

Inhibition of phosphoinositide 3-kinase δ attenuates allergic airway inflammation and hyperresponsiveness in murine asthma model

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ABSTRACT P110 δ phosphoinositide 3-kinase (PI3K) plays a pivotal role in the recruitment and activation of certain inflammatory cells. Recent findings revealed that the activity of p110 δ also contributes to allergen-IgE-induced mast cell activation and vascular permeability. We investigated the role of p110 δ in allergic airway inflammation and hyperresponsiveness using IC87114, a selective p110 δ inhibitor, in a mouse asthma model. BALB/c mice were sensitized with OVA and, upon OVA aerosol challenge, developed airway eosinophilia, mucus hypersecretion, elevation in cytokine and chemokine levels, up-regulation of ICAM-1 and VCAM-1 expression, and airway hyperresponsiveness. Intratracheal administration of IC87114 significantly ($P < 0.05$) attenuated OVA-induced influx into lungs of total leukocytes, eosinophils, neutrophils, and lymphocytes, as well as levels of IL-4, IL-5, IL-13, and RANTES in a dose-dependent manner. IC87114 also significantly ($P < 0.05$) reduced the serum levels of total IgE and OVA-specific IgE and LTC₄ release into the airspace. Histological studies show that IC87114 inhibited OVA-induced lung tissue eosinophilia, airway mucus production, and inflammation score. In addition, IC87114 significantly ($P < 0.05$) suppressed OVA-induced airway hyperresponsiveness to inhaled methacholine. Western blot analyses of whole lung tissue lysates shows that IC87114 markedly attenuated the OVA-induced increase in expression of IL-4, IL-5, IL-13, ICAM-1, VCAM-1, RANTES, and eotaxin. Furthermore, IC87114 treatment markedly attenuated OVA-induced serine phosphorylation of Akt, a downstream effector of PI3K signaling. Taken together, our findings implicate that inhibition of p110 δ signaling pathway may have therapeutic potential for the treatment of allergic airway inflammation.—Lee, K. S., Lee, H. K., Hayflick, J. S., Lee, Y. C., Puri, K. D. Inhibition of phosphoinositide 3-kinase δ attenuates allergic airway inflammation and hyperresponsiveness in murine asthma model. *FASEB J.* 20, 455–465 (2006)

Key Words: allergy • inflammation • cell activation • lung • cytokines • signal transduction

CHRONIC INFLAMMATION, mucus hypersecretion, elevated serum IgE levels, and airway hyperresponsiveness (AHR) are fundamental characteristics of allergic asthma. T helper type 2 (Th2) cells, together with other inflammatory cells such as mast cells, B cells, and eosinophils, are proposed to play critical roles in the initiation, development, and chronicity of this disease (1–6). Upon activation, these inflammatory cells contribute to the production of Th2 cytokines (IL-4, IL-5, and IL-13) and chemokines (eotaxin and RANTES), which are found at elevated levels in asthmatic lungs. Th2 cytokines are pivotal for B cell maturation, IgE synthesis, airway eosinophilia, mucus secretion, and ultimately AHR. The importance of Th2 cytokines in the pathogenesis of allergic asthma has been demonstrated by specific gene knockout and antibody neutralization studies in mouse asthma models (7–9). In addition, RANTES and eotaxin are central to the recruitment of eosinophils to the airways, which is regulated by the interaction of adhesion molecules such as VLA-4 and its ligand VCAM-1 (10, 11). AHR is thought to result from the combination of submucosal edema, infiltration of airway epithelium by leukocytes, damage to epithelial cells, followed by the direct effect of mediators derived from inflammatory cells (1, 6, 12).

Class I phosphoinositide 3-kinases (PI3Ks) play a key role in induction of the Th2 responses (13–16). Structurally, these kinases exist as heterodimeric complexes in which a catalytic p110 subunit (designated as α , β , γ , or δ) is in association with a particular regulatory subunit (designated p85, p55, p50, or p101) (17, 18). Functionally, all class I PI3Ks catalyze the phosphorylation of phosphatidylinositol (4, 5)-bisphosphate (PIP₂) to form phosphatidylinositol (3, 4, 5)-trisphosphate (PIP₃) in response to activation of either receptor tyrosine kinase (RTK) or G-protein-coupled receptors (GPCRs), which ultimately regulate cell growth, differ-

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entiation, survival, proliferation, migration, and cytokine production (18, 19). p110 α and p110 β isoforms are ubiquitously expressed and genetic knockout leads to early embryonic lethality. By contrast, expression of the p110 δ isoform is largely restricted to circulating hematogenous cells and endothelial cells; mice lacking p110 δ exhibit a high degree of normal development and growth (20–22).

