p110δ Phosphoinositide 3-Kinase Represses IgE Switch by Potentiating BCL6 Expression

Ting-ting Zhang, Kennedy J. Makondo and Aaron J. Marshall

*J Immunol* 2012; 188:3700-3708; Prepublished online 14 March 2012;
doi: 10.4049/jimmunol.1103302

http://www.jimmunol.org/content/188/8/3700

---

**Supplementary Material**

http://www.jimmunol.org/content/suppl/2012/03/14/jimmunol.1103302.DC1

**References**

This article cites 42 articles, 21 of which you can access for free at:

http://www.jimmunol.org/content/188/8/3700.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at:

http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at:

http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at:

http://jimmunol.org/alerts
p110δ Phosphoinositide 3-Kinase Represses IgE Switch by Potentiating BCL6 Expression

Ting-ting Zhang,* Kennedy J. Makondo,* and Aaron J. Marshall*†

PI3Ks are key signaling enzymes required for triggering many immunological functions. In B lymphocytes, PI3K signaling is required for Ag-induced proliferation and robust production of most Ab isotypes. Paradoxically, PI3K was found to have a negatively regulatory function regarding Ab class switch recombination, and blockade of PI3K can strongly potentiate IgE switch. In this article, we explore the mechanisms of this unexpected negative regulatory function of PI3K regarding IgE. We demonstrate that p110δ PI3K selectively regulates IgE switch in a B cell-intrinsic manner by controlling germline transcription of the IgE promoter (εGLT). Although p110δ can regulate transcription of activation-induced cytidine deaminase via Akt, repression of εGLT and IgE switch is not dependent on Akt signaling. Inhibition of p110δ, but not Akt, leads to reduced expression of transcriptional repressor B cell lymphoma 6 (BCL6) and concomitant upregulation of εGLT and other BCL6-target genes. p110δ inhibitor treatment strikingly alters the balance between BCL6 and IRF4 (a transcription factor that antagonizes BCL6), leading to increased IRF4 and decreased BCL6 expression levels in germinal center B cells. Ectopic expression of BCL6 can partially overcome the elevated εGLTs and potentiated IgE switching in p110δ-inhibited B cells. To our knowledge, these results provide the first evidence that p110δ PI3K signaling regulates BCL6 expression and indicate that PI3K promotes the germinal center B cell program and selectively represses IgE switch by maintaining sufficient levels of BCL6. The Journal of Immunology, 2012, 188: 3700–3708.

PI3Ks are a family of enzymes that phosphorylate phosphatidylinositol lipids at the D3 position of the inositol ring to generate second messengers, which are critical for a wide range of cellular functions, such as cell survival, proliferation, migration, and the trafficking of intracellular organelles (1, 2). Genetic or pharmacological targeting of PI3K isoform p110δ showed that this isoform plays a dominant role in immune cells (1, 2). Extensive studies have been carried out to target these enzymes, especially p110δ, for the treatment of immune cell-mediated diseases (3). Human allergy and asthma are among those diseases that may benefit from p110δ inhibition, and it was shown that in vivo inactivation of the class IA isoform p110δ is able to reduce AgR function, Th2 cytokine production, Th2-mediated inflammatory reactions, and airway hyperresponsiveness (4, 5).

Allergen-specific IgE is a hallmark of atopic diseases, contributing to the pathogenesis of allergy and asthma (6). IgE is produced by B lymphocytes in a Th2 cell-dependent manner through CD40L and IL-4, which enable B cells to undergo class switch recombination (CSR) to produce IgE. Key events required for IgE CSR include induction of activation-induced cytidine deaminase (AID) and sterile germline transcription of the IgH ε locus (7). Induction of AID is a prerequisite for initiation of CSR to all Ig isotypes, and ε germline transcripts (εGLTs) are specifically required for IgE switch (7). Our previous study and others clearly showed that in vivo inactivation of p110δ leads to selective elevation of IgE, despite diminished generation of Th2 cytokine responses (8–10). Further evidence showed that disruption of p110δ signaling results in increased induction of AID and εGLTs, suggesting that PI3K signaling regulates IgE switch at the molecular level (8). It was demonstrated that activation of PI3Ks can broadly suppress CSR through negative regulation of AID expression, because the PI3K-dependent activation of Akt inactivates transcription factor Foxo1, which is required for the induction of AID (11). However, the PI3K-dependent molecular mechanisms that selectively regulate IgE switch are still unknown.

