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BIOLOGICAL SCIENCES

The B cell antigen receptor integrates adaptive and innate immune signals

Short title: BCR expression licenses B cell mitogenic responses (50 characters with spaces)

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ABSTRACT

B cells respond to antigens by engagement of their B cell antigen receptor (BCR) and of co-receptors through which signals from helper T cells or pathogen-associated molecular patterns are delivered. We show that the proliferative response of B cells to the latter stimuli is controlled by BCR-dependent activation of phosphoinositidyl 3-kinase (PI-3K) signaling. Glycogen synthase kinase 3 β and Foxo1 are two PI-3K-regulated targets that play important roles but to different extents depending on the specific mitogen. These results suggest a model for integrating signals from the innate and the adaptive immune systems in the control of the B cell immune response.

SIGNIFICANCE

Clonal expansion of antigen-specific B cells during an immune response is necessary for effective antibody production. B cells must integrate signals from their clonally-restricted B cell antigen receptor (BCR) and from non-clonal co-receptors that provide contextual cues to the nature of the antigen. How this is accomplished is unclear. We found that B cells require expression of the BCR for mitogenic responses triggered by co-receptors that recognize innate or T cell-derived signals. The signaling pathway used by the BCR to license co-receptor-induced proliferation is similar to the previously described BCR-dependent survival signal and, thus, couples B cell survival and mitogenic responses in an unexpected manner that may have implications for B cell lymphomagenesis.

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INTRODUCTION

Expression of a functional B cell antigen receptor (BCR) is of central importance to nearly every aspect of B cell biology. During early development, B-lineage cells assemble clonally restricted immunoglobulin (Ig) heavy and light chains that are able to recognize a diverse and unpredictable universe of antigens. The proper assembly and expression of a BCR is a prerequisite for entry into the naïve compartment. Furthermore, naïve resting B cells remain dependent on the expression of a correctly assembled BCR. If this is prevented by IgH chain ablation or Ig α truncation, the cells rapidly undergo apoptosis. This has been interpreted to reflect a BCR-dependent ‘tonic’ survival signal, which has recently been shown to involve phosphoinositidyl 3-kinase (PI-3K) activity and subsequent inactivation of the Foxo1 transcription factor (1-3).

As a consequence of the large clonally restricted repertoire generated during development, only a small number of newly generated B cells entering the peripheral mature compartment express a BCR that is able to bind a given antigen. During the early stages of an immune response, antigen-specific B cells proliferate extensively and increase the number of cells that can respond appropriately. The magnitude, duration and quality of the B cell response is guided by signals from co-receptors expressed by B cells such as Toll-like receptors (TLRs) and CD40, which provide information to the B cell about the context of the antigen in T cell-independent and -dependent responses (4, 5). Given that the *raison d’être* of B cells is an antigen-specific response including the production of antibodies, a central role for the BCR in regulating B cell responses to innate or T cell-derived signals seems likely. Indeed, early experiments addressing the *in vitro* response of

mouse B cells to bacterial lipopolysaccharide (LPS) have suggested cooperation of LPS stimulation and BCR engagement in the proliferative B cell response under certain conditions (6, 7). More recent work has uncovered a synergy between TLR and BCR in the control of class-switch recombination in activated B cells (8) and the role of the BCR in the delivery of DNA- or RNA-associated autoantigens to endosomal TLRs in autoimmunity is well established (9). How B cells integrate signals from the BCR and from co-receptors responding to mitogenic stimuli in the induction of the proliferative response is unclear though.

RESULTS AND DISCUSSION

Generation of resting BCR-negative B cells. We generated resting B cells that lack expression of the BCR by transducing splenic B cells from *bcl-2tg* mice that express a loxP-flanked pre-rearranged variable domain targeted into the *Igh* locus (*BI-8f*) (2) with cell-permeable Cre recombinase, TAT-Cre (10). The transduced cells remained alive throughout the culture due to ectopic expression of Bcl-2 and lost BCR expression by day 3 in culture with an efficiency of 30–50%, allowing simultaneous analysis of B cells that either express or lack the BCR (BCR^{pos} and BCR^{neg} B cells, respectively; Fig. 1A).

Western blot analysis of sorted BCR^{pos} and BCR^{neg} B cells confirmed the loss of IgH chain expression in the latter (Fig. 1B). The proportion of BCR^{pos} and BCR^{neg} B cells remained constant for at least 2 weeks, suggesting that the two classes of cells survive equally well *in vitro* (Fig. S1). Although mature BCR^{neg} cells are slightly smaller than their BCR^{pos} counterparts and downregulate some mature B cell markers (e.g. MHC class II and CD22), they largely maintain the surface phenotype of resting, mature B cells (Fig. 1C).

BCR expression is necessary for the mitogenic response of B cells. B cells express receptors such as TLR-9, TLR-4 and CD40 that induce activation and proliferation when triggered by natural ligands or agonist antibodies (11-13). We tested the proliferative response of BCR^{pos} and BCR^{neg} B cells to CpG DNA, LPS or anti-CD40 and found that, in contrast to BCR^{pos} B cells, BCR^{neg} B cells did not proliferate in response to any of these stimuli (Fig. 2A). This defect is not an artifact of protein transduction since *bcl-2tg* BCR^{neg} B cells generated by a tamoxifen-inducible Cre (14) or *in vivo* with a mature B cell-specific Cre-transgene (*CD21-Cre*) (1) showed the same proliferation defect (Fig. S2).

BCR^{neg} B cells are not completely refractory to the mitogenic stimuli, since they activate normal NF-κB DNA binding activity in response to CpG (Fig. S3) and upregulate CD69 and MHC class II when stimulated with CpG DNA, LPS or anti-CD40 (Fig. 2B). Taken together, these data show that mature B cells that have lost expression of the BCR are responsive to innate stimuli but are unable to proliferate.

BCR expression licenses B cell proliferation through PI-3K activation. Previous studies demonstrated that constitutive, low-level signaling, a so-called ‘tonic’ signal, which is dependent on an intact BCR, is necessary for mature B cell survival (1, 2) and is mediated by the PI-3K/Akt/Foxo1 signaling pathway (3). The requirement of BCR expression for mitogenic responses of B cells to innate stimuli raised the possibility that the same ‘tonic’ signal may also regulate B cell proliferation. BCR^{neg} B cells generated *in vivo* with a mature B cell-specific Cre-transgene (i.e. *CD21-Cre*) (1) were used to study Akt status *in vivo*. Indeed, while intracellular staining for phospho-Akt (Ser473) of CpG-treated BCR^{pos} B cells showed prolonged activation of the PI-3K pathway, induced Akt phosphorylation was not seen in BCR^{neg} B cells under these conditions (Fig. 3A). This was confirmed by Western blot analysis of sorted BCR^{pos} and BCR^{neg} B cells cultured with or without CpG-containing medium for 2 hours (Fig. 3B). We conclude that expression of the BCR as mediated by the IgH chain is required for mitogen-induced activation of the PI-3K pathway in response to CpG stimulation. To further test the role of BCR-dependent PI3K signaling in regulating mitogen-induced proliferation of B cells, we isolated BCR^{neg} B cells from *B1-8f*, *CD21-Cre* mice that also carried a transgene for an active PI-3K molecule that was induced by Cre activity (*B1-8f*, *CD21-Cre*, *P110**) (3). Expression of Cre in mature B cells simultaneously ablates expression of the BCR and induces expression of constitutively active PI-3K, which substitutes for the BCR-dependent

survival signal (3). Similar to control BCR^{pos} B cells, BCR^{neg} B cells expressing constitutively active PI-3K proliferated in response to all of the stimuli (Fig. 3C), showing that reconstitution of the PI-3K pathway in BCR^{neg} B cells licenses mitogenic responses to innate stimuli.

