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Self-reactivity on a spectrum: a sliding scale of peripheral B cell tolerance

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Summary :

Efficient mechanisms of central tolerance, including receptor editing and deletion, prevent highly self-reactive B cell receptors (BCRs) from populating the periphery. Despite this, modest self-reactivity persists in (and may even be actively selected into) the mature B cell repertoire. In this review, we discuss new insights into mechanisms of peripheral B cell tolerance that restrain mature B cells from mounting inappropriate responses to endogenous antigens, and place recent work into historical context. In particular, we discuss new findings that have arisen from application of a novel in vivo reporter of BCR signalling, Nur77-eGFP, expression of which scales with the degree of self-reactivity in both monoclonal and polyclonal B cell repertoires. We discuss new and historical evidence that self-reactivity is not just tolerated, but actively selected into the peripheral repertoire. We review recent progress in understanding how dual expression of the IgM and IgD BCR isotypes on mature naïve follicular B cells tunes responsiveness to endogenous antigen recognition, and discuss how this may be integrated with other features of clonal anergy. Finally, we discuss how expression of Nur77 itself couples chronic antigen stimulation with B cell tolerance.

Keywords

anergy; Nur77/Nr4a1; IgM; IgD; tolerance; B cell

1) Introduction

Classic studies of BCR transgenic mice from Nemazee, Goodnow, Weigert and colleagues revealed that immature B cells possess the capacity to edit their light chains in order to reduce self-reactivity that is inadvertently acquired through random somatic VDJ recombination, while clones that fail to do so are eliminated by deletion[1–5]. These

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mechanisms are collectively termed ‘central tolerance’ (recently reviewed by David Nemazee[6]), and are thought to provide a critical safeguard against overt autoimmunity. By directly cloning human BCRs, Wardemann and colleagues subsequently showed that reactivity to DNA and poly-reactivity are indeed disturbingly common among immature human B cells, and that these specificities are efficiently pruned from the maturing B cell repertoire during BM development, presumably by a combination of editing and deletion [7]. Despite elimination of highly self-reactive B cell clones early during BM development, it has been argued that modest self-reactivity persists in (or is actively selected into) the mature B cell repertoire of normal mice and healthy humans. It is posited that layered mechanisms of peripheral B cell tolerance exist to restrain such B cells from mounting inappropriate responses to endogenous antigens. In this review, we discuss studies from our lab and others that have shed light on the positive and negative selection of the primary mature B cell repertoire. We discuss the teleological rationale for selecting and preserving modest self-reactivity in the primary pre-immune B cell repertoire. Finally, we review recent insights into the molecular basis of peripheral tolerance mechanisms that constrain self-reactive B cells, and position these mechanisms in the context of classic foundational work on this topic.

2) Self-reactivity in the mature B cell repertoire

Studies in humans and mice provide independent lines of evidence that some degree of self-reactivity is present in the mature, naturally occurring B cell repertoire. However, the nature, extent, and significance of this has been long-debated, in part because there has been no gold-standard definition of self-reactivity. Published studies can be broken down into those that provide either structural or functional evidence for self-reactivity (i.e. measurement of soluble Ab binding to candidate antigens, and assays of B cell responses to candidate antigens, respectively). This is a crucial distinction because the sensitivity of ELISA, immunofluorescence assay (IFA), or surface plasmon resonance (SPR) for detection of antigen-Ab binding does not faithfully capture biophysical features of interactions between membrane-associated BCRs on naïve B cells and bona fide self-antigens encountered in vivo. Membrane-associated and/or polymeric antigens can trigger BCR signaling with much greater potency than soluble monomeric or oligomeric forms and can compensate for low intrinsic binding affinity with extremely high avidity. Further, even low affinity antigen-BCR interactions, undetectable by ELISA, are biologically relevant and can be sufficient to drive normal humoral immune responses. Indeed, it has been argued that such low affinity interactions characterize most responses to foreign antigens, while only affinity-matured BCRs routinely exhibit detectable binding to monomeric antigen in vitro. Indeed, the HIV literature contains examples of germline-reverted broadly neutralizing antibodies (bNabs) that have no detectable antigen binding[8,9].

Wardemann et al. reported that BCRs cloned from mature follicular (Fo) human B cells, in contrast to immature BM B cells, exhibit little DNA-reactivity and poly-reactivity (defined as binding to more than one antigen among a panel including ssDNA, dsDNA, insulin, and LPS), but a significant fraction (20%) can bind cytoplasmic antigens (assessed by immunofluorescent staining of permeabilized cells using cloned Abs)[7]. Very recently, Kelsoe and colleagues revisited this question (reviewed elsewhere in this volume) in a more

high-throughput manner by establishing Nojima cultures of single human B cells which generate massive expansion and plasma cell differentiation, yielding high concentrations of monoclonal Abs for downstream assays[10]. They subsequently assessed reactivity of monoclonal IgG from these cultures against a large panel of 'self' and 'foreign' antigens using an illuminex bead-based assay. Over 1000 monoclonal Abs from 9 healthy donors were tested against about 25 candidate antigens. Both self-reactivity and poly-reactivity (defined as reactivity to both self and foreign Ag) were reduced by about 50% between transitional and mature B cell populations (and strikingly, as previously reported, this checkpoint is defective in SLE patients[11]). Just under 15% of mature B cells from healthy donors were reactive to at least one self-Ag in this panel, within the avidity limits of detection of this binding assay. Both conclusions are in reasonable agreement with prior work by Wardemann and colleagues despite the distinct methodologies employed[7].

There are, nevertheless, at least three major questions raised by these studies; first, is self-reactivity a feature of only a minor fraction (15–20% as noted above) of the mature repertoire, or is there instead a continuous distribution of self-reactivity, some of which falls below the limits of detection of in vitro binding assays described above? Second, is the degree of self-reactivity in the mature repertoire of functional significance – are these self-antigens actually encountered and 'sensed' by mature B cells in vivo? Third, what are the relevant self-antigens encountered by developing B cells in vivo, and does the normal mature B cell repertoire harbor reactivity to bona fide self-antigens beyond the spectrum of specificities probed in these studies? There is certainly some evidence for the latter possibility; the self-reactive human VH4–34 heavy chain, expressed by 5–10% of the normal mature B cell repertoire, has been reported to recognize Ii antigen on RBCs and B220 on the surface of B cells [12,13]. Given the vast range of potential self-antigens a B cell could recognize through its antigen receptor, how can such varied interactions be explored in an unbiased manner?

Recently, Jumaa and colleagues took a functional approach to assess self-reactivity of the mature B cell repertoire by probing antigen-induced calcium entry, rather than exclusively relying upon direct assays of antigen binding to soluble recombinant Abs [14]. Interestingly, direct comparison of ELISA, SPR, FACS-based detection of antigen binding by membrane BCRs on B cells, and cell-based calcium assay, suggests that the functional calcium assay is the most sensitive of these for certain model antigens. Other differences were noted as well; soluble Abs with detectable binding to dsDNA also trigger calcium entry when expressed as IgM isotype BCRs on B cells, but fail to do so when expressed as IgG1 BCRs. Notably, the authors observe significant differences in Ag binding to previously reported polyreactive BCRs when these were expressed as soluble Ab or as cell surface BCRs. Such profound divergence between in vitro binding assays and 'cell-based' functional assays highlight how critical functional readouts may be in order to define what constitutes biologically relevant self-reactivity in the primary B cell repertoire. However, neither approach overcomes a primary limitation of studying self-reactivity of a diverse repertoire, namely that the identity and nature of endogenous antigens is largely unknown.

Nur77-eGFP BAC Tg reporter of antigen receptor signaling

Previous work from our lab exploiting an in vivo reporter of antigen recognition, Nur77-eGFP BAC Tg, addresses this important point (Figure 1A)[15]. GFP expression in this reporter line is under the control of the regulatory region of *Nr4a1/*Nur77. *Nr4a1–3* encode a small family of orphan nuclear hormone receptors which were originally cloned as signal-dependent primary response genes (a.k.a. immediate-early genes), and are highly upregulated by a range of mitogens, including antigen receptor stimulation [16–18]. Consequently, antigen stimulation rapidly triggers reporter expression in B and T cells in vitro (Figure 1B), and GFP is also induced by infection or immunization in antigen-specific lymphocytes in vivo [15,19–21]. Most strikingly, we observed a broad distribution of reporter expression in mature naïve Fo B cells in the absence of exogenous immune stimuli, and went on to show that endogenous antigen is both *necessary and sufficient* for such expression under steady state conditions in vivo (Figure 1C)[15]. We did so by taking advantage of a BCR Tg that harbors extremely high reactivity towards a foreign antigen, hen egg lysozyme (HEL). Forced expression of the IgHEL BCR Tg in reporter mice in the absence of cognate HEL antigen eliminated most GFP expression in Fo B cells, implying that endogenous antigen recognition is necessary for GFP expression. Conversely, introducing cognate antigen (soluble HEL Tg) into this genetic background was sufficient to reconstitute high GFP expression. We further showed that reporter expression was sensitive to genetic modulation of BCR signal strength via titration of the receptor-like tyrosine phosphatase CD45. These observations led us to hypothesize that Nur77-eGFP served as a functional readout of endogenous antigen encounter in vivo and might therefore represent a bona fide maker of self-reactivity that was extremely sensitive (more so than proximal biochemical events such as calcium entry) but not subject to the limitations of in vitro binding assays (e.g. ELISA, IFA, SPR). Moreover, because the reporter operates in vivo, its expression ought to reflect B cell reactivity to native conformations (and concentrations) of bona fide endogenous antigens, and importantly does not rely upon identification of such antigens. In support of this hypothesis, we observed that reporter expression among mature Fo B cells was correlated with anti-nuclear reactivity and with downregulation of IgM (but not IgD) BCR expression, a well-recognized feature of self-reactive B cells (discussed later in this review; Figures 1D, E)[15,22,23]. We also identified functional evidence of chronic antigen encounter – basal calcium levels were elevated in GFP^{HI} B cells relative to GFP^{LO} B cells[15]. In subsequent work, we further excluded the contribution of other immunoreceptor pathways (especially those mediated by microbial stimuli) as well as commensal flora itself to Nur77 expression in B cells under steady-state conditions [24]. Although NF-κB-dependent mitogenic stimuli can drive reporter upregulation in vitro, in vivo B cell expression of Nur77-eGFP under steady state conditions is specifically regulated by antigen and BCR signaling, but not by other immunoreceptors (including CD40, MyD88-dependent TLRs, Unc93B1-dependent TLRs 3, 7 and 9, BAFF, CXCR4, and Jak-Stat-dependent cytokine receptors; Table 1). Therefore, we propose that Nur77-eGFP expression reflects endogenous antigen encounter and self-reactivity among mature Fo B cells.

To further validate the fidelity of Nur77-eGFP as a reporter of bona fide self-reactivity in vivo, we turned to an independent, polyclonal model of B cell self-reactivity. V_H3H9 is a heavy chain (H chain), originally cloned from the MRL/lpr mouse model of lupus, that

biases the entire BCR repertoire towards DNA-reactivity, most strongly when paired with endogenous λ1 light chain (L chain)[25]. This H chain has been knocked into the endogenous BCR locus to generate site-directed (sd) V_H3H9 Tg mice in which the majority of B cells express this H chain paired with endogenous L chains. This model system is extremely powerful because DNA-reactive B cells can be tracked within a polyclonal repertoire on the basis of surface λ1 L chain expression through development and beyond, and because multiple sequential tolerance mechanisms (receptor editing, deletion, anergy) are triggered and can be probed using this model[25,26]. We generated Nur77-eGFP reporter mice harboring the sd-V_H3H9 Tg, and found that dsDNA-reactive λ1+ B cells from these mice turn on GFP reporter expression very early in development, and express much higher levels in maturity than either WT or V_H3H9/κ B cells, consistent with chronic self-antigen stimulation[27]. Conversely, V_H3H9 Tg B cells expressing Vλ3 L chains (aka λx) that are known to censor DNA-reactivity express much lower levels of GFP, as do rare 'escapee' B cells that express endogenous H chains. Moreover, in this polyclonal model we found that reporter expression also correlated with the degree of functional anergy. Thus, reporter expression correlates with the extent of endogenous antigen recognition under steady state conditions across a broad dynamic range, extending from non-self-reactive 'naïve' IgHEL BCRs (in the absence of cognate antigen) across the normal B cell repertoire, all the way to some of the most highly self-reactive BCR Tgs studied in mice. Most importantly, observations with this reporter demonstrate that the bulk of the physiologic mature Fo B cell compartment in C57Bl/6 mice harbors modest 'self-reactivity' (as functionally defined by reporter expression) that spans a continuum. The Nur77-eGFP reporter therefore represents a unique and very useful tool to facilitate studies of the selection and tolerance of self-reactive B cells.

