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Phosphoinositide 3-Kinase Activity in T Cells Regulates the Magnitude of the Germinal Center Reaction

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After exposure to Ag via immunization or infection, B cells have the capacity to generate plasma cells and develop an extracellular response or, together with follicular dendritic cells, initiate the germinal center (GC) reaction (1). The GC is a microanatomical structure formed in B cell follicles in secondary lymphoid organs where Ag-specific B cells undergo division, isotype switching, somatic hypermutation, and differentiation into memory B cells or plasma cells. Cognate interaction between B and T cells in the GC is essential for the selection of high-affinity Ag-specific B cells, and access to T cell help is thought to be a limiting factor for the positive selection of GC B cells (2).

The PI3K pathway has been implicated in lymphocyte development and activation. It transduces extracellular signals into the production of the second messenger phosphatidylinositol 3,4,5-trisphosphate (PIP3) by phosphorylation of phosphatidylinositol 4,5-bisphosphate. In lymphocytes, PIP3 is generated by the class I PI3K catalytic subunit named p110α, β, γ, δ, and ε. The α, β, δ and ε enzymes form heterodimers with one of five Src homology 2 domain-containing regulatory subunits termed p85α, p85β, p55γ, p50α, and p55ε, which mediate recruitment to phosphotyrosine-containing signalosomes (3). The levels of cellular PIP3 are also regulated by phosphatases: phosphatase and tensin homolog deleted on chromosome 10 (PTEN) directly opposes PI3K by removing the 3′ phosphate from PIP3; the SHIP enzymes generate the second messenger, phosphatidylinositol 3,4-bisphosphate.

Mice with germline mutations of the p110α catalytic subunit have demonstrated its importance for the GC reaction. However, it remains unclear whether this is a reflection of the requirement for p110α in B cells, T cells, dendritic cells (DCs), or other cell types required for the GC response (4, 5). B cells from p110α-deficient mice display in vitro defects in survival and proliferation that correlate with defective signal transduction following stimulation with Abs via the BCR or CD19 (6–8). Whether these in vitro defects are relevant in vivo is unclear since B cell-specific deletion of PTEN impaired class-switch recombination but not the magnitude of the GC response (9). T cell-specific deletion of the class IA regulatory subunits showed that the GC reaction and Ag-specific Ab titers were reduced, implicating a T cell-intrinsic requirement for class IA PI3K but leaving unresolved the nature of the relevant catalytic subunits (10). Using the p110αD910A mouse model, which carries a point mutation that renders p110α catalytically inactive, adoptive transfer experiments revealed impaired Th1/Th2 cytokine production and a 2-fold reduction in clonal expansion (5, 11). In contrast, T cell-specific deletion of PTEN removed the requirement for CD28 costimulation (12) and allowed enhanced IL-4 production (13). Gaining a better understanding of the cell intrinsic role for p110α in Ab responses should prove helpful in determining the mechanism of action of small molecule p110α inhibitors. This is timely, as these inhibitors have shown promise in animal models of inflammatory and autoimmune disease (14, 15), and human studies have been initiated.

There is an increasing understanding of the nature of GC T cells that are synonymous with the specialized population of T cells called T follicular helper (T FH) cells (reviewed in Refs. 16 and 17). This T cell subset represents a novel lineage of effector T cells that is critical for the provision of help to B cells during an immune response.
The development of TFH cells is dependent on the presence of Ag-specific B cells and the transcriptional repressor B cell lymphoma 6 (Bcl-6) (19–21). A defining characteristic of TFH cells is their high expression of the chemokine receptor CXCR5 that allows migration along a gradient of CXCL13 into the B cell follicle (22). Recently, ICOS-mediated PI3K signaling has been shown to be an essential regulator of TFH cell numbers and cytokine production (23). In this study, we show that, unexpectedly, p110β activity in B cells is not necessary for the formation or magnitude of the GC response and that the essential function of p110β in the GC reaction is the T cell-intrinsic regulation of TFH cell formation and function. Moreover, we demonstrate that the p110α subunit is pivotal for ICOS function, thereby acting as a key regulator of the TFH cell lineage at the level of intracellular signaling. The importance of the PI3K pathway in TFH cells is underscored by our finding that the magnitude of PI3K signaling in T cells regulates TFH cell numbers, the numbers of GC B cells, and the number of high-affinity Ab-secreting cells produced upon secondary immunization.