Inactivation of glycogen synthase kinase (GSK) 3 β is necessary for the proliferative mitogenic response of BCR^{neg} B cells to CpG. Next, we investigated the BCR-dependent signaling pathways downstream of PI-3K as required for mitogenic responses of B cells. PI-3K/Akt activates several downstream signaling pathways including pathways utilizing GSK3 β and Foxo1. GSK3 β is a constitutively active serine/threonine kinase that inhibits proliferation by phosphorylating and targeting for degradation proteins that are necessary for mitosis, including c-Myc (15) and D-type cyclins (16). Mitogenic signals activate the PI-3K/Akt pathway, leading to phosphorylation and inactivation of GSK3 β (17), thereby allowing accumulation of cell cycle regulated proteins. Consistent with defective Akt phosphorylation in BCR^{neg} B cells, the levels of phospho-GSK3 β remained low in BCR^{neg} B cells, in contrast to BCR^{pos} B cells, after treatment with CpG (Fig. 4A), suggesting that GSK3 β remained active in BCR^{neg} B cells. To directly assess whether GSK3 β activity prevents the proliferation of mitogen-treated BCR^{neg} B cells, we added the GSK3 β inhibitor BIO (18) to CpG-stimulated cultures and found that inhibition of GSK3 β partially restores the ability of *bcl-2tg* BCR^{neg} B cells to proliferate in response to CpG (Fig. 4B). We also found that CpG-induced c-Myc protein levels were much lower in BCR^{neg} B cells than those seen in BCR^{pos} B cells treated in a similar manner (Fig. 4C). Similar results were seen when LPS or anti-CD40/IL-4 were used as mitogens (Fig. 4C). In contrast, mitogen-induced c-Myc mRNA levels were nearly identical between BCR^{pos} and BCR^{neg} B cells stimulated with CpG (Fig. S4), suggesting that active GSK3 β was

destabilizing c-Myc protein in BCR^{neg} B cells (15). Unfortunately, pharmacological inhibition of GSK3 β activity using BIO, which partially restores proliferation in response to CpG (Fig. 4B), did not substantially restore mitogen-induced c-Myc protein expression in BCR^{neg} B cells (Fig. 4D). Therefore, we conclude that the decreased levels of c-Myc induction in BCR^{neg} B cells, while of potential interest, do not explain the defective proliferation of B cells that have lost expression of their BCR. Furthermore, inhibition of GSK3 β did not rescue proliferation of BCR^{neg} B cells in response to LPS or anti-CD40/IL-4 treatment (Fig. 4E), suggesting that BCR-dependent PI-3K activation regulates additional signaling pathways required for mitogenic responses, depending on the stimulus.

Foxo1 and GSK3 β regulate innate stimuli-induced mitogenic responses downstream of the BCR. Inactivation of the transcription factor Foxo1 by BCR-dependent PI-3K/Akt activation is necessary for mature B cell survival (3). The complete loss of Foxo1 rescues the survival of BCR^{neg} B cells as efficiently as expression of active PI-3K, whereas loss of one Foxo1 allele is sufficient for a partial rescue of BCR^{neg} B cells (3). Since Foxo1 remains active in BCR^{neg} B cells (3) and can inhibit proliferation by up-regulating anti-proliferative genes (19, 20), we tested the effect of Foxo1 ablation on mitogen-treated BCR^{neg} B cells. BCR^{neg} B cells that were either heterozygous or homozygous for a Foxo1-null allele were sorted, and their proliferative responses in the absence or presence of BIO to inhibit GSK3 β were compared to mitogen-stimulated BCR^{pos} cells (Fig. 5). While the complete or partial loss of Foxo1 had only minimal effects on the proliferative response of BCR^{neg} B cells to CpG, the combination of Foxo1 deletion and inhibition of GSK3 β rescued the proliferation of BCR^{neg} B cells in response to CpG to a level comparable to that observed by CpG-stimulated BCR^{pos} B cells. In contrast, while heterozygous deletion

of Foxo1 was sufficient for some anti-CD40/IL-4-treated BCR^{neg} B cells to undergo at least one cell division, homozygous deletion of Foxo1 allowed near-complete rescue of BCR^{neg} proliferation to anti-CD40/IL-4. In both cases, inhibition of GSK3 β had little additional effect, suggesting that different mitogens require PI-3K to activate different signaling pathways for proliferation in the absence of BCR expression (Fig. 5). To further illustrate this point, BCR^{neg} B cells on a Foxo1-null background in the presence of a GSK3 β inhibitor remained unable to proliferate in response to LPS treatment (Fig. 5), suggesting that other or additional pathways downstream of PI-3K/Akt that regulate cell proliferation are involved.

Our experimental model is based on previous evidence that the BCR expressed by resting, mature B cells provides ‘tonic’ survival signals to the cells (1, 2). The mechanism by which such signals are initiated is controversial, but may involve low-affinity interactions of the BCR with environmental antigens, akin to the positive selection of T cells by self-MHC. It is also possible, however, that loss of the BCR induces a danger signal in the cells from which they can be relieved by PI-3K pathway activation. By extending the analysis to the control of B cell proliferation, the present data provide the elements of a molecular mechanism by which the BCR controls the proliferative expansion of B cells upon engagement of invariant, ‘innate’ receptors. Such a crosstalk between these two classes of receptors reflects the physiological requirement of integrating, in any meaningful antibody response, input from the BCR regarding specific antigen recognition and information about the nature and context of the immunizing antigen, the latter being provided to the cells by innate receptors like TLRs recognizing pathogen-associated molecular patterns presented by microbes and the CD40 co-receptor which is triggered by T helper cells. The requirement of this regulatory principle is particularly obvious in the

case of the somatic antibody mutants generated in the germinal center reaction, whose expansion through T cell-derived and innate signals should strictly depend on newly acquired BCR specificities, or BCR expression in the first place.

Although receptor-proximal signaling events in B cells differ among TLRs and CD40, these receptors share common downstream signaling pathways, such as the PI-3K, MAPK and NF- κ B pathways (21, 22). This is supported by the similarity of gene expression profiles of primary B cells stimulated with distinct mitogenic agonists (23). The BCR appeared to specifically control PI-3K signaling in the cells, in that a key PI-3K target, Akt, was not phosphorylated in BCR^{neg} cells upon CpG stimulation (Fig. 3B), and expression of active PI-3K rescued mitogenic responses in the absence of the BCR (Fig. 3C). The BCR is apparently involved in this process in mitogen-activated B cells, conceivably by controlling the levels, activity or localization of inhibitors of the PI-3K pathway. This scenario is supported by results showing that mature B cells undergoing Cre-mediated BCR deletion *in vivo* can be rescued from apoptosis by expression of constitutively active PI-3K as well as by the ablation of PTEN, a lipid phosphatase that directly opposes PI-3K activity (24). Downstream of PI-3K activation, the signaling pathways diverge depending on the specific mitogen. Inactivation of Foxo1 is important for proliferation in response to anti-CD40/IL-4 stimulation, whereas inactivation of GSK3 β plays a greater role in the mitogenic response to CpG. Interestingly, inactivation of both GSK3 β and Foxo1 are not sufficient for LPS-induced proliferation, suggesting that additional BCR-dependent signaling pathways are activated by PI-3K that are necessary for TLR4-mediated proliferation of B cells. The present results indicate that, in addition to its role in the control of B cell survival, BCR modulation of PI-3K signaling licenses the induction of clonal expansion of B cells by mitogens acting on innate receptors expressed

by the cells, a process of crucial importance in the antibody response. In this scenario, BCR engagement by self- or cognate antigen as well as cross-reactivity with mitogens (8) may well play a modulating role. Quite fittingly, the work of Pone *et al.* has identified an interplay between BCR and TLR signaling in the control of class-switch recombination in the activated B cells. This was mediated by NF- κ B signals downstream of both receptors, which, significantly, depended on PI-3K activity in the case of the BCR (8).

