

Role of the phosphoinositide 3-kinase p110 δ in generation of type 2 cytokine responses and allergic airway inflammation

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Phosphoinositide 3-kinases (PI3K) regulate immune activation *via* their roles in signal transduction of multiple classes of receptors. Here, we examined the effect of genetic inactivation of the hemopoietic cell-restricted PI3K isoform p110 δ on systemic cytokine and chemokine responses and allergic airway inflammation. We found that type 2 cytokine responses (IL-4, IL-5 and IL-13) are significantly decreased in p110 δ mutants, whereas type 1 cytokine responses (IFN- γ and CXCL10) were robust. Elevated IFN- γ production during the primary response to ovalbumin (OVA) was associated with reduced production of the regulatory cytokine IL-10. IFN- γ and IL-10 production normalized after secondary OVA immunization; however, type 2 cytokine production was persistently reduced. Type 2 cytokine-dependent airway inflammation elicited by intranasal challenge with OVA was dramatically reduced, with reduced levels of eosinophil recruitment and mucus production observed in the lungs. Induction of respiratory hyper-responsiveness to inhaled methacholine, a hallmark of asthma, was markedly attenuated in p110 δ -inactivated mice. Adoptive transfer of OVA-primed splenocytes from normal but not p110 δ -inactivated mice could induce airway eosinophilia in naive, airway-challenged recipient mice. These data demonstrate a novel functional role for p110 δ signaling in induction of type 2 responses *in vivo* and may offer a new therapeutic target for Th2-mediated airway disease.

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Abbreviations: BALF: bronchoalveolar lavage fluid ·

PAS: periodic acid-Schiff base · Penh: enhanced pause

Introduction

Phosphoinositide 3-kinases (PI3K) are key signaling enzymes regulating immune function [1, 2]. PI3K are activated by stimulation through a variety of receptor types, including antigen receptors, costimulatory receptors and certain cytokine receptors. PI3K function by phosphorylating specific plasma membrane phospholipids to generate short-lived, membrane-integral lipid

second messengers. Higher eukaryotes utilize multiple PI3K types and isoforms that differ in their expression patterns, regulation and lipid substrate specificities. Class 1A PI3K, comprising five distinct regulatory subunits pairing with three different catalytic subunits, appear to be the most relevant to lymphocyte activation.

Studies on mice bearing genetic deletions of the class IA regulatory subunits have revealed critical roles of these molecules in B lymphocyte development and activation [3, 4]. Effects of regulatory subunit deficiencies on T cells are less clear: deficiency of the p85 α regulatory subunit for class 1A PI3K was reported to have no effect on T cell activation *in vitro*, while deficiency in the p85 β regulatory subunit increased T cell proliferation *in vitro* [5]. One group found that p85-deficient mice have enhanced responses to *Leishmania* infection [6], but reduced immunity to nematode infection [7], suggesting that impaired class IA PI3K signaling leads to an immune dysregulation rather than a general immunodeficiency. However, the interpretation of these studies is complicated because regulatory subunits each affect the stability and activity of multiple catalytic subunits and may have adaptor functions in signaling independent of PI3K catalytic subunits.

Recent work has begun to explore the specific roles of different PI3K catalytic subunit isoforms in immune function. Analysis of p110 α and p110 β deficiency has been hindered by the lethality of these mutations [8, 9]. Mice deficient in the class IB subunit p110 γ are viable and have defects in T cell development and activation, as well as severe defects in neutrophil function, while B cell activation appears normal [10]. We have used a targeted mutagenesis approach to generate mice expressing normal levels of p110 δ bearing an inactivating point mutation in the kinase domain [11]. Our previous study found that T cells from p110 δ -D910A mice had reduced antigen-induced proliferation *in vitro*, but this appears in some cases to be overcome by strong signaling through costimulatory receptors [11]. In contrast, p110 δ -deficient B cells have major defects in activation by multiple stimuli *in vitro*, and blunted antibody responses *in vivo* [11–13]. Thus, while PI3K isoforms likely play distinct roles in B cells, T cells and neutrophils, their respective roles in regulating systemic cytokine production patterns, cell-mediated immunity and host defense functions remain unexplored.

Several studies have shown that global inhibition of PI3K signaling using small-molecule inhibitors [14, 15], antagonistic phosphatases [16] or cell-permeable antagonistic proteins [17] leads to a reduction of Th2-dependent airway inflammation in mouse models of allergic asthma. While these results implicate PI3K in asthma pathogenesis at some level, the cellular and molecular mechanisms are poorly understood, and the PI3K isoforms involved remain to be identified. In this

study, we examine the effect of p110 δ inactivation on *in vivo* priming of type 2 cytokine responses, which are known to be required for allergic airway inflammation. Our results implicate p110 δ as a major signaling axis required for the generation of type 2 immunity and Th2-mediated airway inflammation, but not for the generation of type 1 cytokine responses.