Materials and Methods

Mouse models

The pik3cd targeting vector was generated using standard methodology. A single LoxP site was introduced into the Xhol site upstream of the first coding exon. A neomycin cassette flanked by FRT sites and a single LoxP site (FRTLoxP-FRT) was inserted into the EcoRV site between exons 9 and 10. Targeted E14 ES clones were identified by Southern blot analysis of EcoRV digests, which yielded a 5.6-kb fragment for nontargeted clones and a 4.2-kb fragment for targeted clones. FLPe was transiently expressed to mediate recombination excision of the neomycin cassette, which was verified by Southern blot analysis. One of the correct clones was selected and injected into CS12.5 blastocysts to generate chimeric mice. LoxP-flanked exons 1–9 encoding the first 490 aa of p110β, which will be deleted in the presence of cre. Conditional deletion was achieved by crossing with CD1cre, Cd4cre, or Ox40cre mouse strains. Germline p110β−/− mice were described previously (4). Mice were backcrossed at least five times to C57BL/6. Mice with a conditional allele of PTEN, p110α−/− mice, OT-II transgenic RAG−/− recipient mice were performed according to United Kingdom Home Office and local Ethical Review Committee guidelines.

Isolation of DNA, RNA, and quantitative PCR

Genomic DNA was prepared using cell lysis and proteinase K digestion (Genta/Qiagen, Crawley, U.K.). Quantitative PCR was performed to determine the recombination efficiency of genomic DNA using a JOE-labeled probe within exon 2 and a FAM-labeled probe within exon 18 (Supplemental Fig. 1B): p110β forward exon 18, 5′-CACAGGTCTCATCGAGTTGTC-3′; p110β reverse exon 18, 5′-TGGACTTGAGCCAGTTGAGCA-3′; p110α forward exon 18, 5′-CTCGGACACTGCGCAACATCC-3′; p110α reverse exon 18, 5′-CACTTTTGGGCTGAGCTGACA-3′; and p110α probe (FAM) exon 18, 5′-CCTGAGGACGTACGAGGAGGATC-3′. Genomic DNA was prepared using cell lysis and proteinase K digestion. FLPe was transiently expressed to mediate recombination excision of the neomycin cassette, which was verified by Southern blot analysis. One of the correct clones was selected and injected into CS12.5 blastocysts to generate chimeric mice. LoxP-flanked exons 1–9 encoding the first 490 aa of p110β, which will be deleted in the presence of cre. Conditional deletion was achieved by crossing with CD1cre, Cd4cre, or Ox40cre mouse strains. Germline p110β−/− mice were described previously (4). Mice were backcrossed at least five times to C57BL/6. Mice with a conditional allele of PTEN, p110α−/− mice, OT-II transgenic RAG−/− recipient mice were performed according to United Kingdom Home Office and local Ethical Review Committee guidelines.

Flow cytometry

Single-cell suspensions were prepared from lymphoid tissues and the peritoneal cavity. Abs against the following surface markers were used: B220, CD4, CD8, CD21, CD23, CD95, CXCR5, CCR7, ICOS, IgM, programmed cell death 1 (PD-1; BD Biosciences), peanut agglutinin (PNA)-FITC (Vector Laboratories, Peterborough, U.K.), and streptavidin conjugates (eBioscience, Hatfield, U.K.). Dead cells were excluded using DAPI staining. Cells were collected using an LSRII or FACSCalibur (BD Biosciences, Oxford, U.K.) and analyzed using FlowJo software (Tree Star, Ashland, OR).

Immunofluorescence microscopy

Five-micrometer spleen sections were fixed in ice-cold acetone and blocked before incubation with biotinylated primary Abs followed by streptavidin-Alexa 555 (Invitrogen) and then PNA-FITC. The sections were mounted using Aqua-Poly/Mount (Polysciences, Northampton, U.K.). An Olympus IX81 wide-field epifluorescence microscope was used for imaging at ×10–20 magnification with an Olympus ×10 UPLFL (universal plan fluorite) NA 0.3 objective, and images were taken with a Hamamatsu ORCA ER digital camera at room temperature. Color conversion was performed by the Olympus cell software, and minor adjustments were made using Adobe Photoshop. Five images per mouse were taken, and the numbers of B cell follicles and PNA+ GC structures were enumerated. For quantification of CD4+ cells within the B cell follicle and GC, sections were stained with anti-CD86 (BD Biosciences, Oxford, U.K.) at 1:400, anti-IgA, and anti-methylrhodamine isothiocyanate-labeled streptavidin (Jackson ImmunoResearch Laboratories, Newmarket, U.K.). Five fields of view were captured per mouse, and the number of CD4+ cells in the GC (PNA+ and IgD+ areas) was determined by counting cells relative to a defined area (μm2) using Velocity software (Improvision, Coventry, U.K.).