The integration of signals delivered by distinct cellular receptors is an obvious requirement for any cellular response in multicellular organisms. An example is the outside-in signaling of integrins bound to extracellular matrix proteins, which mediate the proliferation of adherent epithelial cells in response to growth factors (25). Notably, the PI-3K pathway plays a role in this context as well, and also provides integrin-activated pro-survival signals to the adherent cells (26, 27). The interplay between the antigen receptor and innate immune receptors, which we have described here for B lymphocytes, may thus be just a variation of an ancient principle of receptor crosstalk in higher cells. It will be interesting to see whether this crosstalk also plays a role in the homeostasis of memory cells and to which extent and by what means the BCR also licenses the proliferative expansion of B cell lymphomas (28).

MATERIAL AND METHODS

Mouse strains. *B1-8^f* (2), *J_HT* (29), *bcl-2tg* (30), *CD21-Cre* (1), *rosa26-stop^f-P110** (3), and Cre-ED4 (14) mice were housed and maintained under specific pathogen-free conditions. All mouse protocols were approved by Harvard University and the Immune Disease Institute Institutional Animal Care and Use Committees.

Cell culture and TAT-Cre transduction. TAT-Cre (also known as HTNC, expression plasmid available from Addgene) was produced as described (10). Purified B cells were washed three times in serum-free, ADCF medium (Hyclone), prewarmed to 37°C, and transduced with 50 µg/ml TAT-Cre in the presence of 50 µg/ml polymyxin B sulfate (Sigma-Aldrich) at a density of 5x10⁶/ml for 45 min at 37°C. Cells were then washed in complete medium [DMEM supplemented with 10% FCS (Biowhittacker), 10 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, 1X penicillin/streptomycin, 1X non-essential amino acids (Cellgro), and 50 µM β-mercaptoethanol] and cultured at 37°C for 3–5 days at 2x10⁶/ml in complete medium. Mock-transduced cells were treated identically except that TAT-Cre was omitted. B cells were labeled with CFSE, as indicated, by washing two times in PBS, prewarming for 10 min at 37°C, and incubation with titrated amounts of CFSE for 10 min at 37°C. Cells were washed in complete medium and stimulated with 1 µg/ml anti-CD40 (1C10, eBioscience), 20 µg/ml ultrapure LPS (InvivoGen), or 645 ng/ml CpG (ODN1826, InvivoGen) at 2x10⁵/ml in complete medium for 3–4 days. The GSK3 inhibitor BIO or the inactive control Meth-BIO (Calbiochem) were added at a concentration of 0.2 µM as indicated.

FACS. Single cell suspensions of *ex vivo* splenocytes or cultured B cells were stained with antibodies to IgM (goat Fab, Jackson ImmunoResearch), IgD (1.3-5), Ig κ (R33-18.10), Ig β (HM79), CD19 (1D3), B220 (RA3-6B2), CD22 (CY34), CD21 (7G6), CD23 (B3B4), CD24 (M1/69), CD95 (Jo2), CD93 (AA4.1), I-A^b (AF6-120), or CD69 (H1.2F3). Dead cells were excluded by addition of ToPro3 (Invitrogen). For intracellular staining, cells were fixed and permeablized with Perm/Fix and Perm/Wash solutions (BD Bioscience) according to manufacturer's instructions, stained with anti-phospho-Akt (S473, Cell Signaling) or anti-phospho-GSK3 α/β (37F11, Cell Signaling) and detected with anti-rabbit-FITC. Data was collected on a FACScalibur (BD Bioscience) and analyzed with CellQuest (BD Bioscience) or FlowJo (TreeStar) software.

Cell separation. Red blood cells were lysed by hypotonic shock from splenocyte suspensions. B cells were purified by CD43-depletion using MACS (Miltenyi). For some experiments, BCR^{neg} B cells were isolated by MACS depletion of IgM⁺ B cells from TAT-Cre-transduced cultures using biotinylated anti-IgM (331-12) and streptavidin-beads (Miltenyi). Purity was >98% as determined by IgD staining. Alternatively, B cells were stained with Fab anti-IgM and anti-B220 before FACS sorting on an Aria2 or FACSVantage (BD Bioscience).

Immunoblot analysis. Whole cell lysates were prepared in lysis buffer [40 mM Tris (pH 7.6), 150 mM NaCl, 2 mM EDTA, 1% NP40, 0.25% sodium deoxycholate, 1X EDTA-free protease inhibitor cocktail (Roche), 1 mM PMSF, 5 mM NaF, 0.5 μ M, 1 mM Na₃VO₄]. The protein concentration was determined by detergent compatible protein assay kit (Bio-Rad). Equal amounts of protein were separated by SDS-PAGE and transferred to PVDF membranes (Millipore). The membranes were immunoblotted, as

indicated, with phospho-Akt (S473), Akt (Cell Signaling), c-Myc (Epitomics), β -actin (Sigma-Aldrich) or biotinylated goat Fab anti-mouse IgM (Jackson ImmunoResearch).

NF- κ B EMSA. Splenic B cells from B1-8f/J_HT, bcl-2tg mice were TAT-Cre-transduced and placed in culture for 3 days. Purified BCR^{pos} and BCR^{neg} B cells were then cultured overnight in 1% FCS-containing medium prior to addition of CpG for the indicated amounts of time. Cells were lysed in buffer A (5 mM HEPES pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT and protease inhibitors) to prepare cytosolic extracts and nuclei. Nuclear extracts were subsequently prepared using buffer C (20 mM HEPES pH 7.9, 25% glycerol, 0.5 M NaCl, 1.5 mM MgCl₂, 0.5 mM EDTA, 0.5 mM DTT and protease inhibitors). Nuclear extracts were incubated with competitor DNA (pdIdC, Sigma) and a ³²P labeled- κ B consensus probe (Promega). Protein-DNA complexes were then resolved by electrophoresis on a non-denaturing polyacrylamide gel.

Quantitative RT-PCR. RNA was purified using mirVana RNA isolation kit (Ambion) and subjected to cDNA synthesis using random hexamer primers (Invitrogen).

Quantitative RT-PCR analysis was performed using *c-myc*- and *hprt*-specific primers on a StepOne Plus (Applied Biosystems) machine. Primers used were: c-MycF1, 5'-gagcccctagtgctgcatgag-3'; c-MycR1, 5'-ttgcctcttctccacagacacc-3'; HPRTF1, 5'-ctataagttctttgctgacctgctggattaca-3'; HPRTR1, 5'-ccagttaaagttgagagatcatctccaccaataact-3'.

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AUTHOR CONTRIBUTIONS

K.L.O., A.W., and K.R. designed research; K.L.O., A.W., E.D., and L.S. performed research; K.L.O., A.W., and E.D. analyzed data; and K.L.O., A.F., and K.R. wrote the paper.

The authors declare no conflict of interest.

REFERENCES

1. Kraus M, Alimzhanov MB, Rajewsky N, Rajewsky K (2004) Survival of resting mature B lymphocytes depends on BCR signaling via the Ig α / β heterodimer. *Cell* 117(6):787–800.
2. Lam KP, Kuhn R, Rajewsky K (1997) *In vivo* ablation of surface immunoglobulin on mature B cells by inducible gene targeting results in rapid cell death. *Cell* 90(6):1073–1083.
3. Srinivasan L, *et al.* (2009) PI3 kinase signals BCR-dependent mature B cell survival. *Cell* 139(3):573–586.
4. Pasare C, Medzhitov R (2005) Control of B-cell responses by Toll-like receptors. *Nature* 438(7066):364–368.
5. Medzhitov R (2007) Recognition of microorganisms and activation of the immune response. *Nature* 449(7164):819–826.
6. Andersson J, Bullock WW, Melchers F (1974) Inhibition of mitogenic stimulation of mouse lymphocytes by anti-mouse immunoglobulin antibodies. I. Mode of action. *Eur J Immunol* 4(11):715–722.
7. Kearney JF, Cooper MD, Lawton AR (1976) B lymphocyte differentiation induced by lipopolysaccharide. III. Suppression of B cell maturation by anti-mouse immunoglobulin antibodies. *J Immunol* 116(6):1664–1668.
8. Pone EJ, *et al.* (2012) BCR-signalling synergizes with TLR-signalling for induction of AID and immunoglobulin class-switching through the non-canonical NF- κ B pathway. *Nat Commun* 3:767.
9. Avalos AM, Busconi L, Marshak-Rothstein A (2010) Regulation of autoreactive B cell responses to endogenous TLR ligands. *Autoimmunity* 43(1):76–83.