Results

Th1-skewed cytokine and chemokine responses in p110 δ -mutant mice

Our previous studies found that p110 δ -D910A mice, bearing a kinase-inactive p110 δ , exhibit selective deficits in lymphocyte proliferative responses to specific stimuli [11, 18]; however, production of type 1 and type 2 cytokines was not assessed. To determine the impact, if any, of p110 δ inactivation on overall cytokine production, p110 δ -mutant mice were immunized intraperitoneally with ovalbumin (OVA) antigen and alum, and splenocytes were restimulated with OVA *in vitro*. We quantified the levels of a panel of cytokines and chemokines in supernatants of antigen-restimulated cultures. p110 δ -D910A mutants developed weak type 2 cytokine production as indicated by two- to fivefold reductions in the OVA-induced production of IL-4, IL-5 or IL-13 (Fig. 1A). In contrast, p110 δ -mutant cells exhibited robust secretion of the type 1 cytokine IFN- γ and the IFN- γ -inducible chemokine CXCL10 (Fig. 1B). Examination of serum CXCL10 levels revealed increases in OVA-immunized p110 δ -mutant mice (Fig. 1C), consistent with a systemic skewing towards type 1 responses. No OVA-induced cytokine production was observed using cells from naive mice, and both type 1 and type 2 cytokine production was blocked by addition of anti-CD4 blocking antibody to the cultures, confirming that these responses are T cell dependent (data not shown). Recall proliferative responses to OVA were reduced by about 50% in p110 δ -D910A mutants (data not shown), consistent with a moderate overall effect on antigen-induced proliferation. These data demonstrate that the absence of p110 δ activity results in a markedly altered balance of type 1 and type 2 cytokine responses.

Reduced IL-10-mediated regulation of IFN- γ in p110 δ -mutant mice

To investigate the unexpectedly strong type 1 cytokine response observed in the absence of p110 δ signaling, we examined production of IL-10, a potent regulator of T cell proliferation and IFN- γ production. We found that IL-10 production was decreased by two thirds in p110 δ mice (Fig. 2A). To address whether this reduction might

partially account for the high IFN- γ responses seen in p110 δ -D910A mutants, we quantified OVA recall responses in cultures carried out in the presence of IL-10-neutralizing antibody (Fig. 2B). As expected, IFN- γ production was markedly increased in both groups in the presence of anti-IL-10 antibody. However, IFN- γ production was only increased by about twofold in p110 δ mutants compared to sixfold in wild-type mice. The result was that similar levels of antigen-driven IFN- γ were produced in the two strains in the presence of anti-IL-10 antibody. Intracellular IFN- γ staining revealed that the frequency of IFN- γ -expressing CD4⁺ cells is not increased in the p110 δ -D910A mutants; rather, the amount of IFN- γ produced per positive cell is increased (mean fluorescence intensity of 614 vs. 333; Fig. 2C). Average IFN- γ staining levels among CD4⁺ cells were increased upon antibody blockade of IL-10, bringing wild-type cultures to a level equivalent to p110 δ mutants

(Fig. 2D). Conversely, addition of exogenous IL-10 decreased average IFN- γ staining for both wild-type and mutants (Fig. 2D). In contrast to the potent regulation of IFN- γ production by IL-10, addition of exogenous IL-4, IL-5 or IL-13, or blocking antibodies against these cytokines, had little or no effect on IFN- γ production (Fig. 2E). These results suggest that lack of regulatory IL-10 production is an important factor in allowing a strong IFN- γ response to develop in p110 δ -D910A mice during the primary response.

