

# Genetic or pharmaceutical blockade of p110 $\delta$ phosphoinositide 3-kinase enhances IgE production

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**Background:** Recent studies indicate that pharmaceutical blockade of phosphoinositide 3-kinase (PI3K) signaling enzymes might be effective in reducing allergic airway inflammation. Signals generated by the p110 $\delta$  PI3K isoform play critical roles in signaling through antigen and cytokine receptors and were shown to be required for induction of type 2, but not type 1, cytokine responses.

**Objective:** We sought to determine the effect of genetic or pharmaceutical inactivation of p110 $\delta$  PI3K on induction of IgE responses.

**Methods:** We determined the effect of p110 $\delta$  inactivation on induction of systemic IgE responses and on the ability of purified B lymphocytes to undergo IgE isotype switch *in vitro*. IgG and IgE germline transcription, postswitch transcription, protein expression, and secretion were measured, as well as cell division and expression of activation-induced cytidine deaminase, an enzyme required for isotype switch.

**Results:** Paradoxically, inactivation of p110 $\delta$  PI3K led to markedly increased IgE responses, despite reduced production of other antibody isotypes. This result was seen by using genetic inactivation of p110 $\delta$  inhibition with IC87114 compound or blockade with the broad-spectrum PI3K inhibitors PIK-90 and PI-103. Significant increases in IgG1/IgE double-positive cells were observed, indicating that inactivation of PI3K leads to uncontrolled sequential switching from IgG1 to IgE. **Disruption**

of p110 $\delta$  signaling results in increased germline transcription at the  $\epsilon$  locus and increased activation-induced cytidine deaminase expression, suggesting deregulation at the level of the isotype switch process.

**Conclusion:** Blockade of PI3K signaling leads to markedly enhanced B-cell switch to IgE and increased IgE levels *in vivo*, despite reduced type 2 cytokine production. (J Allergy Clin Immunol 2008;122:811-9.)

**Key words:** IgE, allergy, B lymphocyte, immunoglobulin class switch, signal transduction, phosphoinositide 3-kinase

Production of allergen-specific IgE antibody is an important component of atopic diseases and is subject to multiple layers of regulation.<sup>1,2</sup> IgE is produced through class-switch recombination (CSR) in association with a germinal center (GC) B-cell differentiation program that includes expression of activation-induced cytidine deaminase (AID), an enzyme required for CSR.<sup>3</sup> Induction of CSR is highly dependent on T cell-derived signals, including CD40 ligand and IL-4, that can promote germline transcription of immunoglobulin heavy-chain constant regions.<sup>4-6</sup> The immunoglobulin isotype specificity of CSR is determined in part by differential transcriptional activation of immunoglobulin constant regions in their germline configurations. The IgE response requires transcriptional activation of the  $\epsilon$  locus through signal transducer and activator of transcription (STAT) 6 and nuclear factor (NF)  $\kappa$ B<sup>7,8</sup>; however, the membrane-proximal signal transduction pathways selectively regulating the IgE response are not well defined.

Phosphoinositide 3-kinases (PI3Ks) are lipid kinase enzymes that mediate key signal transduction reactions during immune and inflammatory reactions.<sup>9-12</sup> Targeting these enzymes pharmacologically holds promise for the treatment of allergy and asthma, inflammatory diseases, and hematologic cancers. As such, the development of PI3K isoform-specific inhibitors is currently an area of intense activity.<sup>9,13,14</sup> *In vivo* inactivation of the class IA isoform p110 $\delta$  has been found to reduce antigen receptor function, T<sub>H</sub>2 cytokine production, and T<sub>H</sub>2-mediated inflammatory reactions.<sup>15-17</sup> These studies suggest that targeting p110 $\delta$  might be effective for the treatment of human allergy or atopic asthma.

Here we have examined the role of p110 $\delta$  signaling in the regulation of B-cell class switching to IgE. When p110 $\delta$  signaling is disrupted, we find a paradoxical enhancement of IgE production *in vivo*, despite diminished T<sub>H</sub>2 cytokine responses and IgG production. Our results identify a novel regulatory function of PI3K signaling in the regulation of immunoglobulin class switch that is relevant to atopic disease and use of PI3K inhibitors in the clinic.

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**Abbreviations used**

AID: Activation-induced cytidine deaminase  
 CFSE: Carboxyfluorescein diacetate succinimidyl ester  
 CSR: Class-switch recombination  
 GC: Germinal center  
 GLT: Germline transcript  
 mTOR: Mammalian target of rapamycin  
 NF: Nuclear factor  
 OVA: Ovalbumin  
 PE: Phycoerythrin  
 PI3K: Phosphoinositide 3-kinase  
 STAT: Signal transducer and activator of transcription  
 WT: Wild-type

**METHODS****Mice**

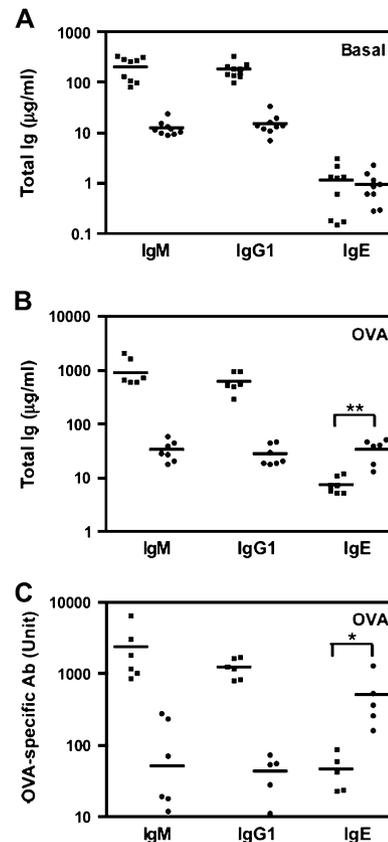
p110 $\delta$ <sup>D910A/D910A</sup> mice<sup>17</sup> (backcrossed to C57BL/6 for 9 generations) were examined between 8 and 14 weeks of age along with age- and sex-matched control mice (bred locally or purchased from Charles River Canada). The p110 $\delta$ <sup>D910A/D910A</sup> mutation completely inactivates catalytic activity.<sup>15,17,18</sup> All animals were housed at the Central Animal Care Facility (University of Manitoba, Winnipeg, Manitoba, Canada) in compliance with the guidelines established by the Canadian Council on Animal Care.