PI3K activity is stimulated after antigen challenge in a murine model of allergic asthma, and administration of wortmannin or LY294002, two broad-spectrum inhibitors of PI3Ks, attenuate inflammation and AHR in this model (23, 24). However, these inhibitors do not distinguish among the four class I PI3Ks (25, 26) and also broadly affect multiple cell types that express these kinases. Previous studies have indicated an important role for p110 δ in B and T cell antigen receptor signaling and activation (20, 21), and in neutrophil migration and activation (22, 27). In addition, p110 δ was reported to be essential for allergen-IgE-induced mast cell degranulation and vascular permeability (28). However, it remains to be determined whether blockade of activity of this leukocyte and endothelial cell-specific PI3K isoform, p110 δ , alone would be sufficient to attenuate allergic inflammation and AHR. Recently, an isoform-specific small molecule inhibitor, IC87114, which is selective for p110 δ catalytic activity, has been identified (22, 26, 27, 29). In this study we investigated the contribution of p110 δ in the pathogenesis of OVA-induced bronchial asthma in mice and provide evidence in support of a major role of this PI3K isoform in the initiation and maintenance of allergic airway inflammation. Our findings suggest the potential therapeutic utility of inhibiting p110 δ for the treatment of allergic airway disorders.

MATERIALS AND METHODS

Animals and experimental protocol

Female BALB/c mice, 8–10 wk of age and free of murine specific pathogens, were obtained from the Korean Research Institute of Chemistry Technology (Daejeon, Korea). Mice were housed throughout the experiments in a laminar flow cabinet and were maintained on standard laboratory chow ad libitum. All experimental animals used in this study were under a protocol approved by the Institutional Animal Care and Use Committee of the Chonbuk National University Medical School.

The allergic phenotype in mice was induced essentially as described for OVA-induced bronchial asthma with minor modifications (24). In brief, mice were sensitized on days 1 and 14 by intraperitoneal injection of 20 μ g ovalbumin (OVA) (Sigma-Aldrich, St. Louis, MO, USA) emulsified in 1 mg of aluminum hydroxide (Pierce Chemical Co., Rockford, IL, USA) in a total volume of 200 μ L. On days 21, 22, and 23 after the initial sensitization, the mice were challenged for 30 min with an aerosol of 3% (wt/vol) OVA in saline (or with saline as a control) using an ultrasonic nebulizer (NE-U12, Omron, Japan). IC87114 (0.005, 0.01, 0.1, or 1 mg/kg body weight/day) or vehicle control (0.05% DMSO) diluted with 0.9% NaCl, was administered in a volume of 50 μ L by

intratracheal instillation twice to each animal: once on day 21 (1 h before the first airway challenge with OVA) and the second time on day 23 (3 h after the last airway challenge with OVA).

Histological studies

At 72 h after the last challenge, mice were killed and the lungs and trachea were filled intratracheally with a fixative (0.8% formalin, 4% acetic acid) using a ligature around the trachea. Lungs were removed and lung tissues were fixed with 10% (vol/vol) neutral buffered formalin. The specimens were dehydrated and embedded in paraffin. For histological examination, 4 μ m sections of fixed embedded tissues were cut on a Leica model 2165 rotary microtome (Leica Microsystems Nussloch GmbH, Nussloch, Germany), placed on glass slides, deparaffinized, and stained with hematoxylin 2, eosin-Y (Richard-Allan Scientific, Kalamazoo, MI, USA) and periodic acid-Schiff (PAS). Three independent blinded investigators graded the inflammation score. The degree of peribronchial and perivascular inflammation was evaluated on a subjective scale of 0 to 3, as described elsewhere (24). A value of 0 was adjudged when no inflammation was detectable, a value of 1 for occasional cuffing with inflammatory cells, a value of 2 for most bronchi or vessels surrounded by thin layer (one to five cells) of inflammatory cells, and a value of 3 when most bronchi or vessels were surrounded by a thick layer (>5 cells) of inflammatory cells.

Measurement of PI3K enzyme activity in lung tissue

Whole lung tissues were homogenized in the presence of protease inhibitors to obtain extracts of lung proteins, as previously reported (24). Protein concentrations were determined using the Bradford reagent (Bio-Rad Laboratories Inc., Hercules, California, USA). The amount of PIP₃ produced was quantified by PIP₃ competition enzyme immunoassays according to the manufacturer's protocol (Echelon, Inc., Salt Lake City, UT, USA). The enzyme activity was expressed as pmol PIP₃ produced by 1 mL of lung tissue extract containing equal amounts of total protein.