The production of εGLTs is controlled by multiple transcription factors, which bind to the promoter regions of Igε exon to either activate or repress transcription. For example, many transcription factors, such as STAT6, NF-κB, and E2A proteins (E12 and E47), positively regulate this promoter activity (12, 13). In contrast, transcriptional repressors, such as B cell lymphoma 6 (BCL6) and ID2, compete with some transcriptional activators to negatively regulate this promoter activity (13, 14). BCL6, which is the key transcription factor controlling the germinal center (GC) B cell program, functions to repress IgE switch within GCs (15–17). Therefore, IgE production is tightly regulated by the balance of positive and negative transcription factors and largely restricted to post-GC stages (18, 19).

In this study, we hypothesize that blockage of PI3K signaling may interfere with this transcription factor network, tilting the balance toward positive regulation of IgE production. Using multiple in vivo and in vitro approaches, we show that PI3K p110δ, but not Akt, is required for full BCL6 expression. Low BCL6 expression resulting from PI3K inhibition is correlated with increased εGLTs and IgE CSR, and these effects of p110δ inhibition could be reversed by boosting BCL6 expression.
Materials and Methods

Mice and reagents

p110<sup>D910A/D910A</sup> mice (20) (backcrossed to C57BL6 for nine generations) were examined between 8 and 14 wk of age along with age- and sex-matched C57BL6 mice (bred locally or purchased from Charles River Canada). B cell-deficient mice (μMT) (Igh-6<sup>tm1Cgn</sup>) were purchased from The Jackson Laboratory. All animals were housed at the Central Animal Care Facility (University of Manitoba) in compliance with the guidelines established by the Canadian Council on Animal Care. The p110<sup>-selective inhibitor</sup> IC87114 was provided by Calistoga Pharmaceuticals (Seattle, WA). AktI-1/2 inhibitor AktI was purchased from Calbiochem.

Adoptive transfers, treatments, and immunization

Splenic B cells were purified by negative selection using CD43 MicroBeads and MACS columns (Miltenyi Biotec) and transferred by i.v. injection (2–3 × 10<sup>7</sup> per recipient) into the tail vein of μMT mice. Six days after transfer, recipient mice were immunized by i.p. injection with 50 μg OVA protein (Sigma) adsorbed onto 2 mg Al(OH)<sub>3</sub> (alum) adjuvant (Imject Alum; Pierce Chemical). Ten days after OVA immunization, sera were collected to measure total and OVA-specific Ab isotypes, as previously described (8). For in vivo inhibition of p110<sup>d</sup>, C57BL6 mice were orally dosed with 25 mg/kg IC87114 or vehicle (PEG400) twice a day for 10 d. Mice were immunized with 20 μg OVA/alum 1 d after initiation of treatment and sacrificed 9 d later.

Cell culture

Purified splenic B cells were cultured in complete medium (RPMI 1640, supplemented with 10% FCS, 2 × 10<sup>-5</sup> M 2-ME) at a final concentration of 1 × 10<sup>6</sup>/ml for 3 d with 2 μg/ml anti-CD40 (BD Bioscience) together with 20 ng/ml IL-4 (R&D Systems) in the presence or absence of pharmacological inhibitors. The p110<sup>-selective inhibitor</sup> IC87114 was used at 10 μM, and the AktI-1/2–specific inhibitor AktI was used at 2 μM.