Th2 deficiency in p110 δ mutants persists after secondary immunization

To determine whether the deficit in type 2 cytokine production can be overcome by repeated immunization, OVA-primed wild-type or p110 δ -D910A mice were given an intraperitoneal booster immunization, followed by respiratory OVA challenge, in a protocol known to evoke strong type 2 responses and type 2-mediated airway inflammation [19]. IL-4 and IL-13 were greatly reduced in p110 δ mutants (Fig. 3A, B), consistent with a persistent inability to develop strong type 2 responses. IFN- γ production by p110 δ mutants was similar to wild-type controls under these conditions (Fig. 3C), perhaps reflecting fully recovered IL-10 production (Fig. 3D).

p110 δ mutants fail to generate Th2-mediated eosinophilic airway inflammation

To assess whether the persistent reduction in type 2 cytokine production is associated with reduced type 2-mediated functional responses, we examined airway inflammation induced after intranasal challenge with OVA. We found that p110 δ mutants had dramatically reduced eosinophilic infiltration in the lung following OVA challenge compared with wild-type mice (Fig. 4A). Bronchoalveolar lavage fluid (BALF) collected from p110 δ mutants contained similar levels of IFN- γ , but substantially reduced levels of IL-4 (Fig. 4B). Lung sections harvested from wild-type or mutant mice were stained to reveal mucous production, a marker for airway inflammation. Mucous production was visibly reduced in p110 δ mutants (Fig. 4C). To determine whether the reduced airway inflammation impacted significantly on lung function, respiratory responsiveness was assessed using whole body plethysmography. In naive mice, no differences in respiratory responsiveness between wild-type mice and p110 δ mutants were observed following administration of graded doses of the bronchoconstrictor methacholine (Fig. 5A). After priming and induction of airway inflammation, wild-type animals showed substantially increased respiratory responsiveness, while p110 δ mutants were resistant to induction of hyper-responsiveness (Fig. 5B). Together,

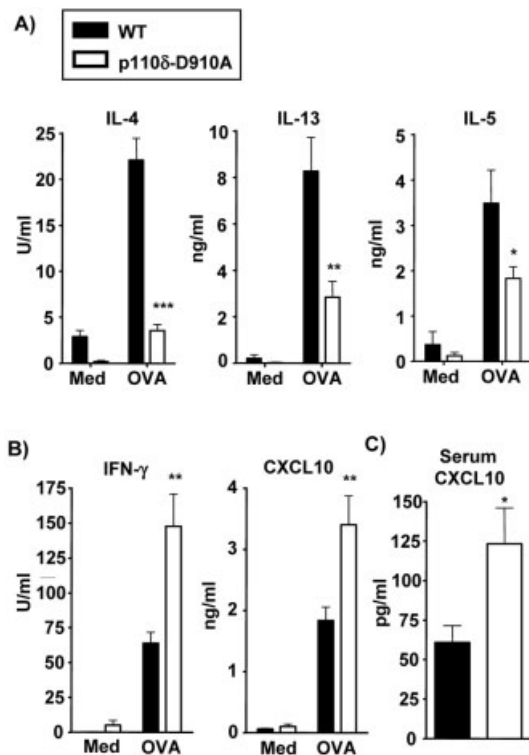


Figure 1. Type 1-skewed cytokines and chemokines in p110 δ -mutant mice. (A) Spleen cells isolated from mice 5 days after OVA immunization were cultured alone or with 300 μ g/mL OVA. Levels of IL-4, IL-13 and IL-5 in restimulated culture supernatants were determined by ELISA. (B) Culture supernatants were analyzed for IFN- γ and CXCL10 by ELISA. (C) Sera collected 5 days after OVA immunization were collected for analysis of CXCL10. In this and subsequent figures, solid bars indicate controls (WT) and open bars indicate p110 δ -mutant mice. Results represent the average and standard error of 17–21 mice per group over five experiments. The statistical significance in this and subsequent figures is denoted as: * $p < 0.05$ as compared to the wild type, ** $p < 0.01$ as compared to the wild type, *** $p < 0.001$ as compared to the wild type.

