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# Regulation of Class-Switch Recombination and Plasma Cell Differentiation by Phosphatidylinositol 3-Kinase Signaling

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## Summary

**Class-switch recombination (CSR) is essential for humoral immunity. However, the regulation of CSR is not completely understood. Here we demonstrate that phosphatidylinositol 3-kinase (PI3K) actively suppressed the onset and frequency of CSR in primary B cells. Consistently, mice lacking the lipid phosphatase, PTEN, in B cells exhibited a hyper-IgM condition due to impaired CSR, which could be restored in vitro by specific inhibition of PI3Kδ. Inhibition of CSR by PI3K was partially dependent on the transcription factor, BLIMP1, linking plasma cell commitment and cessation of CSR. PI3K-dependent activation of the serine-threonine kinase, Akt, suppressed CSR, in part, through the inactivation of the Forkhead Box family (Foxo) of transcription factors. Reduced PI3K signaling enhanced the expression of AID (activation-induced cytidine deaminase) and accelerated CSR. However, ectopic expression of AID could not fully overcome inhibition of CSR by PI3K, suggesting that PI3K regulates both the expression and function of AID.**

## Introduction

Class-switch recombination (CSR) and somatic hypermutation (SHM) have been historically linked to the germinal center (GC). Indeed, AID (activation-induced cytidine deaminase), the B cell-specific factor required for both CSR and SHM, is most highly expressed in GC B cells (Okazaki et al., 2003). However, similar to primary IgM, much of the class-switched IgG antibody generated early after primary immunization with T-dependent antigens is derived from extrafollicular plasmablasts and short-lived antibody-secreting cells (ASCs) prior to the full establishment of GCs (Pape et al., 2003; Peakman and Maizels, 1998). As the immune response progresses, a greater contribution of post-GC-derived long-lived ASCs secreting higher-affinity, class-switched antibody is observed (Blink et al., 2005). These cells ultimately take up residence in the bone marrow and are responsible for long-term high-affinity antibody production (Blink et al., 2005; Manz et al., 2005).

ASC versus GC cell formation result from diametrically opposed genetic programs and differentiation pathways. Induced expression of the transcription factor BLIMP1 is necessary and sufficient to initiate all of the hallmarks of ASC differentiation including cell-cycle withdrawal, shortened lifespan, generation of secretory transcripts, and overall increased secretory capacity associated with endoplasmic reticulum expansion (Shapiro-Shelef and Calame, 2005). A major function of BLIMP1 is to repress the transcription factors Pax5 and BCL6 (Shapiro-Shelef and Calame, 2005). Pax5 is critical for B lineage commitment at both the progenitor and mature B cell stage (Schebesta et al., 2002), but it also represses the expression of genes required for antibody secretion (Shapiro-Shelef and Calame, 2005). BCL6, most highly expressed in GC B cells, is required for GC formation in response to T-dependent antigens (Niu, 2002). Itself a target of BLIMP1 repression, BCL6 also represses BLIMP1, creating a reciprocal negative feedback loop (Shapiro-Shelef and Calame, 2005).

Recent evidence suggests that in addition to GC differentiation, commitment to ASC differentiation also precludes CSR (Hasbold et al., 2004). The contribution of CSR to the maturation of the immune response occurs at many levels; switched isotypes have greater half-lives and tissue access than IgM, specify distinct Fc-mediated effector functions, and display altered B cell antigen receptor (BCR) signaling. Isotype-specific CSR events are induced by distinct extracellular stimuli in the presence of AID. Pax5 and another transcription factor, E2A, are both implicated in the induction of AID transcription, whereas BLIMP1 suppresses AID expression (Gonda et al., 2003; Sayegh et al., 2003; Sciammas and Davis, 2004; Shaffer et al., 2002). In addition, AID may be regulated posttranslationally (Basu et al., 2005; Pasqualucci et al., 2006).

B cell activation in vivo is mediated by many receptors including the BCR, costimulatory molecules, cytokine receptors, and Toll-like receptors. However, it is not well understood how the biochemical pathways triggered by these cell-surface molecules regulate CSR and ASC formation during the course of B cell differentiation. One signaling pathway activated by all of these receptors is the phosphatidylinositol 3-kinase (PI3K) pathway (Deane and Fruman, 2004). Membrane-localized phosphoinositides function as secondary messengers of receptor signaling to regulate diverse cellular functions. These phosphorylated lipid products are inducibly generated by receptor-activated lipid kinases, the most prominent of which is PI3K. PI3K catalyzes the phosphorylation of phosphatidylinositol 4,5 bisphosphate ($PI(4,5)P_2$) on the 3′ position to generate phosphatidylinositol 3,4,5 trisphosphate ($PIP_3$). The class IA PI3Ks are heterodimers composed of a regulatory subunit (p85, p55, or p50) and a catalytic subunit

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(p110α, p110β, or p110δ). p110δ is the catalytic subunit of PI3Kδ, the major isoform activated in B cells downstream of the BCR (Clayton et al., 2002; Jou et al., 2002; Okkenhaug et al., 2002). The lipid phosphastase PTEN directly antagonizes PI3K activity by dephosphorylating $PIP_3$ to regenerate $PI(4,5)P_2$.

Two major pathways are activated downstream of PI3K in B cells, the Btk (Bruton's tyrosine kinase) pathway and the PDK1 (3-phosphoinositide-dependent kinase 1)-dependent Akt (also known as protein kinase B, PKB) pathway (Cantrell, 2002; Schaeffer and Schwartzberg, 2000). Btk, PDK1, and Akt are recruited to the plasma membrane via $PIP_3$ binding pleckstrin homology domains, and both the Btk and Akt pathways contribute to the survival and proliferation of activated B cells. However, the means by which these two pathways effect proliferation and survival are largely distinct. The major target of Btk activation is PLCγ (phospholipase C γ), resulting in $Ca^{2+}$ flux and PKC (protein kinase C) activation (Forssell et al., 1999; Fruman et al., 2000). Activation of PDK1 results in the phosphorylation and activation of Akt and other AGC kinases (containing protein kinase A, protein kinase G, and protein kinase C families), that collectively regulate cellular proliferation, growth, metabolism, and survival (Mora et al., 2004). The involvement of Akt in cellular survival and proliferation may be due largely to its phosphorylation-dependent inactivation of the Forkhead Box (Foxo) family of transcription factors, which results in the silencing of Foxo target proteins that promote cell-cycle arrest and apoptosis (Greer and Brunet, 2005; Yusuf et al., 2004).

The importance of regulated PI3K activity in B cells is exemplified by mice deficient for PI3K or PTEN. Mice deficient for the individual regulatory or catalytic subunits of PI3K have defects in B cell development and immune responses (Deane and Fruman, 2004). Conversely, we and others have found that mice carrying a B cell-specific deletion of PTEN have increased numbers of peripheral B cells, but still exhibit impaired immune responses (Anzelon et al., 2003; Suzuki et al., 2003).

To date, much of the focus on PI3K signaling in B cells has been on mitogenic signaling by the BCR. However, a molecular and biochemical understanding of how PI3K governs other aspects of B cell differentiation is lacking. Here we demonstrate that PI3K inversely regulates ASC formation and induction of CSR by activating Akt and repressing Foxo function.

## Results

### Impaired $PIP_3$ Hydrolysis Blocks CSR

We and others have generated mice deficient for PTEN in B cells (b*Pten*$^{-/-}$) (Anzelon et al., 2003; Suzuki et al., 2003), which produce IgM-dominated immune responses reminiscent of the hyper-IgM (HIGM) condition associated with defects in T cell help or CSR (Durandy et al., 2005; Suzuki et al., 2003). However, although we noted the presence of GCs in b*Pten*$^{-/-}$ mice immunized with sheep red blood cells (SRBC) (Anzelon et al., 2003), others failed to find evidence of GC formation in mice immunized with the hapten-carrier NP-CGG (Suzuki et al., 2003).