Flow cytometry and cell sorting

Cultured B cells were treated for 1 min on ice with 200 μl cold acid buffer (18) to remove cell surface-bound Abs and then neutralized by adding 15 ml RPMI 1640 medium. Before FACS staining, cells were preincubated with Fe blocking Ab (mAb 2.4G2). For intracellular IgG1 and IgE staining, cells were fixed with 2% paraformaldehyde, permeabilized with 0.1% saponin, and then stained with PE-labeled anti-IgE (eBioscience) and FITC or allophycocyanin-labeled anti-IgG1 (BD Bioscience). For nuclear BCL6 staining, cells were fixed and permeabilized with Fixation/Permeabilization buffer (eBioscience) and stained with anti-BCL6 (N3) (Santa Cruz Biotechnology), followed by PE-labeled donkey anti-rabbit IgG (eBioscience). Flow cytometry was performed on a FACSCanto II instrument and analyzed with FlowJo software (TreeStar, Portland, OR).

FIGURE 1. μMT mice repopulated with p110<sup>-D910A</sup> B cells have selectively elevated IgE levels after OVA immunization. μMT mice transferred with splenic B cells from either WT or p110<sup>-D910A</sup> mice were immunized with OVA/alum. At 10 d postimmunization, sera were collected and assessed for the indicated Ab isotypes by ELISA (A) and levels of Ag-binding IgM, IgG1, or IgE Abs (B). Solid squares represent μMT mice repopulated with B cells from WT mice, and open circles represent μMT mice repopulated with B cells from p110<sup>-D910A</sup> mice. *p < 0.05, **p < 0.005, ***p < 0.001, compared with WT (Student t test).

FIGURE 2. p110<sup>-selective inhibitor</sup>, but not Akt inhibitor, potently enhances generation of IgE-switched cells in vitro. WT B cells were stimulated with anti-CD40 + IL-4 in the presence of the indicated doses of p110<sup>-selective inhibitor</sup> IC87114 or AktI/2 inhibitor AktI. At day 3, cells were stained for IgG1 and IgE and analyzed by flow cytometry. (A) FACS plots of intracellular IgG1 and IgE staining, representative of three independent experiments. Percentage of IgG1<sup>+</sup> cells (B) or IgE<sup>+</sup> cells (C) over a range of IC87114 or AktI doses of one representative experiment.
For cell sorting, total splenic B cells of IC87114 or vehicle-treated mice were purified and then stained with FITC-labeled anti-GL7, PE-conjugated anti-Fas, allophycocyanin-labeled anti-IgG1, PerCp-Cy5.5-labeled anti-CD3, and Pacific Blue-labeled anti-B220 (BD Pharmingen). Non-GC B cells (B220+GL7+Fas−CD3−), IgG1−GC cells (B220+GL7+Fas+IgG1−CD3−), and IgG1+GC cells (B220+GL7+Fas−IgG1+CD3+) were purified using MoFlo XDP or BD FACSAria III cell sorters.

**Immunofluorescence microscopy**

For immunohistochemistry, spleens were embedded in OCT compound (Tissue-Tek) and snap frozen in liquid nitrogen. Eight- to ten-micrometer-thick frozen sections were fixed in 4% paraformaldehyde for 10 min, blocked with 10% mouse serum in 0.3% Triton X-100 for 30 min at room temperature, and then stained overnight at 4°C with rabbit anti-BCL6 (N3) (Santa Cruz Biotechnology). After washing with PBS, the slides were stained with FITC-labeled peanut agglutinin, PE–anti-CD4 and anti-rabbit Alexa Fluor 647 (Molecular Probes) for 2 h at room temperature. After extensive washing, the slides were mounted in Prolong Gold anti-fade reagent (Molecular Probes). The sections were then viewed under an inverted confocal microscope (Ultraview LCI; Perkin-Elmer Bioscience). The mean fluorescence intensity (MFI) of BCL6 staining within GC was determined by analyzing images with ImageJ software, as previously described (21).