3) Positive selection of the mature B cell repertoire by antigen recognition

Although modest self-reactivity of mature Fo B cells may persist because it simply falls below the threshold required to trigger central tolerance, it has also been proposed that some degree of self-reactivity is actively selected into the mature compartment and that a minimal threshold of self-reactivity (rather than just tonic BCR signaling) is actually required for optimal B cell development. However, this has been a hotly debated topic among B cell biologists, and unlike thymic development where positive selection imposes MHC restriction, the teleological rationale for positive selection of B cells by antigen is less obvious. Perhaps the clearest and most elegant evidence for antigen-dependent positive selection of B cells comes from studies of the innate-like B1a cell compartment. B1a cells have a fetal origin, are maintained by self-renewal, and exhibit an extremely restricted and highly self-reactive BCR repertoire; more than a third of the repertoire comprises only a handful of BCRs with a shared CDR3 sequence that recognizes phosphatidylcholine, an epitope exposed on the surface of dying cells [28–30]. Forced expression of such BCRs via Tg models greatly expands the pool of B1a cells, and recent provocative work from Rajewsky and colleagues suggests this BCR specificity may even be sufficient to 'convert' B2 cells into "B1a-like" cells [29,31]. Hayakawa and colleagues showed formally that Thy-1 antigen expression is necessary for Thy-1-specific (anti-Thy-1 Ab or ATA) B cells to enter the B1a compartment [32,33]. By contrast, marginal zone (MZ) ATA B cell development is disfavored

both at high levels of antigen and in the complete absence of antigen, but proceeds optimally at low levels of antigen, providing striking evidence not only for a sharp upper threshold for negative selection, but also a minimal degree of self-reactivity required for positive selection into this compartment [34]. Dependence of B1a and MZ B cell development on strong and weak BCR signal strength respectively is likely due to such distinct thresholds for positive and negative selection [30,35]. By contrast to both B1a and MZ B cells, ATA Fo B cells developed even in Thy-1 deficient mice, and IgHEL Fo B cells similarly develop in the absence of cognate soluble HEL antigen, raising questions about whether endogenous antigen recognition is necessary for Fo B cell development, or whether tonic BCR signaling is sufficient [32–34]. Indeed, mixed evidence for and against antigen-dependent positive selection into the Fo compartment exists in the B cell literature [36–40]. Since competition appears to play a role in this process, one challenge has been tracking development of specific B cell clones within a polyclonal repertoire [41]. Another major limitation of many studies has been that the degree of cross-reactivity between BCR Tg cells and endogenous antigen (as opposed to cognate model antigen) is unknown. This has in turn made it hard to draw definitive conclusions about the minimal amount of self-reactivity and antigen-dependent signaling (as opposed to tonic BCR signaling) required for entry into mature B cell compartments, especially the Fo compartment.

Recently, we took a novel approach to address this question by capitalizing on the Nur77-eGFP reporter of endogenous antigen recognition[162]. B1–8i Tg mice express a fixed H chain, but a polyclonal set of endogenous L chains, approximately 5% of which are capable of binding the NP hapten[42]. We identified two minor B cell populations in B1–8i H chain Tg mice that each recognize the NP hapten, but differ in their L chain use and their degree of self-reactivity (as marked both by Nur77-eGFP expression and other indicators). We found that the population with less self-reactivity, NP^+ $Ig\lambda1^+$, displays profoundly impaired entry not only into the B1a compartment, but also exhibits counter-selection during both Fo and MZ B2 cell development in the spleen. By contrast, modest self-reactivity of NP^+ $Ig\lambda2^+$ B cells, while still counter-selected in the B1a compartment, was sufficient to drive efficient development of both MZ and Fo B cells. By titrating CD45 expression in order to genetically modulate BCR signal strength independently of antigen recognition, we established that counter-selection of NP^+ $Ig\lambda1^+$ B cells was due to impaired positive selection rather than negative selection. Moreover, as previously noted in the ATA mode, we found that the degree of self-reactivity required for positive selection into the B1a compartment drives negative selection in the B2 compartment (see model, Figure 2) [32,33]. Our data also suggested that the minimal BCR signaling threshold for entry into MZ is higher than that for entry into Fo, consistent with observations in the ATA model. Since NP^+ $Ig\lambda1^+$ B cells express high levels of a functional BCR on their surface (as demonstrated by their efficient participation in NP-dependent immune responses), these data collectively demonstrate that purely tonic BCR signaling is not sufficient for efficient entry into any mature B cell compartment. Rather, endogenous antigen recognition is required for optimal B cell development.

Where exactly does the antigen-dependent signaling threshold for optimal B2 cell development lie? To establish this, we compared the degree of Nur77-eGFP expression in these two NP-binding populations relative to the physiologic B cell repertoire, and also

relative to naïve IgHEL BCR Tg cells from hosts lacking cognate HEL Ag expression. We found that cross-reactivity of the IgHEL BCR to bona fide endogenous antigens – while quite low relative to the normal B cell repertoire (see Figure 1C) – actually lies in between the two NP-binding populations that we studied. Importantly, Nur77-eGFP seems to mark a cell-intrinsic degree of BCR self-reactivity; altering the constitution of the bulk BCR repertoire via mixed bone marrow chimeras did not change the GFP expression in these clonal populations. We speculate that ATA B cells exhibit a low degree of cross-reactivity to endogenous antigens (just above that of NP^+ $Ig\lambda1^+$ B cells) that is sufficient for Fo B cell development, but below that required for MZ B cell entry. Taken together, we can position these specificities relative to one another in terms of self-reactivity and can identify a minimal degree of self-reactivity required for efficient entry into all mature B cell compartments (Figure 2). These observations also raise provocative questions about how the extremely low degree of self-reactivity of NP-specific $Ig\lambda1^+$ B cells might influence their participation in typical immune responses to this model immunogen; could their behavior diverge from that of more typically self-reactive clones?

Importantly, qualitatively unique structural features of certain self-Ags (such as co-stimulatory capacity), in addition to the avidity of their interaction with BCR, may also regulate selection of specific B cell clones. For example, the profound restriction of the B1a repertoire and unique capacity of PtC-specific BCR Tgs to drive B1a fate suggests that antigen recognized by such BCRs delivers additional signals superimposed on ‘strong’ BCR stimulation[28,31]. Indeed, work from the Barton lab shows that expansion of PtC-specific BCRs in the B1a compartment is regulated by endosomal TLRs that recognize nucleic acids (Kreuk, L. UC Berkeley PhD thesis, 2018; Kreuk, L., B Cell Keystone conference, Dresden, 6/2018). Conversely, endosomal TLRs appear to play a role in mediating central tolerance towards DNA and RNA-reactive BCRs that deliver such TLR ligands to endosomal compartments[43].

The rationale for limiting self-reactivity in mature B cells is self-evident, but why should modest endogenous antigen recognition be actively selected during B cell development? Surely, tonic BCR signaling could ensure successful pairing of edited L chains with H chains to generate a functional BCR? It has been postulated that selection of mild self-reactivity biases the BCR repertoire towards recognition of essential biological patterns displayed on microbes [44,45]. Retention of some self-reactivity in the mature B cell repertoire may serve to limit ‘holes’ in the B cell repertoire generated by central tolerance mechanisms. Such holes could in turn be exploited by pathogens to evade immune recognition; in fact, deletion of self-reactive precursor BCRs is thought to pose a barrier to generation of broadly-neutralizing antibodies (bNAbs) against HIV for this reason [46,47]. Conversely, relaxing the stringency of negative selection and peripheral tolerance may facilitate evolution of HIV bNAbs [48]. Kelsoe and colleagues showed that many self-reactive human B cells are indeed cross-reactive to foreign antigens[10]. Importantly, they show that the frequency of such ‘poly-reactive’ clones declines with B cell maturation, providing direct evidence for generation of such ‘holes’ as a general consequence of tolerance checkpoints, rather than just a special case[10]. A related explanation is that self-reactivity ensures a mild degree of poly-reactivity among the pre-immune B cell repertoire, which may in turn serve to ensure that the primary B cell response to any given foreign antigen is

polyclonal and therefore diverse. Such diversity may facilitate both competition and an efficient search of ‘mutational space’ in the germinal center, thereby optimizing affinity maturation. In contrast to deletion, clonal anergy - which is a reversible state - may be the preferred tolerance mechanism by which mature B cells maintain quiescence in the face of self-antigen recognition in order to retain self-reactivity in the mature repertoire.

4) Clonal anergy in BCR Tg models and naturally occurring self-reactive B cells

BCR Tg mouse models

If (modest) self-reactivity is positively selected into the periphery, how are such B cells restrained from mounting immune responses to self-antigen? One of the first - and still perhaps the most well-studied - genetic models of self-reactive B cells, was developed by Goodnow and colleagues, and takes advantage of the model antigen hen egg lysosome (HEL). When a BCR Tg (IgHEL) specific for HEL is introduced onto a genetic background expressing soluble HEL antigen (sHEL Tg), B cells are not eliminated through deletional tolerance as they are in the presence of membrane-associated HEL (mHEL). Rather, they make it to the periphery, but, in contrast to a background lacking cognate antigen they do not secrete high levels of anti-HEL IgM at steady state or in response to HEL-SRBC immunization [49]. The functional silencing or unresponsiveness of these ‘self-reactive’ B cells is termed clonal anergy, a phenomenon that encompasses a range of independent non-deletional molecular mechanisms of peripheral B cell tolerance[50]. In fact, virtually every associated feature of clonal anergy was first described in this model, including attenuation of BCR signal transduction, selective IgM (but not IgD) BCR downregulation, exclusion from B cell follicles, and markedly reduced lifespan and competitive fitness in the periphery.

The most well-recognized biochemical change that characterizes ‘anergic’ B cells in the IgHEL/sHEL model is elevated basal calcium (presumably indicative of chronic antigen engagement), coupled with a marked impairment in BCR signal transduction. When stimulated with soluble HEL antigen in vitro, anergic IgHEL B cells display reduced calcium entry and impaired global protein tyrosine phosphorylation relative to naïve IgHEL B cells [51]. Because anergic IgHEL B cells exhibit profound downregulation of the surface IgM (but not IgD) BCR, some degree of impaired signaling could be explained by reduced surface receptor expression. However, these phenomena can be uncoupled; after adoptive transfer into non-HEL-expressing mice, formerly anergic IgHEL B cells regain normal levels of surface IgM BCR after 36 hours but still display impaired calcium mobilization in response to antigen stimulation [51]. Similarly, stimulation of anergic IgHEL B cells through IgD also produces impaired global protein phosphorylation and calcium entry despite high surface expression of IgD BCR. Importantly, all downstream signaling pathways are not equally affected; anergic IgHEL B cells exhibit phosphorylation of ERK in response to anti-IgD or HEL Ag, but such stimuli fail to drive JNK1 phosphorylation, NFκB pathway activation, or AKT activation [52,53]. It is thought that these biochemical perturbations in BCR signaling contribute to functional anergy. The molecular basis for some (but not all) of these changes has been dissected in more recent studies reviewed below.

Importantly, many features of anergy (including IgM downregulation and attenuated BCR signal transduction) are shared by several independent self-reactive BCR Tg models, and to varying degrees among naturally occurring 'anergic' B cell populations (reviewed by Cambier and colleagues) [22]. Nevertheless, the extent of anergy varies in proportion to the affinity and avidity of individual BCR clones for self-antigen, and the molecular mechanisms at play differ. Cambier, Wysocki, Manser and colleagues generated Ars/A1 mice harboring a heavy and light chain BCR Tg that recognizes the p-azophenylarsonate (Ars) hapten [54]. However, B cells in these mice do not respond to Ars-KLH immunization, downregulate IgM expression, and display attenuated BCR signaling, suggesting that Ars/A1 B cells cross-react with an endogenous antigen that induces a state of clonal anergy. The affinity of the Ars/A1 BCR for Ars hapten is 2.36×10^{-5} M, and treatment with 10^{-4} M monovalent Ars-conjugated tyrosine is sufficient to compete off endogenous antigen and abolish anergy in vivo, suggesting that affinity of this BCR for cross-reactive endogenous antigen is rather low [55]. Indeed, Ars/A1 B cells cross-react very weakly (2×10^{-3} M affinity) with ssDNA[54]. These measured affinities are many orders of magnitude lower than that of IgHEL for its cognate antigen (2×10^{-9} M) [49]. As such, common features of both models (IgM downregulation and attenuated BCR signaling) are likely to be 'universal' mechanisms of peripheral B cell tolerance operative across a broad spectrum of self-reactivity. However, by contrast to the IgHEL model which requires days after transfer to a host lacking HEL antigen to restore function, clonal anergy of Ars/A1 B cells is much more rapidly reversible; within seconds of treatment with monovalent Ars/Tyr, elevated basal calcium in Ars/A1 B cells falls, and some responsiveness towards anti-IgM is restored after just 20 minutes. This suggests a vastly different rate of antigen dissociation from BCR and likely also points to distinct molecular mechanisms at play in the two models [56].

