PI3K is a negative regulator of IgE production

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Abstract

The production of IgE, a main player in allergic disorders such as asthma and atopic dermatitis, is strictly regulated and the serum concentrations of IgE are normally kept at a much lower level than other isotypes. We found that mice deficient for the p85α regulatory subunit of class IA phosphoinositide 3-kinase (PI3K) produced increasing amounts of serum IgE. Purified p85α−/− B cells produced more IgE than wild-type B cells in vitro in response to anti-CD40 mAb and IL-4. PI3K inhibitors wortmannin and IC87114 enhanced IgE production by wild-type B cells stimulated with anti-CD40 mAb and IL-4. Under the same condition, antigen receptor cross-linking induced the expression of inhibitor of differentiation-2 and suppressed the expression of activation-induced cytidine deaminase and class switch recombination (CSR) in a PI3K-dependent manner. IgE production was also suppressed in a concentrated cell culture condition, which was completely reversed by PI3K inhibition. The selective suppression of IgE production by PI3K was also observed at a protein level after CSR. Our results indicate that PI3K negatively regulates IgE production at both CSR and protein levels.

Introduction

IgE is involved in a defense mechanism against nematode, but at the same time, it is also a main player in allergic disorders such as asthma and atopic dermatitis (1). In normal circumstances, IgE production is strictly regulated and its serum concentration is much less than other isotypes (1). Although IgE has a relatively short half-life in plasma (2), it has been believed that the maintenance of low concentration of plasma IgE is ascribed to a tight control of IgE class switch recombination (CSR) (3, 4).

CSR takes place between two S regions located 5′ to each constant region of Ig heavy chain (C(i)) gene. The regulation of CSR in B cells was collaborated with the germ line transcription (GLT) of C(i) genes and the induction of activation-induced cytidine deaminase (AID) expression. The specificity of C(i) switch is regulated at the level of C(i) GLT (5). IgE CSR is controlled by several molecules, the action of which converges on the regulation of Cε GLT that is induced by TGFβ2 cytokines IL-4 and IL-13 (6) and inhibited by a TGFβ1 cytokine IFN-γ (7). Therefore, TGFβ1/TGFβ2 balance is a critical factor for IgE production.

Several transcription factors are known to regulate the balance between TGFβ1/TGFβ2 differentiation. Those include GATA3 (8), which promotes TGFβ2 cell differentiation and inhibits TGFβ1 cell differentiation, and T-bet (9), which exerts the opposite effects to GATA3. In addition, IL-21 blocks IgE production from LPS-stimulated B cells by inhibiting Cε GLT (10). Several B cell surface receptors, including the B cell receptor (BCR) (11), CD45 (12), cytotoxic T lymphocyte antigen 4 (13) and transcription factors such as Bcl-6 (14) and inhibitor of differentiation-2 (Id2) (15), seem to inhibit this process as well. Furthermore, low-affinity IgE receptor CD23 suppresses IgE production by an unknown mechanism (2, 16). Since IgG1 CSR is also regulated by IL-4 (17), if the efficiency of IgG1 and IgE CSR are the same, IgE-expressing cells must exceed IgG1-expressing cells because IgG1-expressing cells subsequently switch to IgE-expressing cells (18–20). However, as mentioned, IgE production is controlled at a much lower rate than IgG1 production.

Phosphoinositide 3-kinases (PI3Ks) are lipid kinases that phosphorylate inositol phospholipids at the 3′-OH of inositol ring, generating second messengers that provide a binding
site for pleckstrin homology domains of many signaling molecules (21). The PI3K family is divided into four groups (IA, IB, II and III) according to their structural characteristics and substrate specificity. Class IA PI3Ks are dimers containing one of regulatory subunits, p85α, p55γ, p50α, p85β, p55γ and one of catalytic subunits, p110α, p110β and p110δ. p85α is the most abundantly and ubiquitously expressed regulatory subunit of class IA PI3K. We and others previously reported that in mice deficient for the p85α, the number of mature B cells were reduced and the proliferation previously reported that in mice deficient for the p85α expressed regulatory subunit of class IA PI3K. We and others

We demonstrate here that p85α-/- B cells produce more IgE than wild-type B cells and p85α-/- mice have increasing amounts of serum IgE despite the Tn-1-biased immune responses. The inhibition of p110δ, a major catalytic subunit in B cells, enhances IgE production. In addition to the inhibition of IgG1 and IgE CSR, PI3K also suppresses IgE production at a protein level. Our results indicate that PI3K is an isotype selective negative regulator for IgE production.