To determine whether GC formation in b*Pten*$^{-/-}$ mice was unique to SRBC immunization, mice were immunized with the hapten-carrier TNP-OVA in alum and analyzed 8 and 14 days (d8, d14) after immunization. Consistent with previous findings (Suzuki et al., 2003), measurement of antigen-specific antibody titers by ELISA revealed higher amounts of IgM and lower amounts of IgG in sera from b*Pten*$^{-/-}$ mice (see Figure S1A in the Supplemental Data available online). Immunohistochemistry of frozen spleen sections from immunized mice revealed increased numbers of brightly staining IgM$^{hi}$ ASC in b*Pten*$^{-/-}$ mice at d8 and d14 (Figure 1A). In contrast, although a few IgG$^{hi}$ ASCs could be identified in b*Pten*$^{-/-}$ spleen sections at d8, their frequency was extremely low and did not increase markedly over time (Figure 1A). By comparison, brightly staining IgG$^{hi}$ ASCs were identified in the red pulp of wild-type (WT) mice at d8 and d14 after immunization. Consistent with our previous findings with SRBC immunizations (Anzelon et al., 2003), we observed well-formed GCs in b*Pten*$^{-/-}$ mice (Figure 1A), indicating that the lack of IgG$^{+}$ ASCs in these mice is not partially due to the absence of GCs. These effects (CSR, ASC, and GC formation) were not specific to the antigen or adjuvant used; similar results were obtained with different T-dependent antigen and adjuvant pairs (TNP-OVA in alum or NP-KLH in CFA or RIBI; Figure S1B). Given these findings, our focus shifted to the examination of CSR and ASC differentiation in b*Pten*$^{-/-}$ mice.

To assess CSR and ASC formation more quantitatively, splenocytes from d14 immunized mice were analyzed by flow cytometry (FACS) for expression of IgM, IgG, and the ASC marker, Syndecan 1 (Syn1). Consistent with the histology, FACS revealed that b*Pten*$^{-/-}$ mice generated very few IgG$^{+}$ cells (Figure 1B). When the phenotype of the Syn1$^{+}$ cells was assessed, many IgM$^{+}$ and IgM$^{-}$ ASCs were observed in WT mice, whereas b*Pten*$^{-/-}$ mice produced more IgM$^{+}$ ASCs, but much fewer IgM$^{-}$ ASCs. To determine whether the few IgM$^{-}$ ASCs observed in b*Pten*$^{-/-}$ mice contained cells that had switched to an isotype other than IgG (i.e., IgA or IgE), ASC formation was analyzed in an independent cohort of mice with the inclusion of a stain for IgA and IgE. Similar to what was observed for IgG, whereas IgA$^{+}$ ASCs were detected in the IgM$^{-}$ ASC population in WT mice, virtually no IgA$^{+}$ ASCs were observed in b*Pten*$^{-/-}$ mice (Figure S1C). IgE$^{+}$ ASCs were not detected in either WT or b*Pten*$^{-/-}$ mice (data not shown). Lastly, quantitative analysis of antigen-specific ASC formation by ELISPOT confirmed the enhanced formation of IgM-secreting ASCs and decreased formation of IgG-secreting ASCs in b*Pten*$^{-/-}$ mice (Figure 1C). These data suggest that the sustained presence of $PIP_3$ brought on by the loss of PTEN promotes the formation of IgM-secreting ASCs while preventing CSR to other isotypes in response to a T-dependent antigen.

Because in vivo analyses of T-dependent responses can be influenced by B cell-extrinsic variables (e.g., T cell priming or activation), we analyzed ASC formation and CSR under defined in vitro conditions. Splenic B cells were stimulated with LPS (lipopolysaccharide), LPS+IL-4, or αCD40+IL4 and assessed 3 days (d3) later for ASC formation and CSR by FACS. b*Pten*$^{-/-}$ B cells failed to undergo CSR to IgG3 or IgG1 in the presence of LPS or LPS+IL-4, respectively (Figure 1D). However, the formation of ASCs, identified as B220$^{lo}$Syn1$^{hi}$, was enhanced in the absence of PTEN. αCD40+IL-4 did not

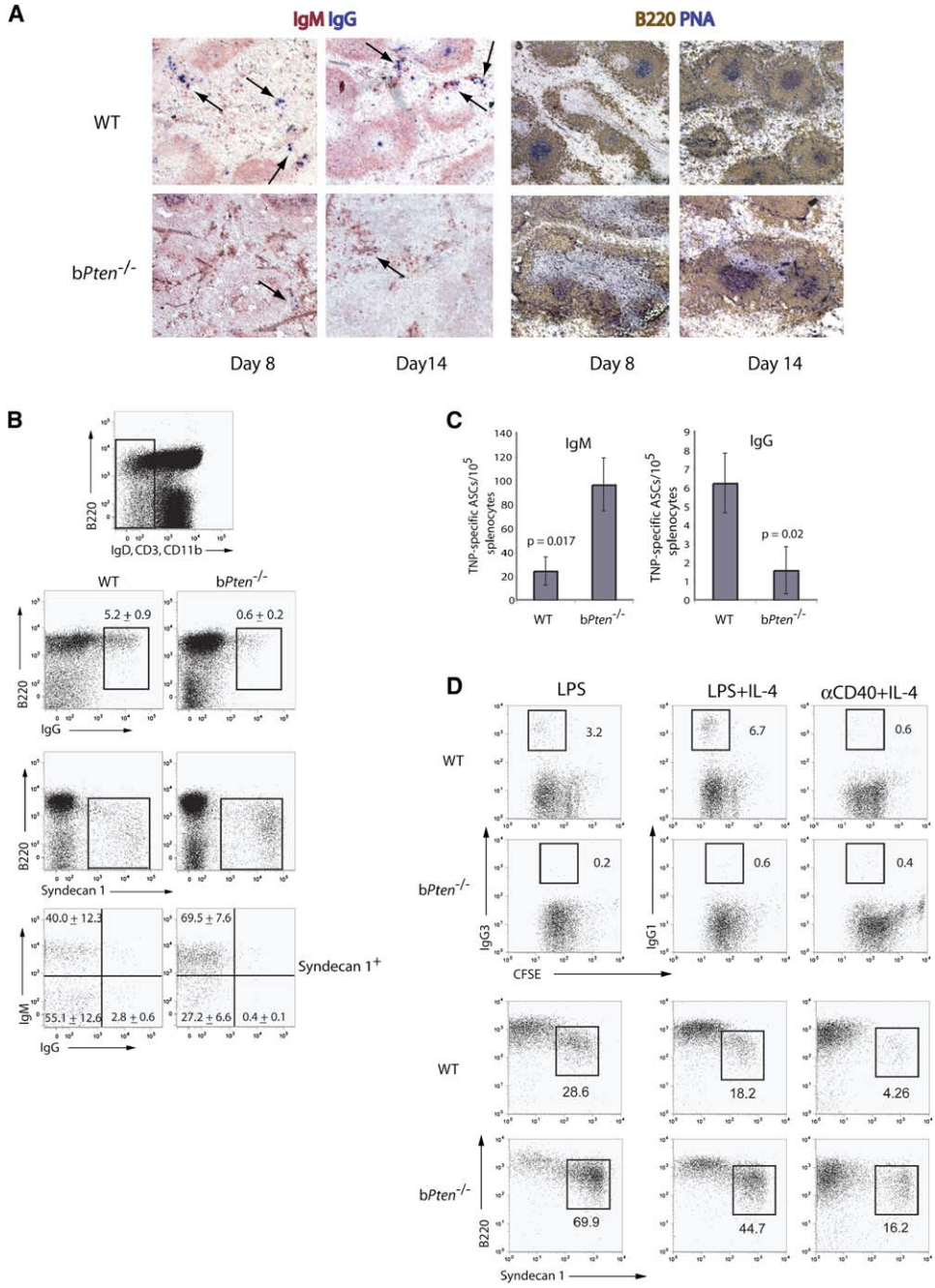


Figure 1. b*Pten*$^{-/-}$ B Cells Display Enhanced ASC Formation, but Fail to Undergo CSR

(A) Spleens from mice immunized with TNP-OVA were stained for ASCs (left) with anti-IgM (red) or a cocktail of anti-IgG1, -IgG2a-b, and -IgG3 (blue); or with PNA (peanut agglutinin, blue) for GCs within B cell (B220, brown) follicles (right) on d8 and d14 after immunization. IgG$^+$ ASC clusters are indicated by arrows.