**Cell culture and inhibitors**

B cells were purified by means of negative selection with the CD43 MicroBeads and MACS columns (Miltenyi Biotech, Bergisch Gladbach, Germany). Unlabeled or 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Eugene, Ore)–labeled purified B cells (>95% CD19<sup>+</sup>) were cultured in complete medium (RPMI 1640 supplemented with 10% FCS,  $2 \times 10^{-5}$  mol/L 2-mercaptoethanol) at a final concentration of  $1 \times 10^6$ /mL for 3 days with 2  $\mu$ g/mL LPS (Sigma, St Louis, Mo) or 2  $\mu$ g/mL anti-CD40 (BD Biosciences, Mississauga, Ontario, Canada) together with 20 ng/mL IL-4 (R&D Systems, Minneapolis, Minn). The CFSE labeling method was specified in a published article.<sup>19</sup> Pharmacologic inhibitors were added at the indicated concentrations at the beginning of the culture period. PI-103, PIK-90, and TGX-115 compounds were synthesized and characterized as previously described.<sup>13</sup> The p110 $\delta$ -selective inhibitor IC87114<sup>20</sup> was provided by Calistoga Pharmaceuticals (Seattle, Wash), and TGX-221<sup>21</sup> was a gift of Simone Schoenwaelder and Shaun Jackson (Monash University). The mammalian target of rapamycin (mTOR) inhibitor rapamycin was purchased from Calbiochem (San Diego, Calif).

**Immunization and ELISA**

Wild-type (WT) or p110 $\delta$ <sup>D910A/D910A</sup> mice were immunized intraperitoneally by means of injection of 2  $\mu$ g of egg ovalbumin (OVA) protein (Sigma) adsorbed onto 2 mg of Al(OH)<sub>3</sub> adjuvant (Imject Alum; Pierce Chemical Co, Cheshire, United Kingdom) and boosted 2 weeks later with a second identical immunization. For *in vivo* pharmacologic PI3K $\delta$  inhibition, WT mice were orally dosed with 25 mg/kg p110 $\delta$  inhibitor IC87114 or an equal amount of vehicle (PEG400) before and after 10  $\mu$ g of OVA immunization twice a day for 12 days. This dosing regimen resulted in peak serum levels of IC87114 in the 2 to 10  $\mu$ mol/L range. To measure total and OVA-specific antibody isotypes, ELISA plates (MaxiSorp; Nunc, Roskilde, Denmark) were coated with purified capture antibodies against different isotypes or 20  $\mu$ g/mL OVA, and then bound serially diluted serum antibodies were detected with corresponding biotinylated detecting antibodies. For determination of OVA-specific IgE, plates were coated with rat anti-mouse IgE (BD Bioscience), and bound IgE was detected with biotinylated OVA. OVA was biotinylated by using the vendor's protocol. Total antibodies were quantified relative to known amounts of monoclonal standard antibodies, and OVA-specific antibodies were quantified relative to a standard curve obtained from serial dilutions of pooled hyperimmune mouse serum.



**FIG 1.** p110 $\delta$ -D910A mice have selectively increased IgE levels after OVA immunization. Mice were immunized with OVA/alum and boosted at day 14. Sera collected before immunization (A) or 7 days after challenge (B) were assessed for the indicated antibody isotypes by means of ELISA. C, Levels of antigen-binding IgM, IgG1, or IgE antibodies were assessed after immunization. Squares represent WT mice, and circles represent p110 $\delta$ -D910A mice.

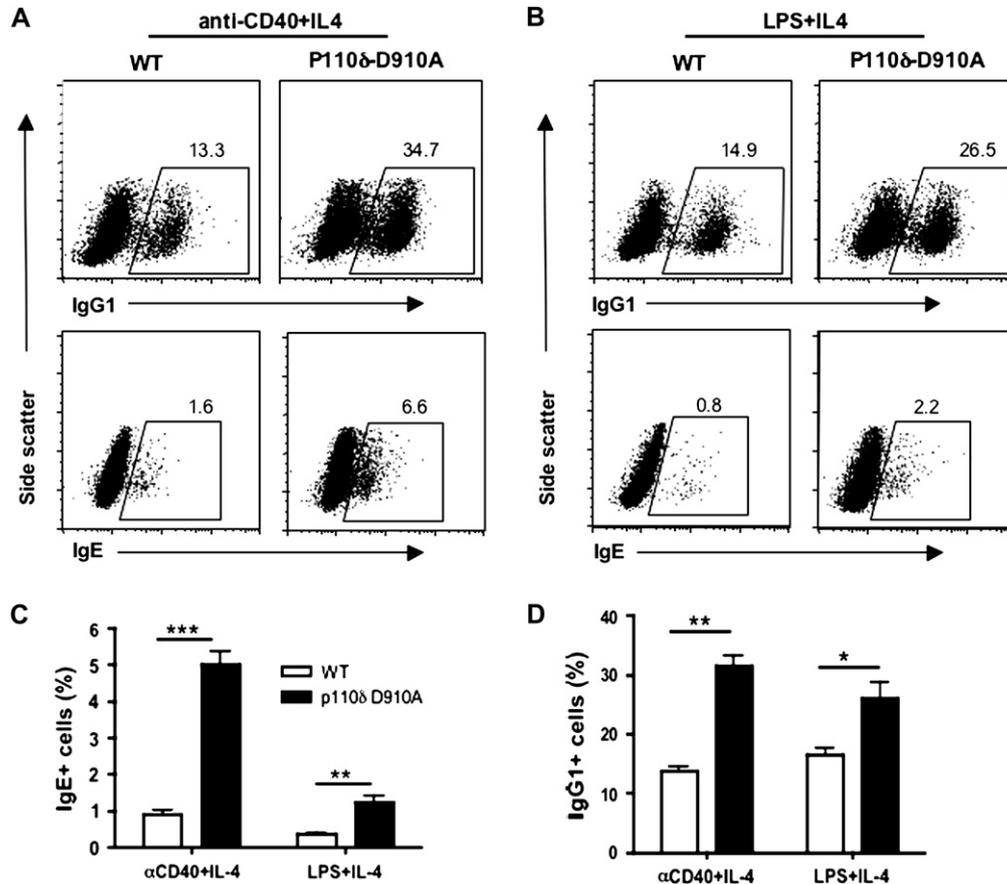
Levels of cytokines in culture supernatants were measured by means of specific ELISA, as previously published.<sup>22</sup>