In vitro IL-21 differentiation

Naïve lymph node CD4+ T cells were FACSAria-sorted to >95% purity. CFSE-labeled, naïve CD4+ T cells (105) were differentiated for 4.5 d in the presence of plate-bound 1 μg/ml anti-CD3, soluble 1 μg/ml anti-CD28, 10 μg/ml anti-IFN-γ (RA-6A2), 10 μg/ml anti-IL-4 (11B-11), and 100 ng/ml IL-6 (PeproTech, London, U.K.) in IMDM media. For intracellular flow cytometry of IL-21, the cells were stimulated with phorbol dibutyrate (50 ng/ml) and ionomycin (500 ng/ml) for 6 h, and 10 μg/ml brefeldin A was added for the last 2 h. The cells were stained using anti-IL-21-allophycocyanin (R&D Systems, Abingdon, U.K.) according to the manufacturer’s protocol. The IL-21+ cells were defined by quadrant gates set using the isotype control staining and the undivided peak of CFSE-labeled cells.

ICS restimulation

CD4+ T cells were purified by MACS and cultured for 2 d in the presence of plate-bound 10 μg/ml anti-CD3 and soluble 1 μg/ml anti-CD28 and then rested in media for 1 d. The cells were restimulated with 0.5 μg/ml anti-CD3, 2 μg/ml anti-CD28, and 20 μg/ml anti-IFN-γ and 100 ng/ml anti-hamster IgG for 6 h.

APC assay

Anti-HEL Ig transgenic B cells were isolated from wild-type and p110β−/− mice using a MACS B cell isolation kit (Miltenyi Biotec, Bisley, U.K.) according to the manufacturer’s protocol (purity >95% B cells). B cells were cultured with increasing doses of HEL (Sigma-Aldrich, Poole, U.K.) and analyzed using FlowJo software (Tree Star, Ashland, OR). The 1-Aα−/− line of HEL-responsive 2G7 T cell line for 24 h at 37°C (24). The quantity of IL-2 secreted by 2G7 cells was measured by ELISA according to the manufacturer’s protocol (R&D Systems).

ELISAs and ELISPOT

ELISA plates (Nunc, Paisley, U.K.) were coated with NP23-BSA, or NP23-BSA, or for high-affinity measurement NP23-BSA, or NP23-BSA at 10 μg/ml in carbonate buffer, washed, and blocked. Serum samples were serially diluted and bound Ig was detected using biotinylated anti-IgM or IgG1 and HRP-conjugated streptavidin (SouthernBiotech, Birmingham, AL). Endpoint titers were calculated to give a measure of relative Ab concentration. For ELISPOT, MultiScreen HA mixed celluloise ester plates (Millipore, Watford, U.K.) were coated with NP23-BSA, or NP23-BSA, or PBS, washed, and blocked before applying serially diluted cells and incubating overnight. Detection was performed using biotinylated IgG1 (SouthernBiotech) and alkaline phosphatase-conjugated streptavidin (R&D Systems) and 5-bromo-4-chloro-3-indolyl carbonimide, wash, and substrate reaction. The absorbance was measured at 490 nm.

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p110α regulates TFH formation

As T cells are known to be critical for the GC reaction (25), the TFH cell population was analyzed in T p110α−/− mice by flow cytometry using CXCR5- and PD-1–specific Abs. In T p110α−/− mice immunized with NP-KLH, the numbers of TFH cells were reduced compared with p110α+/+ controls (Fig. 2A). To extend our analysis to a different Ag, we chose SRBCs, which elicit a robust ICOS-dependent expansion of CXCR5+ TFH cells (26). Four days after immunization with SRBC, TFH cell formation was severely impaired in T p110α−/− mice and, by day 6, the TFH cell population in these mice had returned to baseline (Fig. 2B). These data suggest that p110α is required for TFH cells for their initial expansion or differentiation. Furthermore, the GC reaction elicited by SRBCs was impaired in T p110α−/− mice, as the number of B220+PNA+CD95+ cells was reduced at all time points tested (Fig. 2B), and the histological appearance of the spleen showed fewer and smaller GC structures in the SRBC–immunized T p110α−/− mice (data not shown).

