Endogenous antigen tunes the responsiveness of naive B cells but not T cells

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In humans, up to 75% of newly generated B cells and about 30% of mature B cells show some degree of autoreactivity⁴. Yet, how B cells establish and maintain tolerance in the face of autoantigen exposure during and after development is not certain. Studies of model B-cell antigen receptor (BCR) transgenic systems have highlighted the critical role of functional unresponsiveness or 'anergy'⁵,⁶. Unlike T cells, evidence suggests that receptor editing and anergy, rather than deletion, account for much of B-cell tolerance⁵,⁶, except in the case of strong Ag-specific B cells. However, it remains unclear whether the mature diverse B-cell repertoire of mice contains anergic autoreactive B cells, and if so, whether antigen was encountered during or after their development. By taking advantage of a reporter mouse in which BCR signalling rapidly and robustly induces green fluorescent protein expression under the control of the Nur77 regulatory region, antigen-dependent and antigen-independent BCR signalling events in vivo during B-cell maturation were visualized. Here we show that B cells encounter antigen during development in the spleen, and that this antigen exposure, in turn, tunes the responsiveness of BCR signalling in B cells at least partly by downmodulating expression of surface IgM but not IgD BCRs, and by modifying basal calcium levels. By contrast, no analogous process occurs in naïve mature T cells. Our data demonstrate not only that autoreactive B cells persist in the mature repertoire, but that functional unresponsiveness or anergy exists in the mature B-cell repertoire along a continuum, a fact that has long been suspected, but never yet shown. These results have important implications for understanding how tolerance in T and B cells is differently imposed, and how these processes might go awry in disease.

A new reporter of antigen receptor signalling was generated recently to examine developmental checkpoints during thymic development⁷. This took advantage of the dynamic expression pattern of the orphan nuclear hormone receptor Nur77 (also known as NR4A1), which is induced rapidly in response to negative selection and T-cell receptor (TCR) stimulation, to develop a green fluorescent protein (GFP) reporter bacterial artificial chromosome (BAC) transgenic line of mice⁸. Interestingly, Nur77 is also an immediate early gene that is rapidly transcriptionally upregulated in response to BCR signalling⁹. To visualize antigen receptor signalling in vivo, we obtained independently generated reporter mice from the Gene Expression Nervous System Atlas (GENSAT) consortium in which enhanced GFP (EGFP) expression is under the control of the Nur77 regulatory region⁹. The founders harboured two distinct insertion sites driving (EGFP) expression is under the control of the Nur77 regulatory region, independently generated reporter mice from the Gene Expression Nervous System Atlas (GENSAT) consortium in which enhanced GFP (EGFP) expression is under the control of the Nur77 regulatory region⁹. To visualize antigen receptor signalling in vivo, we obtained independently generated reporter mice from the Gene Expression Nervous System Atlas (GENSAT) consortium in which enhanced GFP (EGFP) expression is under the control of the Nur77 regulatory region⁹. The founders harboured two distinct insertion sites.

Basal expression of GFP in peripheral CD4⁺ and CD8⁺ T cells was higher in both the GFP⁺ and GFP⁺ lines compared to the reporter line described in ref. 6 (Supplementary Fig. 1a). Although basal GFP expression in B cells was substantially higher in the GFPHI line relative to the reporter used in ref. 6, the GFP⁺ line failed to express GFP in B cells, suggesting an isolated positional effect. For this reason, all subsequent B-cell studies have focused on the GFPHI reporter. After stimulation of thymocytes and peripheral T cells with phorbol myristate acetate (PMA) and/or ionomycin, GFP expression was rapidly induced (Supplementary Fig. 1b; data not shown). In vitro stimulation of either the TCR with anti-CD3 or the BCR with anti-IgM also induced GFP expression in a dose-dependent manner (Fig. 1a and Supplementary Fig. 1c; data not shown). GFPHI mice were crossed to the IgHEL BCR transgenic line (MD4; in which the immunoglobulin (Ig) receptor specifically recognizes hen egg lysozyme (HEL)) to generate mice with a monoclonal BCR repertoire. The resulting MD4–GFP mice showed dose-dependent GFP induction after treatment with HEL in vitro (Fig. 1b and Supplementary Fig. 1d).