**Lentiviral plasmids, transduction, and cell sorting**

Full-length murine BCL6 cDNA in pCMV-SPORT6.1 vector was purchased from Open Biosystems. The full open reading frame of murine IRF4 in pCR4-TOPO was purchased from Source Bioscience (Nottingham, U.K.). BCL6 cDNA was subcloned into the NheI/BamHI restriction sites of lentiviral expression vector pCDH-CMV-MSC-EF1-copGFP (pCDH) (System Biosciences), and IRF4 cDNA was subcloned into the EcoRI/NotI restriction sites of pCDH. The lentivirus was packaged by cotransfecting 293T cells with lentiviral plasmid and packaging plasmids pCMV-dR8.2Apro and pVSV-G, as previously described (22). Splenic B cells were cultured in media containing 2 μg/ml ofCD40 for 18–24 h prior to transduction. A total of 4–5 × 10^6 B cells was suspended in 250 μl viral supernatant with 8 μg/ml Polybrene and seeded into a 24-well culture plate. The plate was then centrifuged at 700 × g for 1 h at room temperature. After spin infection, viral supernatant was removed, and fresh media containing anti-CD40 + IL-4, with or without inhibitors, was added. For flow cytometric analysis of isotype switch, transduced cells were restimulated for 2 or 36 h, and EGFP+ cells were purified by sorting with a BD FACSAria before RNA was extracted.

**Real-time PCR**

RNA was extracted from cultured B cells or FACS-sorted B cells using TRIzol reagent. cDNA was synthesized from 1 μg (cultured cells) or 100 ng (sorted cells) RNA using SuperScript II Reverse Transcriptase (Invitrogen). Quantitative RT-PCR was performed using SYBR Green amplification mix on the LightCycler System (Roche). Primer sequences for germline transcripts (GLT; γ1 and ε GLT), ε postswitch transcripts, and AID were as described (23). Additional primer sequences were as described: BCL6 (24), Blimp-1 (24), Pax5 (25), Id2 (13), E2A (26), IRF4 (26), NFIIL3 (27), Cyclin D2 (28), and CD23 (29). β-actin was used as relative expression control to normalize sample variation.

![Figure 3](http://www.jimmunol.org/)

**FIGURE 3.** p110δ signaling selectively regulates IgE switch in an Akt-independent manner. WT B cells were stimulated with anti-CD40 + IL-4 in the presence of optimal doses of IC87114 (10 μM), AktI (2 μM), or IC87114 (10 μM) + AktI (2 μM). After 3 d of culture, cells were collected and stained for IgG1 and IgE. (A) Representative FACs data for IgG1 and IgE from three independent experiments. (B) Percentage (mean ± SEM) of IgG1+ cells (left panel) or IgE+ cells (right panel) from three independent experiments. **p < 0.005, compared with DMSO control group (Student t test).

![Figure 4](http://www.jimmunol.org/)

**FIGURE 4.** p110δ, but not Akt, regulates the induction of eGLTs. WT B cells were stimulated with anti-CD40 + IL-4 in the presence of optimal doses of IC87114 (10 μM), AktI (2 μM), or IC87114 (10 μM) + AktI (2 μM). At 24 h of culture, cells were collected, and RNA was extracted. Levels of transcripts for γ1GLTs (A), eGLTs (B), and AID (C) were determined by quantitative RT-PCR. Data are expressed as normalized expression relative to the corresponding β-actin control, and graphs show the relative expression (mean ± SEM) of at least three independent cultures per condition. *p < 0.05, **p < 0.005, ***p < 0.001, compared with DMSO control group (Student t test).
Results

μMT mice repopulated with p110\(^{D910A/D910A}\) B cells show selectively increased IgE production

Our previous study demonstrated that genetic or pharmacological disruption of PI3K p110\(^d\) signaling leads to markedly potentiated IgE response in vivo, whereas no significant increase in IgG1 production was observed (8). Disruption of p110\(^d\) signaling was found to impair the function of multiple cell types, including T cells and B cells (1). To determine whether disruption of p110\(^d\) signaling in B cells is sufficient to deregulate IgE production in vivo, we adoptively transferred splenic B cells from either wild-type (WT) or p110\(^{D910A/D910A}\) mice into B cell-deficient μMT mice. Ten days after OVA/alum immunization, the μMT mice repopulated with p110\(^{D910A/D910A}\) B cells showed ~4–5-fold lower frequency of B cells in the spleen relative to mice repopulated with WT B cells, and GC responses were also markedly impaired (Supplemental Fig. 1). Examination of Ab responses revealed that μMT mice repopulated with p110\(^{D910A/D910A}\) B cells have significantly attenuated total and OVA-specific IgM and IgG1 production (Fig. 1). In contrast, total and OVA-specific IgE levels in those mice were markedly higher than in μMT mice repopulated with WT B cells (Fig. 1). These results demonstrate that disruption of p1106 signaling in B cells potentiated their IgE production in vivo.