PI3K/ PTEN axis

As alluded to above, after transfer to a HEL-deficient host, anergic IgHEL B cells upregulate IgM expression after 1–4 days, and still display reduced antibody responses even 10 days after transfer [57]. This suggests that some features of anergy are "hard-wired" in these cells. Indeed, anergic IgHEL B cells upregulate expression of the inositol phosphatase PTEN, which normally counteracts the activity of PI3-kinase (PI3K) to generate the second messenger $PI(3,4,5)P_3$ in the plasma membrane (Figure 3) [53]. $PI(3,4,5)P_3$ in turn recruits PH-domain containing proteins to promote signaling downstream of the BCR via Akt, Btk, and PLCγ2[58]. Rickert and colleagues found that deletion of PTEN in anergic IgHEL B cells completely restored antigen-dependent calcium entry, Akt signaling, competitive survival, steady state antibody secretion, and partially restored surface IgM expression[53]. Some of these changes may be secondary to sequestration of HEL Ag by anti-HEL Abs resulting in reduced receptor occupancy on IgHEL B cells, but it is likely that a subset are direct. Indeed, BCR-induced calcium entry is exquisitely dependent upon PI3K catalytic activity and $PI(3,4,5)P_3$ supply, suggesting that $PI(3,4,5)P_3$ is a limiting reagent in this pathway[59].

The PTEN/PI3K axis may be broadly relevant for maintaining tolerance; overexpression of CD19 (which couples BCR ligation to PI3K signaling) is sufficient to break tolerance, suggesting that the regulation of $PI(3,4,5)P_3$ levels may play an important role in toggling between tolerant and activated B cells[60,61]. Inducible deletion of PTEN is also sufficient to

break tolerance of Ars/A1 B cells, although unlike anergic IgHEL B cells, PTEN itself is not overexpressed in this more modestly self-reactive model [62]. These findings may be generalizable to non-Tg B cells; Cambier and colleagues recently showed that PTEN expression varied continuously across the normal human B cell repertoire and correlated with increasing anergy and decreasing IgM expression [63]. More recently, Shlomchik and colleagues identified PTEN overexpression in GC B cells, which serves to redirect AKT kinase activity towards negative regulators of proximal BCR signaling, thereby engaging a negative feedback loop [64]. It will be exciting to explore whether a similar biochemical mechanism might be exploited by anergic B cells to suppress antigen-dependent signal transduction. However, it remains to be determined how PTEN function and expression is regulated in naturally occurring self-reactive B cells. Further, PTEN predominantly operates downstream of the most proximal segment of the BCR signaling cascade, implying additional pathways must be engaged to suppress very early events such as Syk phosphorylation [51].

ITIM-dependent inhibitory signaling

ITIM-dependent inhibitory coreceptors suppress BCR signaling through recruitment of the effector phosphatases SHP-1 and SHIP-1[58]. SHP-1 is a tyrosine phosphatase that targets Syk and other proximal BCR signaling machinery. SHIP-1 is an inositol phosphatase that hydrolyzes $PI(3,4,5)P_3$ to generate $PI(3,4)P_2$, complementing the role of PTEN in suppressing PI3K-dependent signaling (Figure 3). Some inhibitory co-receptors are co-engaged with the BCR by specific autoantigens that serve as ligands for both (e.g. IgG and FcγRIIb; Sm/RNP Ags and CD72), but others like CD22 are thought to function in a more constitutive manner to regulate BCR signaling[65,66]. The Src family kinase Lyn serves as a molecular link to couple antigen receptor stimulation with engagement of ITIM-dependent inhibitory pathways; although other B cell SFKs are competent to mediate BCR signal transduction via ITAMs, Lyn plays a unique non-redundant role in mediating ITIM-dependent signaling[67]. Indeed, via Lyn activation, BCR ligation drives concomitant increases in phosphorylation of both SHP-1 and SHIP-1, ensuring graded inhibitory tone that scales with the extent of antigenic stimulation. Importantly, these pathways are robustly engaged in anergic B cells as indicated by constitutive phosphorylation of SHP-1 and SHIP-1[58].

Biochemical ‘anergy’ in modestly self-reactive Ars/A1 B cells requires continuous BCR occupancy and is therefore rapidly reversible in vitro[56]. In particular, the anergic state of these cells relies upon continuous ITIM-dependent inhibitory tone imposed by both SHIP-1 and SHP-1; Getahun, Cambier and colleagues bypassed central tolerance and showed that inducible deletion of either phosphatase in adoptively transferred mature Ars/A1 B cells leads to robust short-lived, extra-follicular plasma cell differentiation and antibody secretion [62]. Importantly, such plasma cell responses are dependent upon self-antigen stimulation; analogous experiments with naïve IgHEL B cells do not trigger Ab production. Interestingly, while SHP-1 and SHIP-1 act through independent and parallel inhibitory pathways to impose anergy, SHIP-1 and PTEN exhibit cooperative roles. This confirms the significance of their common substrate $PI(3,4,5)P_3$ as a critical gatekeeper of anergy, and suggests that

$PI(3,4,5)P_3$ concentration defines the 'tipping point' between tolerance and autoimmunity in peripheral B cells (Figure 3).

We previously showed that "stepping on the gas" by increasing the expression of the receptor-like tyrosine phosphatase CD45 enhances SFK priming and proximal BCR signal transduction, but ultimately results in an anergic-like state in which B cells exhibit IgM downregulation, impaired upregulation of CD86, poor survival, and fail to break tolerance, even on highly susceptible genetic backgrounds [68,69]. We showed via biochemical analysis and genetic epistasis that this was due to engagement of inhibitory pathways by Lyn [69]. Indeed, mice harboring a constitutively active allele of Lyn ($Lyn^{up/up}$) exhibit chronic engagement of ITIM-dependent signaling pathways and similarly exhibit features of B cell anergy [70]. By contrast, an allele of CD45 that selectively lacks regulatory control over Lyn (and thus partially phenocopies Lyn−/− mice), selectively "disables the break" imposed by ITIM-dependent effector pathways and produces frank autoimmunity in the same genetic context [69,71]. These studies suggest that it is not simply chronic antigen recognition by self-reactive B cells that eventually breaks tolerance and produces overt autoimmunity, but rather uncoupling such recognition from the inhibitory pathways normally engaged as a result of chronic stimulation. Indeed, either global or B cell-specific disruption of these ITIM-dependent pathways is sufficient to produce lupus-like autoimmunity on susceptible backgrounds[61]. Dependence upon these inhibitory pathways to restrain autoantibody production in mice lacking a self-reactive BCR Tg suggests that these pathways are engaged even by modestly self-reactive B cells within a physiologic repertoire.

Naturally occurring self-reactive B cells in humans

These mechanistic studies of B cell anergy rely in large part upon BCR Tg model systems in mice. How is self-reactivity handled in diverse, natural B2 cell repertoires, and by extension, which tolerance mechanisms play a relevant role in such physiologic settings? Wilson and colleagues identified a novel population of human B cells (B_{ND}) that express high surface levels of IgD but no detectable surface IgM [72]. This population makes up about 2.5% of mature recirculating B cells, and importantly lacks any evidence of somatic hypermutation, suggesting that they represent naïve rather than memory B cells. About 16% of BCRs expressed by B_{ND} cells harbor dsDNA reactivity, and 75% are reactive to nuclear antigens. These specificities are markedly enriched in B_{ND} relative to the bulk mature recirculating B cell population [7,10,72]. Consistent with their self-reactivity, B_{ND} cells have elevated levels of basal intracellular calcium. Despite normal surface expression of IgD, B_{ND} cells exhibit reduced tyrosine phosphorylation and calcium entry in response to both anti-IgM and anti-IgD stimulation [72]. However, it is not yet clear which negative regulators are engaged in these cells to achieve attenuated signaling. Sanz and colleagues reported that $IgD^{+}IgM^{LO}$ mature human B cells share many characteristics with B_{ND} cells [73]. However, although signaling is dampened in response to anti-IgM, responses to anti-IgD stimulation are only modestly impaired. This suggests that rewiring of self-reactive human B cells to suppress signal transduction occurs on a spectrum and is most profound in B_{ND} cells with virtually no detectable surface IgM expression, and possibly the greatest degree of self-reactivity. It is tempting to speculate that such cells have exhausted the capacity of IgM downregulation to restrain their self-reactivity, and depend on additional mechanisms (yet to be defined) to

dampen signaling. By contrast, less self-reactive B cell populations may be controlled primarily by IgM downregulation.

Naturally occurring self-reactive B cells in mice

Naturally occurring autoreactive B cells of varying phenotypes have also been described in mice. Self-reactive B cells from several Tg models, including IgHEL and Ars/A1 B cells, retain expression of the immature B cell marker CD93 (marked by the AA4.1 clone) and their surface phenotype corresponds to that of so-called splenic T3 B cells ($B220^{+}CD93^{+}CD23^{+}IgM^{LO}$) that can be identified in WT mice [74]. T3 B cells constitute about 5–8% of splenic B cells in C57Bl/6 mice, but despite expressing a marker of immaturity, do not appear to represent a developmental intermediate [74,75]. Rather, the Cambier lab has speculated that these may represent a compartment of naturally-occurring anergic B cells and termed this population 'An1' B cells [74]. The An1/T3 population is hypo-responsive to stimulation through the IgM BCR, even when gating on equivalent IgM expression in the Fo mature compartment, is enriched for ssDNA and Smith antigen-reactive B cells, and have a short half-life [74,75]. The intrinsically dampened signaling in An1 B cells suggests that they might be rewired, but the specific biochemical changes that account for this have not yet been defined.

The Nur77-eGFP reporter serves as a marker of functional self-reactivity and reveals that increasing self-reactivity is inversely correlated with surface IgM expression in the mature follicular B cell repertoire, while IgD levels remain high and invariant across the repertoire (Figures 1D, E)[15]. Increasing GFP expression correlates with increased basal intracellular calcium, and, similar to An1 cells and other Tg models of anergy, GFP^{HI} Fo B cells exhibit reduced calcium entry in response to IgM ligation (Figures 4A, B)[15]. However, in our hands, even GFP^{HI} B cells at the far end of the self-reactivity spectrum (top 1%) signal normally in response to IgD ligation, implying that IgM downregulation rather than downstream rewiring is the primary mechanism to attenuate BCR signaling in these cells (Figure 4C). Indeed, gating on matched levels of surface IgM confirms that this is the case (Figure 4D), and this may correspond to Tg models with lower avidity for self-antigens [22]. If we gate directly on mature Fo B cells expressing low surface IgM expression (lowest 10%), we obtain very similar results – impaired responses to IgM ligation, but intact responses to stimulation through IgD (Figure 4E). However, we can identify a very small population of cells with the lowest expression of surface IgM (lowest 1%) that do exhibit impaired calcium entry in response to IgD stimulation (Figure 4F). It is tempting to speculate that these cells may correspond to human IgD+IgM- (B_{ND}) cells identified by Wilson and colleagues[72]. These data argue that, despite harboring functional self-reactivity across a continuum, the vast majority of the mature Fo B cell repertoire is not constrained by rewiring of BCR signal transduction. Rather, IgM downregulation may be the primary strategy employed by these cells (together with physiological ITIM-dependent signaling pathways) to maintain tolerance. As with other naturally occurring populations of anergic B cells, it remains to be determined what mechanisms constrain the small (1%) population of classically anergic Fo B cells identified on the basis of extremely low surface IgM expression, and whether these are shared with An1 and/or B_{ND} cells. It is also interesting to explore why Nur77-eGFP

expression does not seem to identify these cells. One possibility is that rewiring of signal transduction markedly reduces reporter transcription.

There may be exceptions to these general observations; Taylor, Jenkins and colleagues characterized OVA-specific B cells in a normal polyclonal repertoire in mice and show that they remain essentially 'ignorant' in the presence of soluble OVA [76]. The most high affinity clones exhibit deletion in the presence of membrane-associate OVA, and the remainder exhibit 'functional anergy', but unexpectedly display completely 'naïve' surface phenotypes without expression of T3 markers or IgM downregulation. Importantly, OVA-specific B cells in mice – especially those that populate the periphery in mOVA hosts - have a vastly lower affinity for cognate antigen than do IgHEL B cells for HEL (more than 3 million-fold lower), and this must account for their relatively 'naïve' state. It would be of great interest to determine expression of Nur77-eGFP reporter in these cells in the presence and absence of mOVA in order to understand where this anergic population lies on the spectrum of self-reactivity across the physiological B cell repertoire.

Relatively intact BCR signal transduction in the vast majority of the natural mature B cell repertoire forms a striking contrast to models such as IgHEL/sHEL that rely upon PTEN upregulation to suppress downstream signal transduction. It is notable – as will be discussed in more mechanistic detail below – that anergic B cells from this double Tg model undergo extremely rapid (2–3 days) elimination in competition with non-Tg B cells[77,78]. Indeed, biochemical studies of these B cells were made possible by generation of a monoclonal repertoire lacking competitor B cells. Highly self-reactive specificities corresponding to 'anergic' IgHEL B cells may be exceedingly rare in the physiological mature B cell repertoire and do not contribute significantly to the GFP^{HI} compartment of a physiologic B cell repertoire. Notably, several BCR Tg models with modest self-reactivity show no evidence of impaired BCR signal transduction[22]. It is tempting to speculate that these are more typical of clones that populate a normal B cell repertoire. We propose that the physiologic spectrum of mature Fo B cells exhibit a mild degree of self-reactivity that is adequately controlled by IgM downregulation and does not trigger biochemical rewiring of signal transduction except at far extremes of the distribution. An independent study came to similar conclusions by studying the functional properties of IgM^{LO} and IgM^{HI} Fo B cells in mice [79]. These data collectively suggest that IgM downregulation is the primary 'first-line' peripheral tolerance mechanism triggered by self-antigen recognition – at least among mature Fo B cells - and additional rewiring of BCR signal transduction machinery to further dampen signaling is triggered when self-reactivity exceeds certain bounds. It is possible that these strategies are intentionally redundant and layered mechanisms, triggered when conditions (transient lymphopenia, excessive BAFF, other genetic variants in signal transduction) permit the entry and persistence of more self-reactive B cells in the mature Fo B cell repertoire. The contrast between humans and mice in this regard may reflect any of these as well as the marked difference in lifespan between our species, perhaps permitting the accumulation of more self-reactive clones in humans that must be restrained by mechanisms beyond IgM downregulation.