To define which antigen-receptor-induced biochemical pathways were required to drive GFP expression, we treated anti-CD3- and anti-IgM-stimulated lymphocytes with a range of small-molecule inhibitors in vitro. These experiments showed a nearly complete dependence on Src family kinases in T cells and Syk kinase in B cells.

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To define whether signals other than antigen-receptor ligation were sufficient to drive GFP expression in B cells, we treated GFPHI B cells in vitro with various stimuli. Toll-like receptor (TLR)-4 and TLR9 ligands, along with anti-CD40, could drive GFP expression in B cells, but this effect was considerably less robust than anti-IGM stimulation (Supplementary Fig. 1g). Notably, B-cell activating factor (BAFF) treatment with doses as high as 200 ng ml⁻¹, sufficient to induce prolonged B-cell survival in vitro, failed to induce GFP-reporter expression in B cells (Supplementary Fig. 1g).

The reporter responded to TCR-dependent signalling in vivo, as shown by GFP expression at TCR-dependent checkpoints during thymic development. Signalling through the pre-TCR, comprised of a recombinated TCR-β chain and the invariant pre-TCR-γ chain, drives developing thymocytes to transit the β-selection checkpoint. We observed abrupt upregulation of GFP expression at the 'double-negative' DN3b stage of development, precisely at the β-selection checkpoint transition (Supplementary Fig. 2a).

After successful transit through the β-selection checkpoint, double-negative thymocytes upregulate the CD4 and CD8 coreceptors, and recombine the TCR-α chain to express a mature αβTCR. These cells then undergo TCR-dependent positive or negative selection. We observed marked GFP upregulation in post-selection CD69HI TCRβHI 'double-positive' thymocytes (Supplementary Fig. 2b), as found in ref. 6.

It has been speculated that, at the border of positive and negative selection, SP4⁺ thymocytes can be rescued from death by adopting the regulatory T-cell (Treg) fate. Indeed, CD25⁺ SP4⁺ thymocytes expressed much higher GFP levels than conventional SP4⁺ thymocytes, indicating that strong TCR signalling favours the Treg fate, in agreement with the results from ref. 6 (Supplementary Fig. 2c).

We reported that titration of CD45 expression in an allelic series of mice regulates TCR signalling during thymic development². We crossed the GFPHI reporter onto a genetic background harbouring two copies of the Lightning (L) CDMS (also known as Ptprc) allele, in which a point mutation in the extracellular domain leads to reduced surface expression of CD45 (15% of expression levels in wild-type mice)². Both the fraction of high-GFPexpressing cells and the average GFP content of post-selection double-positive thymocytes was markedly reduced in so-called L/L GFP mice (Supplementary Fig. 2d).

To identify analogous BCR-dependent signalling checkpoints during B-cell development, we assessed successive stages of bone marrow B-cell development in GFPHI reporter mice¹ (Fig. 2a and Supplementary Fig. 3a, b). We observed virtually no GFP expression except in the mature B cells that recirculate to the bone marrow (Hardy Fraction F; IgM⁺IgD⁺), indicating that GFP upregulation occurs sometime after the early bone marrow stages of development, despite evidence of the contribution of antigen encounter to deletion and receptor editing in the bone marrow².

Splenic B-cell development, which follows maturation in the bone marrow, is subdivided into successive transitional stages¹³–¹⁵. We observed a bimodal distribution of GFP expression among splenic B cells and found that early transitional B cells (T1) are largely GFP negative, but that later transitional stages (T2 and T3) contained a large proportion of GFP-positive B cells (Fig. 2b, c). Mature follicular B cells were mostly GFP positive and showed a broad distribution of GFP expression (Fig. 2c). Notably, a similar pattern of GFP expression, albeit at much lower levels, was evident in an independently generated GFP reporter (Supplementary Fig. 3c). GFP expression across these splenic developmental stages inversely correlated with surface IgM expression (Fig. 2d). Transitional B-cell stages have previously been subdivided into T2 and T3 stages on the basis of surface IgM expression.
downregulation\(^\text{13}\) (Fig. 2e). We observed that GFP upregulation seems to occur at precisely this transition between the T2 and T3 stages (Fig. 2e and Supplementary Fig. 3d, e).