10. Peitz M, Pfannkuche K, Rajewsky K, Edenhofer F (2002) Ability of the hydrophobic FGF and basic TAT peptides to promote cellular uptake of recombinant Cre recombinase: a tool for efficient genetic engineering of mammalian genomes. *Proc Natl Acad Sci USA* 99(7):4489–4494.
11. Clark EA, Ledbetter JA (1986) Activation of human B cells mediated through two distinct cell surface differentiation antigens, Bp35 and Bp50. *Proc Natl Acad Sci USA* 83(12):4494–4498.
12. Poltorak A, *et al.* (1998) Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science* 282(5396):2085–2088.
13. Krieg AM, *et al.* (1995) CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature* 374(6522):546–549.
14. Schwenk F, Kuhn R, Angrand PO, Rajewsky K, Stewart AF (1998) Temporally and spatially regulated somatic mutagenesis in mice. *Nucleic Acids Res* 26(6):1427–1432.
15. Sears R, *et al.* (2000) Multiple Ras-dependent phosphorylation pathways regulate Myc protein stability. *Genes Dev* 14(19):2501–2514.
16. Diehl JA, Cheng M, Roussel MF, Sherr CJ (1998) Glycogen synthase kinase-3 β regulates cyclin D1 proteolysis and subcellular localization. *Genes Dev* 12(22):3499–3511.
17. Cross DA, Alessi DR, Cohen P, Andjelkovich M, Hemmings BA (1995) Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. *Nature* 378(6559):785–789.
18. Meijer L, *et al.* (2003) GSK-3-selective inhibitors derived from Tyrian purple indirubins. *Chem Biol* 10(12):1255–1266.

19. Yusuf I, Zhu X, Kharas MG, Chen J, Fruman DA (2004) Optimal B-cell proliferation requires phosphoinositide 3-kinase-dependent inactivation of FOXO transcription factors. *Blood* 104(3):784–787.
20. Dansen TB, Burgering BM (2008) Unravelling the tumor-suppressive functions of FOXO proteins. *Trends Cell Biol* 18(9):421–429.
21. Donahue AC, Fruman DA (2004) PI3K signaling controls cell fate at many points in B lymphocyte development and activation. *Semin Cell Dev Biol* 15(2):183–197.
22. Siebenlist U, Brown K, Claudio E (2005) Control of lymphocyte development by nuclear factor- κ B. *Nat Rev Immunol* 5(6):435–445.
23. Zhu X, *et al.* (2004) Analysis of the major patterns of B cell gene expression changes in response to short-term stimulation with 33 single ligands. *J Immunol* 173(12):7141–7149.
24. Leslie NR, Downes CP (2002) PTEN: The down side of PI 3-kinase signalling. *Cell Signal* 14(4):285–295.
25. Giancotti FG, Ruoslahti E (1999) Integrin signaling. *Science* 285(5430):1028–1032.
26. Khwaja A, Rodriguez-Viciano P, Wennstrom S, Warne PH, Downward J (1997) Matrix adhesion and Ras transformation both activate a phosphoinositide 3-OH kinase and protein kinase B/Akt cellular survival pathway. *EMBO J* 16(10):2783–2793.
27. Cabodi S, *et al.* (2009) Convergence of integrins and EGF receptor signaling via PI3K/Akt/FoxO pathway in early gene Egr-1 expression. *J Cell Physiol* 218(2):294–303.
28. Young RM, Staudt LM (2013) Targeting pathological B cell receptor signalling in lymphoid malignancies. *Nat Rev Drug Discov* 12(3):229–243.

29. Gu H, Zou YR, Rajewsky K (1993) Independent control of immunoglobulin switch recombination at individual switch regions evidenced through Cre-loxP-mediated gene targeting. *Cell* 73(6):1155–1164.
30. Strasser A, *et al.* (1991) Enforced BCL2 expression in B-lymphoid cells prolongs antibody responses and elicits autoimmune disease. *Proc Natl Acad Sci USA* 88(19):8661–8665.

FIGURE LEGENDS

Fig. 1. *In vitro* generation of BCR^{neg} B cells. (A) Schematic showing Cre-mediated deletion of the loxP-flanked *B1-8* allele and subsequent loss of BCR expression. (B) Splenic B cells from *B1-8f/+*, *bcl-2tg* mice were treated with recombinant TAT-Cre for 45 min, cultured for 9 days, and sorted by FACS to >98% purity based on IgM expression (data are representative of two biological replicates). Serial dilutions of whole cell lysates starting at 300,000 cell equivalents were analyzed for Ig μ and β -actin expression by Western blotting. (C) Splenic B cells from *B1-8f/+*, *bcl-2tg* mice were transduced with TAT-Cre for 45 min, cultured for 5 days in medium, and stained for CD19, IgM, and the indicated surface markers (data are representative of three biological replicates). BCR^{pos} cells (open histograms) and BCR^{neg} cells (closed histograms) were gated as shown.

Fig. 2. Expression of the BCR is necessary for B cell proliferation. BCR^{pos} and BCR^{neg} B cells were generated by TAT-Cre transduction of B cells from *B1-8f/+*, *bcl-2tg* mice. After 3 days in culture, cells were either (A) labeled with CFSE and stimulated for 3–4 days (data are representative of five biological replicates) or (B) stimulated for 24 hrs with the indicated mitogen and analyzed by FACS (data are representative of two biological replicates). Live cells were gated as ToPro3⁻, CD19⁺.

Fig. 3. Defective activation of PI-3K in BCR^{neg} B cells. (A) Splenic B cells from *B1-8f/J_HT*, *bcl-2tg* or *B1-8f/J_HT*, *CD21-Cre*, *bcl-2tg* mice were stimulated with CpG for the indicated times and stained for CD19 and IgM and intracellular phospho-Akt (Ser473).