**Methods**

**Reagents and antibodies**

FITC-anti-IgE, biotin-anti-IgG1, biotin-anti-IgG3, PE-anti-CTLA-4 antibodies and streptavidin–allophycocyanin were purchased from BD Biosciences (San Jose, CA, USA). Propidium iodide and carboxyfluorescein succinimidyl ester (CFSE) were purchased from BD Biosciences (San Jose, CA, USA). Anti-CD19 Magnetic Cell Sorting (MACS) beads were purchased from Miltenyi Biotec (Bergisch, Germany). Anti-CD40 antibody was purchased from eBioscience (San Diego, CA, USA). Recombinant IL-4, IFN-γ and IL-21 were purchased from Peprotech (London, UK). Anti-IgM antibody F(ab)2 fragment was purchased from Jackson ImmunoResearch (Bar Harbor, ME, USA).

**Mice and immunization**

p85α-/- mice (22) on a C57BL/6 background were maintained under specific pathogen-free conditions at Taconic (Germany). Propidium iodide and carboxyfluorescein succinimidyl ester (CFSE) were purchased from Sigma (St Louis, MO, USA). Anti-trinitrophenol-IgE was produced as ascites from a hybridoma and concentrated from Sigma (St Louis, MO, USA) or our animal facility. p85α-/- mice (22) on a C57BL/6 background were maintained under specific pathogen-free conditions at Taconic (Germany). Essentially same results were obtained by both preparations. One hundred thousand B cells were cultured in one well of 96-well plates with 200 μl of complete medium (RPMI 1640 containing 10% FCS, sodium pyruvate, non-essential amino acid, penicillin and streptomycin) unless otherwise stated. For IgG1 and IgE CSR, B cells were stimulated with 5 μg ml⁻¹ anti-CD40 antibodies and 10 μg ml⁻¹ IL-4 for 4 days. For IgG3 CSR, cells were stimulated with 5 μg ml⁻¹ anti-CD40 and 0 μg ml⁻¹ LPS for 5 days.

**Flow cytometric analysis**

PBS containing 0.5% BSA and 10 mM ethyleneglycol-bis(2-aminoethylether)-N,N,N’,N’-tetraacetic acid (EGTA) was used for staining except for the experiment shown in Fig. 1D where EGTA was omitted. Since secreted IgE binds B cell surface via CD23, EGTA treatment that removes bound IgE from CD23 is important to quantitate surface IgE expression. Cells were stained with biotin–anti-IgG1 or anti-IgG3 antibodies in 50% of normal rat serum. After washing, cells were stained with FITC–anti-IgE antibody and streptavidin–allophycocyanin. For intracellular staining, cells were fixed and permeabilized in 70% ethanol and stained with FITC–anti-IgE antibody.

**ELISA and ELISPOT**

NP-specific antibody titers were determined by ELISA using microtiter plates coated with NP–BSA. NP–BSA-coated plates were incubated with 1% BSA for blocking non-specific binding, and diluted serum samples were added to individual wells. Bound antibodies were revealed by HRP-conjugated anti-IgG1, IgG2a, IgM (SouthernBiotech, Birmingham, AL, USA) or IgE (Bethyl, Montgomery, TX, USA) antibodies.

The frequency of IgE-producing cells was determined by enzyme-linked immunospot (ELISPOT) using anti-IgE antibody-coated filter plates. B cells (10⁵ or 10⁶) were plated in one well with culture medium. Plates were incubated at 37°C in a CO₂ incubator for 5 h. Spots derived from IgE-producing cells were visualized with HRP-conjugated anti-IgE antibody.