Interestingly, in vitro BCR stimulation of bone marrow and splenic B-cell subsets resulted in GFP upregulation to differing extents. Minimal GFP upregulation was seen in bone marrow immature and transitional stages, but robust upregulation was evident in splenic T1, T2 and follicular B cells (Supplementary Fig. 4). This indicates that splenic, but not bone marrow, subsets have the capacity to upregulate GFP.

To determine whether the amount of GFP expression in unstimulated B cells reflected BCR signal strength/antigen exposure, we took advantage of our previously characterized allelic series of CD45-expressing mice\(^{10,16}\). In these animals, CD45 expression is genetically varied across a broad range and correlates with BCR signal strength\(^{16}\).

"Notably, the distribution of GFP expression in this compartment varied across a broad range and correlates with BCR signal strength\(^{16}\)."

"...we noted that GFP expression at the T1 stage was unaffected, whereas increasing CD45 expression resulted in a higher proportion of GFP-positive B cells at the T2 stage (Fig. 3a and Supplementary Fig. 5a). Notably, the distribution of GFP expression in this compartment remained bimodal, further supporting the notion that a discrete signalling event occurs at this stage, the threshold of which is regulated by CD45 and BCR signal strength. GFP expression in follicular mature B cells was markedly reduced in L/L mice, consistent with a reduction in BCR signal strength, but was minimally altered in H/− mice with higher CD45 expression (Fig. 3a and Supplementary Fig. 5b). However, modulation of GFP expression by CD45 was much more apparent in the marginal zone compartment, suggesting an exquisite sensitivity to BCR signal strength (Fig. 3a and Supplementary Fig. 5b).

As Nur77-GFP expression is regulated by modulation of BCR signal strength (Fig. 3a and Supplementary Fig. 5b), we proposed that endogenous antigen exposure might drive BCR signalling during maturation of wild-type B cells with a diverse repertoire. To explore this possibility, we took advantage of the IgHEL-soluble (s)HEL double-transgenic system (MD4/ML5), in which MD4 mice with a monoclonal IgHEL BCR can be studied in the presence or absence of sHEL\(^2\). In the Nur77-GFP reporter mice with the IgHEL BCR transgene-restricted repertoire in the absence of antigen, we observed a marked reduction in GFP in splenic B cells (Fig. 3b and Supplementary Fig. 5b, c). Notably, the bimodal distribution of GFP expression observed in the context of a wild-type repertoire was lost in these mice. Further increasing CD45 expression in the context of such a restricted repertoire to increase tonic BCR signalling resulted in increasing GFP expression, but again only in a unimodal rather than a bimodal distribution (Fig. 3b and Supplementary Fig. 5c). Finally, the introduction of sHEL ligand by crossing ML5 (sHEL transgenic) mice to IgHEL transgenic reporter mice resulted in increased GFP expression as expected, and remarkably reconstituted bimodal GFP expression in the transitional splenic stages of development (Fig. 3c and Supplementary Figs 5d and 6). These data indicate that normal B-cell development is characterized by a wide range of antigen experience, and that Nur77-driven GFP distribution in follicular mature B cells serves as a marker of such exposure.