(B) Splenocytes from the same mice were stained for IgM and IgG (cocktail of anti-IgG1, -IgG2a-b, and -IgG3) and Syndecan1 (Syn1) expression on IgD-, CD3-, CD11b-negative cells as indicated by the starting gate. The mean ± SD of the IgG$^+$ populations; and the IgM$^+$, IgM$^-$IgG$^-$, and IgG$^+$ populations within the B220$^{int\text{-}lo}$Syn1$^+$ ASC gate for three mice in each group is shown. Data are representative of at least three experiments of n $\geq$ 3 mice each.

(C) ELISPOT analysis (mean ± SD) of antigen-specific ASCs from mice immunized 14 days prior with TNP-OVA (n = 4 mice each).

(D) Purified B cells cultured with the indicated mitogens were stained for B220, Syn1, and isotype-specific IgG as indicated to measure ASC formation (gated B220$^{lo}$Syn1$^{hi}$ population) and CSR (gated CFSE$^{lo}$IgG$^+$ population). Values indicate the percentage of cells in the gated population. Data are representative of five or more independent experiments.

induce marked CSR to IgG1 in either WT or b*Pten*$^{-/-}$ B cells, but did cause enhanced ASC differentiation in the absence of PTEN. Consistent with our in vivo findings, these data suggest that unchecked $PIP_3$ production supports the development of ASCs while suppressing CSR.

### Inhibition of PI3K Activity Promotes CSR in WT B Cells

Because elevated amounts of $PIP_3$ inhibited CSR in b*Pten*$^{-/-}$ B cells, we reasoned that inhibition of induced PI3K activity would promote CSR. To test this hypothesis, WT B cells were labeled with the cell division tracking dye carboxyfluorescein diacetate (CFSE) and cultured under the conditions described above in the absence or presence of the pan-PI3K inhibitor, LY294002, or the PI3K$\delta$-specific inhibitor, IC87114 (Puri et al., 2004; Sadhu et al., 2003). A titration curve was performed to determine the inhibitor concentrations that had limited effects on survival and proliferations (Figures S2 and S5). These concentrations (5 $\mu$M for LY294002 and 10 $\mu$M for IC87114) were used in all subsequent experiments. Analyses of equal numbers of live cells showed that inclusion of LY294002 in LPS- or LPS+IL-4-stimulated cultures resulted in enhanced CSR to IgG3 or IgG1, respectively, compared to untreated cells receiving the same stimuli (Figure 2A). When IC87114 was included in cultures stimulated with LPS, LPS+IL-4, or $\alpha$CD40+IL-4, a greater increase in the amount of CSR to IgG3 or IgG1, respectively, was observed relative to untreated or LY294002-treated cultures (Figure 2A). An analysis of multiple experiments showed that increased CSR in cultures treated with PI3K inhibitors compared to untreated cultures was highly reproducible and statistically significant (Figure 2B). Variations in cell density, mitogen concentration, source of IL-4 (recombinant or culture supernatant), and switching to nontargeted loci were also tested to verify the generality of these findings (Figure S3). While we cannot rule out that LY294002 had more widespread effects that negatively regulated CSR, which could account for the difference in the effects of the two inhibitors, it is more probable that this difference was due to increased toxicity associated with LY294002 compared to IC87114 (data not shown). At the concentration used, LY294002 strongly inhibits PI3K$\delta$ but also affects PI3K$\beta$; whereas the chosen concentration of IC87114 specifically affects PI3K$\delta$ (Sadhu et al., 2003). Altogether these data indicate that PI3K$\delta$ is the predominant class I PI3K isoform involved in regulating CSR. In addition to their profound effect on CSR, the selected concentrations of PI3K inhibitors also impaired ASC formation both phenotypically as measured by FACS (Figure 2A) and functionally as measured by ELISPOT (Figure S4), suggesting that increased CSR may come, in part, at the expense of ASC formation.

Because treatment of stimulated cultures with IC87114 did result in a generalized decrease in cell survival, it was necessary to ascertain whether the differences in CSR and ASC formation observed in IC87114-treated cultures were due to selective survival or death of the class-switched or ASC populations. When cellular viability in d3 cultures was measured by 7AAD staining, the data showed that there was no evidence for a selective decrease in ASC viability in inhibitor-treated cultures that could account for the decrease in ASC formation, nor selective survival of class-switched cells to account for the increase in CSR (Figure S5).

CSR is targeted by cytokine-induced transcriptional activation of specific heavy chain constant regions (Stavnezer, 1996). To determine whether enhanced CSR in the presence of PI3K inhibitors was applicable to other isotypes or stimuli, WT B cells were stimulated with LPS or LPS+TGF-$\beta$ (Figure S6) or LPS+IFN-$\gamma$ (data not shown) in the absence or presence of IC87114. FACS analyses revealed enhanced CSR to IgG2a or IgG2b (LPS, LPS+TGF-$\beta$, LPS+IFN-$\gamma$) and IgA (LPS+TGF-$\beta$) in the presence of IC87114. Suppression of CSR in the absence of PTEN does not affect the production of sterile transcripts from the heavy chain locus (Suzuki et al., 2003, and our data not shown), so these data indicate that PI3K activity is not acting at the level of locus accessibility, but is involved in regulating the central process of CSR.

### Inhibition of PI3K$\delta$ Increases the Rate and Frequency of CSR

The appearance of class-switched cells and ASCs occurs in a division-linked manner that does not depend on the length of time in cycle (Hasbold et al., 1998, 2004; Hodgkin et al., 1996). To ascertain the reason for increased numbers of class-switched cells in cultures treated with IC87114, the percent IgG$^+$ cells per generation was determined (Figure 2C). In untreated, LPS-, or LPS+IL-4-stimulated cultures, substantial numbers of switched cells were not apparent until the third or fourth division and did not occur to a substantial degree in untreated $\alpha$CD40+IL-4 cultures. In marked contrast, cultures treated with IC87114 produced large numbers of switched cells as early as the first division cycle and steadily increased with each subsequent division (Figure 2C, Figure S3). Similar results were observed when recombinant IL-4 was used instead of IL-4-containing supernatant (Figure S3). The preponderance of switched cells observed at later divisions is likely due to the additive presence of both newly switched cells at a particular division cycle as well as the persistence of cells that had undergone CSR in a previous cell cycle and continued to divide (Hasbold et al., 2004).

Because PI3K seemed to regulate CSR, we wished to determine whether decreasing PI3K activity in stimulated b*Pten*$^{-/-}$ B cells would restore CSR. When CSR was assessed in b*Pten*$^{-/-}$ B cell cultures stimulated with LPS, LPS+IL-4, or $\alpha$CD40+IL-4 in the presence of LY294002 or IC87114, we found that CSR was partially restored (Figure 3). These findings indicate that the inability of b*Pten*$^{-/-}$ B cells to undergo CSR is not a result of altered in vivo differentiation, but due to active suppression of the class-switch machinery in the presence of sustained $PIP_3$. These data strongly support the hypothesis that a reduction in $PIP_3$ or reduced PI3K activity is a necessary prerequisite for initiation of the CSR program during B cell differentiation.

### Inhibition of CSR by PI3K Is Partially Dependent on BLIMP1

The transcription factor BLIMP1 is absolutely required for ASC development, and gene expression arrays suggest that it may repress AID expression and thus CSR (Sciammas and Davis, 2004; Shaffer et al., 2002). Our finding that PI3K inhibitors promote CSR and suppress ASC formation in vitro suggested that BLIMP1 and PI3K have a functional relationship. To explore this possibility, B cells from mice carrying a B cell-specific deletion of BLIMP1 (b*Prdm1*$^{-/-}$) were stimulated in the