TFH cells must form cognate interactions with B cells and, therefore, migration into the B cell follicle is important for TFH cell differentiation (21, 27). The numbers of CD4+ T cells in the B cell follicle were slightly lower in T p110α−/− mice compared with p110α+/+ mice. However, the numbers of CD4+ T cells in the PNA+ IgD− GC area were significantly reduced (Fig. 2C). Thus, the reduced number and size of GCs are reflected by reduced numbers of TFH cells. As CCR7 downregulation is essential for follicular entry and T cell help to B cells (27), we determined the levels of CCR7 on CD4+ T cells. The expression of CCR7 was higher on PD−1+ T p110α−/− CD4+ T cells compared with p110α+/+ mice, which is consistent with a previous study showing that p110α regulated CCR7 on CD8+ T cells (28). We found that PD−1+ TFH cells downregulated CCR7 on their surface similarly in T p110α−/− and p110α+/+ mice (Fig. 2D). Taken together, these data suggest that CD4+ TFH cells residing in the GC were reduced in T p110α−/− mice, but this reduction is unlikely to be a consequence of failure to downregulate CCR7.

p110α function in APCs and B cells is not essential for Ab responses

Although the T p110α−/− mice revealed a T cell-intrinsic role for p110α in the GC reaction, the initial observations on the severe defects GC formation and humoral immunity in the p110α−/− or p110αD910A knock-in mice could also reflect a role for p110α in APCs or B cells (4, 5). To determine whether p110α plays a role in the initial priming of naive T cells by APCs, we used adoptive transfer of transgenic OT-II cells and NP-OVA immunization. The ability of wild-type OT-II T cells to stimulate the production of NP-specific IgG1 was assessed 7 d after adoptive transfer into C57BL/6 and p110α−/− recipients (Fig. 3A). The NP-specific IgG1 titers were not significantly different between the two groups, suggesting that initial T cell priming by APCs is independent of p110α and, moreover, that provision of extrinsic T cell help through adoptive transfer can rescue defects in humoral immunity in the p110α−/− mice.

To explore the B cell-intrinsic role of p110α, we used CD19cre-mediated deletion of p110α in B p110α−/− mice. Deletion was less efficient than that obtained with CD4cre but led to reductions in the number of marginal zone (MZ) and periportal B1, but not follicular B cells (Supplemental Figs. 1, 2); this is a phenotype similar to germline p110α knockout and p110αD910A mice. The absence of p110α in B cells did not cause any reduction in GC B cell numbers after immunization (Fig. 3B). Following NP-CGG immunization, IgM and high affinity NP-specific IgG1 titers were not significantly decreased in the B p110α−/− mice compared with p110α+/+ mice (Fig. 3C). However, we found that the Ag-specific IgE titers were increased 30-fold after immunization in B p110α−/− compared with...
p110δ/fl mice (Fig. 3C). This is in agreement with evidence that PI3K negatively regulates IgE (29, 30) and indicates that this effect is B cell-intrinsic. In the B p110δ/fl mice, there was a trend toward impairment in affinity maturation compared with p110δ/fl mice (Fig. 3D), indicating that p110δ has a modest role in affinity maturation or the survival or functionality of plasma cells. Alternatively, the reduction in IgG1 may be secondary to the enhanced class-switching to IgE. Analysis of high-affinity NP-specific IgG1 titers after booster immunization showed no difference in memory response between B p110δ/fl and p110δ/fl mice (Fig. 3E). The GC imposes a powerful selective environment upon B cells, which should result in accumulation of p110δ-sufficient cells if this enzyme is
The number of CXCR5+PD-1+ TFH cells among CD4+ T cells and the mean, and data are representative of two independent experiments. 

MHC class II-mediated presentation of peptide Ag by B cells to GC T cells is essential for successful selection of high-affinity B cells. Therefore, we tested the ability of p110δ-deficient B cells to process and present Ag using an intravitro T cell stimulation assay. B cells from p110δ−/− mice bearing the anti-HEL Ig transgene (MD4) (31) showed an increased capacity to present Ag over a range of HEL concentrations compared with p110δ-sufficient controls (Fig. 3G). Taken together, these data suggest that p110δ in B cells plays an essential role in limiting class-switching to IgE, but it is dispensable for the GC response.

TFH cell and GC B cell numbers are regulated by p110δ and PTEN activity in T cells

The deletion of p110δ in developing T cells by Cd4cre would be expected to influence early activation events that were dependent on this lipid kinase. To investigate whether p110δ was required for the GC reaction subsequent to the initial activation of T cells, we generated mice in which deletion of p110δ was mediated by OX40cre. The costimulatory molecule OX40 is expressed following TCR- and CD28-mediated T cell activation and has been found to be expressed on CXCR5+CD4+ T cells, suggesting that the OX40cre transgene will be expressed in TFH cells (32, 33). Following immunization, we analyzed TFH cell numbers at the peak of the GC reaction in the OX40cre-p110δ−/− model and found that TFH cells were significantly diminished in these mice compared with p110δfl/fl controls (Fig. 4A). The number of GC B cells and the ratio of GCs to B cell follicles were concomitantly reduced (Fig. 4B). The B cell memory response was analyzed at day 7 after boosting in the OX40cre models. In the OX40cre-p110δ−/− mice, the high-affinity Ab titers were modestly reduced and there was a significant reduction in the numbers of NP2-binding AFCs in the bone marrow and spleen (Fig. 5A).