To determine whether antigen recognition during splenic B-cell development had functional effects on signalling, we selectively gated on the extremes of GFP expression. We observed that high-GFP-expressing B cells had dampened 40S ribosomal protein S6 (RP56) phosphorylation (a PI(3)K-dependent event) and calcium entry relative to low-GFP-expressing B cells in response to IgM ligation (Supplementary Figs 4a and 7a). Moreover, we observed that basal calcium levels were elevated in high-GFP-expressing B cells, reminiscent of anergic B cells identified in various model BCR transgenic systems\(^{17,18}\). Dampened inducible signalling and increased basal calcium were not isolated properties of very-high-GFP-expressing B cells, but rather seemed to represent continuous functional properties across the entire spectrum of GFP expression of mature follicular B cells (Fig. 4a).

Furthermore, restricting the BCR repertoire in the absence of ligand ablated differences in functional responsiveness, but not in basal calcium (Supplementary Fig. 7b). Notably, neither inducible calcium responses nor basal calcium levels correlated with GFP expression in naïve CD25\(^+\) CD4\(^+\) T cells, indicating that only in B cells does antigen exposure tune functional responsiveness (Supplementary Fig. 8a).

Mature B cells express two isotypes of the BCR, IgM and IgD. We wanted to determine whether the functional responsiveness in GFP B cells was modulated in response to stimulation through the IgD BCR in the same manner as it is to the IgM BCR. We found that this was not the case (Fig. 4b, c); responsiveness to IgM BCR stimulation was markedly blunted in cells with high GFP expression, whereas IgD responsiveness remained intact. Stimulation with anti-κ antibodies to ligate both surface IgM and IgD resembled isolated IgD stimulation (Supplementary Fig. 8b). By simultaneously staining for surface IgM expression with a nonstimulatory monovalent Fab fragment and assessing calcium responses in GFP B cells, we show that differences in surface IgM expression largely accounted for the functional differences at different levels of GFP expression (Supplementary Fig. 8c). However, basal calcium differences were independent of surface IgM expression (Supplementary Fig. 8d).

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**Figure 3** | Expression of the Nur77-GFP BAC transgenic reporter is sensitive to genetic modulation of BCR signal strength and to antigen.

**a.** CD45 allelic series (low to high CD45 expression: L/L, L/+ , +/+ and H/−) GFP\(^{13}\) transgenic splenic B cells were stained to identify B-cell subsets as in Fig. 2b, c. Overlaid histograms represent GFP expression in splenic subsets as gated in Supplementary Fig. 5a, b. CD45\(^{+/+}\) GFP\(^{13}\) transgenic and H/− GFP\(^{13}\) transgenic splenic B cells with an unrestricted (no IgHEL transgene; IgHEL\(^−\)) or restricted (IgHEL\(^+\)) repertoire in the absence of SHEL antigen were analysed as in a. Overlaid histograms represent GFP expression in splenic subsets as gated in Supplementary Fig. 5c. c, d. CD45\(^{+/+}\) GFP\(^{13}\) transgenic splenic B cells with an unrestricted or restricted repertoire in the presence or absence of sHEL antigen were analysed as in a. Overlaid histograms represent GFP expression in splenic subsets as gated in Supplementary Fig. 5d. All animals in these experiments were generated through genetic crosses. All data are representative of at least five independent experiments.
Additional characteristics of monoclonal BCR transgenic models of anergic B cells include a failure to upregulate activation markers in response to various stimuli. We stimulated sorted high- and low-GFP-expressing B cells and observed that activation marker upregulation in response to IgM stimulation is impaired in high-GFP-expressing B cells (Supplementary Figs 9, 10). Importantly, responses to lipopolysaccharide (LPS) and CD40 were unaffected, as was in vitro survival in the presence or absence of BAFF (data not shown).

Finally, to determine directly whether the BCR repertoire of mature B cells with high GFP expression and impaired functional responses was indeed autoreactive, sorted high- and low-GFP-expressing B cells were polyclonally stimulated in vitro with LPS, and secreted antibody was assessed for anti-nuclear antibody (ANA) reactivity. (Fig. 4d, e and Supplementary Fig. 9). Notably, neither cell proliferation nor antibody secretion following LPS stimulation were impaired in high-GFP B cells (data not shown). We found a significant increase in ANA reactivity, suggesting auto- or polyreactivity in the repertoire of such naturally occurring anergic B cells (Fig. 4d, e).