5) Dual expression of IgM and IgD BCRs on Fo B cells: a unique strategy to impose peripheral tolerance

Downregulation of IgM (but not IgD) BCRs is a nearly universal feature of self-reactive, anergic B cells both in BCR Tg models, and in the physiologic B cell repertoire[22]. Our studies of the Nur77-eGFP reporter reveal that the degree of IgM downregulation correlates with self-reactivity across the Fo B cell repertoire in mice, and suggests that this may represent a primary mechanism of peripheral B cell tolerance[15]. However, it has been challenging to understand how IgM downregulation could suppress responses to endogenous antigens in the face of high IgD expression since these BCR isotypes are co-expressed and, on any given B cell, contain identical antigen-binding domains. Indeed, the reason for co-expression of these two BCR isotypes on mature naïve follicular B cells has been a very long-standing puzzle in B cell biology.

IgM is the first isotype expressed on the surface of developing B cells, and it can be expressed in all known B cell lineages[80]. By contrast, the IgD BCR is co-expressed with IgM during a relatively narrow window of development, beginning in the late splenic transitional stages, and peaking on mature naïve Fo B cells (IgD expression is much lower on innate-like B cell lineages). Unlike other BCR isotypes, IgD is not predominantly generated through genomic class-switch recombination (although it can be in limited settings); rather, its expression on Fo B cells is the product of a developmentally-regulated alternative splicing event, and abruptly ends once naïve B cells are activated. Not only do IgM and IgD BCRs on a given B cell share an identical antigen-binding domain, they also have an identical intracellular domain that consists of three amino acids (lysine-valine-lysine), and both rely upon pairing with Igα/β for initiation of signaling, and to a more variable extent, transport to the surface of the cell [81,82]. However, they differ notably in their extra-cellular constant domains; IgD uniquely harbors a long and flexible hinge c-terminal relative to the $C_{\delta}1$ Ig domain [83]. X-ray scattering reveals that this long hinge, along with heavy O-glycosylation, gives IgD a T-shaped structure, in contrast to the more rigid Y-shaped structure of IgM [84].

These data suggest that IgM and IgD BCRs ought to play non-redundant functions on mature naïve B cells. However, generation of two independent IgD-deficient mouse lines and one IgM-deficient mouse line in the 1990s failed to uncover such a non-redundant function for either of these BCR isotypes; B cells harboring one or the other could traverse development, enter all B cell lineages, and mount both T-independent and T-dependent immune responses [85–87]. Similarly, an earlier study comparing HEL-specific IgM or IgD BCRs reported comparably efficient development, functional responses, and clonal anergy on a self-reactive genetic background [88]. The only prominent difference between these two IgHEL Tg lines was that acute and chronic antigen stimulation induced receptor downregulation to quite a different extent; IgM downregulation was much more dramatic than IgD downregulation, mirroring the observations made with the dual isotype-expressing Tg line. More recently, two additional IgD-deficient mouse lines were generated by Goodnow and colleagues; one harbored deletion of ZFP318, an obligate mediator of IgD splicing, while the other (*dmit*) was generated via ENU mutagenesis and impaired

trafficking of IgD to the cell surface [89,90]. Notably, *dmit* mutants, by contrast to other IgD-deficient models, lost surface IgD expression without a compensatory increase in surface IgM. Collectively, these studies revealed that IgD-deficient Fo B cells, most obviously in *dmit* mutants, exhibit an impairment in competitive survival under steady state conditions [85,86,90]. This is consistent with a well-appreciated role for tonic BCR signaling in B cell survival[91–93], and in turn implies that one role for co-expression of IgD may be to deliver 'tonic' survival signals that preserve self-reactive IgM^{LO} B cells in the mature Fo B cell compartment, perhaps to serve as a reservoir of protective BCR specificities that limit 'holes' in the repertoire. Surprisingly, no defect in competitive fitness was apparent in a recent analysis of a small family of $IgD^{+/-}$ patients in which IgD-sufficient and IgD-deficient B cells from the same patient could be tracked [94]. However, it remains possible that the BCR repertoires of these two competing B cell populations differed and might compensate via altered antigen-dependent signaling. These studies, nevertheless, do not establish how IgM downregulation could serve as a tolerance mechanism when a signaling competent IgD BCR remains on the surface of self-reactive B cells.

Parallel lines of investigation into differences between the in vitro signaling capacity of IgM and IgD BCRs have begun to bear fruit as well. Work from Batista, Reth and colleagues have suggested that IgM and IgD are not homogeneously distributed across the plasma membrane, but exist in pre-formed and distinct "nano-clusters" [82,95–97]. Differential clustering, mobility, and association of IgM and IgD BCRs with co-receptors such as CD22 and CD19 could in turn confer distinct signaling capacity despite shared use of the canonical Igα/β-dependent BCR signaling apparatus [97–99]. The CD22 co-receptor deliver signals via an ITIM/LYN/SHP-1 inhibitory pathway, while CD19 is critical to drive PI3K activation, and differential association of IgM and IgD with these co-receptors on the cell surface could have important implications for the physiological roles of these BCR isotypes in B cell anergy, survival, and differentiation. Interestingly, it has been argued that IgD is associated with CD19 at rest, but IgM associates with CD19 upon stimulation [82,97]. This could provide a mechanistic basis for a tonic survival function of IgD, and may also help explain why IgM downregulation is an effective strategy to suppress antigen-dependent $PI(3,4,5)P_3$ generation, and impose tolerance. Recently, Maity and colleagues identified an obligate role for IgD expression to facilitate signaling via CXCR4, a receptor that was not previously known to interact with the BCR pathway[100]. This finding opens the floodgates to a host of other potential isotype-specific interactions on B cells.

Recently, Jumaa and colleagues supplied a very important insight into how direct 'sensing' of antigen by IgM and IgD BCRs may differ, suggesting that the flexible hinge region of IgD renders it unresponsive to monovalent antigens [101]. To support this model, they took advantage of pro-B cells from so-called 'triple-deficient' mice (lacking Slp65, surrogate light chain L5, and RAG2) that are blocked at an early stage of development, do not express endogenous BCR, but can be transduced with model BCRs for use in signaling assays. Polyvalent forms of either NIP hapten conjugated to peptides or HEL could trigger signaling through both IgM and IgD antigen-specific BCRs introduced into this system, while monovalent variants could signal only through IgM. Moreover, deleting the hinge region from IgD or introducing it into IgM was sufficient to confer or eliminate sensitivity to monovalent antigens, respectively. More recently, this group has extended these findings to

the Ramos B cell line and has made analogous observations using both human and murine BCR isotypes [102]. These data suggest that self-reactive Fo B cells (IgD^{HI} IgM^{LO}) retain the capacity to make responses only towards multivalent antigens (e.g. immune complexes aggregated by preformed IgM Ab or via FcRs on APCs). However, recent work from Goodnow and colleagues argues against this model; isotype-specific IgHEL Tg mature Fo B cells expressing either IgD or IgM alone exhibit comparable responsiveness of both to soluble HEL Ag in vitro [90]. Moreover, it was previously shown that both BCR isotypes were rendered functionally and phenotypically 'anergic' in the context of a host expressing soluble HEL Tg [88]. Both isotypes could also mediate a similar transcriptional 'anergy' program in vivo, further implying comparable sensitivity to sHEL in vivo [90]. Unexpectedly, IgM-only cells in the latter study induced transcription of a subset of anergy-associated genes more strongly than cells expressing both IgM and IgD BCR isotypes, suggesting that IgD "attenuates" the anergy program. Whether and how IgD could directly interfere with IgM signaling remains to be clarified.

It is currently uncertain how to reconcile the discrepancy between these reports, although conclusions may differ, at least in part, because of distinct cellular contexts, antigen doses and preparations investigated. The actual sensitivity of IgD BCRs towards monovalent antigens may in fact lie somewhere between these two extremes. Regardless, certain limitations remain: both of these key studies investigated predominantly soluble, high affinity model antigens. However, the affinity, avidity, and structural features of bona fide endogenous antigens are largely unknown and likely differ significantly from model antigens. Indeed, as noted earlier, perhaps the most well-defined naturally occurring self-reactive B cell population identified in humans, expressing the VH4–34 H chain, is argued to recognize highly abundant cell-surface antigens on RBCs and B cells [12,13]. It is not yet known whether IgM and IgD BCRs might be differentially sensitive to antigen affinity. Nor is it clear how these isotypes respond to membrane-associated antigens of varying affinity and density. Yet differences in this respect are likely as it has been reported that IgM is more sensitive to high density antigens while IgD binds more tightly to low density epitopes [103].

Our laboratory became interested in understanding how IgM and IgD BCRs might differ in their responsiveness to bona fide endogenous antigens because we had observed in Nur77-eGFP reporter mice that IgM but not IgD was downregulated continuously across the entire mature Fo B cell repertoire in proportion to endogenous antigen encounter[15]. This strongly suggested to us that IgM BCRs might be relatively more sensitive to endogenous antigens, and as has long been speculated, that IgM downregulation could serve as an elegant and efficient tolerance mechanism if this were the case. Both the appeal and the challenge to us was to understand whether this could be the case with a diverse, naturally occurring B cell repertoire that was exposed to a complex range of bona fide endogenous antigens (that might and likely did vary tremendously from any particular model antigen studied).

We took several independent approaches to determine the sensitivity of IgM and IgD for bona fide endogenous antigens across a diverse B cell repertoire[24]. We introduced the Nur77-eGFP reporter into both $IgM^{-/-}$ and $IgD^{-/-}$ genetic backgrounds, and showed across multiple B cell compartments that IgD BCRs are less efficient than IgM BCRs at driving reporter GFP expression in vivo. This difference was most apparent when the BCR

repertoire was either unconstrained (via BAFF overexpression), or single specificities were compared (PtC-specific B1a cells), suggesting that the selection of BCR repertoires compensate for and partially mask signaling differences in individual isotype-deficient mice. In parallel, we took advantage of allelic exclusion in $IgM^{+/-}$ $IgD^{+/-}$ mice to generate an immature B cell pool comprised of 50% IgM-only and 50% IgD-only B cells. As discussed earlier, MZ and B1a development proceed optimally in response to low and high self-reactivity, respectively [35,104]. We found that IgD-only B cells outcompete IgM-only cells in the MZ compartment, but the result is reversed in the B1a compartment [24]. Collectively, these observations suggest that IgM is more sensitive to bona fide endogenous antigens in vivo than IgD. Importantly, in vitro signal transduction in response to L chain cross-linking of IgM-only and IgD-only B cells revealed no corresponding defects, suggesting that 'sensing' of endogenous antigens, rather than differential coupling to the downstream signaling apparatus, accounted for these in vivo differences.

These studies of IgM and IgD biology collectively support an exciting model to account for peripheral B cell quiescence despite variable engagement by endogenous antigens. Mature naïve Fo B cells downregulate surface IgM (but not IgD) in proportion to their degree of functional self-reactivity. Physical properties of IgM that render it more sensitive to endogenous antigens than IgD (rigid structure with short hinge region, and possibly unique association with distinct co-receptors) ensure that these cells remain unresponsive in the face of chronic self-antigen stimulation. Preservation of IgD expression, conversely, may sustain survival of self-reactive clones in a competitive environment (perhaps by promoting PI3K-dependent signaling via association with CD19) in order to patch 'holes' in the mature B cell repertoire that would otherwise arise if such clones were eliminated entirely. As discussed further below, IgD may also facilitate participation of B cells in GC-dependent immune responses upon receipt of appropriate cross-linking and co-stimulatory cues by foreign pathogens. This remarkable dual BCR isotype mechanism of peripheral tolerance may suffice to control the majority of the mature B cell repertoire, but clones that exceed the buffering capacity of this elegant system may go on to trigger a range of post-translational and even transcriptional mechanisms that further attenuate downstream signal transduction.

However, many important questions remain unanswered, including the relative responsiveness of IgM and IgD to membrane-associated and low affinity antigens, as well as the relative contribution of the IgD hinge and coreceptor association to such isotype-specific differences. Moreover, the molecular mechanism that couples chronic antigen recognition to IgM downregulation remains an open area of investigation. Prior work suggests that the cbl and cbl-b ubiquitin ligases drive Ag-dependent ubiquitination and degradation of the Igα BCR subunit, and appear to be critical for IgM downregulation in anergic IgHEL B cells, although how they are linked to antigen stimulation is not clear [105]. Recent and as yet unpublished work suggests that the E3 ubiquitin ligase GRAIL may play a similar role via ubiquitination and degradation of Igα, and may be coupled to antigen recognition via PTEN (R. Nurieva, June 2018 Dresden B Cell Keystone Conference presentation; Nurieva et al. AAI abstract 2018). In conjunction, antigen-dependent upregulation of ZFP318 may favor splicing of the primary IgM/D transcript towards IgD expression in anergic B cells, and this may also be promoted by PTEN [90,102].