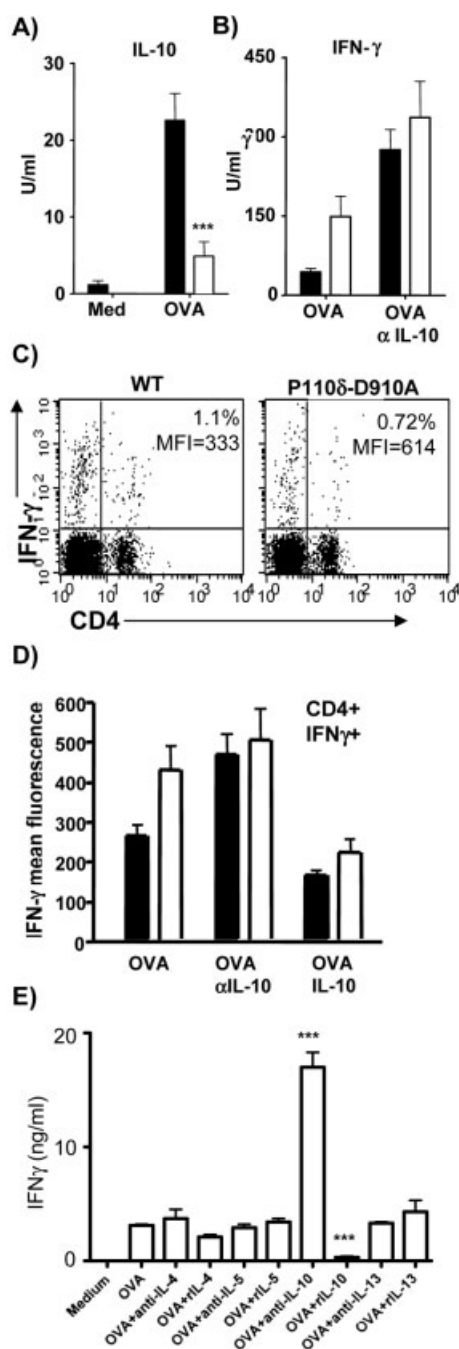


Figure 2. Reduced IL-10-mediated counter-regulation of IFN- γ in p110 δ -mutant mice. (A) Culture supernatants of OVA-restimulated spleen cells were analyzed for IL-10. Results represent the average and standard error of 17–21 mice per group over five experiments. *** $p < 0.001$ as compared to wild-type cultures. (B) Spleen cells from OVA-immunized mice were cultured with 300 μ g/mL OVA alone or OVA + 5 μ g/mL anti-IL-10 antibody. Culture supernatants were analyzed for IFN- γ by ELISA. (C) Spleen cells from OVA-immunized mice were restimulated with 300 μ g/mL OVA, stained for surface CD4 and intracellular IFN- γ and analyzed by flow cytometry. (D) Graph of mean fluorescence intensities of IFN- γ staining, gated on CD4 $^{+}$ populations from the indicated groups. (E) Spleen cells from OVA-immunized p110 δ -mutant mice were restimulated with 300 μ g/mL OVA in the presence of the indicated exogenous cytokines or anti-cytokine blocking antibodies. Resulting IFN- γ production was determined by ELISA. *** $p < 0.001$ as compared to cultures containing OVA antigen only.

these results demonstrate that inactivation of p110 δ has a dramatic impact on the development of Th2-mediated airway inflammation and respiratory hyper-responsiveness to inhaled methacholine.

Primed p110 δ -mutant splenocytes fail to transfer airway eosinophilia response

To determine whether the difference in airway eosinophilic infiltration is due to a lack of p110 δ activity in hematopoietic cells or airway structural cells, we transferred spleen cells from OVA-immunized p110 δ

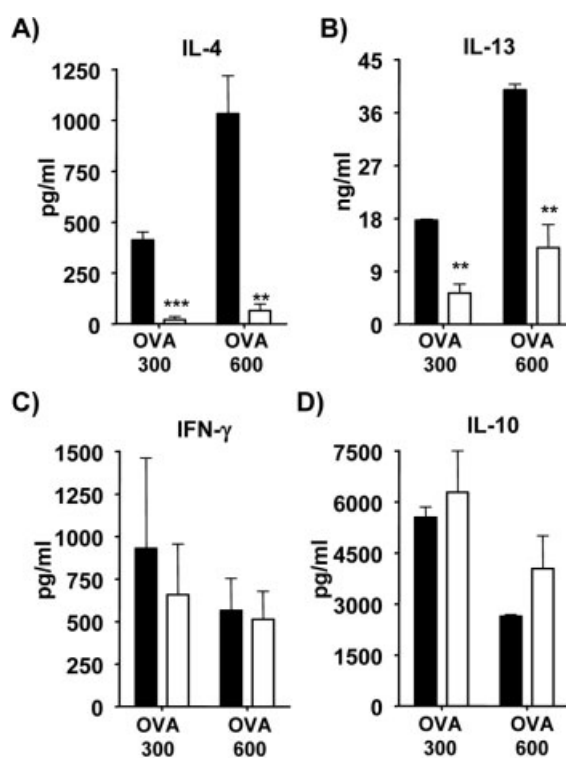


Figure 3. The secondary immune response reveals persistently reduced type 2 cytokine production in p110 δ -mutant mice. Spleen cells were isolated from wild-type BALB/c and p110 δ -D910A mice after a second OVA immunization and intranasal challenge. This immunization protocol has been shown to give strong type 2 responses leading to airway inflammation in Balb/c mice. Spleen cells were cultured with 300 and 600 μ g/mL OVA and supernatants were analyzed for IL-4 (A), IL-13 (B), IFN- γ (C) and IL-10 (D) by ELISA. ** $p < 0.01$ as compared to the wild type, *** $p < 0.001$ as compared to the wild type.