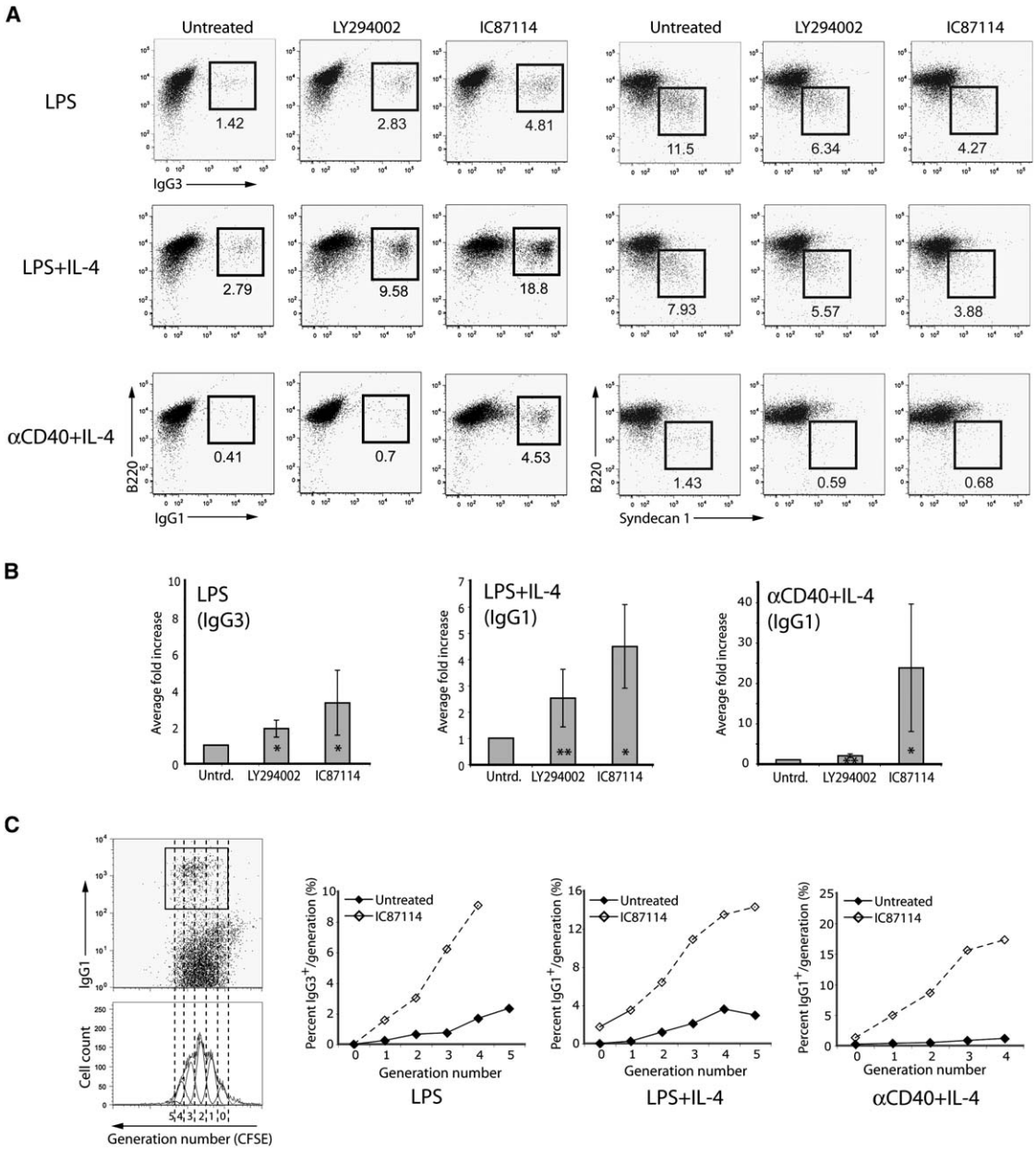


Figure 2. Inhibition of PI3K Activity Increases the Generation of Class-Switched Cells

(A) WT B cells cultured with the indicated mitogens in the absence or presence of the pan-PI3K inhibitor, LY294002, or the PI3Kδ-specific inhibitor, IC87114, were stained for B220, Syn1, and isotype-specific IgG as indicated to identify class-switched cells ($B220^+IgG^+$, left) and ASCs ($B220^{lo}Syn1^+$, right). Values indicate the percentage of cells in the gated population. Data are representative of 4–10 independent experiments.

(B) Average fold increase (mean ± SD) in CSR of PI3K inhibitor-treated cultures over untreated cultures of 4–10 independent experiments. p values are indicated by asterisks (*$p < 0.005$, **$p < 0.05$).

(C) Division-linked CSR was assessed in WT B cells cultured with the indicated mitogens in the absence or presence of IC87114. Graphs show the proportion of isotype-specific $IgG^+$ cells present in each cell division. Each division peak was gated with the proliferation tool in FlowJo (Treestar), and a representative gating profile for WT B cells stimulated with LPS+IL-4 in the presence of IC87114 is shown. Data are representative of 4–10 independent experiments.

absence or presence of IC87114 and analyzed for CSR and ASC formation. As previously reported (Shapiro-Shelef et al., 2003), ASC development was completely blocked in cultures of b*Prdm1*$^{-/-}$ B cells (data not shown). However, stimulation of b*Prdm1*$^{-/-}$ B cells with LPS or LPS+IL-4, even in the absence of IC87114, resulted in increased CSR (Figure 4A). To determine whether suppression of CSR by PI3K was dependent on BLIMP1 expression, CSR was assessed in B cells lacking both PTEN and BLIMP1 (b*Pten*$^{-/-}$b*Prdm1*$^{-/-}$). The absence of BLIMP1 resulted in a partial restoration of CSR in B cells that also lacked PTEN (Figure 4B). Together with the observation that IC87114 enhanced CSR in b*Prdm1*$^{-/-}$ B cells to levels that surpassed those achieved in IC87114-treated WT B cells (Figure 4A), these data suggest that BLIMP1 activation downstream

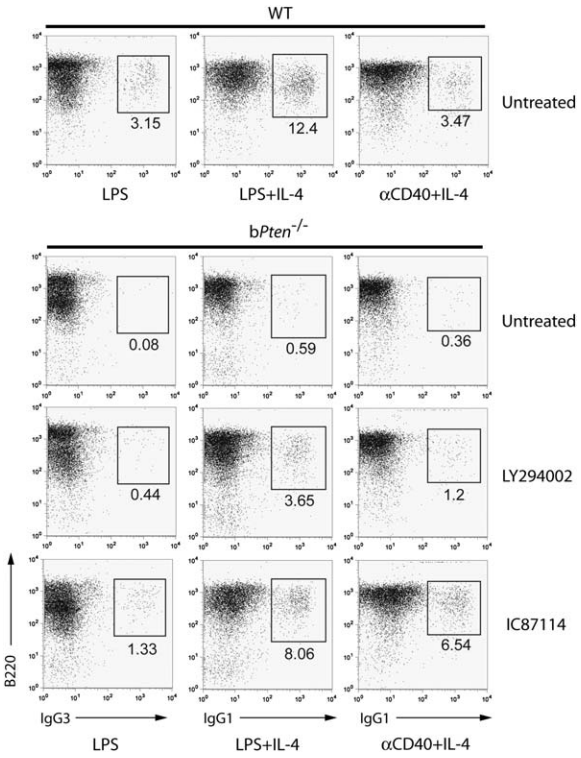


Figure 3. Inhibition of PI3K Activity Partially Restores CSR in b*Pten*$^{-/-}$ B Cells

Isotype-specific CSR and ASC formation were assessed in b*Pten*$^{-/-}$ B cells cultured with the indicated mitogens in the absence or presence of LY294002 or IC87114. WT B cells stimulated in the absence of inhibitors are shown for comparison. Values indicate the percentage of cells in the gated population. Data are representative of four independent experiments.

of PI3K is partially responsible for suppression of CSR in the absence of PTEN.

The ability of BLIMP1 deficiency alone to increase CSR correlated with culture conditions that promote the generation of ASCs in WT cells. These data are consistent with the observation that WT B cells cultured in the presence of IC87114 have a decrease in ASC generation (see Figure 2). These data suggest that under conditions where an uncommitted, activated precursor is prevented (chemically or genetically) from becoming an ASC, those cells are more prone to undergo CSR. In support of this hypothesis, when CSR was analyzed with respect to division number, the combined effect of BLIMP1 deficiency and PI3Kδ inhibition was additive in cultures stimulated with LPS or LPS+IL-4 (Figure 4C). These results imply that BLIMP1 expression contributes to PI3Kδ-dependent inhibition of CSR by coordinating ASC commitment (see Discussion).