Although most self-reactive BCR Tg models exhibit selective downregulation of the IgM BCR, a few exceptions show reduced expression of both IgM and IgD beginning during immature stages of BM development [6]. Included among these are DNA-reactive V_H3H9 / λ1 B cells and an independent model (HKIR) described by Manser and colleagues in which DNA-reactive Tg B cells can also bind Ars hapten (similar to Ars/A1 model) [106]. It has been suggested that this mode of BCR downregulation is associated with receptor editing, but that is not thought to be the case in the HKIR model. Rather, BCR downregulation in that model was shown to be reversible via displacement of endogenous antigen with the Ars hapten, and was associated with transcriptional repression of BCR components[107]. However, the precise molecular mechanism is still not clear, and likely differs from selective IgM downregulation seen in mature self-reactive B cells [6]. Downregulation of both BCR isotypes clearly contributes to anergy of self-reactive Tg clones by desensitizing them to antigen, but appears to constitute a rather rare tolerance mechanism triggered in highly self-reactive clones early in development; its relative contribution to the physiological B cell repertoire may be very minor.

6) Competitive fitness of self-reactive B cells in the periphery

IgM downregulation and modulation of the downstream BCR signaling apparatus are not the only mechanisms of tolerance engaged by self-reactive B cells in the periphery. B cell clones also undergo competitive elimination in proportion to their self-reactivity; in early studies, it was noted that “anergic” IgHEL B cells turn over rapidly in vivo, and are rapidly lost after transfer into a sHEL Tg host with competing WT B cells [77,78,108]. By contrast to an estimated half-life ($t_{1/2}$) for naïve mature Fo B cell on the order of several weeks, the $t_{1/2}$ of anergic IgHEL B cells is several days in the absence of clonal competition, and about half a day with such competition. After adoptive transfer into hosts expressing soluble HEL Ag, loss of “anergic” IgHEL B cells depends upon the presence of both competitor B cell clones and self-antigen, but is genetically separable from follicular exclusion [109,110]. Interestingly, transfer of naïve IgHEL B cells into hosts expressing cognate sHEL Ag gives a nearly identical cell loss, delayed only by 24 hours relative to that of anergic IgHEL B cells, suggesting that this phenomenon is separable from other features of anergy[77]. After discovery of the critical B cell survival factor BAFF/BLyS, it was shown that competition for a limiting supply of this factor accounts for this phenomenon; highly self-reactive B cells are rapidly eliminated under conditions of limiting BAFF (as in a normal polyclonal B cell repertoire), while an excess supply of BAFF rescues their survival [111–114]. Importantly, this phenomenon appears to exists along a continuum such that self-reactive B cells strike a balance between the degree of endogenous antigen recognition and their relative dependence upon BAFF supply.

While a limiting supply of BAFF plays a critical role in the competitive elimination of self-reactive B cells in the periphery, BAFF is not thought to regulate central deletion because immature B cells do not upregulate the BAFF receptor (BAFFR) until later in splenic stages of development. We previously showed that increasing CD45 expression in B cells (because of co-engagement of both BCR and ITIM-dependent inhibitory pathways) generates an anergic-like state characterized by a very short $t_{1/2}$, profound downregulation of BAFFR, and resistance to BAFF[68]. This mechanism may contribute to poor survival of some anergic

B cell populations. However, although anergic IgHEL B cells modestly downregulate BAFFR, they remain extremely responsive to BAFF supplementation both in vitro and in vivo. This implies the existence of another cell-intrinsic change in these anergic cells that does not render them insensitive to, but rather dependent on, BAFF.

One such change may be upregulation of the pro-apoptotic BH3-only Bcl2 family member BIM; anergic IgHEL B cells, in competitive settings, upregulate BIM in response to chronic antigen stimulation, and this may partially account for their increased dependence upon BAFF [111]. Deletion of BIM rescues survival of anergic IgHEL B cells in mice expressing soluble HEL and renders these cells less dependent on BAFF for survival in vitro, but has only a modest effect on central deletion triggered by membrane-associated HEL [115,116]. Importantly, deletion of BIM also indirectly overwhelms other peripheral tolerance mechanisms such that BCR signal transduction is no longer attenuated and tolerance is broken[116]. It is unclear whether this is a direct consequence of BIM deletion, or rather represents an indirect effect of reduced BCR occupancy as the number of surviving IgHEL B cells goes up and the supply of HEL protein is reduced by serum Ab.

Upregulation of BIM may not be the only mechanism that renders anergic B cells dependent upon BAFF for survival. We recently identified a specific function for Nur77 itself in the competitive elimination of mature Fo self-reactive B cells by taking advantage of *Nr4a1*$^{-/-}$ mice[27,117]. Nur77 belongs to a three-member family of orphan nuclear hormone receptors (Nur77, Nurr1, and Nor1 encoded by *Nr4a1-3* respectively) that share significant structural similarities in their DNA-binding and ligand-binding domains [118]. Nr4a family members are thought to function as constitutively active transcription factors with no known endogenous ligand [119]. It has been argued that, in addition to their role as transcriptional regulators, Nr4a family members can also trigger apoptosis independently of DNA-binding by translocating to the mitochondria, binding directly to Bcl2, and inducing a conformational change that exposes the pro-apoptotic BH3-only domain of Bcl2 [120–122]. Indeed, Nur77 misexpression promotes TCR-induced apoptosis in T cell hybridomas and thymocytes, while a dominant negative construct lacking the n-terminal transactivation domain produces the opposite phenotype, implicating this family as mediators of thymic negative selection [123–125]. These observations raise the possibility that Nur77 plays an analogous role in B cell tolerance.

We recently showed that Nur77 expression correlates with self-reactivity, counter-selection, and anergy among individual B cell clones from both the IgHEL and the V_H3H9 models of self-reactivity [27]. Although Nur77 itself was largely dispensable for receptor editing, central deletion, follicular exclusion and anergy, we discovered that Nur77 restrains the survival of mature self-reactive B cells in vitro and in vivo, particularly in settings where the B cell survival factor BAFF is limiting, and this can be rescued entirely by supplemental BAFF [27]. Nur77-deficiency also results in the progressive accumulation of self-reactive B cells in the mature repertoire with age, and is sufficient to break B cell tolerance in V_H3H9 Tg mice. Importantly, since Nur77 is upregulated in self-reactive B cells in proportion to the degree of chronic antigen stimulation, we argue that it serves as a direct molecular link between self-reactivity and B cell survival in the periphery; Nur77 gradually prunes B cell clones from the mature repertoire in proportion to their self-reactivity and thereby imposes a novel layer of peripheral B cell tolerance.

7) Tolerance of self-reactivity in innate-like B cells

By contrast to Fo B cells which express high levels of IgD and variable levels of IgM BCRs on their surface (that vary with self-reactivity), innate-like MZ and B1a cells express predominantly IgM and do not downregulate this receptor in proportion to their self-reactivity, suggesting that they must rely upon other tolerance strategies (unpublished data, Zikherman lab). MZ B cells are exceptionally sensitive to negative selection by strong BCR signaling, and, although there is a minimal amount of mild self-reactivity that facilitates MZ B cell development, strongly self-reactive clones are stringently excluded from that compartment (Figure 2). By contrast to MZ B cells, B1a cells are highly resistant to deletional tolerance and the threshold for positive selection of B1a cells overlaps with that for negative selection of Fo B2 cells resulting in an extremely self-reactive compartment (Figure 2). Perhaps for this reason, ITIM-dependent inhibitory tone is especially important to restrain B1a cells from activation by self-antigen; deletion of Siglec-G, CD22, SHP-1, CD5, PirB (or combinations thereof) in B1a cells results in enhanced BCR signal transduction along with concomitant expansion of the B1a compartment [126–130]. In addition to this critical reliance upon inhibitory co-receptors, a number of other unique tolerance mechanisms are engaged by B1a cells. It has long been observed that B1a cells are sensitive to immobilized, but not soluble antigen, perhaps because the latter clusters BCRs and can exclude inhibitory co-receptors. A related observation is that soluble antigen stimulation of B1a cells – in contrast to B2 cells – fails to trigger downstream NF-κB signaling and proliferation, but the molecular mechanism for this is not well understood[131,132].

Recently, we showed that Nur77-eGFP reporter expression was highly upregulated by chronic antigen stimulation in self-reactive B1a cells, particularly in highly self-reactive PtC-binding clones [133]. Unexpectedly, we found that deletion of *Nr4a1* markedly increased generation of B1a-derived natural IgM plasma cells, but did not expand the size of the B1a compartment. By contrast to ITIM-dependent inhibitory tone, Nur77 seems to regulate a distinct, downstream checkpoint by an unknown mechanism. Further delineation of this and other B1a-specific tolerance mechanisms is needed.

8) The two-signal model of B cell activation and self/non-self discrimination

In vivo B cell responses to T-dependent stimuli such as hapten-protein conjugates are characterized by an initial phase of antigen-dependent events such as entry into cell cycle, upregulation of peptide-loaded MHC-II, adhesion, and costimulatory molecules, and secretion of chemokines that collectively facilitate recruitment of T cell help, along with migration away from the B cell follicle and towards the T-B border for the purpose of accessing such help [134]. Of necessity, there is a physiological delay between receipt of signal one (antigen), and acquisition of signal two in the form of T cell help (including components such as CD40L, the cytokines IL-4 and IL-21, as well as other co-stimulatory molecules on the surface of T cells). Even a “single round” of antigen stimulation is sufficient to prime B cells to seek out and respond to T cell help within a defined temporal window[135,136]. B cells that fail to do so may revert to a naïve-like state if the antigenic stimulus was sufficiently

transient [136,137]. However, B cells that receive a strong or prolonged BCR signal are 'hard-wired' to make a very abortive immune response, ending in apoptosis, when they fail to acquire signal two within a defined span of time [58,138]. Hodgkin and colleagues have quantitatively characterized this so-called 'death timer', and more recently, Pierce and colleagues described the metabolic changes that accompany failure to recruit signal two [137,138]. Interestingly, they identify a role for antigen-dependent chronic calcium 'leak' via membrane CRAC channels, and progressive mitochondrial dysfunction that can be rescued in its early stages by appropriate help, but functions as a metabolic 'death timer' in the absence of help. Yet the full molecular program that produces such abortive and non-productive responses to signal one (in the absence of signal two) is not completely clear.

In a number of ways, chronically antigen-stimulated self-reactive B cells exhibit behavior that resembles an abortive response to acute BCR signaling. While self-reactive B cells may not divide, in a number of models they upregulate activation markers, including MHC-II and CD86, downregulate IgM, and migrate out of the B cell follicle to the T/B border or the T cell zone itself [22]. This last phenomenon is termed follicular exclusion, depends upon the chemokine receptors CXCR5 and CCR7, and has been described not only in the IgHEL model, but also in the V_H3H9 and Ars/A1 models [58,78,109,139]. In other words, anergic B cells exhibit antigen-induced changes that normally serve to recruit signal two in the form of T cell help. Importantly, both acute and chronic antigen stimulation triggers a program of antigen-induced cell death in B cells that can be rescued by an excess supply of the survival factor BAFF and/or a relevant costimulatory signal. Indeed, "anergic"-like phenotypes can be induced in B cells by presenting antigen (aka signal one) in the absence of signal two; transfer of naïve IgHEL B cells into sHEL-expressing hosts is sufficient to recapitulate several features of anergy following a round of rather abortive proliferation, including both IgM downregulation and markedly reduced survival [77]. Conversely, provision of signal two under inappropriate circumstances can be sufficient to break B cell tolerance (e.g. $Roquin^{san/san}$ mice with over-expression of co-stimulatory molecules on Tfh cells, or co-engagement of BCR and endosomal TLRs by nucleic acid-containing autoantigens) to produce frank autoimmune disease in mice and humans[140,141]. It is possible (perhaps likely) that the state of clonal 'anergy' and the abortive responses of a B cell that receives signal one without signal two exist on a continuum with one another, rely upon overlapping molecular mechanisms, and serve a similar purpose: to restrain inappropriate self-reactive B cell responses to endogenous antigens. Indeed, the two signal model of B cell activation was initially proposed by Bretscher and Cohn in part as a strategy to discriminate self from non-self [142].

Nur77 is not only upregulated in response to chronic antigen stimulation, but, along with its family members, is an immediate-early or so-called 'primary response gene' that is rapidly and potently (but transiently) induced by acute BCR stimulation. We found that Nur77-deficient B cells exhibit enhanced survival and proliferation relative to WT B cells in response to acute BCR stimulation, but exhibit no competitive advantage in the context of co-stimulation, in the form of either TLR stimuli or signals that mimic T cell help (unpublished data, Zikherman lab)[27]. We postulate that negative regulation by Nur77 of both naïve and self-reactive B cells (which exist on a continuum!) serves to confer dependence upon a second signal and thus helps maintain tolerance by restricting survival of B cells that

receive signal one either acutely or chronically in the absence of signal two. The downstream effectors that mediate this role, and other molecular pathways that contribute to this important tolerance mechanism (i.e. the other components of the death timer) remain to be fully dissected, but may include upregulation of Bim by BCR engagement [111].