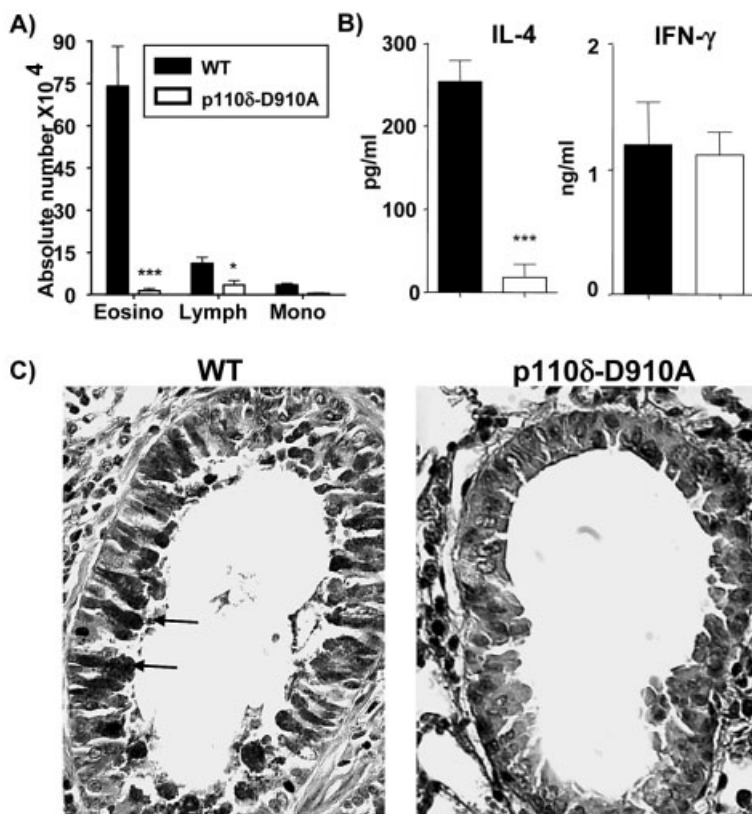


Figure 4. Reduced induction of airway inflammation in p110 δ -mutant mice. (A) BALF cells were collected 6 days after intranasal challenge with OVA. Percentages of eosinophils, lymphocytes and monocytes were determined by histology staining and absolute numbers were calculated based on total cell recoveries. Results represent the average and standard error of 14 mice per group over four experiments.

*** $p < 0.001$ as compared to the wild type. (B) IL-4 and IFN- γ levels in the BALF, showing reduced IL-4 levels in p110 δ -D910A mice.

(C) Representative lung sections, showing reduced mucous production in p110 δ -mutant mice, as determined by PAS staining.

mutants or wild-type mice to naive mice of either genotype and then challenged intranasally with OVA (Fig. 6A). The results showed that primed spleen cells from wild-type mice induced similar levels of eosinophil migration when adoptively transferred to naive wild-type or p110 δ -mutant recipients (Fig. 6B). This demonstrates that p110 δ -D910A mice can support inflammatory responses within their airways given a source of appropriately primed immune cells. In contrast, primed spleen cells from p110 δ mutants only induce one-fifth the level of eosinophil migration, regardless of whether they are transferred into wild-type or mutant mice (Fig. 5B). These results demonstrate that the resistance to airway inflammation is due to the requirement for p110 δ signaling in hematopoietic cells.

Discussion

Our results identify a novel biological function of p110 δ signaling in determining the quality of the cytokine response induced *in vivo*. The observed 75–95% reduction in type 2 response is maintained even after multiple immunizations with strongly type 2-promoting conditions, and can be observed both locally (in the lung) and systemically (in the spleen), suggesting a critical requirement for p110 δ signaling in the generation of type 2 responses. Consistent with defective

induction of type 2 responses, we found a striking reduction in the expression of Th2-dependent airway inflammatory responses. The reduced airway inflammation in p110 δ -inactivated mice is most likely to result from defective induction of type 2 responses, since type 2 cytokines such as IL-4, IL-5 and IL-13 are known to play critical roles in the induction of airway eosinophilia [20–23]. Notably, reduced Th2 airway inflammation had significant effects on lung function, as p110 δ -inactivated mice were resistant to the development of methacholine-induced respiratory hyper-responsiveness after allergen challenge. In addition, we found that primed splenocytes from p110 δ -inactivated mice have a greatly reduced ability to transfer airway inflammatory responses to naive wild-type mice, confirming that they are functionally impaired in this regard.