**Reverse transcription–PCR and digestion circularization–PCR**

Total RNA was purified with Trizol reagent (Invitrogen, San Diego, CA, USA). Two micrograms of total RNA was used.
for reverse transcription. The amounts of mRNAs for AID, C<sup>c1</sup>-GLT, γ<sup>c1</sup>-circle transcript (CT), C<sup>e</sup>-GLT, I<sup>l</sup>-C<sup>e</sup>-post-switch transcript (PST), Id2 and β-actin were measured by semi-quantitative PCR. Digestion circularization (DC)–PCR was previously described (7, 26). Briefly, genomic DNA was digested with EcoRI. Self-ligated DNA fragments were used for PCR. PCR was done with the following primer pairs: AID, CAATTTTCAGATCTTTTGTAG and GGCCCTTGCTCCTTTCTCTACA; γ<sup>1</sup>-CT, GGCCCTTCCAGATCTTTTGTAG and AATGGTGCTGGG-CAGGAAGT; Cγ<sup>1</sup>-GLT, GGCCCTTCCAGATCTTTTGTAG and GGATCCAGAGTTCCAGGTCACT; C<sup>e</sup>-GLT, CATCTGGGCATTGAATTAATGGTTACTA and GTAGCTCCAAGGTGGGCTCAGT; Id2, CAGCCATTTCACCAGGAGAACA and CAGCATTCAGTAGGCTCGTGTCA; I<sup>l</sup>-C<sup>e</sup>-PST, CTCTGGCCCTGCTTATTGTG and GTAGCTCCAAGGTGGGCTCAGT; β-actin, GTGGGC-GCGCTCTAGCCACCAA and TCTTTGATGTCACGCACGATTTC;

Fig. 1. Enhanced IgE production in p85<sup>x<sup>±</sup>/−</sup> mice. Mice were immunized with alum-precipitated NP-CGG and boosted with soluble NP-CGG after 71 days. Open and filled circles in (A) and (C) show the titers of p85<sup>x<sup>±</sup>/−</sup> (n = 6), p85<sup>x<sup>−</sup>/−</sup> mice (n = 7), respectively. (A) NP-specific IgM, IgG1 and IgG2a responses were measured by ELISA. RU; relative unit. (B) Spleen sections from immunized p85<sup>x<sup>±</sup>/−</sup> and p85<sup>x<sup>−</sup>/−</sup> naive mice. Cells were stained with peanut agglutinin and hematoxylin. Brown is PNA. (C) NP-specific IgE (left) and total IgE (right) were measured by ELISA. **<i>P</i> < 0.01, *<i>P</i> < 0.05. (D) Spleenocytes from p85<sup>x<sup>±</sup>/−</sup> and p85<sup>x<sup>−</sup>/−</sup> naive mice were stained with anti-CD19 and anti-IgE with or without EGTA pre-treatment. (E) Three hundred micrograms of trinitrophenol-specific IgE was injected intravenously to p85<sup>x<sup>±</sup>/−</sup> and p85<sup>x<sup>−</sup>/−</sup> mice (n = 4). After 1, 3, 5 days, serum trinitrophenol-specific IgE concentrations were measured by ELISA. Open and filled circles show the IgE titer of p85<sup>x<sup>±</sup>/−</sup> and p85<sup>x<sup>−</sup>/−</sup> mice, respectively.
Enhanced IgE production by p85α−/− mice

To investigate antibody response to T cell-dependent antigen in p85α−/− mice, mice were immunized with alum-precipitated NP-CGG and boosted with soluble NP-CGG on day 71. NP-specific IgM, IgG1, IgG2a and IgE titers were analyzed by ELISA (Fig. 1). In p85α−/− mice, IgM, IgG1 and IgG2a responses to NP were comparable to or slightly less than those of p85α+/+ mice (Fig. 1A). Germinal center formation after immunization was impaired in p85α−/− mice compared with wild-type mice (Fig. 1B). These results are consistent with our previous observation that mature B cell numbers are reduced in p85α−/− mice and BCR- and LPS-mediated activation is partially impaired in p85α−/− B cells (22).