The enzymatic function of p110δ is directly opposed by the lipid phosphatase PTEN that converts PIP3 to phosphatidylinositol 4,5-bisphosphate. Deletion of PTEN is associated with hyperresponsiveness of cells to stimulation and malignant transformation (13). To understand the contribution of PTEN in TFH cells, we again used the OX40cre system. Following immunization of OX40cre-PTENfl/fl mice, the TFH population was increased ~3-fold compared with PTENfl/fl control mice (Fig. 4C). The number of GC B cells was also significantly increased and there was a trend toward a higher ratio of GCs per B cell follicle (Fig. 4D). In the OX40cre-PTENfl/fl mice, the high-affinity Ab titers were increased and the numbers of NP2-binding AFCs tended to be higher (Fig. 5B).

PI3K activity in T cells and the subsequent effect on TFH cell numbers are highly correlated with GC B cell numbers in the T p110δ−/− and OX40cre mouse models (Fig. 4E), suggesting that PI3K activity in T cells determines the magnitude of the GC response. Thus, the GC reaction and subsequent effects on B cell memory function is influenced by the amount of PI3K activity in T cells.

FIGURE 2. TFH cells and GC T cells are diminished in T p110δ−/− mice. A, Flow cytometric analysis of CXCR5+PD-1+ TFH cells among CD4+ T cells in the spleen at day 10 following NP-KLH immunization of p110δ−/− compared with T p110δ−/− mice and the numbers of TFH cells. For A and C, each symbol represents one mouse, horizontal bars indicate mean ± SD, and data are representative of two independent experiments. B, The number of CXCR5+PD-1+ TFH cells among CD4+ T cells and the absolute number of GC B cells in NI and on days 4, 5, and 6 after SRBC immunization are shown as mean ± SD of four to five mice per time point in p110δ−/− (■) compared with T p110δ−/− (●) mice. Nonparametric ANOVA. C, The number of CD4+ cells within B cell follicles or GCs was determined 5 d following SRBC immunization by immunofluorescence using mAbs to CD4 (red), IgD (blue), and PNA (green) (original magnification ×20). The left scatter plot shows the numbers of CD4+ cells in the B cell folicle per 0.01 mm2, and the right scatter plot shows the numbers of CD4+ cells per 0.01 mm2 of GC. D, Representative histograms of the MFI of CCR7 on PD-1+CD4+ T cells and TFH PD-1+CD4+ T cells day 4 after SRBC immunization. Isotype (black), T p110δ−/− (gray), and p110δ−/− (open) histograms. MFI, mean fluorescence intensity; NI, nonimmunized mice.
FIGURE 3. The B cell-intrinsic role of p110δ in humoral immunity. A, In vivo priming of \(3 \times 10^5\) OT-II T cells after adoptive transfer into B6 recipients (\(n = 7\)) or p110δ-/- recipients (\(n = 5\)) was measured as NP3-specific IgM titers day 7 after transfer and NP-OVA immunization. B6 (\(n = 4\)) and p110δ-/- (\(n = 2\)) mice that did not receive donor cells are shown. For A–C, each symbol represents one mouse and horizontal bars indicate the mean. Data are representative of two independent experiments. B, Numbers of GC (B220+CD95+) B cells at days 7 and 14 following NP-CGG and analyzed for NP3-specific IgM at days 7 and 14, NP3-specific IgG1 at day 7, NP3-specific IgG2a at day 14, and NP3-specific IgE at day 14. C, Mice were immunized with NP-CGG and analyzed for NP3-specific IgM at days 7 and 14, NP3-specific IgG1 at day 7, NP3-specific IgE at day 14. D, Affinity maturation presented as the mean ratio (±SD) of the NP3 titer IgG1-to-NP3 titer IgG1 following NP-CGG immunization in p110δ-/- (\(n = 8\)) compared with B p110δ-fl/fl mice (\(n = 8\)). The mean high-affinity NP3 IgG1 titers (±SD) in p110δ-fl/fl (\(n = 8\)) compared with B p110δ-fl/fl mice (\(n = 8\)) was measured as mean (±SEM) of the HEL-2-secreted by the HEL-responsive CD4+ T cell line. Nonparametric ANOVA, F and C57BL/6 mice.
role of p110δ in ICOS downstream signaling, we analyzed the constitutively active kinase GSK3 that is phosphorylated and thereby inhibited by p-Akt. We found that the levels of phosphorylation of the isoform GSK3β was reduced in p110δ−/− T cell blasts compared with B6 after ICOS-stimulation (Fig. 7D).