**Flow cytometry**

Antibodies used for flow cytometric staining were purchased from BD Bioscience, except for those specified. After preincubation with Fc blocking antibody (mAb 2.4G2), stimulated B cells were fixed, permeabilized, and stained for intracellular IgM, IgG1, and IgE, essentially as described.<sup>23</sup> phycoerythrin (PE)–labeled anti-IgE (eBioscience, San Diego, Calif) was used in all figures for consistency, but results were validated with an independent fluorescein isothiocyanate–labeled anti-IgE mAb (BD Bioscience; see Fig E1 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). Before intracellular staining for anti-IgE, cells were treated for 1 minute with ice-cold acid buffer to remove passively absorbed IgE bound to CD23.<sup>24</sup> For GC B-cell staining, splenocytes isolated from spleens of immunized mice were stained with combinations of fluorescein isothiocyanate–labeled anti-GL7, PE-conjugated anti-Fas, and allophycocyanin-labeled anti-B220. Stained cells were analyzed with a FACSCalibur flow cytometer, and data were plotted by using FlowJo software (TreeStar, Portland, Ore).

**Real-time PCR**

RNA was extracted from the indicated B-cell populations with Trizol reagent. cDNA was synthesized from 1  $\mu$ g of RNA by using SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, Calif). Quantitative RT-PCR was performed with the SyberGreen amplification mix on the LightCycler System (Roche, Mannheim, Germany). Primer sequences used for detection of



**FIG 2.** p110δ-D910A B cells show increased class switch to IgE and IgG1 *in vitro*. WT or p110δ-D910A B cells were stimulated with anti-CD40 plus IL-4 (A) or with LPS plus IL-4 (B). After 3 days of culture, cells were stained for intracellular IgG1 or IgE. The average percentage of IgE<sup>+</sup> (C) or IgG1<sup>+</sup> (D) cells and SEMs from 3 independent experiments are shown.

germline transcripts (GLTs;  $\mu$ ,  $\gamma 1$ , and  $\epsilon$ ), postswitch transcripts, and AID were previously published.<sup>23</sup>  $\beta$ -Actin was used as relative expression control to normalize sample variation.

### Statistics

In all figures statistical significance was determined by using the Student *t* test, comparing the WT group with no inhibitors with the corresponding p110δ-D910A or inhibitor-treated group. The standard labeling for significance is as follows: \**P* < .05, \*\**P* < .005, and \*\*\**P* < .001.

## RESULTS

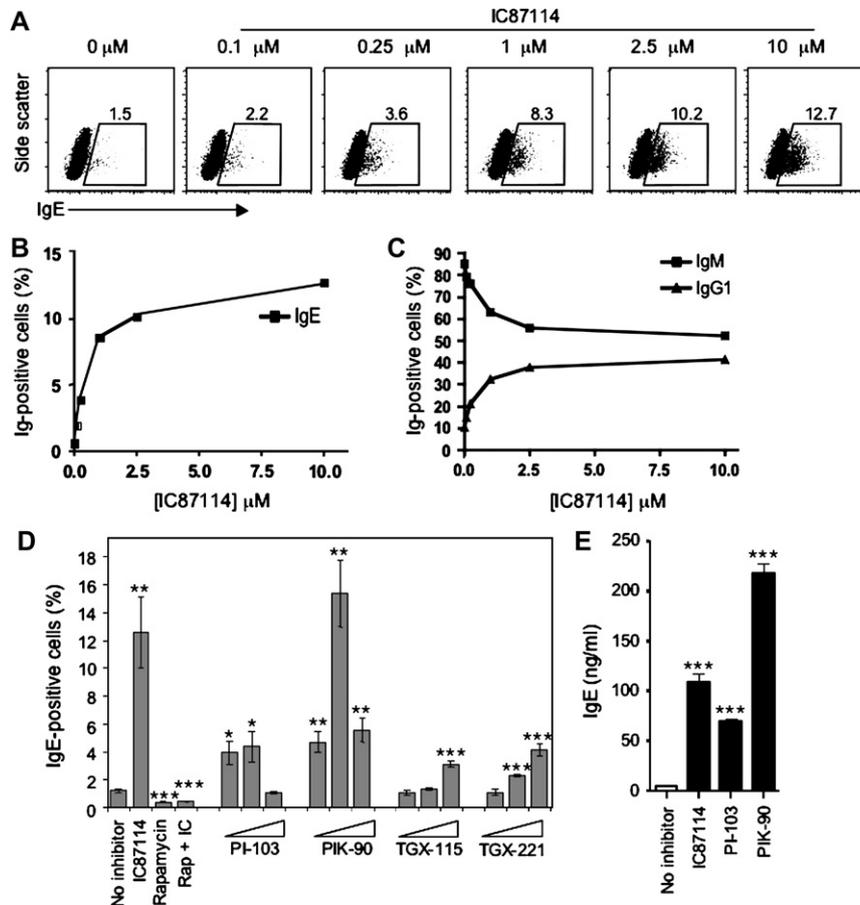
### Selectively increased IgE production in p110δ<sup>D910A/D910A</sup> mice