9) Differentiation of self-reactive B cells into short-lived plasma cells and germinal center B cells

Although the classic hallmark of clonal anergy is failure to generate rapid plasma cell responses to antigen-specific immunization, anergic B cells can – with appropriate T cell help - be recruited into the GC [90,143–145]. Indeed, anergic B cells may enter the GC more efficiently than naïve B cells in some contexts[145]. The mechanism that underlies this preferential recruitment of self-reactive B cells towards GC fate has not been fully elucidated but may relate, in part, to differences in IgM and IgD expression; predominant IgD expression on anergic cells that downregulate IgM might serve to transmit a quantitatively (or qualitatively) distinct signal relative to IgM. Indeed, Goodnow and colleagues reported that IgD-only anergic B cells are more efficiently recruited into the GC than IgM-only anergic B cells, postulating greater survival of the former [90]. Recently, Jumaa and colleagues also proposed that IgD expression enhances GC entry, although prior work suggests that such results may vary according to the immunogen studied and such assays must be controlled for varied precursor B cell frequency conferred by the distinct VDJ loci of WT and isotype-deficient animals [24,85,86,102].

We made related observations in our studies of IgM-deficient and IgD-deficient mice [24]. Because $Lyn^{-/-}$ mice lack ITIM-dependent inhibitory tone, they develop a dramatic and spontaneous expansion of short-lived plasma cells that are not class-switched (similar to conditional deletion of PTPases in this pathway)[62,67,70,146]. Genetic disruption of ITIM-dependent signaling results in downregulation of the transcription factor ETS1 which suppresses PC differentiation[147]. This is thought to be due in part to excessive BTK-dependent signal transduction in response to endogenous antigen stimulation in the absence of inhibitory tone [147,148]. Deletion of ETS1 in B cells is sufficient to drive spontaneous PC differentiation even with intact inhibitory tone and even in the absence of self-antigen stimulation, while over-expression of ETS1 can suppress such differentiation in Lyn-deficient and SHP-1-deficient B cells [147,149,150]. On the $Lyn^{-/-}$ genetic background, we observed that IgM-only B cells spontaneously differentiate into these short-lived ‘unswitched’ plasma cells, but IgD-only B cells do not, and showed that this is associated with reduced downregulation of ETS1 by IgD-only B cells[24]. This implies that IgD BCRs are more inert than IgM in the face of chronic endogenous antigen stimulation, even without the safeguard of ITIM-dependent inhibitory signals. The corollary of this is that IgM downregulation on self-reactive B cells could, to some extent, compensate for loss of inhibitory tone. By contrast, in our work and in prior studies of IgM-deficient mice, IgD-only B cells are quite efficiently recruited into the GC in response to either T-D immunization (both SRBC and NP-protein conjugates, controlled for VDJ locus) or in the context of spontaneous autoimmunity in Lyn-deficient animals [24,87]. This suggests that IgD

does not efficiently drive short-lived plasma cell differentiation by self-reactive B cells with low IgM expression, but is sufficient for effective GC responses.

Persistent ETS1 expression may not be the only mechanism diverting anergic B cells away from short-lived PC fate and towards GC entry. Brink and colleagues have shown that short-lived PC expansion is much more highly dependent upon antigen affinity than is GC entry [151,152]. By virtue of predominant IgD expression and attenuated signal transduction, self-reactive anergic B cell clones may be unable to drive efficient expansion of short-lived PCs, but antigen stimulation can still generate sufficient signaling to support GC entry.

It is possible that qualitative differences in IgM- and IgD-dependent signaling in anergic and naïve B cells may also contribute to their differential propensity to support an extra-follicular short-lived PC response. For example, differential association of IgM and IgD with CD19 and CD22 or other coreceptors could drive distinct engagement of SHP-1, SHIP1, and PI3K pathways by these BCR isotypes. PI3K signaling can greatly facilitate PC differentiation, so preferential engagement of $PI(3,4,5)P_3$ phosphatases by anergic B cells may repress PC fate to provide a second layer of restraint independently of IgM and IgD expression[59].

The rationale for shunting self-reactive B cells away from PC differentiation and into the GC may be to avoid generation of autoantibodies. But what happens once self-reactive B cells enter the GC? Manser and colleagues showed that an Ars hapten-reactive BCR that was also self-reactive, could be recruited into the GC by immunization with Ars-conjugated protein, but these cells appeared to be censored in the memory compartment that emerged from the GC[106]. However, it was not clear how this was achieved, and moreover, these observations did not explain why we would evolve elaborate peripheral tolerance mechanisms that facilitate retention of self-reactive clones in the pre-immune B cell repertoire only to dispose of them during humoral immune responses. More recently in a series of elegant studies, Goodnow and colleagues postulated and demonstrated that self-reactive BCRs can undergo SHM and mutate away from self-reactivity in a process termed ‘clonal redemption’ (reviewed elsewhere in this volume)[143–145]. This phenomenon explains how self-reactive BCRs can be ‘used’ but simultaneously neutralized during a humoral immune response, and could help to explain why such clones are constrained by anergy and preserved in the peripheral repertoire, rather than deleted.

10) Tolerance in the germinal center and beyond

Not only does the host need to handle pre-existing self-reactive clones that seed the GC, but the threat of de novo self-reactivity, inadvertently acquired via random SHM, must be neutralized. Indeed, Nussenzweig and colleagues identified examples of de novo self-reactivity in the IgG+ memory compartments of healthy humans, implying that this represents a real and significant threat to B cell tolerance[153]. What are the cellular and molecular mechanisms of tolerance in the GC and what are its limitations?

It has long been known that soluble antigen stimulation in the absence of co-stimulation results in GC B cell apoptosis, and it has been argued that this represents a tolerance

mechanism to delete self-reactive clones that inadvertently arise during the process of somatic hypermutation (SHM) and affinity maturation[154–156]. With a very strong BCR stimulus, such cell death occurs rapidly (within 8 hours), can be at least partially rescued by Bcl2 overexpression, but proceeds independently of Fas and CD40[54–56]. More recently, both the Brink and Goodnow labs elegantly demonstrated counter-selection of self-reactivity in the GC using the IgHEL model system coupled with genetic manipulation of neo-self-antigen[143,157]. The molecular mechanisms that underpin this selection pressure to lose self-reactivity in the GC remain to be worked out, but may include competition between autoantigen and foreign antigen for binding, and/or Ag-induced death of GC B cells triggered in the absence of T cell help. This strong counter-selection of self-reactivity in the GC, along with prior studies, collectively imply that self-reactive clones in the GC that cannot be redeemed via SHM will be deleted and points to the existence of a GC-intrinsic tolerance mechanism. The physiologic stringency and frequency of redemption and/or deletion of self-reactive B cells during and after the GC response will be important to explore further, as will the molecular mechanisms at play.

Importantly, Brink and colleagues showed that GC clones with cross-reactivity to tissue-restricted self-antigens that were not present within the GC escaped tolerance[157]. It was postulated that molecular mimicry between microbes and self-antigens could drive generation of high affinity autoantibodies in this way and it is clear that this is a clinically important vulnerability. Together with detection of self-reactivity in the human memory B cell compartment, these observations suggest the need for post-GC tolerance. Whether and how this might work is unknown. It is possible that some variation on tolerance mechanisms that constrain the Fo mature B cell compartment might be relevant.

In previously published work, we showed that a subset of B cells in the germinal center light zone upregulate Nur77-eGFP expression [20]. Since the light zone contains FDC presenting antigen and Tfh cells, this suggests that GFP^{HI} GC B cells may be actively signaling in response to such stimuli. As expected, BCR ligation of GC B cells in vivo could increase Nur77-eGFP (unpublished data, Zikherman lab), while in vivo treatment with the Btk inhibitor ibrutinib decreased GFP expression [20]. By contrast, an anti-CD40L blocking Ab (MR1 clone) rapidly begins to dissolve the GC, but does not alter GFP expression (unpublished data, Zikherman lab). Together, these data suggest that Nur77-eGFP expression in GC B cells in vivo is dependent upon BCR signaling but independent of CD40 signaling. Since Nur77 mediates antigen-induced cell death in Fo B cells in response to either acute or chronic antigen stimulation, we hypothesize that the Nr4a family may play an analogous role in the context of the germinal center (or even post-GC memory B cells) where some mechanism must exist to restrain persistence or de novo generation of ‘rogue’ self-reactive clones. This and other molecular mechanisms at play in GC tolerance are of great interest to explore further.

11) Conclusions and future directions:

Modest self-reactivity is an essential feature of the mature Fo B cell compartment; some degree of self-reactivity is not merely tolerated, but is actively selected into and retained in the repertoire for future use. The teleological rationale for selecting a self-reactive repertoire

remains uncertain. Modest self-reactivity may facilitate protective immune responses by promoting recognition of essential biological patterns shared by both host and microbe. Self-reactivity may also represent an inevitable byproduct of the quest for clonal diversity and may limit holes in the repertoire that would otherwise arise as a result of deletional tolerance mechanisms. Importantly, self-reactivity among mature B cells spans a broad continuum, and we postulate a “sliding scale” of tolerance mechanisms that maintain quiescence of individual B cell clones in the face of self-antigen encounter (Figure 5). The modes of peripheral B cell tolerance - IgM downregulation, attenuation of BCR signal transduction, and reduced survival – can be thought of as “quantitative traits” that vary continuously across the B cell repertoire. They are also inter-dependent; impairing one applies greater pressure to the others, and when this is excessive, their collective buffering capacity may be overwhelmed.

We propose that selective downregulation of the IgM but not the IgD BCR represents the primary layer of control to handle the pressure of irrelevant or benign antigen encounter in vivo, allowing clones to adapt to their environment while retaining functional capacity. Importantly, IgM downregulation scales continuously across the entire Fo B cell repertoire in proportion to antigen encounter. Because the IgD BCR is less sensitive to endogenous antigens, cells expressing predominantly IgD are restrained from activation and from direct extra-follicular plasma cell differentiation, but IgD expression facilitates survival and preserves (or perhaps enhances) the capacity of such clones to mount a germinal center (GC) response. Entry into the GC can then redeem self-reactivity of such clones via SHM and counter-selection, but the molecular strategies that drive such efficient counterselection and similarly constrain de novo acquisition of self-reactivity are largely unknown.

However, many components of this mechanism remain to be explored. The sensitivity of IgM and IgD BCRs to antigens ‘seen’ in different contexts is not fully defined; beyond differential sensitivity to valency, do these isotypes respond in different ways to antigens displayed on membranes? Do they exhibit different sensitivity to antigen density or affinity? These questions are important, as is the more challenging task of identifying relevant bona fide self-antigens in vivo. The molecular mechanism that couples antigen recognition to IgM downregulation also needs to be more fully elucidated.

A second layer of control seems to reside in the very wiring of proximal BCR signal transduction; antigen-induced ITAM- and ITIM-dependent signaling are coupled, such that inhibitory phosphatases are activated in proportion to BCR stimulation. Interestingly, IgM-deficient B cells that express only IgD seem to be inert enough to self-antigen stimulation that they are less dependent upon inhibitory tone supplied by Lyn. However, the vast majority of mature Fo B cells have downregulated, but not eliminated IgM expression, and such clones appear to require both layers of control. Importantly, defects at this level may play a role in genetic susceptibility to lupus, and harnessing inhibitory coreceptors to reinforce or induce antigen-specific tolerance remains a promising therapeutic avenue.

Superimposed on this is another network of negative regulatory pathways that can be engaged or even upregulated in response to overwhelming self-reactivity. The most well-characterized among these is PTEN. How PTEN expression is regulated by chronic antigen

stimulation, and how dependent modestly self-reactive B cells are upon this pathway remains to be explored further. We propose that this 'third' layer of control (which represents the most well-recognized feature of clonal anergy, namely attenuation of BCR signal transduction) may be largely reserved for more highly self-reactive clones that exceed the buffering capacity of layers one and two.

Finally, some of the same molecular strategies that render Fo B cells dependent upon signal two also restrain survival of the most self-reactive clones in the periphery, gradually pruning these specificities from the mature repertoire. We showed recently that Nur77/*Nr4a1* provides a novel link between antigen stimulation and impaired survival, and may represent an exciting new druggable target to treat autoimmunity and modulate adaptive immune responses.

The Nr4a family may play functionally analogous roles in both T and B cells in response to chronic antigen stimulation. Indeed, recent work shows that the Nr4a family plays a role in down-modulating T cell responses to chronic antigen stimulation by engaging the exhaustion program in CD8 T cells and the 'anergy program' in CD4 T cells that receive signal one without co-stimulation[160,161]. This raises the possibility that Nr4a family members collectively may play additional undiscovered roles in B cell tolerance. To unmask redundancy among this three-member family, conditional elimination of multiple family members will be necessary.