### Inhibition of CSR by PI3K Occurs via Akt-Mediated Inhibition of Foxo Transcription Factors

Akt is a central downstream target of PI3K; therefore, we wished to determine whether it contributed to PI3K-mediated inhibition of CSR. Immunoblot analysis of WT B cells stimulated with LPS+IL-4 in the presence of IC87114 showed strong inhibition of Akt activation at 1 hr poststimulation that was sustained to 24 hr (Figure 5A). To directly address the role of Akt in PI3K-mediated inhibition of CSR, retroviral gene transfer was used to express constitutively active Akt (myrAKT, membrane targeted) in WT B cells prestimulated with LPS+IL-4. Where noted, IC87114 was added to cells immediately after infection. Cells were harvested 2 days after infection, and infected cells (GFP$^+$) were analyzed for CSR and ASC formation (Figure 5B). Strikingly, myrAKT strongly blocked CSR and caused a modest increase in LPS+IL-4-induced ASC formation. Moreover, myrAKT also blocked enhanced CSR promoted by IC87114, consistent with the $PIP_3$-independent targeting of myrAKT to the membrane. Quantitative RT-PCR (QT-PCR) of GFP$^+$ sorted cells revealed that inhibition of CSR by myrAKT correlated with the reduction of AID message.

To further elucidate the Akt-dependent pathway leading to inhibition of CSR, we focused on the Foxo family of transcription factors that are negatively regulated by Akt phosphorylation (Greer and Brunet, 2005). Mutation of the regulatory serine and threonine residues in Foxo proteins results in a constitutively nuclear protein that can no longer be phosphorylated and inactivated by Akt (Greer and Brunet, 2005). Both Foxo1 and Foxo3a are expressed at demonstrable amounts in primary B cells; however, Foxo1 appears to be more abundant (Yusuf et al., 2004). To determine whether the Foxo proteins were responsible for promoting CSR when PI3K-Akt activity are attenuated, WT and b*Pten*$^{-/-}$ B cells were infected with retroviruses expressing wild-type (Foxo1) or constitutively nuclear Foxo1 (Foxo1(A3)), and infected cells (Thy1.1$^+$) were analyzed for CSR and ASC formation (Figure 5C). Expression of wild-type Foxo1 did not induce higher CSR in either WT or b*Pten*$^{-/-}$ B cells. However, consistent with the involvement of Akt in the inhibition of CSR downstream of PI3K activation, Foxo1(A3) caused a small increase in CSR in WT B cells and a significant increase in CSR in b*Pten*$^{-/-}$ B cells. Further, Foxo1(A3) decreased ASC formation in both WT and b*Pten*$^{-/-}$ B cell cultures (Figure 5C). Interestingly, Foxo1(A3) caused an even greater increase in CSR in b*Pten*$^{-/-}$ B cells treated with IC87114 than was observed in similarly treated WT B cells. Consistently, QT-PCR analyses of infected cells showed strong induction of AID message in cells infected with Foxo1(A3)-expressing virus. These data suggest that PI3K activity supports ASC differentiation and inhibits CSR in uncommitted precursors via Akt activation and nuclear exclusion of Foxo1 resulting in poor induction of AID expression.

It has been proposed that BCL6 is a downstream target of Foxo3a and Foxo4 (Fernandez de Mattos et al., 2004; Tang et al., 2002). We have shown that forced expression of constitutively nuclear Foxo1 is able to repress ASC formation and promote CSR (Figure 5C). To determine whether BCL6 acts downstream of Foxo1 to promote CSR, WT and b*Pten*$^{-/-}$ B cells were infected with retroviruses expressing BCL6 (Figure 5D). Ectopic BCL6 strongly inhibited ASC formation in response to LPS+IL-4 as previously demonstrated (Reljic et al., 2000; Tunyaplin et al., 2004); however, no enhancement of CSR was observed in WT or b*Pten*$^{-/-}$ B cells, suggesting that BCL6 does not promote CSR even though it is required for GC formation (Niu, 2002). Hence,

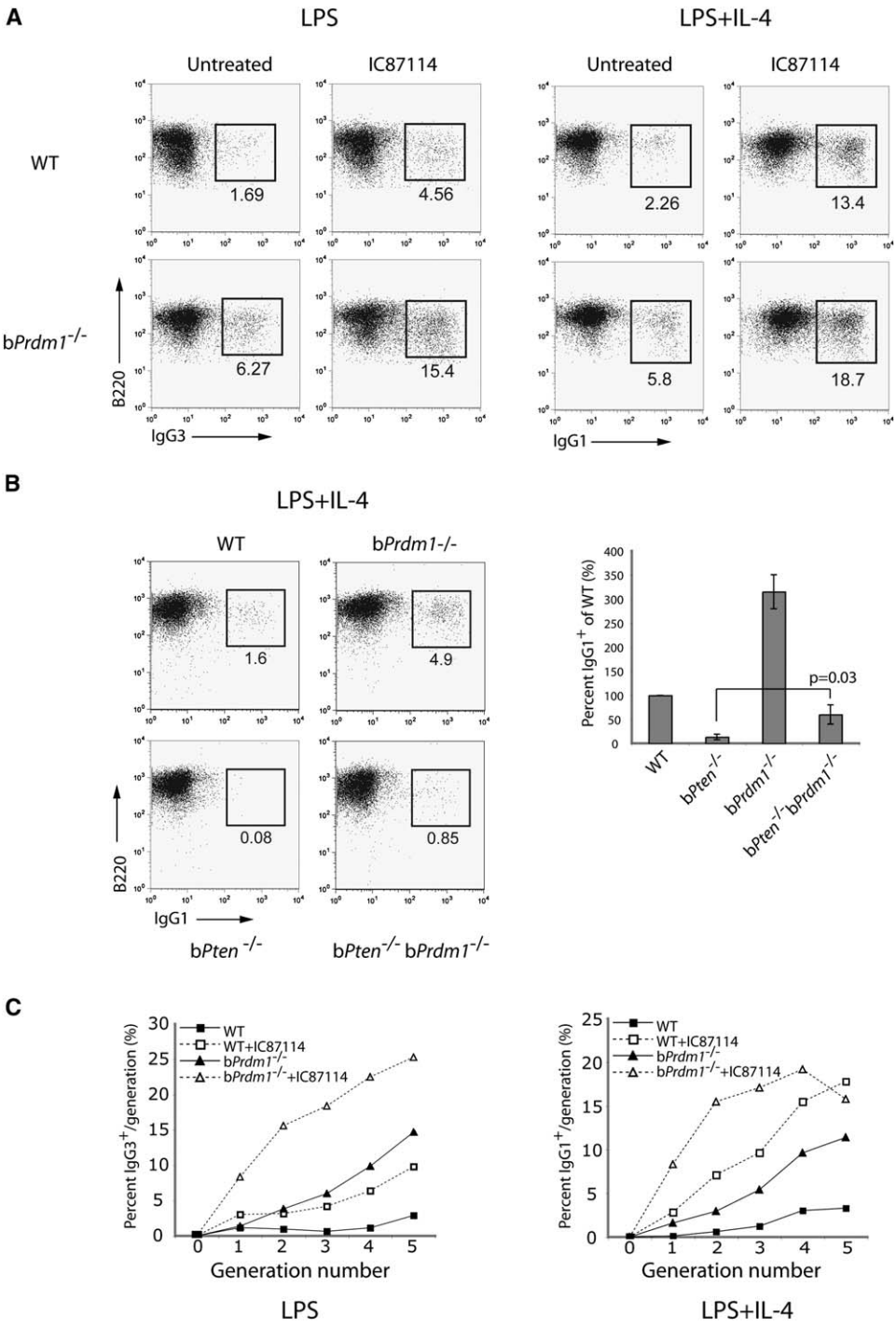


Figure 4. Inhibition of PI3Kδ in b*Prdm1*$^{-/-}$ B Cells Causes Synergistic Enhancement of CSR

(A) Isotype-specific CSR was assessed in WT and b*Prdm1*$^{-/-}$ B cells cultured with the indicated mitogens in the absence or presence of IC87114 as described previously. Values indicate the percentage of cells in the gated population. Data are representative of four independent experiments.

(B) CSR in B cells deficient for both PTEN and BLIMP1 (b*Pten*$^{-/-}$b*Prdm1*$^{-/-}$). A representative FACs profile is shown with percentage of cells in the gated population indicated. Bar graph shows the mean ± SD of percent IgG$^+$ compared to percent IgG$^+$ observed in WT B cells (set to 100% in each experiment) for three independent experiments. p value for b*Pten*$^{-/-}$ compared to b*Pten*$^{-/-}$b*Prdm1*$^{-/-}$ is indicated on the graph.