Mice bearing an inactivating mutation in the p110δ isoform of PI3K were previously shown to have markedly reduced IgM and IgG antibody responses after immunization with T-dependent or T-independent antigens<sup>17</sup>; however, IgE responses were not determined. Consistent with previous findings, unimmunized mice had substantially lower basal levels of IgM and IgG1 (Fig 1, A). In contrast, basal levels of IgE were within the normal range (Fig 1, A). On immunization of p110δ<sup>D910A/D910A</sup> mice with OVA, total IgE levels were markedly higher than normal, whereas IgM and IgG1 responses were markedly attenuated (Fig 1, B). Strong IgE responses are also observed with TNP-KLH immunization (data not shown). Levels of OVA-binding IgE were also increased

in p110δ<sup>D910A/D910A</sup> mice (Fig 1, C), which is consistent with increased antigen-specific induction of this antibody isotype. These results demonstrate that disruption of p110δ signaling leads to enhancement of IgE production. This is paradoxical given that type 2 cytokine responses induced by means of OVA immunization, including production of the major IgE switch factor IL-4, were markedly inhibited in these mice.<sup>16</sup>

### p110δ<sup>D910A/D910A</sup> B cells generate an increased frequency of IgE-switched cells

To test whether p110δ<sup>D910A/D910A</sup> B cells are intrinsically predisposed toward IgE isotype switching, we assessed isotype switching of purified B cells *in vitro*. Splenic B cells purified from WT or p110δ<sup>D910A/D910A</sup> mice were stimulated with anti-CD40 plus IL-4, and then IgG1- or IgE-expressing cells were detected by means of flow cytometry (Fig 2, A). Strikingly, p110δ<sup>D910A/D910A</sup> B cells showed an approximately 5-fold higher frequency of IgE-positive cells in anti-CD40 plus IL-4 cultures. IgG1-switched cells were also present at a higher frequency in p110δ<sup>D910A/D910A</sup> cultures; however, the increase is only 2.5-fold for this isotype (Fig 2, A). Proportionally similar increases were observed in cultures containing LPS plus IL-4 (Fig 2, B). Increased IgG1 and IgE expression was consistently observed in p110δ<sup>D910A/D910A</sup> cultures and was highly significant (Fig 2, C and D).



**FIG 3.** p110 $\delta$  inhibitor potently enhances generation of IgG1- and IgE-switched cells. **A**, WT B cells were stimulated with anti-CD40 plus IL-4 in the presence of the indicated doses of the p110 $\delta$  selective inhibitor IC87114. At day 3, cells were stained for IgM, IgG1, or IgE. FACS data are representative of 3 independent experiments. **B**, The percentage of IgE<sup>+</sup> cells from 1 representative experiment is shown. **C**, The percentage of IgG1<sup>+</sup> and IgM<sup>+</sup> cells is shown. **D**, PI-103, PIK-90, TGX-115, and TGX-221 inhibitors were added at 0.1, 1, or 10  $\mu$ mol/L, and the percentage of IgE<sup>+</sup> cells was assessed. IC87114 was used at 10  $\mu$ mol/L and rapamycin was used at 0.01  $\mu$ mol/L. **E**, Effect of PI3K inhibitors on secreted IgE levels was determined by means of ELISA.

### Pharmacologic inhibition of p110 $\delta$ potentiates IgE switching *in vitro*

To examine the effect of acutely disrupting p110 $\delta$  signaling in WT B cells, we titrated the IC87114 inhibitor into B-cell cultures stimulated with anti-CD40 plus IL-4 and examined the generation of IgE-switched cells (Fig 3, A and B). Significant effects were observed at doses as low as 0.25  $\mu$ mol/L IC87114, and increases in IgE-switched cells of up to 10-fold were observed at 10  $\mu$ mol/L (Fig 3, B). IC87114 also potentiated switching to IgG1, with observed increases up to 4-fold (Fig 3, C). Similar increases were seen when IC87114 was added to LPS plus IL-4 cultures (data not shown). To determine the effect of inhibiting a broader spectrum of class I PI3Ks, we tested a number of additional PI3K inhibitors to determine their effect on isotype switch. Several chemically distinct inhibitor compounds with a range of target selectivities<sup>13,21</sup> were able to potentiate switching to IgE to varying degrees (Fig 3, D). TGX115 and TGX221 preferentially inhibit p110 $\beta$  PI3K,<sup>21</sup> but they can also effectively inhibit p110 $\delta$  and other isoforms at doses of greater than 1  $\mu$ mol/L.<sup>13,25</sup> Both TGX115 and TGX221 had no effect on IgE switching at doses of less than 1  $\mu$ mol/L but significantly enhanced IgE switch at 10  $\mu$ mol/L (Fig 3, D). The broad-spectrum PI3K inhibitors

PIK-90 or PI-103 potently enhanced IgE switch in the 0.1 to 1  $\mu$ mol/L dose range; however, at doses of greater than 2  $\mu$ mol/L, they inhibited IgE production. This is likely due to the known ability of these compounds to inhibit the mTOR at these doses.<sup>13</sup> Consistent with this interpretation, the mTOR inhibitor rapamycin strongly suppressed IgE switching, even in the presence of IC87114 (Fig 3, D). Cultures containing IC87114 or 1  $\mu$ mol/L PIK-90 or PI-103 also produced increased levels of secreted IgE (Fig 3, E). Together, these pharmacologic data concur with the results using genetic inactivation of p110 $\delta$ , providing strong evidence that signaling through class I PI3Ks functions in normal control of IgE isotype switch.

### PI3K signaling controls sequential switching from IgG1 to IgE

A substantial proportion of the IgE response is generated through sequential switch recombination from IgG1 to IgE.<sup>26,27</sup> This process normally occurs after the IgG1<sup>+</sup> cells have undergone several additional cell divisions<sup>28</sup> and generates small but detectable IgG1<sup>+</sup>IgE<sup>+</sup> intermediate populations.<sup>29</sup> We thus performed double-staining experiments to determine the frequencies

of IgE<sup>+</sup>IgG1<sup>+</sup> cells (Fig 4, A). In WT B-cell cultures, about 2% of IgG1<sup>+</sup> cells coexpressed IgE, whereas in PI3K inhibitor-treated cultures or in p110δ<sup>910A/D910A</sup> cultures, the percentage of IgG1<sup>+</sup> cells coexpressing IgE increased to 5% to 15% (Fig 4, A). B cells were labeled with CFSE before culture and stained with PE-labeled anti-IgE at day 3 to examine the relationship between IgE switch and cell division (Fig 4, B). This analysis revealed a striking deregulation of division-linked IgE switch under conditions in which PI3K signaling is disrupted, with significant populations of IgE<sup>+</sup> cells appearing by the second cell division and increasingly disproportionate levels of switch appearing at each subsequent division (Fig 4, C). These data indicate that inhibition of PI3K signaling does not markedly alter cell division but leads to deregulated sequential switching from IgM to IgE.