How is tolerance breached in B cell-mediated autoimmune disease? A well-recognized possibility is inappropriate provision of signal two (TLR stimulation or T cell help). An important question is whether self-reactive, anergic B cells are the source of autoantibodies and/or provide help for pathogenic T cells in various autoimmune diseases, or whether relevant specificities are in fact generated de novo in the GC. Evidence in SLE and other rituximab-sensitive diseases like GPA suggest that extra-follicular SLPCs are the origin for at least some pathogenic autoAbs [158] (discussed by Sanz and colleagues elsewhere in this volume).

This reinforces the significance of clonal anergy and its breakdown for the origins of (some) autoimmune diseases. It also suggests that targeting self-reactive clones in the periphery is a valid strategy to treat or even prevent autoimmune disease, one borne out by the development of clinical BAFF antagonists for the management of patients with SLE. We propose that depletion of self-reactive B cells may also be accomplished by synthetic agonist ligands of Nur77 (or modulators of its interaction with Bcl2), and by inhibition of PI3K signaling. The latter may serve to reinforce anergy and may selectively inhibit survival of self-reactive clones; new work from Cambier and colleagues suggests that low dose p110δ inhibition does so not only in mice with reduced $PI(3,4,5)P_3$ phosphatase expression (as might be expected), but also in a B cell-dependent mouse model of diabetes[59].

Handling of self-reactivity in the peripheral B cell repertoire remains a point of incredible vulnerability as revealed by the prevalence of B cell-driven autoimmunity, ranging from diseases characterized by pathogenic autoantibodies (such as SLE), to those in which self-reactive B cells provide inappropriate help to pathogenic T cells (such as MS and T1D). To

intervene effectively in such diseases, a greater understanding of both normal tolerance and its dysregulation are essential. Continuing to explore the relevance of these pathways to human B cells will be critical to translate advances driven by the power and elegance of mouse genetics.

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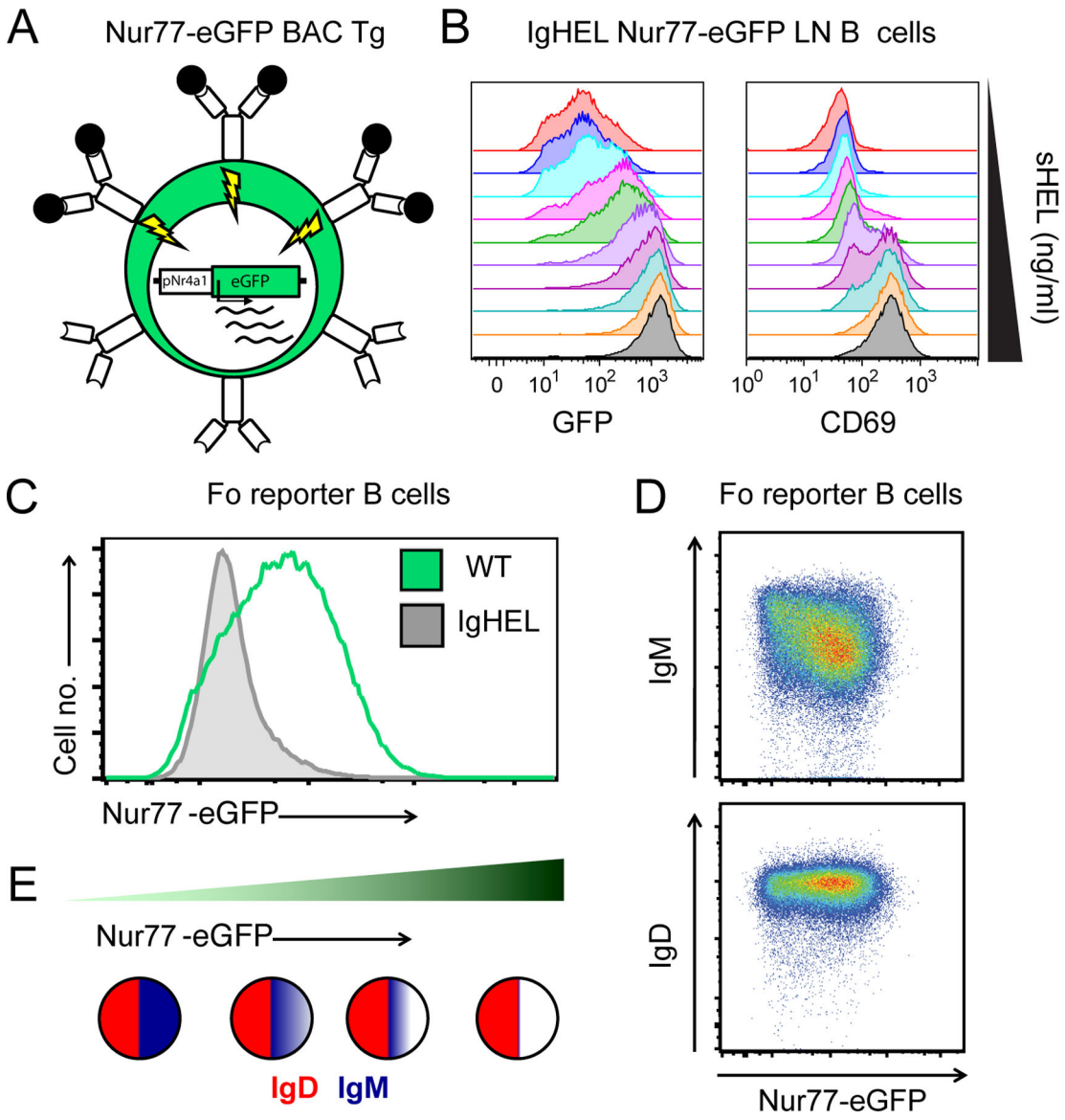

Figure 1. Nur77-eGFP BAC Tg reporter of antigen receptor signaling marks self-reactive B cells in vivo.

A. Schematic of Nur77-eGFP BAC Tg depicts eGFP transcript under the control of the regulatory region of Nr4a1. Since Nr4a1 is a primary response gene (PRG) that is rapidly transcribed in response to antigen receptor signaling, antigen encounter results in rapid GFP induction in reporter B cells.

B. IgHEL BCR Tg B cells harboring the Nur77-eGFP BAC Tg were stimulated in vitro with varying doses of soluble HEL antigen, and both GFP and CD69 expression were assessed via FACS after 24 hours.

C. Mature follicular (Fo) splenic B cells ($CD23^{HI}$CD93-B220+) from Nur77-eGFP BAC Tg mice with or without IgHEL BCR Tg (in the absence of cognate HEL Ag) were assessed for GFP expression via FACS immediately ex vivo. Restricting the BCR repertoire to restrict endogenous antigen recognition markedly reduces GFP expression.

D. Surface IgM and IgD BCR expression on mature Fo splenic B cells from Nur77-eGFP BAC Tg mice was assessed via FACS, revealing that IgM expression but not IgD expression is inversely correlated with GFP. Dynamic range of IgM across the repertoire is large, varying by more than an order of magnitude along a $\log_{10}$ scale.

E. Schematic depicts relative surface expression of IgM (blue) and IgD (red) BCRs on B cells with increasing expression of GFP (and therefore increasing self-reactivity) across the mature Fo B cell repertoire.

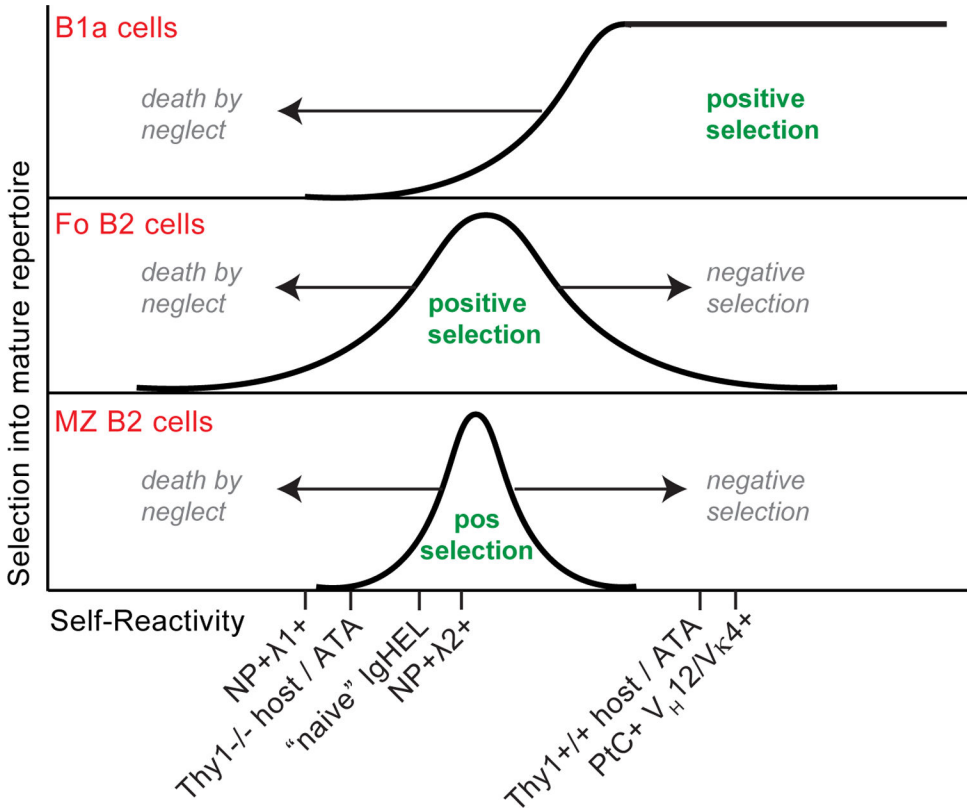

Figure 2. Model: Antigen-dependent signaling thresholds for positive and negative selection of mature B cell lineages.

Y-axis depicts efficiency of selecting individual BCRs of varying self-reactivity (x-axis) into B1a, Fo, and MZ mature B cell compartments. Area under each curve corresponds to range of BCRs that are positively selected into each compartment. Area to the left of each curve corresponds to BCRs that are counter-selected because of insufficient endogenous antigen recognition ("death by neglect"), while BCRs to the right of the curve are counter-selected because of excess self-reactivity (negative selection). Putative degree of self-reactivity and selection pressures of particular Tg BCR specificities is depicted. As previously described in the ATA model, antigen receptor signaling that normally drives negative selection of B2 cells is required for positive selection of B1 cells. Further, entry into the Fo B2 cell compartment is more permissive - at both the low and high end - than that for development of MZ B2 cells.

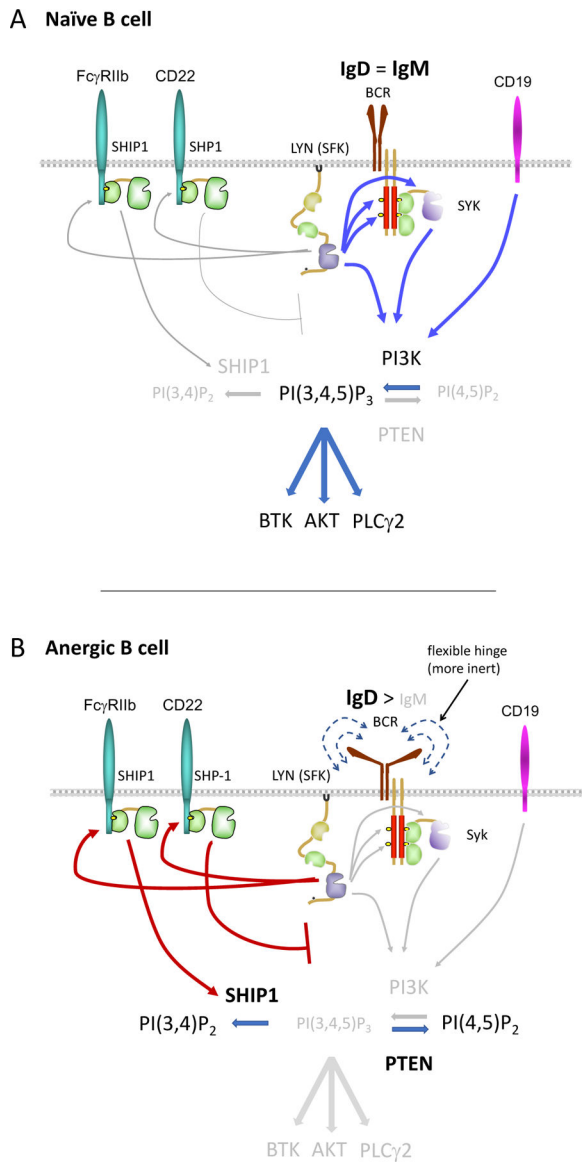

Figure 3. Model: Differences in antigen-dependent signal transduction between naïve and anergic B cells.