(C) Graphs show the proportion of IgG$^+$ cells present in each cell division for the experiment shown in (A). Division gating was performed as shown in Figure 2C. Data are representative of four independent experiments.

although suppression of ASC development by Foxo1 may be due to BCL6 expression, increased CSR by Foxo1 is not.

## Elevated PI3K Activity Affects AID Function

We showed that pharmacologic inhibition of PI3K activity in b*Pten*$^{-/-}$ B cells partially restored CSR (Figure 3).

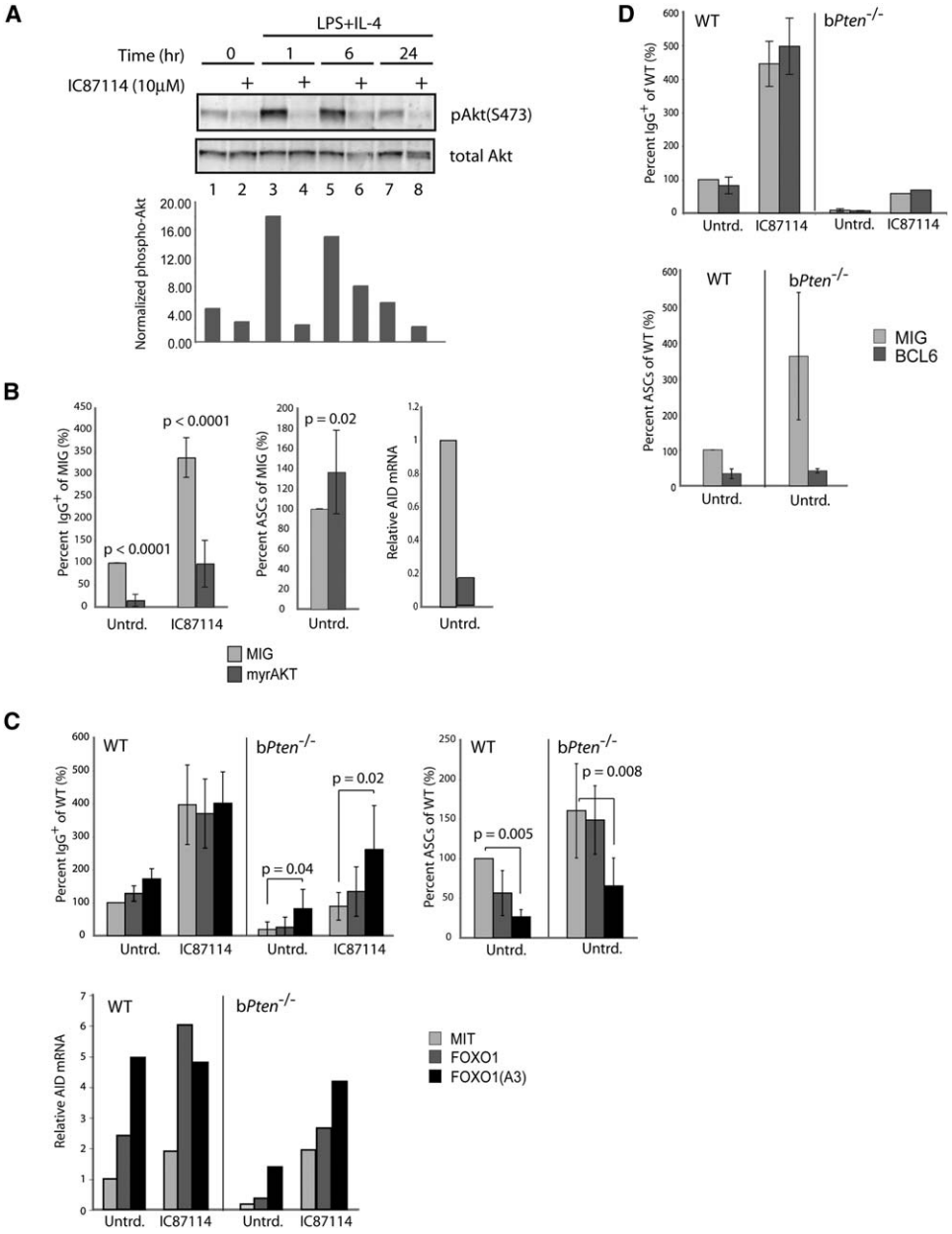


Figure 5. CSR Is Regulated by the Akt/Foxo Signaling Axis

(A) Akt activation was assessed in purified B cells stimulated with LPS+IL-4 in the absence or presence (+) of IC87114 for the indicated times. The bar graph shows the amount of phospho-Akt normalized to total Akt in each sample.

(B–D) B cells were infected with bicistronic retroviruses expressing (B) constitutively active Akt (myrAKT), (C) wild-type Foxo1 (Foxo1) or constitutively nuclear Foxo1 (Foxo1(A3)), (D) BCL6, or empty virus control (MIG or MIT). After infection, cells were cultured for 2 additional days in the absence or presence of IC87114, after which CSR and expression of ASC markers were assessed in infected cells (GFP$^+$, myrAKT, or BCL6; Thy1.1$^+$, Foxo1, or Foxo1(A3)). Bar graphs show the mean ± SD of the percent IgG1$^+$ (or B220$^{lo}$Syn1$^{hi}$ ASCs) of myrAKT-, Foxo1-, Foxo1(A3)-, or BCL6-infected cells compared to WT cells infected with empty virus cultured in the absence of inhibitor (set at 100% in each experiment) for at least six independent experiments (B, C) or two independent experiments (D). p values for the bracketed populations are indicated on each graph. Typical infection efficiency for all viruses was 30%–70%. Relative AID message levels were determined by QT-PCR on sorted GFP$^+$ (B) or Thy1.1$^+$ (C) infected cells, and graphs are representative of three experiments. Untrd, untreated.

QT-PCR of RNA from stimulated b*Pten*$^{-/-}$ B cells showed impaired AID expression (see Figure 5C), consistent with previous work (Suzuki et al., 2003), whereas inhibition of PI3K caused an increase in AID expression. To determine whether impaired CSR in b*Pten*$^{-/-}$ B cells is solely due to negative regulation of AID expression by PI3K, AID was ectopically expressed in stimulated b*Pten*$^{-/-}$ B cells by retroviral infection (Figure 6A). Ectopic expression of AID in b*Pten*$^{-/-}$ B cells resulted in increased CSR to levels similar to those observed in b*Pten*$^{-/-}$ B cells infected with empty virus (TAC) and treated with IC87114, suggesting that PI3K is regulating CSR by controlling AID expression. However, these levels were still lower