Unexpectedly, p85α−/− mice produced significantly more NP-specific IgE than p85α+/+ mice from 14 days after immunization and the higher titers were sustained for up to 70 days (Fig. 1C, left panel). Upon the secondary immunization with soluble NP-CGG, the concentration of NP-specific serum IgE was increased and the titers were higher in p85α−/− than p85α+/+ mice. Total serum IgE of p85α−/− mice was also higher than that of p85α+/+ mice at 14 days after immunization (Fig. 1C, right panel). These results indicate that the lack of p85α leads to higher IgE response. Since alum is a strong inducer of T cell-independent IL-4 production (27), CpG-based adjuvant was used to examine if enhancement of IgE production is due to alum-based immunization. Although CpG-based adjuvant barely induced IgE in p85α−/− mice, the adjuvant strongly induced IgE production in p85α−/− mice (data not shown), further demonstrating that the lack of p85α results in a higher IgE response.

Before immunization, serum IgE titer was extremely low and close to or below detection sensitivity because free IgE is trapped by tissue mast cells and B cells via the high-affinity IgE receptor FceRI and the low-affinity IgE receptor CD23, respectively. When B cells from unimmunized mice were stained with anti-IgE antibody, substantial amounts of IgE were detected on the surface of most splenic B cells from p85α−/− mice, while only low amounts were detected on B cells from p85α+/+ mice (Fig. 1D). Such surface IgE was removed by treating cells with EGTA, confirming that these IgE molecules bound B cells via CD23. These results indicate that p85α−/− mice produce more IgE than p85α+/+ mice even under naive conditions.

Since IgE is rapidly cleared from the serum compared with other isotypes, it is possible that IgE clearance is impaired in p85α−/− mice. To test this possibility, IgE was exogenously injected to p85α−/− and p85α+/+ mice and serum IgE concentrations were measured (Fig. 1E). There was no difference in the kinetics of IgE clearance between p85α−/− and p85α+/+ mice. These results collectively indicate that IgE production is accelerated in p85α−/− mice without changing IgE clearance from the serum.

Enhanced IgE production by p85α−/− B cells

To determine whether the dysregulation of IgE production in p85α−/− mice is B cell autonomous, splenic B cells from p85α−/− and p85α+/+ mice were stimulated with anti-CD40 and IL-4 to induce CSR to IgG1 and IgE in vitro. The amounts of IgM and IgG1 produced by p85α−/− B cells in the supernatant were lower than or comparative to those of p85α+/+ B cells. In contrast, the production of IgE from p85α−/− B cells was higher than that of p85α+/+ B cells (Fig. 2A). Flow cytometric analysis and ELISPOT assay also demonstrated that higher percentage of B cells expressed IgE in p85α−/− B cells than p85α+/+ B cells (Fig. 2B and C). These results indicate that p85α deficiency in B cells enhances IgE production.

Kinase activity of PI3K is required for IgE suppression

The major catalytic subunit of class IA PI3K expressed in B cells is p110α and the lack of p85α, which stabilizes p110α (28, 29), greatly reduced the expression of p110α (30). To determine whether the kinase activity of PI3K is required for IgE suppression, PI3K was inhibited with pharmacological inhibitors and cell surface expression of IgG1, IgG3 and IgE was examined by flow cytometry (Fig. 3A). IC87114, a specific inhibitor of p110α (31), enhanced the number of cells expressing IgE but not those expressing IgG1 or IgG3, indicating that PI3K activity is required for IgE-selective suppression. It is possible that the high percentages of IgE-positive cells are caused by the specific survival of IgE-positive cells compared with B cells expressing other isotypes in the presence of PI3K inhibitor. However, IC87114 treatment for 3 days increased absolute number of IgE-positive cells (Fig. 3B) without affecting cell division as examined by the dilution of fluorescence intensity of CFSE-labeled B cells (Fig. 3C), confirming that the inhibition of PI3K enhances IgE CSR rather than the selective survival or proliferation of IgE-expressing B cells.