The human B-cell repertoire is characterized by a high prevalence of polyreactive and autoreactive BCRs,1.9. Anergy or functional unresponsiveness may serve to keep such autoreactive clones in check. Array data have shown that wild-type B cells have an intermediate phenotype between antigen-naïve and anergic B cells, suggesting the possible presence of anergic B cells in the wild-type mature repertoire.20,21. It has recently been argued that the so-called T3 splenic subset may in fact represent sequestered anergic B cells rather than an intermediate developmental stage.22,23 However, the prevalence of anergy in the normal mature B-cell repertoire has not been clear.23,24 We show that there is a continuum of anergy or unresponsiveness to anti-IgM stimulation in the mature B-cell compartment, and that this responsiveness is, in turn, tuned by developmental antigen recognition.

It has long been observed that marked IgM downregulation is seen in BCR transgenic systems in the presence of either antigen or enhanced BCR signal strength.21,16,19,24,26 IgD, by contrast, remains relatively unmodulated in these systems. Here, we show that, in the wild-type B-cell repertoire, IgM downregulation correlates with the extent of antigen recognition during development and accounts for dampened B-cell responses to anti-IgM stimulation, whereas IgD expression and responses are intact. We suggest that this constitutes a general mechanism to modulate BCR signalling in autoreactive B cells, but permits them to persist as a pool of extended antibody specificity for purposes of protective immunity. Indeed, we demonstrate an increased proportion of ANA-reactive BCR specificities in high-GFP-expressing B cells, suggesting that these cells are auto- or polyreactive. It is tempting to speculate that this large reservoir of dormant autoreactive B cells in the mature BCR repertoire may serve as the source of pathogenic autoantibodies that characterize rheumatic diseases such as systemic lupus erythematosus.

METHODS SUMMARY

The following mouse strains have been previously described: the CD45 allelic series including Lightning (L/L), H/HE (HE) mice,16,19,27,35. IgHEL (MD4) and sHEL (ML5) mice.16,19,27. Nurr77–EGFP BAC transgenic mice were obtained from the GENSAT consortium.16,19,27 Nurr77–EGFP reporter mice described in ref. 6 were supplied by the Hoggist laboratory. All strains were backcrossed to the C57BL/6 genetic background at least six generations and were maintained in the University of California, San Francisco animal facility in accordance with institutional regulations. In vitro lymphocyte-stimulation assays were performed as previously described on plates containing either soluble anti-IgM Fab2, precoated with anti-CD3, and/or containing various stimuli and inhibitors.28 Calcium assays were performed as previously described,29 except that Indo-1 dye (Invitrogen) was used to load cells, and an ultraviolet laser on the BD Fortessa was used for detection. Intracellular phospho-S6 staining and stimulation was performed as previously described.30 Sorting of GFP-high and low-expressing B cells using a MoFlo cell sorter was performed as follows: splenic and lymph node cells were pooled and stained to identify DAPI (4',6-diamidino-2-phenylindole)–CD23+ AA4.1 mature B cells. The highest and lowest 15% of GFP-expressing B cells were retrieved and were incubated with varying stimuli. Sorted cells were plated at a concentration of 1.5 × 10^6 cells per ml in complete DMEM media and were stimulated with anti-IgM Fab2 at varying doses for 16 h to assess activation marker upregulation. Alternatively, sorted cells were incubated with 10 μg ml^-1 LPS at a concentration of 6 × 10^6 cells per ml in complete DMEM to drive polyclonal antibody secretion. Supernatants were then collected and subjected to enzymelinked immunosorbent assay (ELISA). The assay to detect total IgM was
performed as previously described. The ANA ELISA kit obtained from Inova Inc. was used as per manufacturer’s instructions.