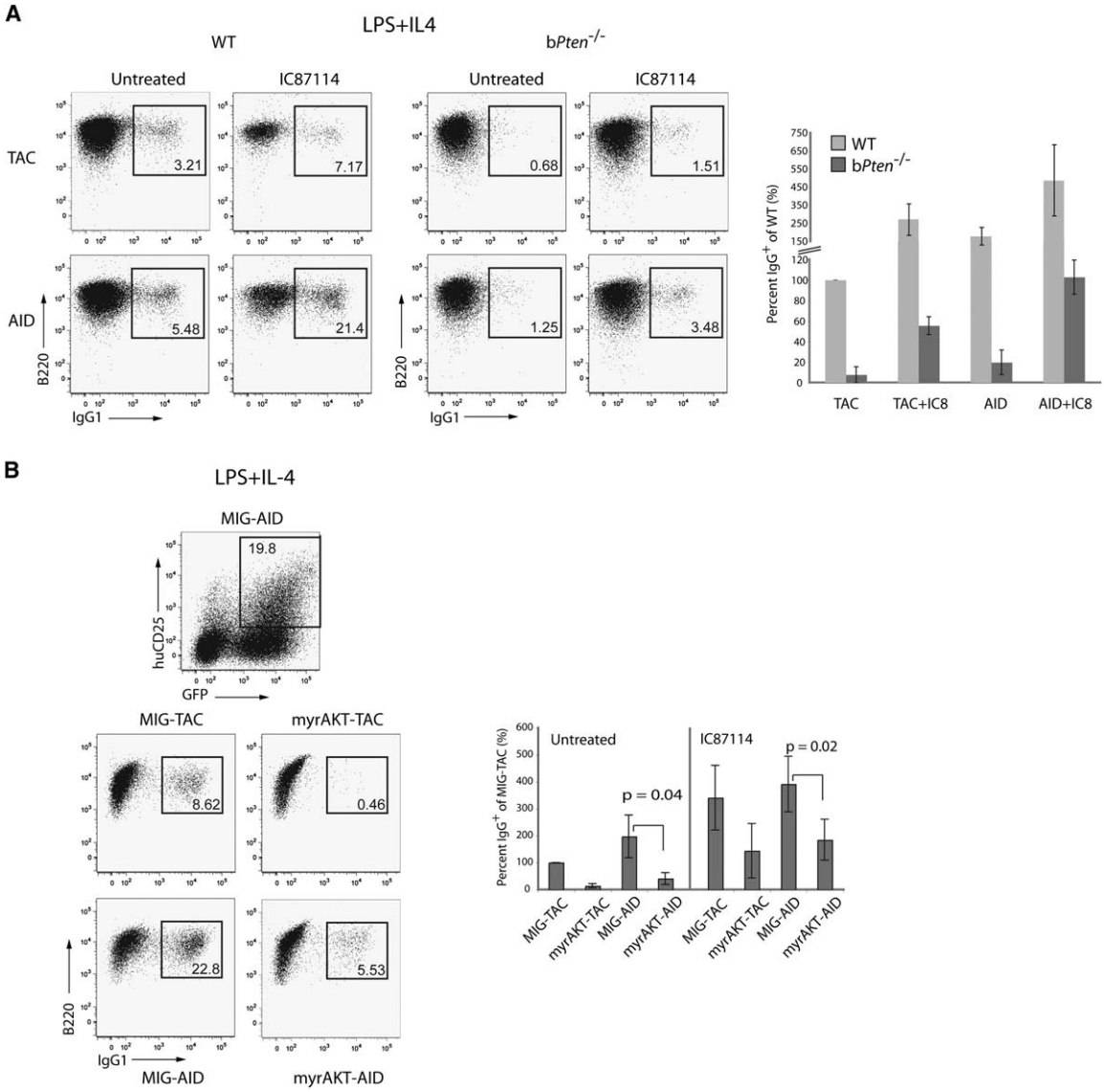


Figure 6. AID Expression only Partially Rescues CSR in b*Pten*$^{-/-}$ B Cells

(A) WT and b*Pten*$^{-/-}$ B cells were infected with AID-expressing retrovirus or empty virus control (TAC) and recultured in the absence or presence of IC87114 for 3 days. Infected cells identified by huCD25$\alpha$ expression were assessed for CSR and ASC formation. A representative FACS plot is shown with the percentage of cells in the gated population indicated, and the bar graph shows the mean ± SD of the percent IgG1$^{+}$ AID-infected cells compared to WT cells infected with empty virus cultured in the absence of inhibitor (set at 100% in each experiment) for four independent experiments. p values for the bracketed populations are indicated on each graph.

(B) WT B cells were infected with both myrAKT (see Figure 5B) and AID-expressing retroviruses and recultured in the absence or presence of IC87114 for 3 days. Doubly infected cells were identified by dual expression of GFP (myrAKT) and huCD25$\alpha$ (AID) as indicated for the MIG-AID sample and assessed for CSR. Empty virus controls are MIG (for myrAKT) and TAC (for AID). A representative FACs profile for untreated cells is shown with the percentage of cells in the gated population indicated. The bar graph shows the mean ± SD of the percent IgG1$^{+}$-infected cells compared to WT cells infected with both empty viruses (MIG-TAC) cultured in the absence of inhibitor (set at 100% in each experiment) for three independent experiments. p values are indicated on each graph. Infection efficiency was typically 15%–40%. IC8, IC87114.

than those achieved in WT B cells infected with empty virus and stimulated in the absence of inhibitor. Furthermore, when b*Pten*$^{-/-}$ cells infected with the AID-expressing virus were allowed to recover in the presence of IC87114, an additional enhancement of CSR was observed. These data suggest that expression of AID does not completely restore CSR in the presence of high PI3K activity, implying that PI3K may also inhibit AID function in addition to regulating its expression.

To test this hypothesis, WT B cells stimulated with LPS+IL-4 or $\alpha$CD40+IL-4 (Figure 6B and data not shown) were doubly infected with constitutively active Akt (myrAKT) and AID-expressing retroviruses. Analysis of CSR showed that cells containing both myrAKT and AID displayed increased CSR compared to cells infected with AKT alone, but significantly lower levels of CSR compared to cells infected with AID alone. These data support our hypothesis that sustained PI3K-Akt

activity prevents CSR by inhibiting AID at the transcriptional and posttranscriptional levels.

## Discussion

Primary antibody responses are dominated by low-affinity IgM that provides neutralizing activity to limit dissemination of toxic or infectious agents. As clonal expansion proceeds, some B cells undergo ASC differentiation, while others undergo CSR or form GCs. Class-switched antibody is a major component of primary antibody responses as the humoral response progresses and contributes more effectively to host defense through diversified effector functions, and later post-GC through enhanced affinity. The necessity for CSR and effective immune responses is underscored by deficiencies noted in patients with HIGM syndrome. These patients have increased susceptibility to infections resulting in part from an inability to undergo CSR (Etzioni and Ochs, 2004). Our data support the view that PTEN deficiency in B cells may also represent a HIGM condition. Thus, an interesting prospect is that cases of HIGM that currently have no genetic classification may carry mutations in genes that regulate PI3K activity.

It has been proposed that ASC formation and CSR are regulated by stochastic mechanisms at the level of the individual cell (Hasbold et al., 2004), and while terminal gene targets required for these processes have been identified (BLIMP1, BCL6, and AID), the signaling pathway(s) involved are largely unknown. We show here that commitment to ASC formation or CSR is intimately linked to the degree of PI3K activation elicited by surface receptors. Inhibition of PI3Kδ strongly promotes CSR by increasing the generation of switched cells at earlier cell divisions. These data support the hypothesis that repression of CSR and simultaneous promotion of ASC formation by strong PI3K activity prevents premature CSR during the early stages of antigenic encounter when antigen load is high and BCR-dependent clonal expansion is vigorous. Such a mechanism would allow for the successive generation of IgM-secreting ASCs to promote initial clearance of antigen. Indeed, it has been shown that multivalent crosslinking of the BCR (mimicked in vitro with anti-IgM $(Fab')_2$ fragments) causes a delay in CSR in response to CD40L+IL-4 stimulation (Rush et al., 2002). Based on our data, we propose that this effect is caused by strong PI3K activation through the combined contributions of the BCR, CD40, and IL-4R (IL-4 receptor). As antigenic load decreases, changes in surface receptor signaling resulting in reduced PI3K signaling together with depletion of $PIP_3$, presumably by PTEN, causes an overall reduction in $PIP_3$ levels, allowing for CSR and the generation of more specialized class-switched ASC effectors.

By using retroviral gene transfer, we demonstrate that regulation of CSR and ASC formation by PI3K occurs via the PI3K→Akt→Foxo signaling axis. Constitutively active Akt (myrAKT) inactivates Foxo transcription factors and strongly suppresses CSR, whereas constitutively nuclear Foxo1 (Foxo1(A3)) enhances CSR and suppresses ASC formation. A potential mechanism for suppression of ASC formation by Foxo proteins is the activation of BCL6, a target of Foxo transcription factors in other systems (Fernandez de Mattos et al., 2004; Tang et al., 2002). Since BCL6 expression appears to be confined to the GC, such a mechanism may operate only in the late follicular response. However, enhanced ASC formation by *BCL6*$^{-/-}$ B cells in vitro suggests that a regulatory role for BCL6 in the extrafollicular ASC response cannot be ruled out (Tunyaplin et al., 2004). In addition, our data show that while ectopic BCL6 is sufficient to suppress ASC development, it does not induce or enhance CSR in WT B cells regardless of PI3K activity. Hence, BCL6 is likely not the Foxo target in B cells that induces CSR, providing further support that CSR is not a BCL6-driven process.

It was recently reported that the expression of AID is regulated by cell division and that its overexpression in B cells from AID transgenic animals results in the appearance of switched cells at a higher frequency and earlier cell divisions (Rush et al., 2005). These findings are reminiscent of what we observe here with the PI3Kδ inhibitor, implying that PI3K regulates division-linked CSR by controlling the expression of AID. However, while forced expression of constitutively nuclear Foxo1 caused a modest rescue of CSR in b*Pten*$^{-/-}$ B cells, when combined with reduced PI3K activity, a synergistic effect on CSR was observed. Furthermore, expression of AID in b*Pten*$^{-/-}$ B cells resulted in a much stronger rescue of CSR when combined with inhibition of PI3K activity, and expression of active Akt decreased CSR induced by ectopic AID expression in WT B cells. Together, these data suggest that PI3K is acting to suppress CSR by interfering with AID transcription as well as its function.