To further explore the role p110δ in ICOS signaling, we re-stimulated ICOS-expressing T cell blasts in vitro with anti-CD3 and anti-ICOS and measured selected cytokine and transcription factor mRNAs in these cells. The transcription factor c-Maf (42) is known to be induced by ICOS (43). In T cells lacking p110δ, the amount of c-Maf, IL-21, and IL-4 mRNA was reduced compared with control p110δfl/fl T cells, showing that p110δ is crucial for ICOS-dependent gene expression (Fig. 7E). The mRNA levels of the mutually antagonistic transcriptional repressors Bcl-6 and B lymphocyte-induced maturation protein 1 (Blimp-1) were analyzed after ICOS restimulation. Bcl-6 was induced by ICOS restimulation in p110δ−/− T cell blasts, but in the T p110δfl/fl blasts Bcl-6 expression was already high. Blimp-1 expression in CD4+ T cell blasts has previously been shown to be lower in stimulated CD4+ T cells from p110δD910A mice compared with controls (44),
and we found that Blimp-1 mRNA levels were also lower in T p110δ/δ blasts after ICOS restimulation (Fig. 7E).

**Discussion**

In this study, we show that the essential cell-intrinsic role of p110δ required for the GC reaction to proceed resides within T lymphocytes. Moreover, our results suggest that the activity of the PI3K pathway in T cells is a critical determinant of GC size and output. The numbers of GC B cells was dependent on PI3K activity in T cells, which is compatible with the idea that access to TFH cells; however, we found that their expression was p110δ-activity is attenuated in vitro (29, 30).

When extrinsic T cell help was provided, p110δ was dispensable in other cells for the generation of an isotype-switched Ab response. Furthermore, in B p110δ/δ mice the magnitude and output from GC reaction was intact. As there was no evidence for selection of nondepleted cells, we conclude that p110δ signaling in B cells is not essential for the GC magnitude. This was unexpected given the many reports, including our own, of defective signaling by p110δ-deficient B cells and challenges the widely held view that p110δ is the main PI3K isoform involved in B cell activation (46). These previous findings may reflect the key role of p110δ in the development of MZ B cells, as the in vitro studies using splenic B cell populations did not take account of the hyperresponsiveness of MZ B cells (47, 48) which were missing in the p110δ mutants. Similarly, previous suggestions of defective Ag presentation by p110δD910A B cells (49) may reflect the paucity of MZ B cells, which are known to be more effective at presenting Ags than follicular B cells (47). Indeed, we found Ag processing and presentation was enhanced in the absence of p110δ when Ag-specific B cells were tested. Our study also revealed that a B cell-intrinsic role of p110δ was to suppress class-switch recombination. This is consistent with PTEN inactivation in B cells inhibiting class-switch recombination (9) and the enhanced switching to IgE when p110δ activity is attenuated in vitro (29, 30). Taken together, the data show the nonredundant role of p110δ in B cells to control the differentiation or survival of the MZ and B1 B cell subsets and during the TD response to suppress Ig class-switch recombination. Other PI3K catalytic subunits, such as the p110α isoform that has been shown to be associated with CD19 after BCR stimulation, may contribute in a redundant manner to the GC reaction (6).

Multiple priming/activation events are required for a successful GC reaction. First, T and B lymphocytes individually need to recognize Ag and initiate signaling through the TCR or BCR, respectively. In a second phase, the T and B cells home to the T cell–B cell border zone within secondary lymphoid tissues, form conjugates, and exchange signals that regulate selection and survival. Subsequently, continued interactions between B and T cells are required for the sustenance of the GC reaction. The T cell-intrinsic requirement for p110δ was characterized by a paucity of the T FH cell subset, which is specialized in providing help to B cells. Although p110δ has previously been shown to be important for FoxP3” regulatory T cell function (50), the T FH cell defect is unlikely to be secondary to a defect in these cells since adoptively transferred regulatory T cells do not contribute to GC formation in the spleen (51). CD4+ T cells lacking p110δ could enter primary follicles but were substantially reduced in GCs. The downmodulation of surface expression of CCR7, a prerequisite for follicular entry, suggested that some early activation events in T cells were independent of p110δ. The importance of the signaling lymphocyte activation molecule (SLAM) family members CD84 and Ly108 for cognate interactions between T FH cells and GC B cells (52) led us to analyze their presence on CD4+ T cells; however, we found that their expression was p110δ-independent (data not shown). Deletion of p110δ after the initial priming event, by expression of OX40cre, suggested that T FH cells required the continual presence of p110δ. This finding may reflect the key role of p110δ in ICOS signaling.