Western blot analysis

Protein extracts from lung tissue homogenates (30 μ g of protein per lane) were electrophoresed in polyacrylamide gels (Invitrogen Life Technologies, Carlsbad, CA, USA), transferred electrophoretically to a PVDF membrane (Immobilon-P; Millipore, Billerica, MA, USA), and incubated overnight at 4°C with antibodies to IL-4 (Serotec Ltd, Oxford, UK), IL-5 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), IL-13 (R&D Systems, Minneapolis, MN, USA), RANTES and eotaxin (Abcam Ltd., Cambridge, UK), Akt and Ser-473 phosphorylated Akt (p-Akt) (Cell Signaling Technology, Beverly, MA, USA), then with horseradish peroxidase-conjugated secondary antibodies. Bound antibody was detected by chemiluminescence according to the manufacturer's instructions (Amersham Biosciences, Piscataway, NJ, USA). The membranes were stripped and reblotted with anti-actin antibody (Sigma-Aldrich) to verify equal loading of protein in each lane. Where indicated, immunoreactive and phosphorylation signals were analyzed by densitometric scanning (LAS-3000; Fuji Film, Tokyo, Japan). Data were expressed as mean $\pm$ SE.

Collection of blood and BAL samples

At 72 h after the last challenge, mice were killed with an overdose of pentobarbital-Na (100 mg/kg of body weight,

administered intraperitoneally). Blood was drawn by puncture of the vena cava and centrifuged. Serum was shock frozen in liquid nitrogen and stored at -70°C for IgE measurements. BAL was performed as described (24). Briefly, the chest cavity was exposed to allow for expansion, after which the trachea was carefully intubated and the catheter secured with ligatures. Prewarmed 0.9% NaCl solution was slowly infused into the lungs and withdrawn. Total BAL cells were counted using a hemocytometer. Differential cell counts were obtained from BAL cells spun down onto slides with a cytocentrifuge (Shannon Scientific Ltd., Cheshire, UK) and treated with Diff-Quik solution (Dade Diagnostics of Puerto Rico Inc. Aguada, Puerto Rico). Two independent, blinded investigators counted the cells using a microscope. Approximately 400 cells, in each of four different random locations, were counted by two independent, blinded investigators. Inter-investigator variation was $<5\%$. The mean number from the two investigators was used to estimate the cell differentials. For cytokine and leukotriene measurements, supernatants of BAL were shock frozen in liquid nitrogen and stored at -70°C until use.

Examination of bronchoalveolar lavage fluid

Levels of IL-1 β , TNF α , IL-4, IL-5, IL-13, and RANTES were quantified in the supernatants of BAL fluids by enzyme immunoassays according to the manufacturer's protocol (IL-1 β , TNF α , IL-4, and IL-5; Endogen, Inc., Woburn, MA, USA; IL-13 and RANTES; R&D Systems, Inc.). The lower detection limit for IL-1 β , TNF α , IL-4, IL-5, IL-13, and RANTES in these assays was 3, 10, 5, 5, 1.5, and 2 pg/mL, respectively.

Total serum IgE and OVA-specific IgE

OVA-specific IgE levels were measured by capture ELISA as described previously (30). Briefly, microtiter plates were coated with 2 $\mu\text{g/mL}$ of purified monoclonal anti-mouse IgE (BD PharMingen, San Diego, CA, USA). After blocking with PBS-10% FCS, appropriate dilutions of serum samples in PBS-10% FCS were added to the plate and incubated for 2 h at room temperature. After washing with PBS-Tween, biotinylated OVA (10 $\mu\text{g/mL}$) and HRP-conjugated streptavidin were added to the wells and incubated for 1 h. The plates were washed, followed by addition of the HRP substrate, 3,3',5,5'-tetramethylbenzidine substrate (TMBS, Sigma). After incubation for 30 min in the dark at room temperature, the plates were read at 450 nm on a microplate reader (Molecular Dynamics, Sunnyvale, CA, USA). Total serum IgE was measured by capture ELISA in a manner similar to the detection of OVA-specific IgE. A biotinylated rat anti-mouse IgE (PharMingen) was used to detect captured IgE in place of the biotinylated OVA.

Measurement of BAL leukotriene C₄

Levels of LTC₄ were quantified in the supernatants of BAL fluids by enzyme immunoassay according to the manufacturer's protocol (Cayman Chemical Co., Ann Arbor, MI, USA). The lower detection limit for LTC₄ in this assay was 10 pg/mL.