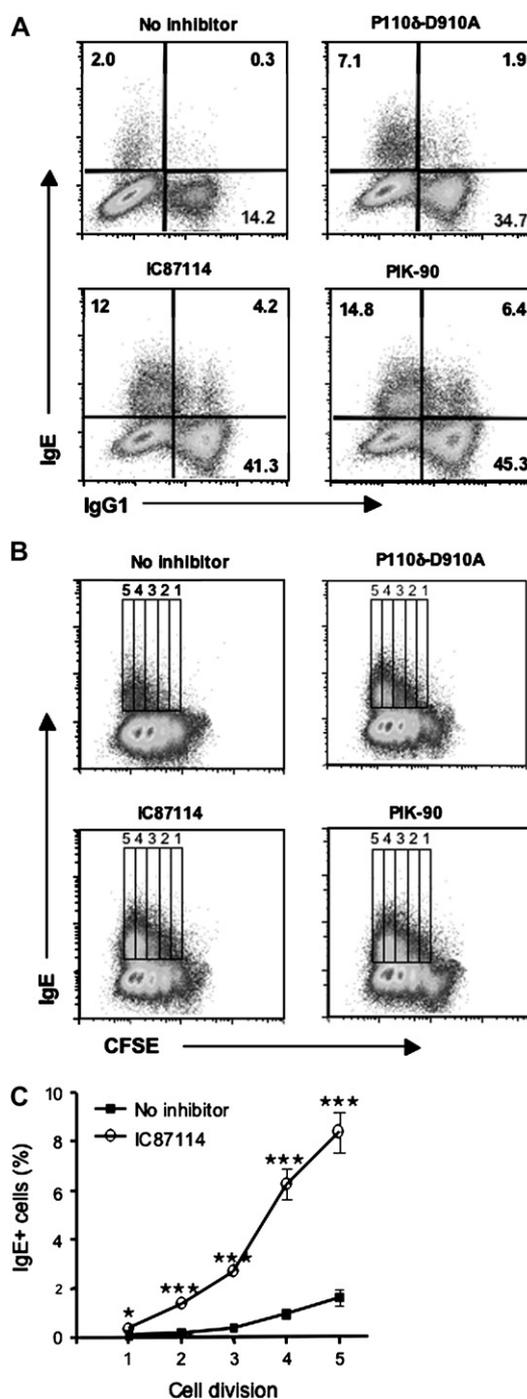
### p110δ regulates ε germline transcription and AID expression

CSR is associated with germline transcription of constant regions that is thought to be important in determining the accessibility of loci to switch recombination machinery.<sup>30</sup> We examined levels of εGLTs using quantitative RT-PCR. It was found that εGLTs are significantly increased in p110δ<sup>910A/D910A</sup> B-cell cultures, and this increase is apparent as early as 24 hours after culture initiation (Fig 5, A). A similar increase in εGLT levels was seen in cultures of WT B cells after addition of IC87114 and was observable in both anti-CD40 plus IL-4 cultures and LPS plus IL-4 cultures (Fig 5, A). Disruption of p110δ signaling also increased γ1GLT levels within the first day of culture, but this increase is no longer apparent by day 3 of culture (Fig 5, B), presumably in part due to deletion of the γ1 switch region in cells undergoing sequential switch. Postswitch transcripts were also measured in cells of day 3 cultures. Consistent with the IgE and IgG1 staining data, both ε and γ1 postswitch transcripts were increased when p110δ activity was inhibited, with a more significant fold increase of ε postswitch transcripts (Fig 5, C).

We also examined expression of AID, an enzyme critical for CSR.<sup>3</sup> In WT B-cell cultures AID transcripts were present at low levels at the 24-hour time point and then increased by more than 100-fold by day 3 of culture. However, in p110δ<sup>910A/D910A</sup> B cells or WT B cells treated with IC87114, AID expression was initiated earlier, with significant expression at 24 hours (Fig 5, D). After 3 days, anti-CD40 plus IL-4 cultures with impaired p110δ activity expressed about 2-fold more AID transcripts than WT B cells. Cultures stimulated with IL-4 alone also showed increased εGLT and AID expression when p110δ was inhibited, although substantial AID expression requires costimulation with anti-CD40 (see Fig E2 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). Together, these data support the model that class switching to IgE is enhanced from the outset of B-cell activation in the presence of IL-4 because of initial deregulation in both εGLT and AID expression.