**Full Methods** and any associated references are available in the online version of the paper.

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1. Wardemann, H. et al. Predominant autoantibody production by early human B cell precursors. *Science* **301**, 1374–1377 (2003).
2. Goodnow, C. C. et al. Altered immunoglobulin expression and functional silencing of self-reactive B lymphocytes in transgenic mice. *Nature* **334**, 676–682 (1988).
3. Gambier, J. C., Gauld, S. B., Merrell, K. T. & Vilen, B. J. B-cell anergy: from transgenic models to naturally occurring anergic B cells? *Nature Rev. Immunol.* **7**, 633–643 (2007).
4. Lang, J. et al. Enforced Bcl-2 expression inhibits antigen-mediated clonal elimination of peripheral B cells in an antigen dose-dependent manner and promotes receptor editing in autoreactive, immature B cells. *J. Exp. Med.* **186**, 1513–1522 (1997).
5. Halverson, R., Torres, R. & Pelanda, R. Receptor editing is the main mechanism of B cell tolerance toward membrane antigens. *Nature Immunol.* **5**, 645–650 (2004).
6. Moran, A. E. et al. T cell receptor signal strength in Treg and iNKT cell development demonstrated by a novel fluorescent reporter mouse. *J. Exp. Med.* **208**, 1279–1289 (2011).
7. Winoto, A. & Littman, D. R. Nuclear hormone receptors in T lymphocytes. *Nature Rev. Immunol.* **7**, 953–962 (2006).
8. Mittelstadt, P. R. & DeFranco, A. L. Induction of early response genes by cross-linking membrane Ig on B lymphocytes. *J. Immunol.* **150**, 4822–4832 (1993).
9. The Gene Expression Nervous System Atlas (GENSAT) Project. NINDS Contract #N01NS02331 to The Rockefeller University. http://www.gensat.org/index.html (New York, USA).
10. Zikherman, J. et al. CD45–Csk phosphatase–kinase titration uncouples basal and inducible T cell receptor signaling during thymic development. *Immunity* **32**, 342–354 (2010).
11. Hardy, R. R., Carmack, C. E., Shinton, S. A., Kemp, J. D. & Hayakawa, K. Resolution and characterization of pro-B and pre-pro-B cell stages in normal mouse bone marrow. *J. Exp. Med.* **173**, 1213–1225 (1991).
12. Goodnow, C. C., Sprent, J., Fazekas de St Groth, B. & Vinuesa, C. G. Cellular and genetic mechanisms of self tolerance and autoimmunity. *Nature* **435**, 590–597 (2005).
13. Loder, F. et al. B cell development in the spleen takes place in discrete steps and is determined by the quality of B cell receptor-derived signals. *J. Exp. Med.* **190**, 75–90 (1999).
14. Chung, J. B., Silverman, M. & Monroe, J. G. Transitional B cells: step by step towards immune competence. *Trends Immunol.* **24**, 342–349 (2003).
15. Allman, D. et al. Resolution of nonproliferative immature splenic B cell subsets reveals multiple selection points during peripheral B cell maturation. *J. Immunol.* **167**, 6834–6840 (2001).
16. Zikherman, J., Doan, K., Parameswaran, R., Raschke, W. & Weiss, A. Quantitative differences in CD45 expression unmask functions for CD45 in B-cell development, tolerance, and survival. *Proc. Natl Acad. Sci. USA* **109**, E3–E12 (2012).
METHODS

Mice. The CD45 allelic series including Lightning (L/L) and H/H, H/H (HE) mice have been previously described\(^{10,16,26,27}\), as have IgHEL (MD4) and sHEL (ML5) mice\(^2\). Nur77–GFP reporter mice were obtained from the GENSAT consortium\(^9\). Nur77–GFP reporter mice described in ref. 6 were supplied by the Hogquist laboratory. All strains were backcrossed to the C57BL/6 genetic background at least six generations. Mice were used for all functional and biochemical experiments at age 5–9 weeks. All mice were housed in a specific pathogen-free facility at University of California, San Francisco in accordance with the University’s Animal Care Committee and National Institutes of Health guidelines.