Inhibition of Akt activity in B cells cannot account for the potent effect of PI3K p110\(^d\) inhibition on IgE switch

Addition of PI3K p110\(^d\) inhibitor IC87114 into B cell cultures stimulated with anti-CD40 + IL-4 greatly enhances isotype switching, especially IgE switching (8). One mechanism by which PI3K can suppress CSR is through activation of Akt, which controls transcription of AID (11). To test whether disrupting Akt activity results in a similar potentiation of IgE switching, we titrated IC87114 or AktI inhibitor (30) into WT B cell cultures and examined generation of switched cells by flow cytometry. AktI enhanced IgG1 switching as potently as did IC87114 and in a dose-dependent manner (Fig. 2). However, only subtle increases in the frequency of IgE\(^+\) cells could be observed in the presence of AktI, and a dose-dependent increase was not seen (Fig. 2). When both p1106 and Akt inhibitors were added into B cell cultures, the frequency of IgE\(^+\) cells reached levels comparable to cultures with...
IC87114 only, indicating that AktI has neither an enhancing nor a significant inhibitory effect on IgE switch (Fig. 3). Together, these results suggest that PI3K p110δ does not signal exclusively through Akt to selectively suppress IgE switching.

**PI3K p110δ signaling regulates transcription of εGLTs independently of Akt**

The process of CSR is exquisitely controlled at multiple levels. Accessibility of Ig loci to switch-recombination machinery is controlled at the level of sterile germline transcription of switch regions (7), and introduction of double-strand breaks within switch sequences requires expression of the AID enzyme (7). We examined levels of GLTs and AID mRNA in B cells at 24 h after culture initiation using quantitative RT-PCR. Consistent with IgG1-switching results, we observed slightly increased transcription of γ1GLTs in B cell cultures containing IC87114 and/or AktI (Fig. 4A). However, εGLTs were only significantly increased in cultures after addition of IC87114 alone or both IC87114 and AktI but not AktI alone (Fig. 4B). In contrast, AktI was effective in markedly increasing AID transcripts to a similar level as IC87114 (Fig. 4C). Addition of both IC87114 and AktI led to a further increase in AID transcription, which may be due to the greater combined capability to block Akt activation than either inhibitor alone. Together, these data suggest that blockade of p110δ PI3K signaling can potentiate IgE switch at the level of εGLT expression, which is independent of Akt activity.

**Expression of the transcriptional repressor BCL6 is significantly reduced in B cells cultured with p110δ selective inhibitor**

To understand how εGLTs are negatively regulated by PI3K activity, we examined whether p110δ inhibitor treatment influences expression of transcription factors known to bind to the ε promoter. No significant differences in mRNA levels of E2A, PAX5, and NFIL3, which promote transcription of εGLTs (13, 27, 31), were observed in B cell cultures containing IC87114 and/or AktI (Supplemental Fig. 2). However, mRNA levels of BCL6, a transcriptional repressor that suppresses GLTs (14), were significantly reduced in IC87114-treated B cells but not in AktI-treated B cells (Fig. 5A). A similar reduction in the BCL6 transcript could be observed in cultured p110δ-D910A mutant B cells (Fig. 5A). Intracellular staining confirmed that BCL6 protein expression is also lower in IC87114-treated cultures (Fig. 5B). In addition to εGLTs, BCL6 targets and represses many genes critical for B cell differentiation, activation, and cell cycling (32). Consistent with the BCL6-expression pattern, we found that transcription of BCL6 targets Cyclin D2 and ID2 were significantly increased in B cell cultures inhibited with IC87114 but not AktI (Fig. 5C). Another reported BCL6 target (CD23) was not significantly affected by IC87114 (Fig. 5C), which may suggest that CD23 transcription is less sensitive to variation in BCL6 levels (14).