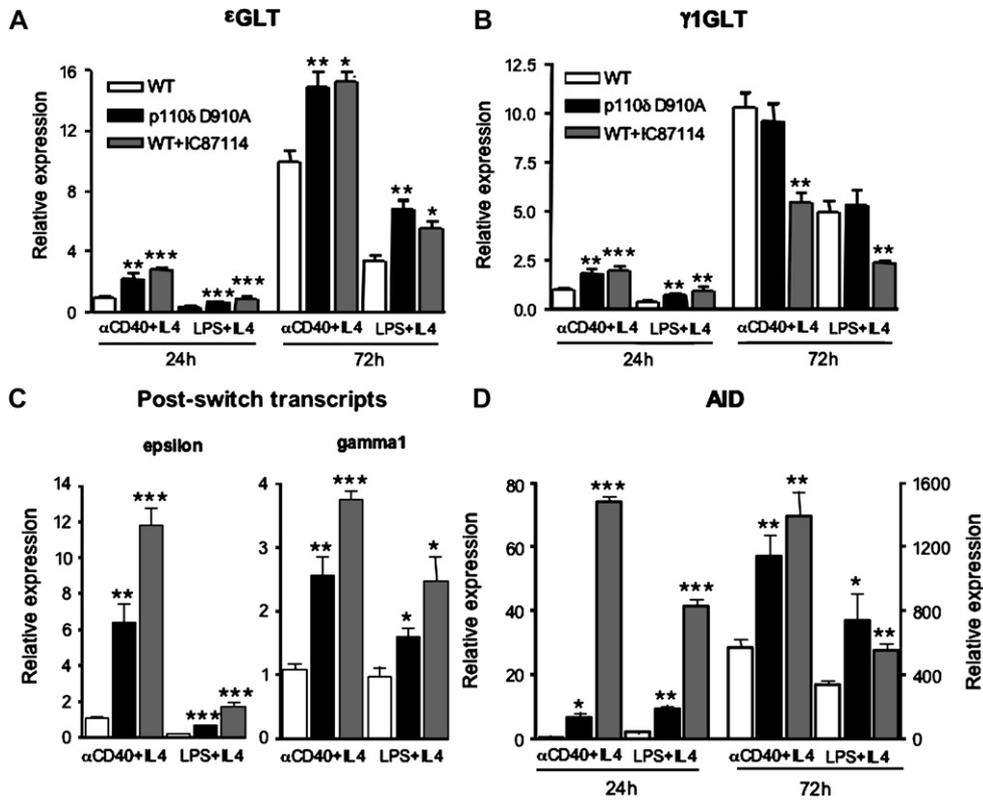
### Pharmacologic p110δ inhibition leads to higher IgE production *in vivo*

Mice were orally treated with IC87114 before and during OVA immunization to evaluate whether *in vivo* administration of p110δ inhibitor compound can also cause enhanced IgE production. At day 12 after immunization, a significantly lower percentage of



**FIG 4.** Blockade of p110δ leads to deregulated sequential switching from IgG1 to IgE. **A**, WT or p110δ-D910A B cells cultured for 3 days with anti-CD40 plus IL-4 were double-stained for IgE and IgG1. The indicated inhibitors were used at 1 μmol/L. **B**, WT or p110δ-D910A B cells were labeled with CFSE before culture. At day 4, cells were harvested and stained with PE-labeled anti-IgE. Vertical lines mark cell divisions, as assessed by means of 2-fold dilutions of CFSE. **C**, Graph showing the frequency of IgE<sup>+</sup> cells at divisions 1 to 5. Data represent means and SEs from 3 independent experiments.

splenic B220<sup>+</sup>Fas<sup>+</sup>GL7<sup>+</sup> GC cells were present in IC87114-treated mice (Fig 6, A), indicating that this oral treatment was effective in reducing the GC response, as seen in p110δ<sup>910A/D910A</sup> mice.<sup>17</sup> Sera were also collected for measurement of the total and OVA-specific antibodies. In contrast to p110δ<sup>910A/D910A</sup>



**FIG 5.** p110 $\delta$  regulates  $\epsilon$  germline transcription and AID expression. RNA was extracted from B cells stimulated under the indicated conditions, and levels of the indicated transcripts were determined by using LightCycler RT-PCR. **A**,  $\epsilon$ GLTs measured at 24 or 72 hours of culture. **B**,  $\gamma$ 1GLTs measured at 24 or 72 hours of culture. **C**,  $\epsilon$  and  $\gamma$ 1 postswitch transcripts measured at 72 hours of culture. **D**, AID transcripts measured at 24 and 72 hours of culture. Data are expressed as normalized expression relative to the corresponding  $\beta$ -actin control and represent the average and SEM of at least 3 independent cultures.

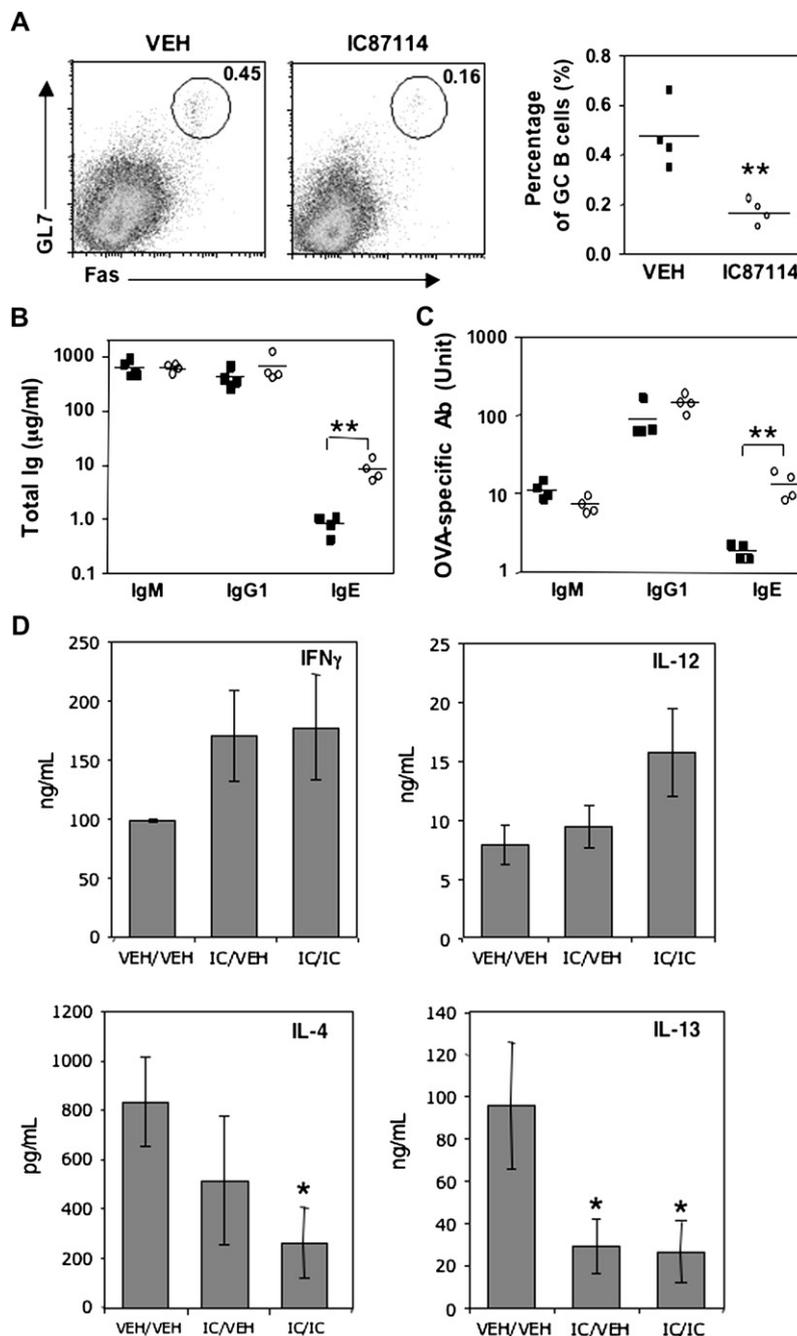
mice, IC87114-treated mice did not have reduced levels of total and OVA-specific IgM or IgG1 (Fig 6, B and C). However, both total and OVA-specific IgE levels were significantly increased in IC87114-treated mice, by 10- and 7-fold, respectively (Fig 6, B and C). Thus in the absence of lymphopenia or other developmental abnormalities known to be present in the genetic inactivation model,<sup>17</sup> acute inhibition of p110 $\delta$  has a marked selective effect on IgE responses *in vivo*. To determine whether oral IC87114 treatment might affect production of cytokines regulating CSR, we assessed levels of IFN- $\gamma$ , IL-4, IL-12, and IL-13 produced after OVA restimulation *in vitro*. The results indicate that IC87114 treatment leads to reduced IL-4 and IL-13 production, whereas production of IFN- $\gamma$  and IL-12 is similar or increased compared with that seen in the vehicle-treated groups (Fig 6, D). Together, these results indicate that acute pharmacologic inhibition of p110 $\delta$  leads to a selective increase in IgE responses *in vivo*, despite reduced production of the critical IgE switch factor IL-4.