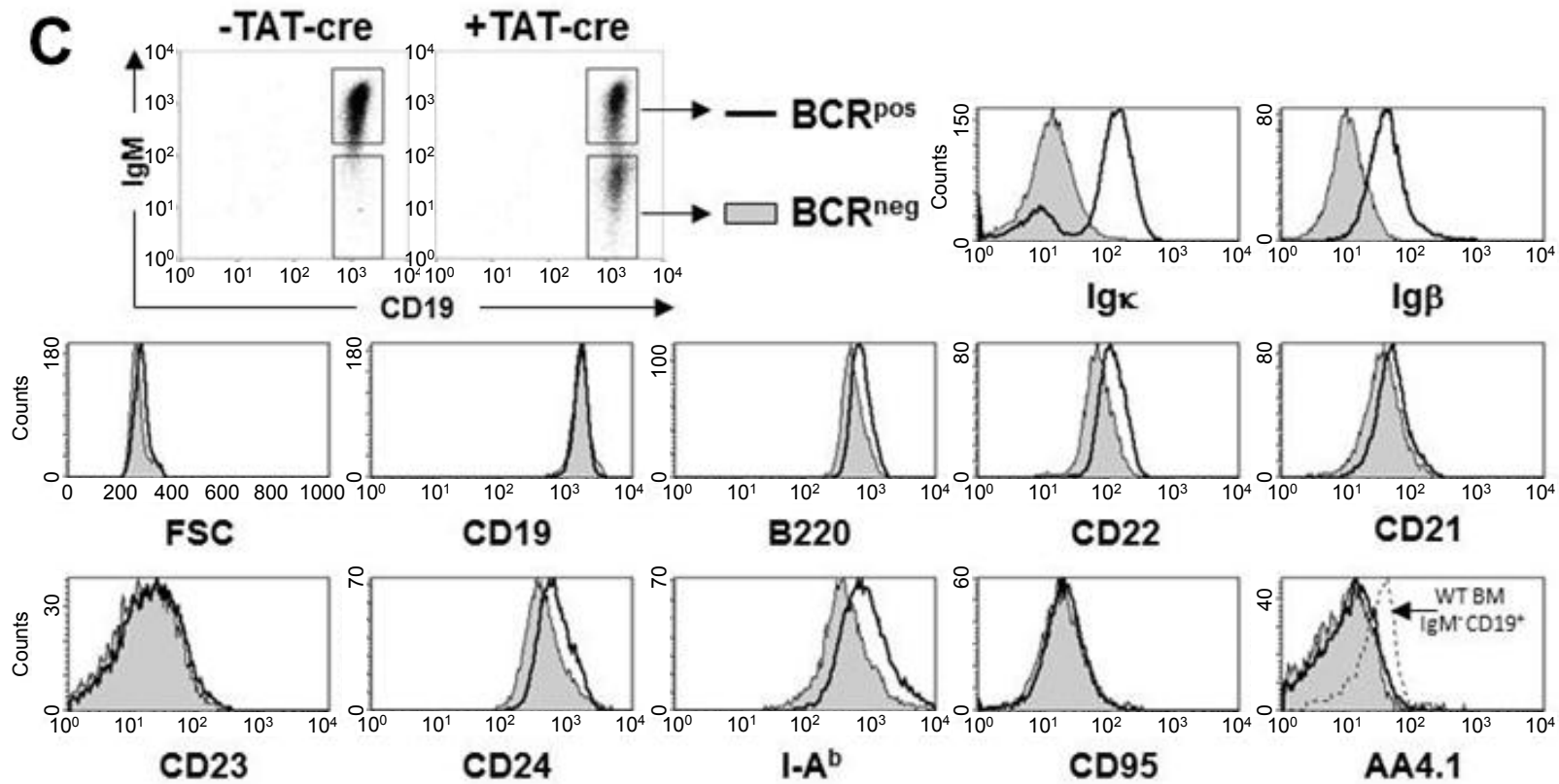
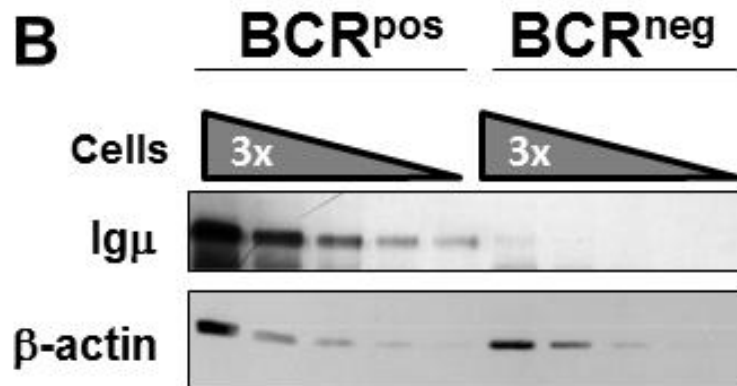
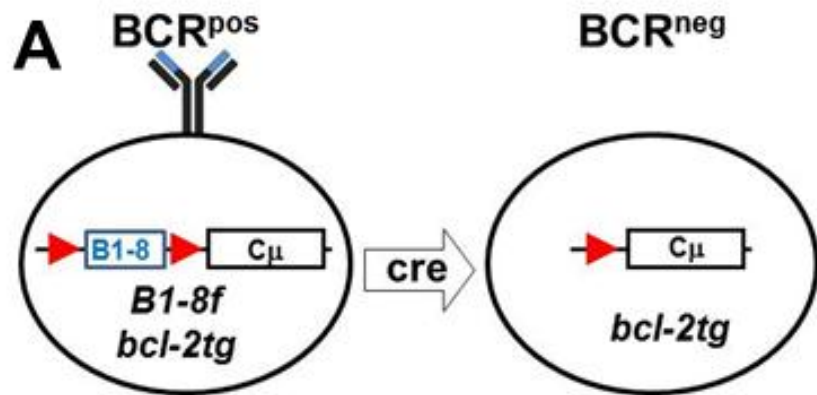
BCR^{pos} (green) cells were gated from *B1-8f/J_HT*, *bcl-2tg* cells, while Cre⁺ BCR^{pos} (red) or Cre⁺ BCR^{neg} (blue) B cells were gated from *B1-8f/J_HT*, *CD21-Cre*, *bcl-2tg* cells.