BCL6 transcription in B cells was shown to be repressed by the transcription factor IRF4 (33). We examined the mRNA level of IRF4 in B cell cultures with or without inhibitors and found that inhibition of p110δ, but not Akt, led to increased IRF4 transcription, a reciprocal pattern relative to BCL6 (Fig. 5D). These data indicate that PI3K blockade can disrupt the balance between IRF4 and BCL6 expression, impacting the expression of some BCL6 target genes, including εGLTs.

**p110δ inhibition in vivo leads to reduced BCL6 expression in GC B cells**

To examine whether inhibition of p110δ in vivo affects the expression of BCL6 in GC B cells, mice were treated orally with IC87114 and immunized. By day 10 after immunization, serum total and OVA-specific IgE were selectively increased in IC87114-treated mice, consistent with previously published results (8). GC B cells purified from treated mice were found to have reduced BCL6 expression in both the IgG1+ and IgG1− fractions (Fig. 6A). On the contrary, expression levels of IRF4 mRNA were elevated in both non-GC and IgG1+ GC B cells derived from IC87114-treated mice (Fig. 6A). εGLT and AID expression were also significantly elevated in GC cells from IC87114-treated mice.

**FIGURE 6.** Pharmacological p110δ inhibition in vivo leads to increased IgE switch and reduced BCL6 expression in GC B cells. Mice were treated orally with 25 mg/kg p110δ inhibitor IC87114 or vehicle (PEG400) 1 h before OVA immunization. After immunization, IC87114 or vehicle was delivered twice per day for 10 d. At day 10, mice were sacrificed to harvest splenocytes for FACS staining and FACS sorting of IgG1+ GC, IgG1− GC, and non-GC B cells. (A) RNA was extracted from sorted cells, and levels of the indicated transcripts were determined by quantitative RT-PCR. The average and SEM of replicate quantitative RT-PCR data are shown and are representative of cells sorted from two independent groups of treated mice. (B) B220+ Fas+GL7+ cells (GC B cells) were further analyzed for IgG1 and IgE staining by FACS. Representative FACS data from six mice in each group are shown. (C) Average percentage of IgG1+ IgE+ and IgG1− IgE+ cells in GC B cells, illustrating a marked increase in the IC87114-treated group (right panel). The left panel shows that the expression pattern of postswitch ε transcripts is consistent with levels of IgE+ cells.
Flow cytometric analysis revealed that the frequencies of both IgG1+IgE+ and IgG1+IgE+ B cells are significantly increased within the GC population of IC87114-treated mice (Fig. 6B, 6C). Both postswitch ε transcripts and IgE expression are markedly increased within IgG1+ GC B cells (Fig. 6C), suggesting that PI3K signaling in vivo may predominantly suppress sequential switch from IgG1 to IgE.

Protein levels of BCL6 and IRF4 within GC of IC87114- or vehicle-treated mice were assessed by immunofluorescence staining (Fig. 7). Previous studies revealed differential distribution patterns of BCL6 and IRF4, showing that BCL6 is highly expressed in GC B cells, whereas IRF4 is expressed broadly in follicular mantle B cells but only in a small fraction of GC cells (34, 35). As quantified by MFI, GCs of IC87114-treated mice showed significant reductions in BCL6 protein levels (Fig. 7A), whereas IRF4 expression was significantly increased (Fig. 7B).

Flow cytometry also revealed a significant reduction in BCL6 expression in GC B cells (B220+Fas+GL7+) from IC87114-treated mice compared with that in GC B cells from vehicle-treated mice (Fig. 7C). Collectively, these results indicate that acute disruption of p110δ signaling in vivo reduces BCL6 expression and potentiates IgE switch in GC B cells.