The robust production of IFN- γ generated after primary immunization of p110 δ mutants is unexpected and could suggest that p110 δ -deficient T cells are intrinsically predisposed towards Th1 differentiation. However, we have found no evidence for such a mechanism using an *in vitro* differentiation model with purified TCR-transgenic cells [24]. Rather, we find here that p110 δ -D910A mice mount a weak regulatory IL-10 response, which in turn contributes to high IFN- γ expression. Thus, in the presence of the physiological regulatory circuits, reduced IL-10 allows p110 δ -deficient

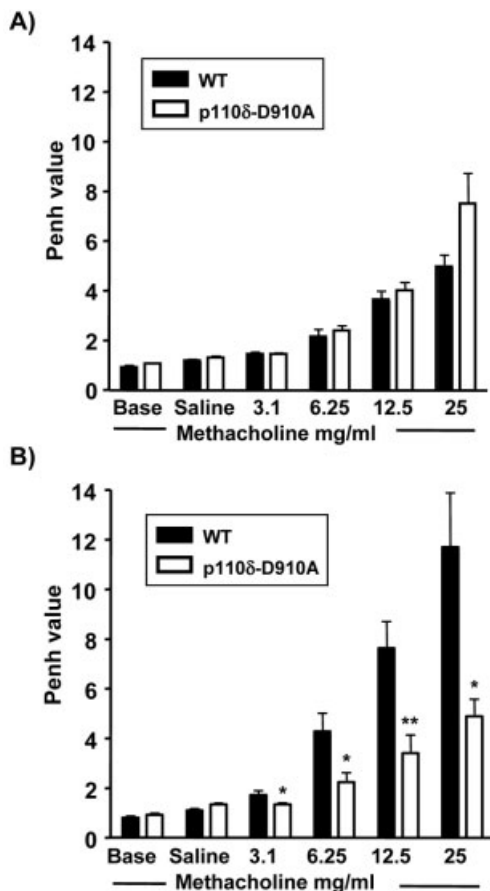


Figure 5. Reduced induction of respiratory hyper-responsiveness in p110δ-D910A mice. Measurement of respiratory responsiveness to increasing concentrations of methacholine was performed on naive and airway-challenged Balb/c and p110δ-mutant BALB/c mice. Results are expressed as Penh values and represent the average and standard error of eight mice per group over two experiments. (A) Naive p110δ-mutant mice have similar respiratory responsiveness to controls. (B) After airway challenge, p110δ-mutant mice have significantly reduced respiratory hyper-responsiveness compared to controls. Responses were measured 5 days after initial intranasal challenge. * $p < 0.05$ as compared to the wild type. ** $p < 0.01$ as compared to the wild type.

T cells to develop a predominant, unopposed type 1 response. This interpretation is consistent with the previously described sporadic inflammatory bowel disease (IBD) phenotype occurring in older p110δ-inactivated mice [11], since IBD is known to involve deregulated Th1 responses and is opposed by IL-10 [25].

The persistent inability of p110δ-inactivated mice to generate type 2 cytokines and effector responses suggests that PI3K signaling plays a critical role in the generation of these responses that cannot be compensated by other signaling pathways. As such, it is one of the first examples where deletion of a signaling molecule results in a clear qualitative shift in antigen-driven