Our studies using hapten protein and SRBCs, both of which elicit ICOS-dependent GC responses, implicate p110δ as being an essential catalytic subunit for ICOS signaling. ICOS is a key cell-surface receptor regulating the generation and function of T FH cells. ICOS signals principally by recruitment of PI3K to the tyrosine within its cytoplasmic YFMF motif (40, 53). Mutation of this tyrosine has revealed the requirement for this interaction in...
the generation and function of T<sub>FH</sub> cells (23). The similarities between the recently reported ICOS-Y181F mutant and the T<sub>p110<sup>d</sup></sub>fl/fl mice are striking. Both of these mouse models resemble ICOS knockout mice or ICOS inhibitory Ab treatment in terms of induction of T<sub>FH</sub> cells and the GC response (23, 26). The p110<sub>d</sub> dependence of ICOS signaling suggests that p110<sub>a</sub> and p110<sub>b</sub>, which are expressed in T cells, are either quantitatively or qualitatively unable to substitute for p110<sub>d</sub>. The latter possibility may reflect the growing awareness of selective recruitment and activation of PI3K isoforms by particular receptors. ICOS signaling is known to generate high levels of PIP3 (54) in the immunological synapse via recruitment of the p50<sub>a</sub> regulatory subunit (55). The selective requirement for p110<sub>d</sub> downstream of ICOS signaling may reflect a preferential molecular association between p50<sub>a</sub> and p110<sub>d</sub>, rather than p110<sub>a</sub> or p110<sub>b</sub>.

We found that p110<sub>d</sub> was required for ICOS-mediated increases in IL-21, IL-4, and c-Maf mRNA. These observations are in accordance with ICOS regulating c-Maf (43), which in turn regulates IL-21 (56) and IL-4 (42). Our data are consistent with results showing ICOS signaling regulates NFAT-dependent expression of cytokine genes in a PI3K-dependent manner (57) and with observations that Kruppel-like factor 2, which is suppressed by the PI3K pathway, attenuates IL-4 expression (58). The kinase GSK3 is known to be inhibited by ICOS costimulation (55) and to negatively regulate at least 50 target proteins, including several transcription factors that are critical for T cell effector functions and also molecules controlling cell growth.

**FIGURE 7.** ICOS downstream signaling is dependent on p110<sub>d</sub>. For A–D, CD<sup>4+</sup> T cell blasts were stimulated with anti-CD3 and anti-ICOS and cross-linked using anti-hamster IgG and the i.i. of p-AktS473 or GSK3<sub>a</sub>S21 or pGSK3<sub>b</sub>S9, and total Akt or GSK3<sub>a</sub> or GSK3<sub>b</sub> was determined by Western blot. The mean and SD i.i. of the phosphorylated protein was normalized to i.i. of the total protein (n = 3 independent experiments). The dotted line represents the baseline levels. Nonparametric ANOVA statistical analysis used for multiple measurements displayed in A–D. A. Time course of stimulation and (B) titration of anti-ICOS Ab concentrations and (C) time course of stimulation using B6 CD4<sup>+</sup> T cells blasts pretreated with 1 μM IC87114. D, p-GSK3 and total GSK3 during a time course of stimulation. E, The levels of IL-21, IL-4, c-Maf, Bcl-6, and Blimp-1 mRNA after ICOS-dependent restimulation were detected by RT-PCR. The mean (±SD) amount of mRNA relative to β<sub>2</sub>-microglobulin as 2−ΔΔ<sub>Ct</sub> for p110<sub>d</sub>fl/fl (filled bars, n = 3) and T<sub>p110<sub>d</sub>fl/fl</sub> (open bars, n = 4) mice. i.i., integrated intensity.
and apoptosis (reviewed in Ref. 59). Thus, p110δ may orchestrate multiple transcription factor activities downstream of ICOS to control Tfh cell function through Gsk3β, which may be the nexus between PI3K signaling and downstream targets. The PI3K pathway also regulates the expression of the mutually antagonistic transcriptional repressors Bcl-6 and Blimp-1, which play a central role in Tfh cell differentiation. p110δ was not required for the induction of Bcl-6 mRNA, and our data rather suggested that P13K repressed Bcl-6 mRNA. This may reflect the loss of P13K inhibition of the FOXO family of transcription factors, as these activate transcription of the Bcl-6 gene (60). The low levels of Blimp-1 mRNA in T p110δ−/− T cells may further reflect the effect of the P13K pathway on the Bcl-6–Blimp-1 axis that regulates Tfh cell differentiation (21). Although p110δ may negatively regulate Bcl-6 mRNA levels, the increased Bcl-6 mRNA levels in T p110δ−/− T cells is insufficient to overcome the requirement for p110δ during Tfh cell formation in vivo.