**Antibodies and other reagents.** The following antibodies were used: antibodies to murine CD1d, CD4, CD5, CD8, CD11b, CD11c, CD19, CD21, CD23, CD24, CD25, CD43, CD44, CD69, CD93 (AA4.1), BP-1, IgD, IgM, pNK, γ/δTCR and TCR-β were conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyll protein complex (PerCP)-Cy5.5, PE-Cy5.5, PE-Cy7, Pacific blue, allophtococyanin (APC) or Alexa647 for fluorescence-activated cell sorting (FACS) staining (eBiosciences or BD Biosciences), phospho-S6 Alexa488 (2E9) antibody for intracellular staining, unconjugated CD3ε (2C11) antibody (Harlan), goat anti-Armenian hamster immunoglobulin (H+L), goat anti-mouse IgM Fab’2 for stimulation and Fab fragment coupled to Alexa647 for surface staining (Jackson ImmunoResearch), streptavidin–horseradish peroxidise (HRP) conjugate (1:4,000) were used for detection in both assays for signal amplification instructions. Biotinylated anti-mouse IgM (1:5,000) and streptavidin–HRP conjugate (1:4,000) were used for detection in both assays for signal amplification instructions. Slow kinetic tetramethylbenzidine (Sigma) was used as substrate. Molecular devices SpectraMax and SoftMax Pro software were used to read plates. ANA IgM quantification was normalized to total IgM for each sample.

In vitro lymphocyte stimulation (+/- inhibitor). Single cell suspensions of lymphocytes were plated at a concentration of 1.5 × 10^6 cells per ml in complete DMEM and were incubated in the presence of various stimuli and/or inhibitors at the doses described above for 16 h. Assays were performed as previously described\(^{26}\). Calcium measurements. Assays were performed as previously described\(^{26}\), except that Indo-1 dye (Invitrogen) was used to load cells, and an ultraviolet laser on the BD Fortessa was used for detection. Before stimulation and analysis, splenocytes were surface stained for expression of CD23 and AA4.1 to identify B-cell subsets. Where noted, cells were also pre-stained with anti-IgM Fab fragments to identify surface IgM expression without inducing BCR stimulation. Stimulation was carried out using either varying doses of anti-IgM Fab’2, anti-κ antibody or biotinylated anti-IgD followed by streptavidin crosslinking (15 μg ml\(^{-1}\)), or varying doses of anti-CD3ε followed by goat anti-Armenian hamster immunoglobulin crosslinking (50 μg ml\(^{-1}\)). Intracellular phospho-S6 staining. Staining and stimulation was performed as previously described\(^{26}\). B-cell sorting and stimulation. GFP-high- and -low-expressing B cells were sorted using a MoFlo cell sorter. Spleen and lymph node cells were pooled and stained for CD23 and AA4.1 as well as DAPI (4’,6-diamidino-2-phenylindole) to identify CD23^+ AA4.1^- mature B cells. The 15% highest and lowest GFP-expressing B cells were retrieved and incubated with varying stimuli. Sorted cells were plated at a concentration of 1.5 × 10^6 cells per ml in complete DMEM and were stimulated with anti-IgM Fab’2 at varying doses for 16 h. Cells were then stained for CD69 expression in order to assess activation-marker upregulation. Alternatively, sorted cells were incubated with 10 μg ml\(^{-1}\) LPS at a concentration of 6 × 10^6 cells per ml in complete DMEM media in order to drive polyclonal antibody secretion. Supernatants were then collected and subjected to ELISA.

**ELISA.** The ELISA to detect total IgM was performed as previously described\(^{26}\). The ANA ELISA kit obtained from Inova, Inc. was used as per manufacturer’s instructions. Biotinylated anti-mouse IgM (1:5,000) and streptavidin–HRP conjugate (1:4,000) were used for detection in both assays for signal amplification (Southern Biotech), and slow kinetic tetramethylbenzidine (Sigma) was used as substrate. Molecular devices SpectraMax and SoftMax Pro software were used to read plates. ANA IgM quantification was normalized to total IgM for each sample.