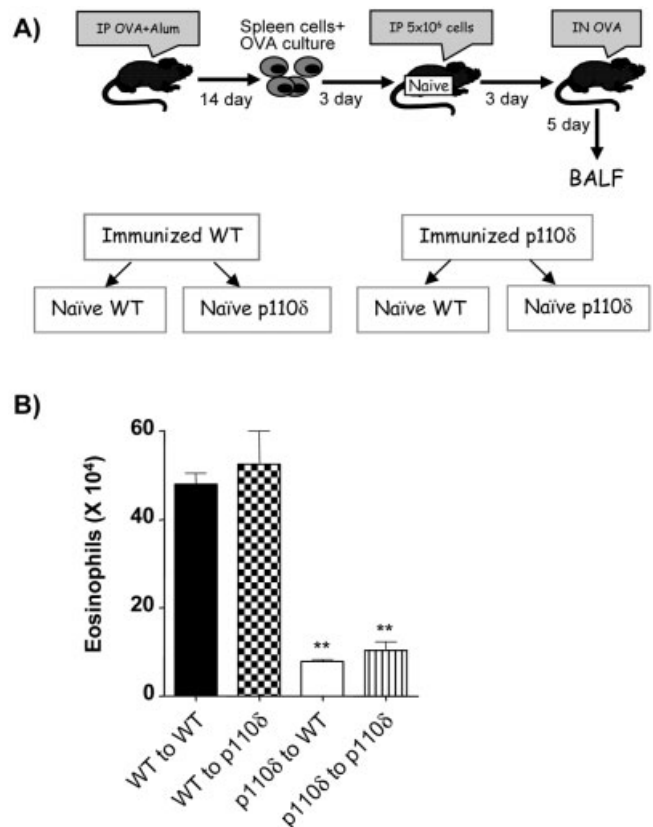


Figure 6. Adoptive transfer of primed p110δ-mutant splenocytes induces much less airway eosinophilia than primed wild-type cells. (A) Schematic illustrating the adoptive transfer system. Splenocytes from OVA-primed mice were restimulated with OVA for 3 days and then transferred to naive mice. At 3 days after transfer, recipient mice were challenged with OVA intranasally. At 5 days after challenge, BALF was harvested and eosinophils were counted. (B) Eosinophil counts in BALF from all genotype combinations of primed cell donors and naive recipient mice, showing that p110δ-mutant donor cells are unable to induce strong airway eosinophilia. ** $p < 0.01$ as compared to the wild-type adoptive transfer group.

type 1 vs. type 2 response patterns. An extensive literature examines the role of the MyD88 adaptor protein in Th1 vs. Th2 activation [26–28]. This led to a working consensus that MyD88-dependent signaling is preferentially required for Th1 responses, although it can also play a role in Th2 induction under some circumstances [29]. The MyD88-independent signaling pathways preferentially eliciting development of Th2-biased responses are not well characterized. Our results clearly implicate p110δ as a major signaling axis in Th2 induction.

Signaling through the IL-4 receptor is known to activate PI3K signaling [30, 31], and we have recently shown a requirement for p110δ in IL-4 signaling in B cells [18]. The phenotype we describe here is similar to that seen in STAT6-deficient mice, which are defective in IL-4 signaling [32, 33]. Since IL-4 signaling is critical in

feed-forward amplification of type 2 responses, defective IL-4 signaling in T cells, in combination with excessive IFN- γ production secondary to the reduction in IL-10 responses demonstrated here, could at least partially explain the reduced type 2 priming *in vivo*. While the evidence supports a possible T cell intrinsic requirement for p110 δ signaling, present data cannot rule out additional T cell extrinsic roles for p110 δ in Th2 induction. Thus, in analogy with MyD88 which promotes Th1 induction *via* its role in signaling DC maturation, p110 δ may promote Th2 induction through its recently described signaling functions in mast cell activation [34].

Our results provide clear evidence that p110 δ signaling is required for Th2-mediated airway inflammation. Previous studies found that transient blockade of all PI3K isoforms at the airway challenge stage led to substantial reductions in airway inflammation [16, 17]. Additionally, a recent report found that administering a p110 δ -specific inhibitor compound at the airway challenge stage led to significant reductions in local type 2 cytokine production and airway inflammation [35]. This evidence is consistent with roles for PI3K signaling at the stage of Th2-mediated effector function, in addition to the role in priming that we demonstrate here. Given the known critical role for type 2 cytokines in initiation and maintenance of airway inflammation, it is likely that the block in type 2 cytokine production we have identified contributes significantly to the inhibited airway responses in these mice. However, our data do not rule out the possibility that p110 δ inactivation may additionally impact upon other functions relevant to airway inflammation, such as cell migration and homing. One study found that p110 γ , but not p110 δ , was required for neutrophil migration into the lungs after intranasal chemokine administration [36]; however, no analogous studies have been reported for eosinophils. Our data show that adoptive transfer of wild-type splenocytes (which contain very few eosinophils) can induce similar levels of airway eosinophilia in p110 δ -D910A and wild-type hosts, arguing against a defect in eosinophil mobilization in these mutants.

PI3K signaling is also known to be important in the regulation of airway-resident smooth muscle cells [37], which play critical roles in airway inflammation and airway hyper-responsiveness [38]. The p110 δ isoform is unlikely to be the major PI3K isoform expressed in airway smooth muscle, however, and our data show that p110 δ mutants exhibit airway inflammation equivalent to that of wild-type controls if provided with primed wild-type immune cells. Thus, the major defect arising from p110 δ deficiency lies at the level of qualitatively altered immune capacity rather than structural cells.