Determination of airway responsiveness

Three days after the last ovalbumin challenge, airway responsiveness was assessed as a change in airway function after challenge with aerosolized methacholine via airways, using invasive assessment technique as described elsewhere (31,

32). Anesthesia was achieved with 80 mg/kg of pentobarbital sodium injected intraperitoneally. The trachea was then exposed through midcervical incision, tracheostomized, inserted with an 18-gauge metal needle. Mice were connected to a computer-controlled small animal ventilator (flexiVent, SCIREQ, Montreal, Canada). The mouse was quasi-sinusoidally ventilated with tidal volume of 10 mL/kg at a frequency of 150 breaths/min and a positive end-expiratory pressure of 2–3 cm H₂O to achieve a mean lung volume close to that during spontaneous breathing. This was achieved by connecting the expiratory port of the ventilator to water column. Methacholine aerosol was generated with an in-line nebulizer and administered directly through the ventilator. To determine the differences in airway response to methacholine, each mouse was challenged with methacholine aerosol in increasing concentrations (2.5–50 mg/mL in saline). From 20 s up to 3 min after each aerosol challenge, the data of airway resistance (R_L) were continuously collected. Maximum values of R_L were selected to express changes in airway function, which was represented as a percentage change from baseline after saline aerosol.

IC87114 level in plasma and lung tissue

Animals received a single intratracheal dose of either IC87114 (0.1 or 1 mg/kg) or drug vehicle (0.05% DMSO). Whole lung tissues were removed at 30 min, 1 and 6 h after compound administration and homogenized using BeadBeater (BioSpec Products, Inc., Bartlesville, OK, USA). Blood samples were also drawn at the same time points. Concentration of the compound in plasma and lung tissue homogenate was determined after liquid-liquid extraction by liquid chromatography/mass spectroscopy. The lower quantification limit was 50 ng/mL. Lung tissue homogenate and plasma samples from control animals (vehicle alone) were used as the blank control.

Statistical analysis

Statistical comparisons were performed using one-way ANOVA, followed by the Fisher's test. Significant differences between groups were determined using the unpaired Student's *t* test. Statistical significance was set at $P < 0.05$.

RESULTS

Allergen-induced airway inflammation leads to increased activity of p110 δ in lung tissue

In OVA-exposed mice, class I PI3K activity was increased ~ 4.6 -, 6.1-, 9.5-, and 9.6-fold at 1, 24, 48, and 72 h respectively, after a single OVA challenge, compared with the pre-challenge period (Fig. 1A). In contrast, no significant changes in PI3K activity were observed after saline inhalation. Activation of these kinases has been linked to phosphorylation of Ser-473 of Akt, an event crucial for Akt enzymatic activation (33). Consistent with increased PI3K activity, levels of p-Akt protein in the lung tissues were also increased 72 h after OVA inhalation compared with levels in the control animals that received saline inhalation (Fig. 1B). No significant changes in total Akt protein levels were observed. Intratracheal administration of IC87114 blocked Akt phosphorylation similar to saline control

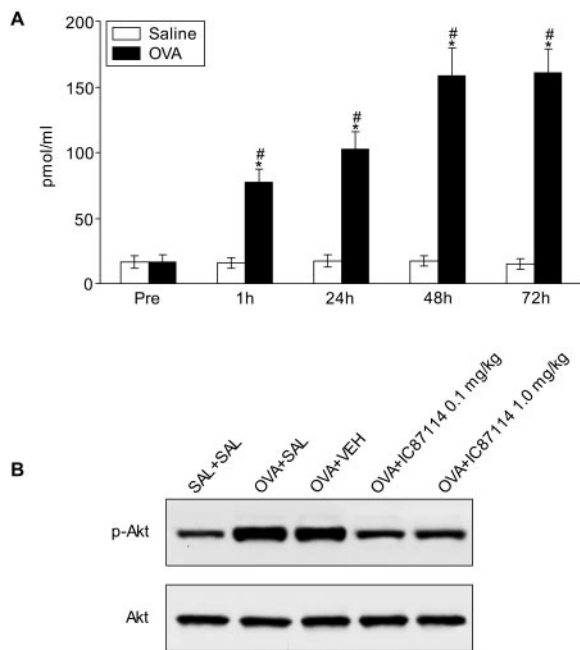


Figure 1. PI3K enzyme activity and the effect of IC87114 on Akt phosphorylation in lung tissue extracts of OVA-sensitized and -challenged mice. *A*) PIP_3 generation by PI3Ks in lung tissue extracts from OVA-sensitized mice challenged with OVA or with saline. Pre, before challenge; 1, 24, 48, and 72 h are time periods after a single OVA challenge. Bars represent mean $\pm$ SE from 6 independent experiments. $\#P < 0.05$ vs. SAL+SAL; $*P < 0.05$ vs. Pre. *B*) P-Akt and Akt protein expression in lung tissue extracts was measured 72 h after a single challenge in saline-inhaled mice with the administration of saline (SAL+SAL), OVA-inhaled mice with the administration of saline (OVA+SAL), OVA-inhaled mice with the administration of drug vehicle (OVA+VEH), OVA-inhaled mice with the administration of either 0.1 mg/kg of IC87114 (OVA+IC87114 0.1 mg/kg), or 1 mg/kg of IC87114 (OVA+IC87114 1 mg/kg). Results were similar in 6 independent experiments.