BCL6 overexpression partially reverses potentiated IgE switching in p110δ-inhibited B cells

To directly address whether restoration of BCL6 expression can overcome deregulated IgE switch in p110δ-inhibited B cells, BCL6 was ectopically expressed in primary B cell cultures. Both RT-PCR and flow cytometry results confirmed that BCL6 was overexpressed in GFP+ cells transduced with BCL6 vector but not empty vector (Supplemental Fig. 3A, 3B). BCL6 overexpression did not affect the expression level of AID, γ1GLT, or IRF4 in...
activated B cells under any conditions; however, it markedly inhibited transcription of εGLTs and reversed the effect of PI3K inhibition (Fig. 8A). Flow cytometry analysis also revealed that overexpression of BCL6 was able to reverse potentiated generation of IgE⁺ cells, with minimal effect on IgG1⁺ cells (Fig. 8B, 8C). These results demonstrate that restoration of BCL6 expression can overcome the requirement for PI3K signaling in regulating εGLT and IgE switch. In contrast, ectopic expression of IRF4 led to enhanced εGLTs and increased numbers of IgE⁺ cells, albeit not to the levels reached in the presence of IC87114 (Supplemental Fig. 4). Together, these data suggest that εGLT expression and IgE switch can be directly modulated by IRF4 and BCL6.

**Discussion**

It has been well documented that blockage of PI3K signaling selectively potentiates IgE production both in vivo and in vitro (8–10, 36). Studies by Dr. Rickert’s group (11) indicated that PI3Ks globally suppress CSR through Akt downstream transcription factor FOXO1 by regulating AID induction. We studied the additional selective effects of PI3K on IgE switch and found, using multiple approaches, that p110δ signaling represses IRF4 and potentiates BCL6 expression, impacting on the transcription of εGLTs and IgE switching. Thus, our results have implications for both regulation of IgE and the role of PI3K in controlling the GC B cell-differentiation program.

Although Akt appears to be the primary target of p110δ controlling AID expression, transcription of εGLTs and IgE CSR are p110δ dependent but Akt independent. Our findings strongly implicate BCL6 as a relevant p110δ-dependent factor controlling Ig switch. BCL6 is thought to compete with STAT6 to bind to the IgE promoter, repressing transcription of εGLTs (14). BCL6 is a key player in GC B cell development and differentiation (15–17). BCL6 needs to be tightly regulated to guarantee normal T-dependent Ab responses, as well as to prevent lymphomagenesis (37). Once GC B cells have acquired high-affinity BCRs and

![FIGURE 8. Ectopic expression of BCL6 partially reverses potentiated IgE switching in p110δ-inhibited B cells. anti-CD40–preactivated WT B cells were infected with BCL6-expressing lentivirus (BCL6-PCDH) or empty virus control (PCDH) and then recultured with anti-CD40 + IL-4 in the absence or presence of Akt or IC87114. (A) At 24 h of culture, GFP⁺ cells were purified by cell sorting. RNA was extracted from GFP⁺ cells, and levels of εGLTs, γ1GLTs, AID, and IRF4 were determined by quantitative RT-PCR. Data are expressed as normalized expression relative to the corresponding β-actin control and represent the average and SEM of at least three independent experiments. (B) At 48 h of culture, cells were fixed, permeabilized, and stained for IgG1 and IgE. Representative FACS data are shown, indicating IgG1⁻ and IgE-staining pattern in GFP⁺ cells. (C) Percentage of IgE⁺ cells (left panel) or IgG1⁺ cells (right panel) within GFP⁺ cells (mean ± SEM) from three independent experiments. *p < 0.05, **p < 0.005, compared with empty virus-infected cells (Student t test).](http://www.jimmunol.org/)

Downloaded from http://www.jimmunol.org/ by guest on May 1, 2017
undergone CSR, BCL6 downregulation is required for GC B cells to exit GCs for terminal differentiation. Stimulation by costimulatory molecules expressed on T follicular helper (Tfh) cells, leading to IRF4 expression, is thought to be one cause of BCL6 downregulation in GC B cells (33). Our results demonstrate that inhibition of p110δ markedly alters the balance between BCL6 and IRF4 expression. We found that several reported BCL6 target genes were affected. Enforced BCL6 expression was sufficient to restore eGLT and IgE switch to relatively normal levels, despite p110δ inhibition, consistent with the role of BCL6 as a key PI3K-dependent regulatory factor selectively repressing IgE switch.