There are many points at which PI3K activity could be regulating the function of AID in CSR. AID contains a nuclear export sequence (NES) that prevents its nuclear accumulation (McBride et al., 2004; Shinkura et al., 2004). However, forced nuclear accumulation of AID did not result in increased CSR (McBride et al., 2004), implicating a requirement for other limiting factors. Along these lines, CSR and SHM require separate domains of AID and the recruitment of specific cofactors (Barreto et al., 2003; Shinkura et al., 2004; Ta et al., 2003), and AID activity is dependent upon phosphorylation by protein kinase A (Basu et al., 2005; Pasqualucci et al., 2006). Hence, PI3K could be directly affecting AID by regulating its nuclear localization, phosphorylation, and/or association with cofactors, and/or by regulating the nuclear localization and/or expression of cofactors or upstream effectors.

Our data suggest that PI3K acts to suppress CSR in both a BLIMP1-dependent and BLIMP1-independent manner. This hypothesis is supported by the fact that the absence of BLIMP1 in b*Pten*$^{-/-}$ B cells partially restores CSR. One explanation as to why the level of rescue in these cells is not higher is that these cells retain a high level of activated Akt acting to suppress CSR in a PI3K-dependent, likely Foxo1-dependent, and BLIMP1-independent manner. These data are consistent with the observation that increased CSR observed in b*Prdm1*$^{-/-}$ B cells can be further augmented by inhibiting PI3K. Under conditions that efficiently promote ASC development, BLIMP1 expression is sufficient to promote the early entry of some uncommitted precursors toward ASC formation, suppressing further CSR (Hasbold et al., 2004; Sciammas and Davis, 2004;

Shaffer et al., 2000). However, in the absence of BLIMP1, the number of uncommitted precursors is increased (due to the unavailability of the ASC pathway), thereby increasing the potential frequency of cells available to undergo CSR. Our data suggest that enhanced CSR due to inhibition of PI3K is the result of increased recruitment of undifferentiated precursors into the CSR pathway, in part due to the inhibition of ASC formation.

We propose that during the early stages of the antibody response, ASC formation is promoted and CSR is suppressed in uncommitted precursors by high PI3K activity. Suppression of CSR by PI3K is dependent on Akt activity and inhibition of Foxo family transcription factors. As the local antigenic load decreases, PI3K activity is reduced and Foxo proteins are released from Akt-mediated repression. Activation of Foxo proteins results in the repression of ASC development, potentially through inhibition of BLIMP1, allowing the expression and activation of AID to enable CSR. Subsequently, class-switched cells re-enter the differentiation cycle and can reinitiate the ASC genetic program or undergo another round of CSR. Whether the signals that trigger ASC formation of a class-switched cell are the same as those required for ASC formation of a naive B cell (i.e., high PI3K activity) remains to be determined.

## Experimental Procedures

### Mice

B cell-specific deletion of PTEN and BLIMP1 and genotyping has been described previously (Anzelon et al., 2003; Shapiro-Shelef et al., 2003). WT littermate controls were used whenever possible to control for background variation. No differences were observed between *CD19*$^{+/+}$ or *CD19cre*$^{+/-}$ control animals. Experiments utilizing only WT animals were performed with 129 Sv/J mice bred in the BIMR animal facility. All experiments were performed on animals 8–20 weeks old and approved by the BIMR Animal Review Committee.

### Immunizations and Immunohistochemistry

Mice were immunized by intraperitoneal injection with 50 μg of $TNP_{12}$OVA (Biosearch Technologies) in alum (Imject, Pierce). Frozen sections or spleens from immunized animals embedded in OCT compound (Sakura, Finetek) were stained as described in Qian et al. (2004). Images were captured at 5× magnification.

ELISPOTS were performed as described previously (Shapiro-Shelef et al., 2003), with the exception that both IgM and IgG ASCs were differentially detected in the same well with anti-mouse IgM IR700 and anti-mouse IgG IR800 (Rockland Immunochemicals) and the Odyssey Infrared Imaging System (Licor).

### Cell Culture

After hypotonic lysis of RBCs with ACK buffer, splenic B cells were purified by negative selection with anti-CD43 magnetic beads (Miltenyi Biotec, >90% purity), labeled with 2.5 μM CFSE (Molecular Probes), and cultured in complete RPMI 1640 at 2 × $10^6$/ml for 3 days unless otherwise noted. Mitogen concentrations were: LPS (Sigma) 20 μg/ml, anti-CD40 (IC10 clone, eBiosciences) 4 μg/ml, IL-4 from cell culture supernatant 1:50 dilution. For inhibitor studies, LY294002 (5 μM in DMSO, Calbiochem) or IC87114 (10 μM in DMSO, ICOS Corporation) was added once at the beginning of the culture period unless otherwise noted. In some experiments, DMSO was added to "untreated" controls (1:1000 dilution). No differences between cells treated with or without DMSO alone were observed (data not shown).

### Flow Cytometry

Cells were preincubated with FcBlock (anti-CD16/32) prior to staining with biotinylated IgG1, IgG2a-b, IgG3, or Syndecan1 (Syn1) antibody followed by incubation with streptavidin (SA)-conjugated PerCP-Cy5 or APC, anti-Syn1-PE or -IgA-PE or -IgE-PE, and -B220-APC or -APC-Cy7. For immunized animals, splenocytes were stained with a cocktail of biotinylated anti-IgG1, -IgG2a-b, and -IgG3 followed by anti-IgD-FITC, -CD3-FITC, -CD11b-FITC, -IgM-APC, and SA-PerCP-CY5. Other antibodies used: anti-Thy1.1-APC and -human CD25α-PECy7 or -APC. All antibodies were from BDPharmingen or eBioscience. Flow cytometry was performed on a FACSCanto and analyzed with FlowJo (Treestar) analysis software. Values shown for gated populations represent percentage of total live lymphocytes based on FSC versus SSC and with doublet discrimination.

### Statistics

When noted, p values were calculated with the Student's t test.

### Retroviral Plasmids and Transduction

Bicistronic, retroviral plasmid backbones used in this work are pMIG (MSCV-IRES-GFP), pMIT (MSCV-IRES-Thy1.1), and pCSretTAC (pTAC, MMLV-IRES-huCD25α). Retroviral supernatants for primary B cell infections were obtained by cotransfecting the packaging cell line, Phoenix-eco, with a 2:1 ratio of viral plasmid to pCL-ECO packaging plasmid with Lipofectamine 2000 (Invitrogen) at a 1:2.5 ratio with a coplating protocol (Escobedo and Koh, 2003). For spinfections, primary B cells were cultured for 18–24 hr with mitogens prior to infection. Viral supernatants (1 ml/1 × $10^6$ cells) were incubated with 8 μg/ml polybrene (Sigma) for 10 min on ice prior to addition to cells and spinfection at 930 × g for 90 min at 30°C. After infection, cells were resuspended in fresh media containing mitogens with or without PI3K inhibitors.

### Western Blot

Western blots of total cell lysates were probed with rabbit anti-phosphoAkt (S473) and mouse anti-Akt (Cell Signaling Technologies) and developed with anti-rabbit AF680 and anti-mouse IR800 (Molecular Probes). Blots were imaged and band intensities calculated with the Odyssey Infrared Imaging System (Licor).

### QT-PCR

DNase-treated RNA was isolated from GFP$^+$ or Thy1.1$^+$ sorted cells by the RNeasy method (Qiagen). cDNA was prepared with oligodT-primed Sprint Advantage Single Shots (Clontech). QT-PCR was performed with SybrGreen (Applied Biosystems) on an ABI Prism7000 under standard conditions with specificity reinforced via the dissociation protocol via published primer sequences (Sayegh et al., 2003). Relative abundance of mRNA was determined with the relative quantification method and normalized to Igβ message.

### Supplemental Data

Six Supplemental Figures can be found with this article online at http://www.immunity.com/cgi/content/full/25/4/545/DC1/.

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