## DISCUSSION

p110 $\delta$  has well-established functions in antigen receptor signaling in both B and T lymphocytes; however, the importance of PI3K signaling in T-dependant B-cell differentiation is not yet clear. p110 $\delta$  inactivation does result in severely impaired IgM and IgG antibody responses; however, this is likely at least in part due to impaired helper T-cell function.<sup>31,32</sup> Here we show that the ability of p110 $\delta$ -deficient B cells to mount antigen-specific IgE

responses *in vivo* is not impaired. At face value, this result suggests that, at least for cells switching to this antibody isotype, B-cell activation and differentiation to antibody secretion can proceed relatively efficiently in the absence of p110 $\delta$  signaling. It is particularly striking that increased IgE responses can be generated despite the weak T<sub>H</sub>2 cytokine responses generated in these mice.<sup>16</sup>

Our results indicate that PI3K inhibition greatly increases IgE switching in a B-cell autonomous manner, probably because of upregulation of AID and  $\epsilon$ GLT levels. In normal B cells AID expression is induced when T-dependant activation signals are received, and expression correlates spatially and temporally with CSR and somatic hypermutation. With p110 $\delta$  inhibition, B cells still express AID in an activation-dependant manner, but dramatic increases of AID mRNA were observed early after activation. AID transcription in normal B cells is initiated by synergistic signaling through NF- $\kappa$ B (originating from CD40 or Toll-like receptor ligation) and STAT6 (originating from IL-4 receptor).<sup>33</sup> Evidence generated to date does not support any role of p110 $\delta$  in CD40/IL-4 activation of STAT6 or NF- $\kappa$ B<sup>18</sup>; thus the precise signaling mechanisms linking p110 $\delta$  to transcription of AID and  $\epsilon$ GLT remains to be determined. A recent study found that LPS plus IL-4-induced switching to IgG1 is inhibited by PI3K signaling in part due to regulation of AID.<sup>34</sup> This study provides evidence that the effect of PI3K on AID expression is mediated by Akt inactivation of forkhead transcription factors; however, it is presently unclear whether the AID gene is a direct transcriptional target or whether Akt/forkhead might indirectly regulate AID through other factors.



**FIG 6.** Pharmacologic p110 $\delta$  inhibition *in vivo* selectively generates higher IgE production after OVA immunization. Mice were orally treated with IC87114 1 hour before OVA immunization and then twice per day for 12 days. At day 12, sera and splenocytes were collected. *Solid squares* represent vehicle-treated mice, and *open circles* represent IC87114-treated mice. **A**, The frequency of B220<sup>+</sup>Fas<sup>+</sup>GL7<sup>+</sup> GC cells in the spleen was analyzed by means of FACS, illustrating a significant reduction in the IC87114-treated group. **B**, Sera were analyzed by means of ELISA to determine total IgM, IgG1, and IgE levels. **C**, Sera were analyzed by means of ELISA to determine OVA-binding IgM, IgG1, and IgE levels. **D**, Spleen cells were restimulated *in vitro* with 300  $\mu$ g/mL OVA with or without IC87114. Culture supernatants were analyzed for IFN- $\gamma$ , IL-12p40, IL-4, and IL-13 by means of ELISA. *VEH/VEH*, Cells from vehicle-treated mice cultured with no inhibitor; *IC/VEH*, cells from IC87114-treated mice cultured with no inhibitor; *IC/IC*, cells from IC87114-treated mice cultured in the presence of 10  $\mu$ mol/L IC87114; *Ab*, antibody.