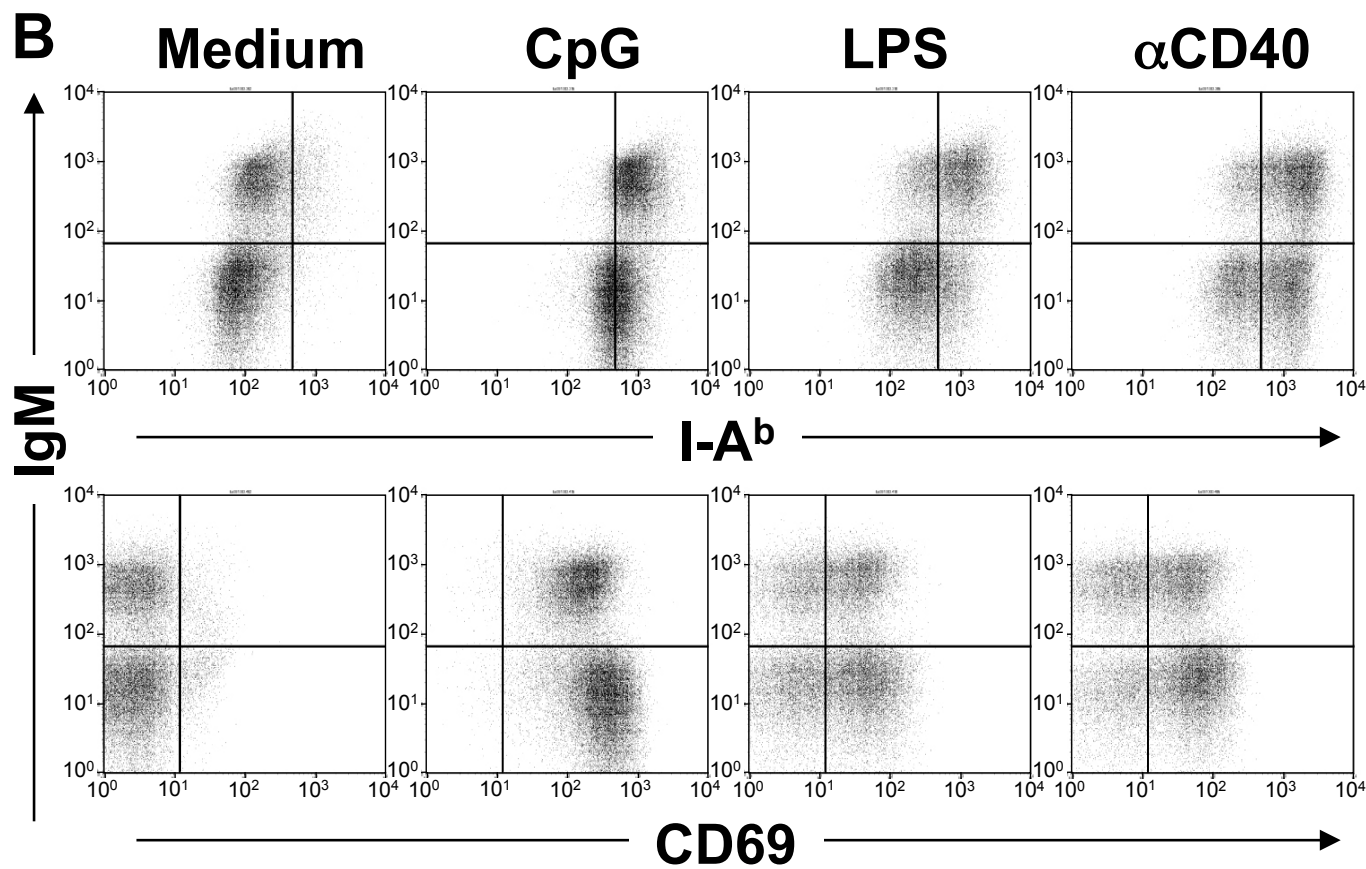
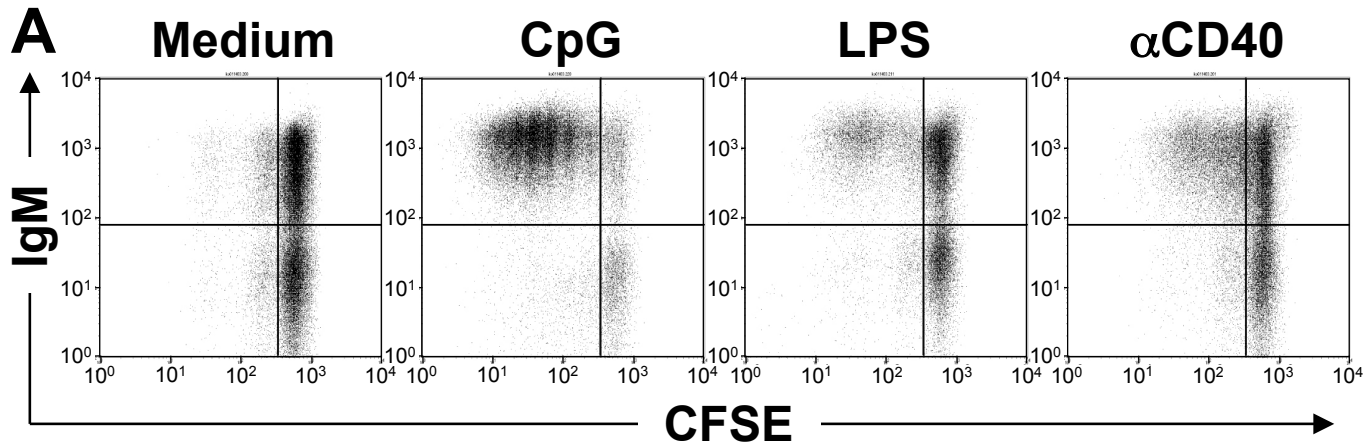
Normalized mean fluorescence intensity values for phospho-Akt (Ser473) are shown. Data represent three biological replicates. (B) BCR^{pos} and BCR^{neg} B cells were sorted from *B1-8f/J_HT*, *bcl-2tg* splenic B cells 5 days after transduction with TAT-Cre, placed in culture overnight and stimulated with CpG for 2 hrs. Equal amounts of cytoplasmic lysates were blotted for phospho-Akt (Ser473) and Akt. Data are representative of two biological replicates. (C) Splenic BCR^{pos} and GFP⁺ BCR^{neg} B cells were sorted from Rag2^{Δ/Δ}, cγ^{-/-} mice that had been reconstituted with fetal liver from *B1-8f/J_HT*, *CD21-Cre*, *P110** mice, labeled with CFSE and cultured for 3 days in medium (black) or medium plus the indicated stimulus (red). Data are representative of three biological replicates.

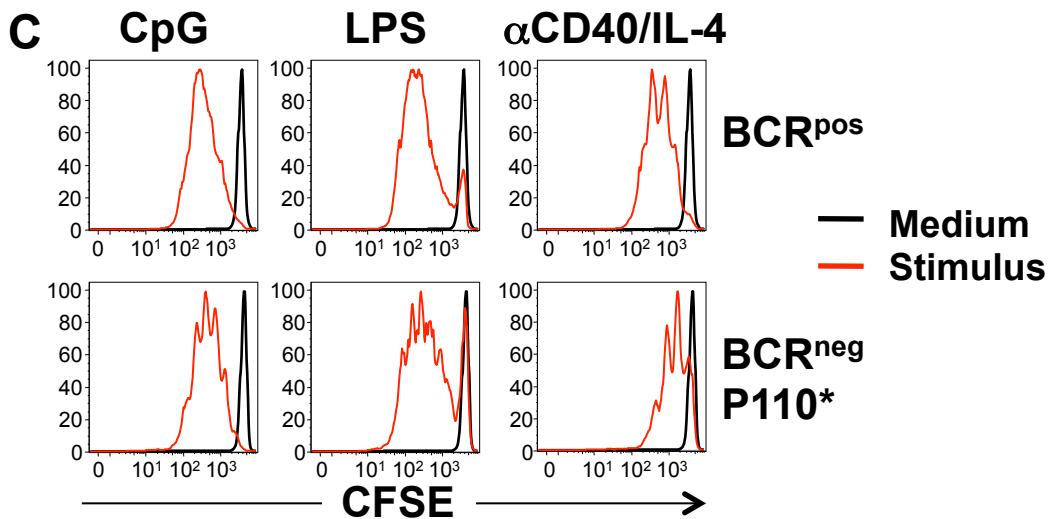
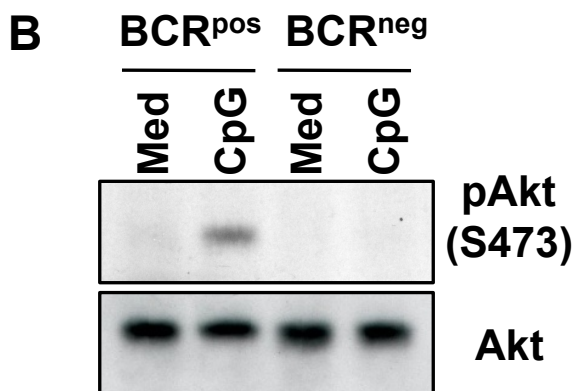
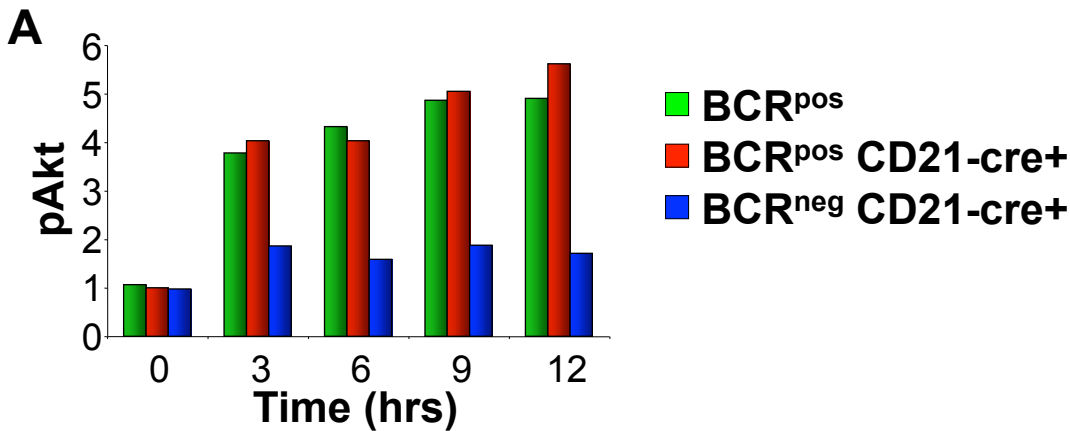
Fig. 4. GSK3β activity prevents the proliferation of CpG-treated BCR^{neg} B cells. (A) Splenic B cells from *B1-8f/J_HT*, *bcl-2tg* or *B1-8f/J_HT*, *CD21-Cre*, *bcl-2tg* mice were stimulated with CpG for the indicated times and stained for CD19 and IgM and intracellular phospho-GSK3α/β (S21/9). BCR^{pos} (green) cells were gated from *B1-8f/J_HT*, *bcl-2tg* cells, while Cre⁺ BCR^{pos} (red) or Cre⁺ BCR^{neg} (blue) B cells were gated from *B1-8f/J_HT*, *CD21-Cre*, *bcl-2tg* cells. Normalized mean fluorescence interval values for phospho-GSK3α/β (S21/9) are shown. Data represents three biological replicates. (B) B cells from *B1-8f/J_HT*, *CD21-Cre*, *bcl-2tg* mice or TAT-Cre transduced B cells from *B1-8f/J_HT*, *bcl-2tg* mice were CFSE labeled and stimulated for 3 days with CpG alone (green) or with Meth-BIO (inactive control inhibitor, red) or BIO (GSK3 inhibitor, blue). Cells were gated on ToPro3⁻, CD19⁺ and IgM⁺ or IgM⁻. Data are representative of three biological replicates. (C) Splenic B cells from *B1-8f/+*, *bcl-2tg* mice were TAT-Cre