CD23 is implicated both as a BCL6 target gene and as a regulator of IgE production; however, we did not observe any effect of p110δ inhibition on CD23 expression. Upregulation of CD23 upon anti-CD40 + IL-4 stimulation is controlled by many factors that bind to the CD23 promoter regions, including BCL6. In a cotransfection system, only overexpression of a very high amount of BCL6 is able to repress CD23 expression (14). In a microarray study comparing cells expressing full-length BCL6 or BCL6 null, no difference in the expression levels of CD23 was observed (32). Thus, regulation of CD23 expression is complex, involves multiple factors, and may be less sensitive to alterations in BCL6 expression.

IRF4 is elevated in p110δ-inactivated B cells both in vivo and in vitro, suggesting that PI3K signaling could be an important factor in repressing IRF4 and, thus, maintaining high BCL6 levels. BCL6 and IRF4 were suggested to be cross-inhibitory; however, when we overexpressed BCL6 in mitogen-stimulated B cells, no changes in IRF4 levels were observed (Fig. 8A), indicating that low BCL6 does not account for deregulated IRF4 in this context. How PI3K signaling can negatively regulate transcription of IRF4 is still an open question. It is tempting to hypothesize that PI3Ks can negatively regulate NF-κB activation under certain circumstances, because PI3K-pathway inhibition has been associated with increased expression of other NF-κB target genes, such as IL-12 (38–40).

IL-21, a cytokine secreted predominantly by Tfh cells, was demonstrated to upregulate BCL6 expression in B cells (24, 41). However, we found that IL-21–induced BCL6 expression was not significantly inhibited by IC87114 (data not shown). This suggests that PI3K signaling does not act at the level of IL-21 signaling. However, p110δ was found to play a role in Tfh function (10); thus, it is possible that p110δ inhibition might lead to reduced IL-21 in vivo, impacting on BCL6 induction in GC B cells. Interestingly, this study found that p110δ was not required for BCL6 expression by Tfh, suggesting that T cell expression of BCL6 may not be subject to the PI3K-dependent regulatory circuit described in this article for B cells. Interestingly, deletion of p110δ in B cells using CD19-Cre did not have a marked impact on GC responses, in contrast to our findings with adoptive transfer of p110δD910A B cells (10). This may be due, in part, to differential survival or homing of p110δD910A B cells, leading to a greater impact on GC responses in the adoptive-transfer system. Another possibility is that incomplete CD19cre-mediated p110δ deletion or functional compensation by other PI3K isoforms occurs in the p110δ-flox system, reducing the observed impact on GC responses. However, this study concurs with our finding that p110δ has a B cell-intrinsic role in IgE production.

There is evidence for constitutive PI3K activity in GC B cells (42, 43), whereas only very rare GC B cells are NF-κB active (44). It was suggested that limiting factors in the GC microenvironment may dampen PI3K signaling in some GC B cells (42). Based on our findings and the studies mentioned above, our working model is that, within GCs, PI3K signaling maintains the GC B cell-activation program while keeping differentiation in check by modulating expression levels of AID and BCL6. Attenuation of PI3K signaling due to limiting microenvironmental factors, in addition to costimulatory signals from Tfh interaction, may upregulate IRF4 and downregulate BCL6 to allow further differentiation. Our data suggest that when PI3K signaling is inhibited, GC B cells expressing low BCL6 more frequently undergo uncontrolled CSR to become IgE+ cells. Because IgE+ cells were shown to be more closely related to plasma cells than GC B cells, based on their location, morphology, and genetic signature (18), we hypothesize that p110δ inactivation may trigger GC B cells to undergo an abortive differentiation program, resulting in excessive generation of IgE+ cells.

Acknowledgments
We thank Dr. Klaus Okkenhaug for providing p110δ-D910A mice and critical reading of the manuscript; Dr. Kamal D. Puri for providing IC87114; Monroe Chan for cell sorting; Rhonda Kelley for assistance with mouse gavage; and Hongzhao Li, Sen Hou, Manli Zhang, and Dr. Dong Liu for helpful discussions and experimental troubleshooting.

Disclosures
The authors have no financial conflicts of interest.

References