Mutation of the Y500 phosphotyrosine motif in the IL-4 receptor  $\alpha$  cytoplasmic tail was shown to block IL-4-induced phosphorylation of insulin receptor substrate 2 and Akt, suggesting that this motif is required for IL-4-induced PI3K activation.<sup>35</sup>

Interestingly, Y500 mutants also showed increased IgE responses associated with increased transcription of  $\epsilon$ GLT,<sup>35</sup> which is consistent with our conclusion that PI3K activity antagonizes IgE switch. Y500 mutants show normal IL-4-induced STAT6

activation,<sup>35</sup> as do p110 $\delta$  mutants<sup>18</sup>; thus PI3K likely regulates IL-4–induced  $\epsilon$ GLT levels through STAT6-independent mechanisms. Although the IL-4 receptor Y500F mutation and p110 $\delta$  mutation have similar effects regarding IgE responses, they have opposite effects on induction of airway inflammation, with Y500F showing enhanced airway inflammation<sup>35</sup> while p110 $\delta$  mutation markedly attenuates airway inflammation.<sup>16</sup> This suggests that p110 $\delta$  is important for additional IL-4–independent processes affecting allergen priming and airway inflammation, such as antigen receptor and CD28 signaling.<sup>10</sup> p110 $\delta$  mutants also showed a 2-fold increase in the frequency of IgG2a-switched cells in cultures stimulated with anti-CD40 or LPS plus IFN- $\gamma$  (data not shown), indicating that p110 $\delta$  signaling can affect CSR, even in the absence of a strong PI3K activator, such as IL-4. Our *in vivo* data show that the net effect of blocking all p110 $\delta$  signaling generated by various receptors engaged during the immune response is enhanced IgE production.

Our pharmacologic inhibitor data largely concur with the results obtained by using genetic inactivation of p110 $\delta$ , providing strong evidence that signaling through this PI3K isoform is essential for B cell–intrinsic control mechanisms limiting IgE isotype switch. Our results show that a variety of structurally diverse inhibitor compounds with a range of target selectivity share the ability to potentiate switch to IgE. TGX115 and TGX221 inhibitors did not show an effect on IgE switch at submicromolar doses reported to selectively inhibit p110 $\beta$ <sup>21</sup>; however, an effect was observed at higher doses known to block p110 $\delta$ .<sup>25</sup> This could indicate either that p110 $\beta$  is not linked to class switch or that B cells have low expression or activity levels of this isoform. Interestingly, the potent broad-spectrum PI3K inhibitors PIK-90 and PI-103 had reduced effects on IgE when used at higher doses. Although this could suggest that a minimum level of PI3K signaling is required to support isotype switch, it is also known that off-target effects occur at these doses. Consistent with the latter interpretation, both of these compounds are known to directly inhibit mTOR, whereas IC87114 does not,<sup>13</sup> and we found that the mTOR inhibitor rapamycin potently inhibits isotype switch. Although even highly specific PI3K inhibitors, such as IC87114, could indirectly inhibit mTOR through decreased Akt activity,<sup>18</sup> B cells can also activate mTOR through PI3K-independent mechanisms.<sup>31</sup> Our data indicate that sufficient mTOR activity is present under conditions of p110 $\delta$  blockade to permit high levels of isotype switch.

Our *in vivo* results show a disconnect between IgG1 and IgE responses in both p110 $\delta$  mutant mice and IC87114-treated mice, with a marked increase in IgE, but not IgG1, levels. In contrast, short-term *in vitro* studies show that p110 $\delta$  blockade leads to increases in both IgG1 and IgE switching. One possible reason for this disconnect is that p110 $\delta$  inactivation leads to uncontrolled sequential switch from IgG1 to IgE, resulting in lower IgG1 secretion responses that are not reflective of the high proportion of cells that initially switched to IgG1. Our *in vitro* data show substantial increases in IgG1/IgE double-positive cells and also a relative decrease of  $\gamma$ 1GLT level over time, both of which are indicative of increased sequential switch. Although this was observed for both p110 $\delta$  D910A B cells and IC87114-treated WT B cells, markedly higher levels of IgG1<sup>+</sup>/IgE<sup>+</sup> cells and larger relative decreases in  $\gamma$ 1GLT levels were seen with IC87114 treatment for reasons that are not clear. Sequential switch from IgG1 to IgG4 to IgE has been shown to contribute to IgE responses in allergic individuals,<sup>36</sup> and thus regulation of sequential switch is likely relevant to human

allergic disease. It was recently demonstrated that IL-21 can suppress sequential switching from IgG1 to IgE,<sup>37</sup> and IL-21 deficiency led to increased IgE levels but impaired IgG1 production,<sup>38</sup> implicating this cytokine as a selective regulator of IgE versus IgG1 that can act at the level of sequential switch. It will be of interest to investigate whether inhibition of sequential switching directed by the IL-21 receptor or other receptors is PI3K pathway dependent.

A variety of evidence suggests that the GC program of B-cell activation promotes AID-mediated somatic hypermutation and isotype switch to IgG but tends to disfavor IgE responses.<sup>39</sup> A recent study provided direct evidence that IgE<sup>+</sup> cells are located outside of GCs and tend to express plasma cell characteristics soon after their generation, suggesting that IgE production is associated with a unique quasisplasmablast genetic program that is opposed by the transcriptional repressor Bcl6.<sup>37</sup> Bcl6 is a key regulator for the GC B-cell transcriptional program, and Bcl6-deficient mice, such as p110 $\delta$ -inactivated mice, lack robust GC responses and generate high IgE responses,<sup>40,41</sup> supporting the idea that IgE switch is actively suppressed as an integral part of the GC B-cell program. Because IL-21 can be produced by GC T cells, local production of IL-21 can represent an additional mechanism for exogenous repression of sequential switch to IgE within GC. Thus it is possible that partial inhibition of the GC program under conditions of PI3K blockade can disrupt normal mechanisms that keep IgE responses in check, further contributing to enhanced IgE but suppressed IgG levels.

Our results provide clear evidence that signaling through PI3Ks provides a B cell–intrinsic brake on IgE isotype switch. This PI3K-dependant regulatory mechanism is potent enough to substantially influence IgE levels generated *in vivo*, with removal of this brake allowing generation of enhanced IgE responses despite low IL-4 production. The data outline a new and unexpected immunomodulatory activity associated with multiple PI3K inhibitor compounds being developed for potential clinical use.<sup>13</sup> Clearly, this unexpected function of PI3K adds an additional layer of complexity that will need to be considered in efforts to target this pathway in allergic diseases.

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**Clinical implications: PI3K enzymes have an unexpected function in restraining IgE production that will need to be considered in current efforts to target this signaling pathway in allergic diseases.**

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