Our results demonstrating the profound impact of p110 δ deficiency on the normal development of type 1-

vs. type 2-biased immunity raise the possibility that therapies targeting PI3K signaling may be beneficial in the context of pre-established Th2-mediated diseases such as asthma or allergic rhinitis. Such a strategy has the potential to target the pathogenic process at several levels, including the generation of type 2 cytokine responses. Indeed, development of isoform-specific inhibitors of PI3K with varied pharmacologic properties is being actively pursued [39, 40], which should allow the assessment of such strategies in the near future.

Materials and methods

Mice and immunizations

P110 $\delta^{D910A/D910A}$ mice have been described [11]. They were backcrossed on a C57BL/6 or Balb/c background for nine generations. Mice were immunized by intraperitoneal injection of 2 μ g OVA protein (ICN Biochemicals, Montreal, Canada) adsorbed onto 2.0 mg Al(OH) $_3$ (alum) adjuvant. For airway challenge experiments, mice were immunized twice with OVA in alum, 2 wk apart, and then challenged intranasally twice with 50 μ g OVA in 40 μ L sterile saline (on days 28 and 31 after initial immunization). Adoptive transfer experiments were carried out essentially as described [41]. Briefly, splenocytes were harvested at day 14 after a single OVA immunization, restimulated *in vitro* with 600 μ g/mL OVA for 3 days and transferred to non-irradiated naive recipient mice (5×10^6 cells per recipient). At 3 days after transfer, mice were challenged intranasally with OVA and the resulting airway eosinophilia was quantified as above.

Cytokine and chemokine quantification

The mice were sacrificed 5 days after immunization. Spleen cells were harvested and cultured at 7.5×10^6 cells/mL alone or with 300 μ g/mL OVA. Supernatants were harvested after 2–4 days. In each experiment, duplicate spleen cell suspensions from three to five mice per group were cultured and analyzed independently. IL-4 and IFN- γ levels were determined by ELISA as described [42]. Other cytokines and chemokines were measured using specific antibody pairs from the following sources: IL-5 (BD PharMingen), IL-10 (BD PharMingen), CXCL10 (Peprotech), IL-13 (R&D Systems, Minneapolis, MN). In the indicated experiments, anti-cytokine blocking antibodies (BD PharMingen) were added to the cultures at 5 μ g/mL. Recombinant (r) IL-10 or IL-4 (Peprotech) was used at 50 ng/mL, rIL-5 at 5 ng/mL, and rIL-13 at 1 ng/mL. For intracellular IFN- γ staining, 500 ng/mL ionomycin (Sigma, St. Louis, MO), 50 ng/mL phorbol myristate acetate (PMA; Sigma) and 10 μ g/mL brefeldin A (BFA; Sigma) were added during the last 4–6 h of the OVA restimulation culture. Cells were then surface-stained with FITC-conjugated anti-mouse CD4 antibody (BD PharMingen), fixed, permeabilized, intracellularly stained with APC-conjugated anti-mouse IFN- γ antibody (BD PharMingen) and analyzed by flow cytometry.

Analyses of airway inflammation and methacholine responsiveness

Airway analyses and lung function assessment were performed 5–6 days after the first intranasal challenge. BALF cell numbers were counted and prepared cytopspins were stained to determine eosinophil, lymphocyte and monocyte percentages. Lungs were formalin-fixed, paraffin-embedded, sectioned and stained for mucous production using periodic acid-Schiff base (PAS) stain. As a functional correlate to the degree of airway inflammation, respiratory responsiveness was measured in unrestrained animals using whole body plethysmography (Buxco, Troy, NY). After system equilibration for 30–60 min, a model 5500D De-Vilbiss Electronic Nebulizer was used to expose mice to aerosolized physiological saline for 3 min and then increasing concentrations of nebulized methacholine. The settings for dilution flows and aerosol time period were selected according to the manufacturer's recommendations. Mice were allowed to stabilize for at least 5 min to allow breathing patterns to return to baseline levels between each aerosol exposure. Recordings were taken for 3 min after each nebulization. During plethysmography, pressure differences measured between a mouse chamber and a reference chamber during expiration and inspiration were used to calculate enhanced pause (Penh), a parameter that is a function of total pulmonary airflow during the respiratory cycle. Penh is dependent on breathing pattern and can correlate well with airway resistance measured in ventilated mice [43, 44]. Respiratory responsiveness was calculated from the correlation between changes in Penh and the concentration of inhaled methacholine from 0–25 mg/mL.

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