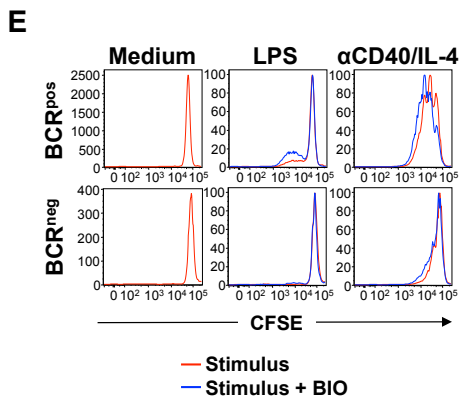
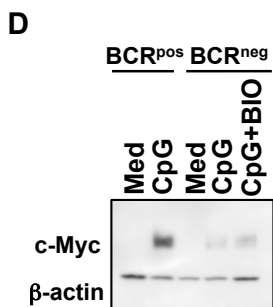
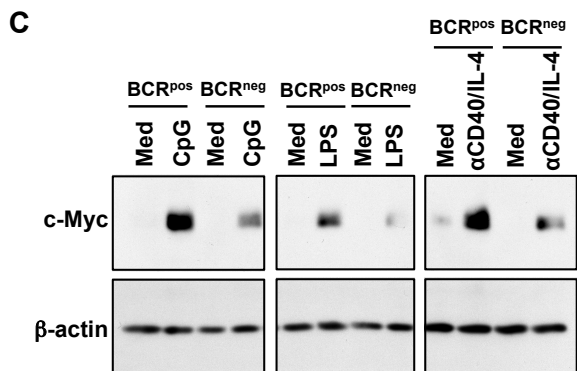
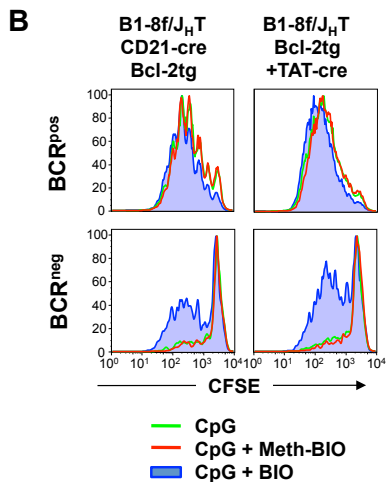
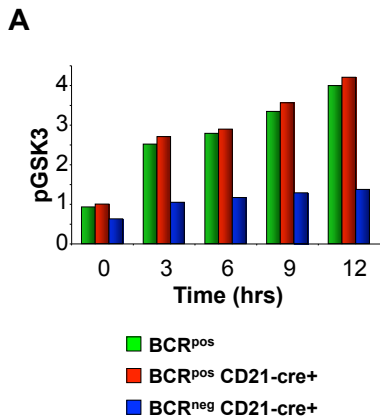
transduced and cultured for 3 days. Purified BCR^{pos} and BCR^{neg} B cells were placed in culture overnight and stimulated with CpG, LPS or anti-CD40/IL-4 for 6 hrs. Equal amounts of protein from whole cell lysates were used for Western blotting with the indicated antibodies. Data are representative of three biological replicates. (D) Sorted BCR^{pos} and BCR^{neg} B cells from *B1-8f/+*, *CD21-Cre*, *bcl-2tg* mice were cultured in medium, CpG or CpG and BIO for 6 hrs. Lysates were analyzed by Western blot for c-Myc and β -actin levels. Data are representative of three biological replicates. (E) Splenic BCR^{pos} and BCR^{neg} B cells from *B1-8f/+*, *CD21-Cre*, *bcl-2tg* mice were sorted, labeled with CFSE and cultured in media or with LPS or anti-CD40/IL-4 alone (red) or with BIO (blue). Data are representative of three biological replicates.

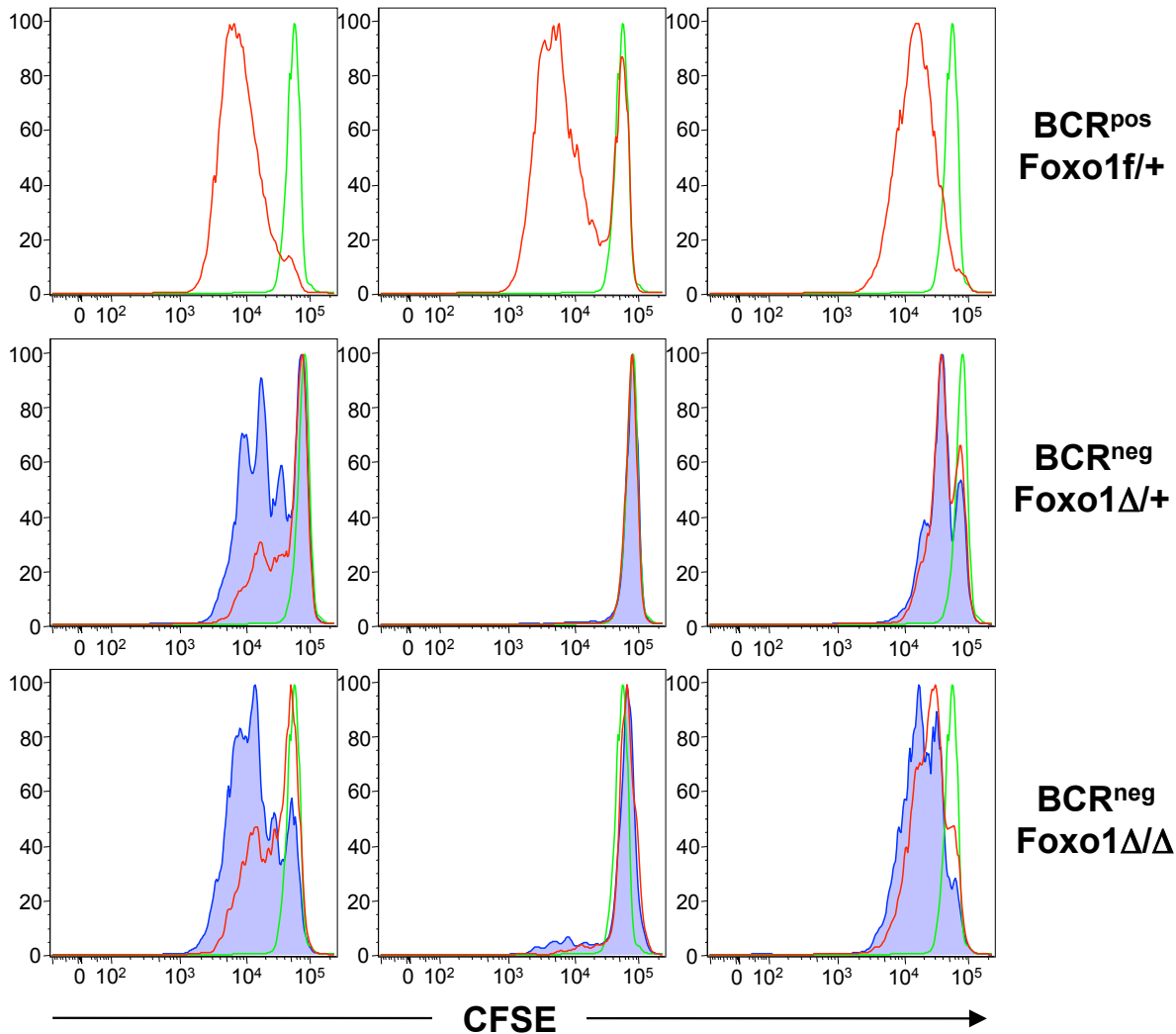
Fig. 5. Foxo1 deletion plus GSK3 β inhibition rescues proliferation in CpG-treated BCR^{neg} B cells. Splenic B cells were sorted, labeled with CFSE and cultured either in medium (green) or with the indicated stimuli, with (blue) or without (red) the GSK3 β inhibitor BIO. BCR^{pos}, Foxo1^{f/+} and BCR^{neg} Foxo1 Δ /⁺ cells were sorted from *B1-8f/J_HT*, *CD21-Cre*, *Foxo1^{f/+}* mice, while BCR^{neg}, Foxo1 Δ / Δ cells were sorted from *B1-8f/J_HT*, *CD21-Cre*, *Foxo1^{ff}* mice. Data are representative of three biological replicates.