Inhibition of PI3K enhances CSR to IgE and IgG1

We next examined the mechanisms of enhanced IgE production by inhibiting PI3K. First, CSR was assayed by DC-PCR and it was revealed that the PI3K inhibitor enhanced both IgE and IgG1 CSR induced by anti-CD40 and IL-4 (Fig. 4A), indicating that PI3K activity directly suppresses IgE production by blocking IgE CSR. It has been known that BCR signal suppresses IgE and IgG1 CSR (11). We then tested the effect of PI3K inhibitor on the BCR-mediated suppression of CSR. As shown in Fig. 4B, BCR cross-linking suppressed IgE CSR at the Cε GLT level. Interestingly, the same signal suppressed IgG1 CSR as examined by γ1-CT but Cγ1-GLT was unaffected (Fig. 4B). CSR examined by
Cγ1-CT and Cε-GLT was partially recovered by wortmannin. Partial recovery of IgG1 CSR was confirmed by flow cytometric analysis (Fig. 4C). BCR signal also suppressed the expression of AID but induced Id2. Such inhibition of AID and induction of Id2 were partially reversed with PI3K inhibitor wortmannin, suggesting that PI3K is also involved in the BCR-mediated effects on CSR.

It is known that a Th1 cytokine IFN-γ suppresses IgE production but IC87114 had no effect on IFN-γ-mediated suppression of IgE CSR (Fig. 4D). It has been reported that IL-21 specifically inhibits IgE CSR induced by a combination of LPS and IL-4 (10). However, IL-21 enhanced IgE CSR in B cells stimulated by a combination of anti-CD40 and IL-4. IC87114 treatment killed B cells in the presence of IL-21 (Fig. 4D).

**Fig. 2.** Enhanced IgE production in p85α−/− B cells. (A) Purified splenic B cells were stimulated with 5 μg ml−1 anti-CD40 and indicated concentrations of IL-4. After 4 days, IgE, IgG1 and IgM titers in culture supernatants were measured by ELISA. White and black bars show the titers of p85α+/+ and p85α−/− B cells, respectively. (B) The indicated numbers of p85α+/+ and p85α−/− B cells were stimulated with 5 μg ml−1 anti-CD40 and 10 ng ml−1 IL-4 in 200 μl culture medium. The expression of IgG1 and IgE on the cell surface was analyzed by flow cytometry. The percentages of IgG1+ and IgE+ cells are indicated at each gate. (C) Data are representatives of three independent experiments; data are shown as mean ± SD.

*PI3K-mediated cell density-dependent IgE suppression at post-translational level*

It is known that IgE CSR is sensitive to cell density and IgE production is suppressed in high-density cell cultures (32). Enhanced IgE induction by p85α−/− B cells was more prominent in high-density cultures (Fig. 2B) as p85α−/− B cells were more sensitive to cell density than p85α+/+ B cells. This observation prompted us to test the involvement of PI3K in cell density-dependent IgE suppression. IgE secretion and the percentages of IgE+ cells decreased as cell density increased (Fig. 5A and B: thin lines). The inhibition of PI3K cancelled this suppression as IgE production became proportional to the cell numbers in the presence of IC87114 (Fig. 5A). In addition, the percentages of IgE+ B cells were
**Discussion**

Mast cells play a central role in allergic responses by releasing inflammatory substances. The activation of mast cells is triggered by the binding of allergen in complex with antigen-specific IgE to the high-affinity IgE receptors FcεRI. Since the pharmacological inhibition of p110δ reduced mast cell activation and protected mice against passive systemic anaphylactic allergic responses, p110δ was proposed to be a new target for therapeutic intervention in allergic diseases (33). As shown here, however, the inhibition of PI3K including p110δ augments IgE responses, raising the possibility that the inhibition of PI3K pathway *in vivo* may not be beneficial for the protection of allergic disorders. As demonstrated by the binding of higher amounts of IgE on p85α−/− B cell surface via CD23 compared with those of wild-type B cells, the lack of p85α leads to higher level of IgE production even without immunization. In addition, we sometimes observed significant amounts of IgE in the serum of unimmunized p85α−/− mice, indicating that IgE production is generally enhanced in the absence of p85α. Although it has been reported that the basal level of serum IgE in p110δ−/− mice is comparable to that of wild-type mice (34), it will be of interest to examine the IgE response to exogenous antigen in those mice. *In vitro* induction of IgE CSR in p110δ−/− B cells will also be informative to compare the phenotype observed in p85α−/− mice in future studies.