Schematic depicts differences in signal transduction between naïve (top) and anergic (bottom) B cells as gleaned from studies of BCR Tg models in mice. The extent of rewiring depends upon the specific BCR Tg and likely corresponds to avidity for self-antigen. Fo B cells express both IgM and IgD BCRs. While IgD expression is constant across the repertoire, IgM expression is downregulated in proportion to self-reactivity, such that the most anergic cells express much more IgD than IgM BCR on their surface. IgD BCR harbors a flexible 'hinge' domain that may render it less sensitive to endogenous antigens. In addition, IgD and IgM BCRs may exhibit differential association with co-stimulatory receptors (e.g. CD19) and inhibitory co-receptors (e.g. CD22). Increased engagement of inhibitory co-receptors by chronically antigen-stimulated anergic B cells results in activation of SHP-1 which represses proximal signal transduction, and of SHIP1 which hydrolyzes $PI(3,4,5)P_3$. Increased expression of PTEN in anergic B cells similarly depletes $PI(3,4,5)P_3$

in the plasma membrane. Collectively, reduced $PI(3,4,5)P_3$ levels in anergic B cells impairs recruitment of PH-domain containing downstream signaling molecules to the plasma membrane and their subsequent activation, including Btk, Akt, and PLCγ2, which couple proximal BCR signaling to NF-κB, mTORC1 complex, and calcium entry respectively.

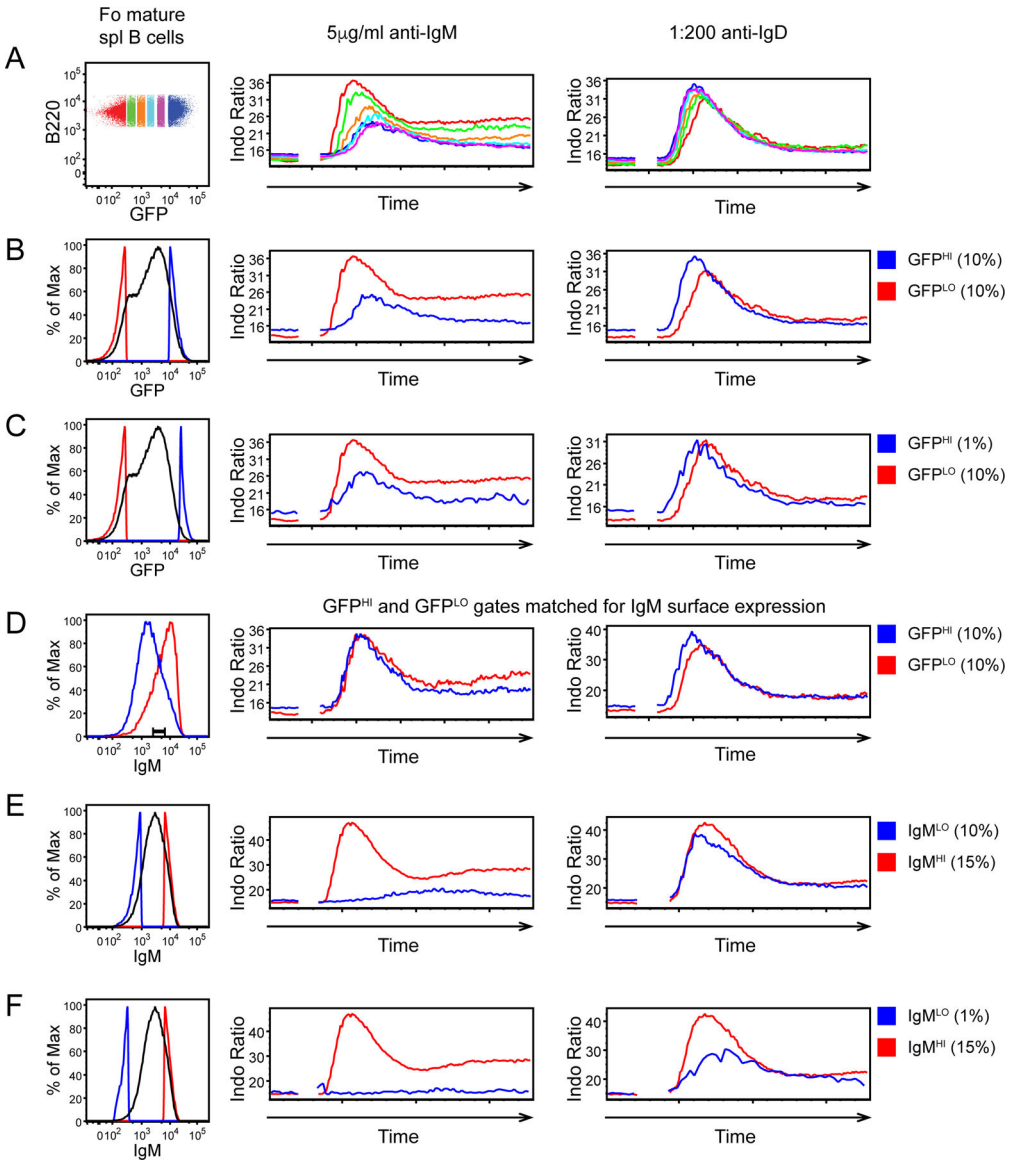

Figure 4. Most Fo B cells are constrained by IgM downregulation, while only a minor fraction exhibits rewiring of downstream signal transduction machinery.

Splenocytes were loaded with Indo-1 to detect intra-cellular calcium, and stained with B220, CD23, and CD93 to identify Fo mature B cells (B220+ $CD23^{HI}$ CD93-), and with anti-IgM $F(ab')_1$ to label surface IgM BCR without triggering a signal. After subsequent stimulation with either anti-IgM $F(ab')_2$ or anti-IgD, cells were collected by FACS for a total of 3 minutes. Indo-1 ratio is plotted to depict calcium entry into mature Fo B cells as a function of time. Left-hand histograms depict gates drawn either on the basis of Nur77-eGFP expression (A-C) or surface IgM expression (E, F) or both (C).

A. Fo mature B cells were gated on the basis of Nur77-eGFP along a continuum. Anti-IgM responses vary across this spectrum but IgD responses are invariant.

B, C. 10% lowest (GFP^{LO}) and either 10% (B) or 1% (C) highest (GFP^{HI}) gates are drawn. Anti-IgM responses are dampened in GFP^{HI} populations, but anti-IgD responses are not, even among the B cells with 1% highest GFP expression.

D. 10% GFP^{LO} and 10% GFP^{HI} populations as gated in (B), were further gated to identify cells with matched surface IgM expression. This completely corrected differences in response to anti-IgM stimulation.

E, F. Fo mature B cells were gated to identify those with 15% highest surface IgM expression and either 10% (E) or 1% (F) lowest IgM. Anti-IgM responses are almost undetectable in cells with extremely low IgM, but IgD responses are intact in 10% lowest IgM gate. Only the 1% lowest IgM gate contains cells that exhibit impaired responses to IgD, suggesting intrinsically attenuated signal transduction

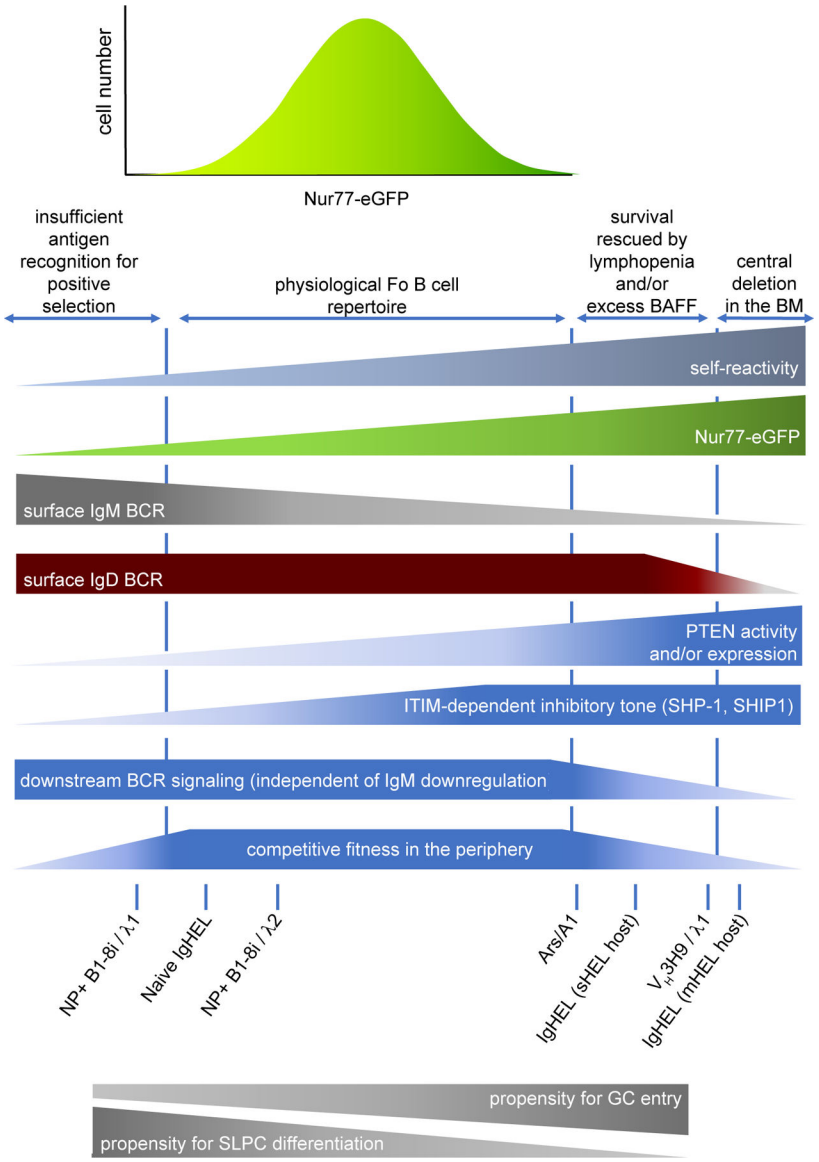

Figure 5. Model: Self-reactivity and peripheral tolerance mechanisms in mature Fo B cells exist along a continuum.

Schematic depicts, from top to bottom, distribution of functional self-reactivity in the normal mature Fo B cell repertoire as detected by Nur77-eGFP expression. At either end of this distribution lie BCR clones whose self-reactivity falls outside the thresholds for positive and negative selection. IgM but not IgD expression declines continuously with increasing self-reactivity. Among the most highly self-reactive clones (that do not typically populate the mature B cell repertoire) lie those that may downregulate both IgM and IgD BCRs. PTEN enzymatic activity and/or expression may increase continuously with increasing self-reactivity and serves to reduce $PI(3,4,5)P_3$ levels and downstream signal transduction. Increasing engagement and activation of ITIM-dependent inhibitory phosphatases is seen as chronic antigen receptor occupancy increases. Collectively, these strategies restrain activation of B cells in response to self-antigen, but only in the most highly self-reactive B cells is downstream signal transduction rewired such that it is attenuated in response to ex

vivo ligation of the IgD BCR. Examples of specific BCR transgenes with varying degrees of self-reactivity and engagement of varied tolerance mechanisms are depicted along with their putative location along this spectrum. Finally, propensity of B cells to differentiate into either short-lived PCs or to enter the GC varies such that anergic B cells are restrained from the former fate, but may have an advantage in entering the GC, where self-reactivity can be ‘redeemed’ and censored.

Table 1.

Regulation of Nur77/*Nr4a1* in B cells

Conclusion	Stimulus / perturbation	Pathway	Read-out	Cell type	Ref.
Does not modulate Nur77 at steady state in vivo	CD40L$^{-/-}$	CD40	Nur77-eGFP	B cells	24
	TLR7$^{-/-}$	TLR7	Nur77-eGFP	B cells	24
	Un93b1$^{3d/3d}$	TLR3/7/9	Nur77-eGFP	B cells	24
	MyD88$^{fl/fl}$MB1Cre	MyD88	Endogenous Nur77/ Nr4a1	PerC B1a cells, spleen	24
	Germ-free mice	MyD88/TRIF	Endogenous Nur77/ Nr4a1	Spl B cells, spleen	24
	Constit. active STAT5	JAK/STAT	Nur77-eGFP	thymocytes	21
Does not induce Nur77 in vitro	BAFF	BAFFR	Nur77-eGFP	B cells	15
	IL-4	JAK/STAT	Nur77-eGFP	B cells	24
	IL-2, IL-15	JAK/STAT	Nur77-eGFP	CD8 (IL-2, 15) CD4 (IL-2)	159
	CXCL12/SDF1	CXCR4	Nur77-eGFP	B cells	24
Induces Nur77 in vitro	LPS	TLR4 / Rp150	Nur77-eGFP	B cells	15,24
	CpG	TLR9	Nur77-eGFP	B cells	15,24
	PAM3CSK4	TLR1/2	Nur77-eGFP	B cells	24
	Anti-Igk; sHEL Ag	BCR	Nur77-eGFP	B cells	15,24
Modulates Nur77 at steady state in vivo	IgHEL BCR Tg	Ag / BCR	Nur77-eGFP	B cells	15,24
	IgHEL BCR Tg / sHEL	Ag / BCR	Nur77-eGFP	B cells	15,24
	Lyn−/−	BCR via ITIMs	Nur77-eGFP	B cells	24
	CD45 allelic series	BCR via SFKs	Nur77-eGFP	B cells	15