isotype-switching, germinal centers, and the formation of a Tbet^{-} memory pool. The outcome of detecting nucleic acid molecular patterns in the internalized antigen depends on the innate sensor that is engaged. If TLR7 alone is activated, the cell is primed to become an effector or memory Tbet^{+} ABC. If TLR9 is engaged, with or without TLR7, the cell is also primed to adopt an effector or memory Tbet^{+} ABC fate, but the default pathway is cell cycle arrest and apoptosis unless circumvented by costimulation.

Conclusion

The connection of innate pattern recognition sensors such as TLR9 in maintaining peripheral B cell tolerance is illustrated by ABCs; the signals that poise activated B cells for this fate also initiate a programmed death program that must be skirted to enable survival and ABC effector differentiation. However, many questions remain regarding the apparently unique role of DNA sensors, the intracellular processes that parse these signals, and the points in B cell activation and differentiation where they are active.

A lingering question is the apparent lack of tolerogenic pathways activated by TLR7 in B cells. Although formal theories have yet to be experimentally tested, observations about the endosomal trafficking of TLR7 and TLR9 by Unc93B1 provide grounds for speculation. Certain mutations in Unc93B1 cause preferential shuttling of TLR7 into signaling endosomes making cells hyperresponsive to TLR7 ligands, but hyporesponsive to TLR9 ligands [49]. Moreover, RNA is more labile than DNA and subject to nuclease digestion. Thus, TLR9 may act as a dominant negative regulator of TLR7 responses when DNA and RNA are both present.

Whether cytoplasmic DNA arising from endogenous sources can trigger the same tolerogenic cell death program in B cells is unknown. Moreover, the role of other DNA sensors in this process remains an open question. Cytoplasmic DNA resulting from genomic stress as well as mitochondrial DNA can be detected by cyclic guanosine monophosphate (GMP)–adenosine monophosphate (AMP) synthase (cGAS) to activate STING for the production of type-1 interferons and proinflammatory cytokines in innate cells for antiviral immunity [50]. Although there does not seem to be a need to limit a B cell response to endogenously derived DNA, perhaps cGAS recognition of exogenously acquired DNA also plays a tolerogenic role by distinguishing antigen class? Supporting this notion, STING-deficient MRL.Fas^{lpr} mice have accelerated disease onset and increased autoantibody formation and STING-agonists are cytotoxic in primary B cells [51,52].

These ideas also raise the question as to how long such pattern sensing tolerogenic mechanisms remain active during an unfolding immune response. In particular, whether this go/no go decision continues through the germinal center reaction is a crucial question, inasmuch as many autoantibodies acquire autoreactivity over the course of somatic hypermutation (reviewed in Ref. [53]). Indeed, TLR9 signals provided by BCR internalization and trafficking of antigen contributes to the germinal center response and improves antibody affinity to the antigens carrying this DNA cargo [54].

Conflict of interest statement

Nothing declared.

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