Since the augmentation of IgE production by p85α−/− B cells was reproduced by the inhibition of p110δ kinase activity with an isof orm-specific inhibitor IC87114, it is likely that p85α suppresses IgE production by recruiting p110δ catalytic subunit rather than functioning as a GTPase activating protein (GAP) activity (35) that is independent of the kinase activity of PI3K. In addition, these results indicate that the PI3K activity in B cells autonomously regulates IgE production. It is of note that B cells deficient for phosphatase and tensin homolog deleted on chromosome 10 (PTEN), a negative regulator of PI3K, are unable to induce AID and CSR (36, 37), which is consistent with our observation. Downstream target of PI3K to suppress IgE production is elusive at the moment. BCR signal and cell–cell interaction must transduce signals through PI3K for IgE inhibition because CD40 and IL-4 receptor also activate PI3K via tumor necrosis factor receptor associated factor 6 (TRAF6) (38) and insulin receptor substrate 1 (IRS1) (39) molecules, respectively.

Our results demonstrate that PI3K negatively regulates IgE production through two different mechanisms. First...
mechanism is the suppression of CSR for both IgG1 and IgE. It has been reported that the cross-linking of BCR inhibits CSR to IgG1 and IgE mediated by CD40 and IL-4 regardless of their effect on proliferation (11, 40) and the inhibition of PI3K partially restored the effect of BCR cross-linking (Fig. 4). In this context, it is of interest to note that high-dose allergen exposure specifically prevents IgE production and allergic responses (41–44). The mechanism of

Fig. 4. BCR cross-linking inhibits IgG1 and IgE CSR via PI3K. B cells (1 × 10^5 in 200 μl) were stimulated with 5 μg ml⁻¹ anti-CD40 and 10 ng ml⁻¹ IL-4. (A) B cells were stimulated for 2 or 3 days with or without 5 μM IC87114. Sμ-Sγ1 and Sμ-Sc recombination was detected by DC–PCR. Nicotinic acetylcholine receptor (nAchR) was used as internal control for DC–PCR. (B) B cells were stimulated with or without 1 μg ml⁻¹ anti-μ antibody with or without 100 nM of wortmannin for 2 days. The expression of β-actin, AID, γ1-CT, γ1-GLT, Ce-GLT and Id2 were measured by real-time PCR. PCR was done in duplicate and data are shown as mean ± SD, and representatives of three independent experiments are presented. (C) Splenic B cells were stimulated for 3 days with or without 100 nM wortmannin and the expression of IgG1 and IgE on the cell surface was analyzed by flow cytometry. (D) Flow cytometric analysis of stimulated B cells in the presence of 100 U ml⁻¹ of IFN-γ or 20 ng ml⁻¹ of IL-21 with or without 5 μM IC87114 for 4 days. The percentages of IgG1⁺ and IgE⁺ cells are indicated at each gate.
BCR-mediated CSR inhibition for IgE seems different from that for IgG1 as BCR cross-linking blocks Ce GLT but not Cγ1 GLT. BCR cross-linking induces the expression of Id2, which inhibits Ce-GLT (15) and, to a lesser extent, AID expression (45). While the block of AID induction results in the inhibition of IgG1 CSR in B cells stimulated by anti-CD40 and IL-4, IgE CSR is likely regulated at both AID induction and Ce GLT levels. As shown here, BCR-mediated Id2 induction was partially dependent on PI3K, implying the involvement of Id2 in the PI3K-mediated negative regulation of IgE CSR. Although Id2 is known to inhibit IgE CSR, Id2 is unlikely a main factor of PI3K-mediated IgE suppression.