CpG**LPS** **α CD40/IL-4****BCR^{pos}
Foxo1f/+****BCR^{neg}
Foxo1 Δ /+****BCR^{neg}
Foxo1 Δ / Δ** **CFSE** →

— **Medium**
— **Stimulus - BIO**
— **Stimulus + BIO**

SUPPORTING INFORMATION FIGURE LEGENDS

Fig. S1. Expression of Bcl-2 rescues survival of BCR^{neg} B cells *in vitro*. Purified Splenic B cells were transduced with TAT-Cre for 45 min and cultured *in vitro*. After 4 or 14 days, cells were analyzed for a change in the proportion of ToPro3⁻, CD19⁺ B cells that lost expression of IgM and IgD. Data are representative of three biological replicates.

Fig. S2. Defective proliferation of BCR^{neg} B cells. (A) B cells from *B1-8f/+*, *ED-Cre*, *bcl-2tg* mice were purified and treated with 4-OH Tamoxifen for 7 days prior to CFSE labeling and stimulation with the indicated mitogen for 3 days. Data are representative of three biological replicates. (B) *Ex vivo* isolated B cells from *B1-8f/JHT*, *CD21-Cre*, *bcl-2tg* mice were labeled with CFSE and cultured in medium or with CpG for 3 days. ToPro3⁻, CD19⁺ cells are shown. Data are representative of two biological replicates.

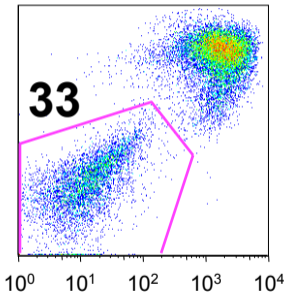
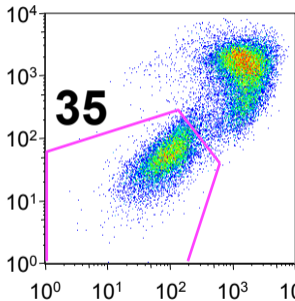
Fig. S3. Stimulation with CpG induces normal NF-κB DNA-binding activity in BCR^{neg} B cells. BCR^{pos} and BCR^{neg} B cells from *B1-8f/+*, *bcl-2tg* mice were cultured overnight in 1% FCS-containing medium and stimulated for the indicated times with CpG. Nuclear lysates were used for EMSA. The data presented here was biologically replicated once using the 0 and 120 minutes time points.

Fig. S4. Normal induction of *c-myc* transcription in CpG-stimulated BCR^{neg} B cells. Sorted BCR^{pos} and BCR^{neg} B cells generated by TAT-Cre transduction from *B1-8f/+*, *bcl-2tg* mice were stimulated for 3 hrs with medium or CpG. cDNA from purified RNA was used for quantitative rtPCR analysis of *c-myc* and *hprt* expression. Data for two biologically independent experiments (average of technical duplicates shown for each biologically independent experiment) represent fold-change of *c-myc* expression relative to BCR^{pos} B cells cultured in medium.

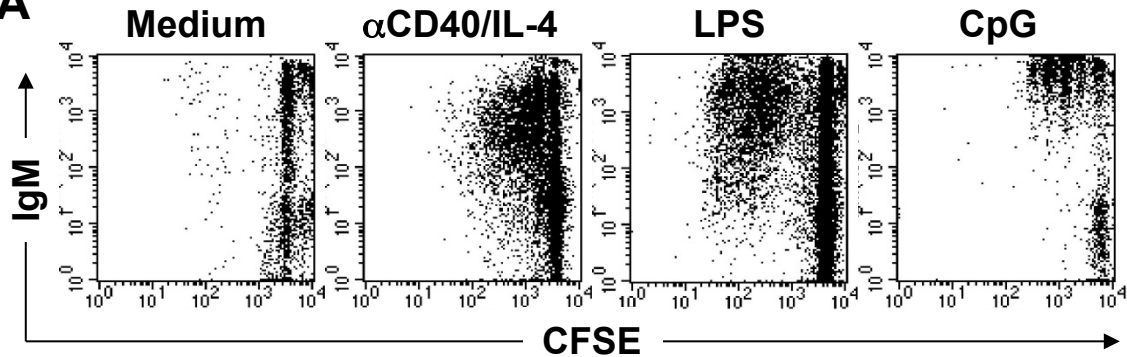
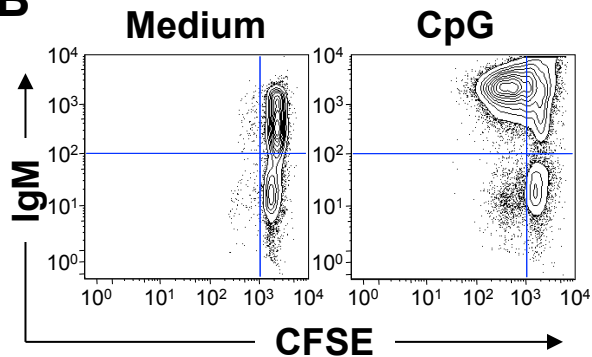
Day 4

Day 14

IgM ↑



IgD →

A**B**

BCR^{pos}

BCR^{neg}

0

30

60

120

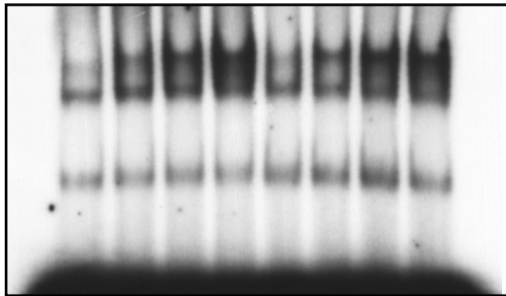
0

30

60

120

min



NF-κB

***c-myc* induction (fold)**