In summary, we propose that the amount of PI3P generated is crucial for Tfh cell formation and for optimal T cell help to B cells. This suggestion is supported by our observation that Tfh cell numbers were reciprocally regulated by the p110δ isoform of PI3K in B cells. This suggestion is supported by our observation that Tfh cell numbers were reciprocally regulated by the p110δ isoform of PI3K in B cells.

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Disclosures

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References


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**Supplementary data**

**Figure S1.** Conditional deletion of p110δ in B and T lymphocytes.

(A) The genomic structure of p110δ is shown, closed boxes represent exons. Restriction sites are marked, E: EcoRV; E*: EcoRV introduced by the targeting vector; K: KpnI; △ represents LoxP sites, ▲ represents FRT sites and ⇨ represents the neomycin resistance cassette.

(B) Strategy for assessing genomic deletion by quantification of exon 2 and exon 18 of p110δ.

(C) The genomic DNA deletion efficiency of CD19cre was determined by quantitative real-time PCR as the ratio of the excised p110δ exon 2 to the non-excised exon 18. The mean exon 2/exon18 ratio and SEM using DNA from purified lymph node B cells from p110δ\(^{fl/fl}\) (n=4 mice, black bars) compared to B p110δ\(^{fl/fl}\) (n=4 mice, white bars). p110δ deletion by CD19cre detected at the protein level by western blot in purified LN B cells from p110δ\(^{fl/fl}\) (n=4 mice, black bars) compared to B p110δ\(^{fl/fl}\) (n=4 mice).

(D) The mean exon 2/exon18 ratio and SEM using DNA from purified lymph node T cells from p110δ\(^{fl/fl}\) (n=4 mice, black bars) compared to T p110δ\(^{fl/fl}\) (n=4 mice, white bars). p110δ deletion by CD4cre detected at the protein level by western blot in purified LN T cells from p110δ\(^{fl/fl}\) (n=3 mice) compared to T p110δ\(^{fl/fl}\) (n=4 mice).

**Figure S2.** Effect of conditional targeting of p110δ on peripheral lymphocyte subsets.

(A) Representative FACS plots of CD21\(^{hi}\)CD23\(^{lo}\) (marginal zone B); CD21\(^{-}\)CD23\(^{+}\) (follicular B) cells in the spleen and CD5\(^{+}\)IgM\(^{+}\) peritoneal B cells, displaying the percentages among live lymphocytes. The bar graphs indicate the absolute numbers of CD21\(^{hi}\)CD23\(^{lo}\) and CD21\(^{-}\)CD23\(^{+}\) cells in the spleen and CD5\(^{+}\)IgM\(^{+}\) peritoneal B cells among control p110δ\(^{fl/fl}\).
(black bars, n=6 mice for spleen, n=8 for peritoneal B cell analysis) compared to B p110δ−/− mice (white bars, n=7 mice for spleen and n=6 for peritoneal B cell analysis). Values shown are mean and s.d.

(B) Representative FACS plots of CD4+ and CD8+ cells in the spleen in p110δ−/− compared to T p110δ−/− mice, displaying the percentages among live lymphocytes. The absolute numbers of CD4+ cells and CD8+ cells in the spleen and pooled inguinal and brachial lymph nodes among p110δ−/− (black bars, n=5 mice) compared to T p110δ−/− (white bars, n=6 mice). Values shown are mean and s.d.
Suppl figure 1

A
Targeting construct

WT

5.6 kb

Targeted ES cell

Exon 2

Exon 18

Targeted allele after FLP

Mutant allele

5.0 kb

3.0 kb

2.6 kb

5.6 kb

B

C

D

β-actin

β-actin

β-actin

β-actin

p110δ

p110δ

p110δ

p110δ

p110δ

p110δ