Fig. 5. Cell density-dependent IgE suppression is mediated by PI3K activity. The indicated concentrations of B cells were stimulated with 5 µg ml⁻¹ anti-CD40 and 10 ng ml⁻¹ IL-4 for 4 days with (square) or without (diamond) 5 µM IC87114. (A) The concentration of each isotype in the supernatant was measured by ELISA. (B) The percentages of IgE⁺ and IgG1⁺ were analyzed by flow cytometry. B cells were cultured in duplicate. Data are the mean ± SD. (C) The indicated numbers of cells were stimulated with 5 µg ml⁻¹ anti-CD40 and 10 ng ml⁻¹ IL-4 for 2 days. Il-Çe-PST was detected by reverse transcription-PCR. The amount of cDNA was normalized by ß-actin. Five-fold serial dilutions of cDNAs were amplified. (D) The indicated numbers of cells were stimulated with (IC) or without (--) 5 µM of IC87114 for 4 days. Intracellular IgE was stained and analyzed by flow cytometry. The percentages of IgE⁺ cells are indicated at each gate. The mean fluorescence intensities of IgE are indicated under each panel. Data are representatives of three independent experiments.
because IC87114 also enhanced IgE CSR in Id2<sup>−/−</sup> B cells (T. Doi, K. Obayashi and S. Koyasu, unpublished observation). In addition, it is known that IgE production is still lower than IgG1 in Id2<sup>−/−</sup> mice (15).

If the efficiency of IgG1 and IgE CSR is the same and IgG1 and IgE CSR occur independently, the percentages of IgG1-expressing cells must be lower than those of IgE-expressing cells because a part of IgG1-expressing cells subsequently switch to IgE-expressing cells (18–20). The fact that both IgG1 and IgE CSRs are negatively regulated by PI3K (Fig. 4A) yet the percentage of IgE-expressing cells is much lower than that of IgG1-expressing cells suggests the presence of another IgE-selective suppression mechanisms. Such second mechanism seems operative at the protein level as PI3K reduces IgE protein expression. It has previously been reported that IgE production is suppressed in a concentrated cell culture in vitro (32), which may explain the fact that IgE-expressing B cells are >1000 times more frequent in the nasal mucosa, which contain fewer B cells, than other lymphoid tissues (46). Our present results indicate that such density-dependent suppression is completely dependent on PI3K signaling (Fig. 5). The amount of IgE mRNA was not suppressed in high-density cultures, suggesting that the density-dependent suppression is controlled at a protein level. Although the mechanism is unclear at the moment, there are several possibilities. IgE expression may be suppressed at the level of protein synthesis, intracellular trafficking, internalization or degradation. Decrease of IgE<sup>+</sup> cells at high density may be due to internalization of IgE as has been shown for CTLA-4 in naive T cells (47). If this were the case, anti-IgE antibody added to the culture medium of IgE-expressing cells would accumulate inside the cells. To test this possibility, we compared the accumulation of FITC-conjugated anti-IgE antibody by IgE-expressing B cells at 37 or 4°C. No accumulation of anti-IgE antibody in B cells was observed after 3 h incubation at concentrated cell culture, while anti-CTLA-4 antibody accumulated in T cells at 37°C as reported (T. Doi, K. Obayashi and S. Koyasu, unpublished observation). Thus the lack of surface IgE is unlikely due to enhanced internalization. The total amount of IgE examined by intracellular staining was much lower in the absence of PI3K inhibitor than that in the presence of the inhibitor (Fig. 5). Therefore, PI3K-dependent degradation and/or block of IgE protein synthesis are more likely to explain IgE reduction at the protein level.

It has been reported that basal signal or tonic signal through BCR is critical for the survival of peripheral B cells and that B cells are eliminated from body shortly after BCR ablation (48). It is possible that PI3K-dependent suppression of surface IgE expression leads to the specific elimination of IgE-positive cells after IgE production, which should be examined in future studies.

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**Abbreviations**

| AID | activation-induced cytidine deaminase |
| BCR | B cell receptor |
| CGG | chicken yglobulin |
| CFSE | carboxyfluorescein succinimidyl ester |
| CSR | class switch recombination |
| CT | circle transcript |
| DC | digestion circularization |
| GLT | germ line transcript |
| Id2 | inhibitor of differentiation-2 |
| NP | (4-hydroxy-3-nitrophenyl) acetyl |
| PI3K | phosphoinositide 3-kinase |
| PST | post-switch transcript |

**References**

Immune cell responses are regulated by various signaling pathways, including those involving phosphoinositide 3-kinase (PI3K). PI3K is crucial for B cell development and differentiation, influencing class switching and antibody production.


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