levels suggesting that p110 δ contributed significantly to the overall class I PI3K activity in allergen-induced Akt activation (Fig. 1*B*). Levels of the inhibitor in plasma and lung tissue were within the range that effectively blocked p110 δ biochemical activity but not the other class I isoforms of PI3K.

P110 δ contributes to OVA-induced eosinophil recruitment in BAL fluid

Having established that p110 δ contributed to allergen-induced Akt activation, we sought to determine the contribution of this kinase in eosinophil recruitment after allergen challenge. BAL fluid was collected 72 h after the last OVA aerosol challenge, and total leukocyte and differential cell counts were performed. OVA inhalation significantly ($P < 0.05$) increased the absolute numbers of eosinophils, lymphocytes, and neutrophils, compared with saline control (Fig. 2). Intratracheal administration of IC87114 (0.1 mg/kg) reduced the number of eosinophils, lymphocytes, and neutro-

phils detected in BAL fluids 72 h postchallenge by 79.8%, 63.5%, and 80%, respectively, compared with mice treated with vehicle control (0.05% DMSO). In contrast, the numbers of macrophages were not affected by IC87114. No eosinophils were found in unsensitized control animals, whereas many eosinophils were present in BAL fluids of allergen-treated mice. Reduction in the total cell number recovered in BAL fluid in IC87114-treated mice compared with vehicle control, was mainly due to a significant ($P < 0.05$) reduction in eosinophils in the IC87114-treated mice. These results suggest that p110 δ activity contributed to eosinophil recruitment during the allergic inflammatory response.

P110 δ contributes to OVA-induced tissue eosinophilia, mucus production, and airway inflammation

Lung tissues were collected 72 h after the last OVA challenge. Histological analyses revealed typical pathologic features of asthma-like inflammation in the OVA-exposed mice. In contrast to the saline controls (Fig. 3*A*), OVA-exposed mice showed numerous inflammatory cells in the peribronchiolar zone and accumulation of mucus and cellular debris within the lumen of the bronchioles (Fig. 3*B*). In contrast, IC87114-treated mice showed substantial attenuation in the eosinophil-rich leukocyte infiltration in the peribronchiolar regions and in the amount of debris present in the lumen (Fig. 3*C, D*). In addition, Fig. 3*E–H* shows the representative sections of each group stained with periodic

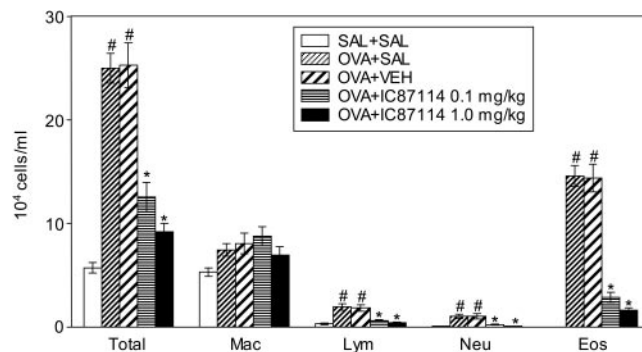


Figure 2. IC87114 reduces inflammatory cell infiltration in BAL fluid of OVA-sensitized/challenged mice. The number of total cells and differential cellular component of BAL fluids from saline-inhaled mice administered saline (SAL+SAL), OVA-inhaled mice with the administration of saline (OVA+SAL), OVA-inhaled mice with the administration of drug vehicle (OVA+VEH), OVA-inhaled mice with the administration of either 0.1 mg/kg of IC87114 (OVA+IC87114 0.1 mg/kg), or 1 mg/kg of IC87114 (OVA+IC87114 1 mg/kg) were counted 72 h after the last challenge. Differential cell count was performed on a minimum of 400 cells in each of four different random locations to identify macrophage (Mac), lymphocyte (Lym), neutrophils (Neu), and eosinophils (Eos). Bars represent mean $\pm$ SE from 6 independent experiments. $\#P < 0.05$ vs. SAL+SAL; $*P < 0.05$ vs. OVA+SAL.

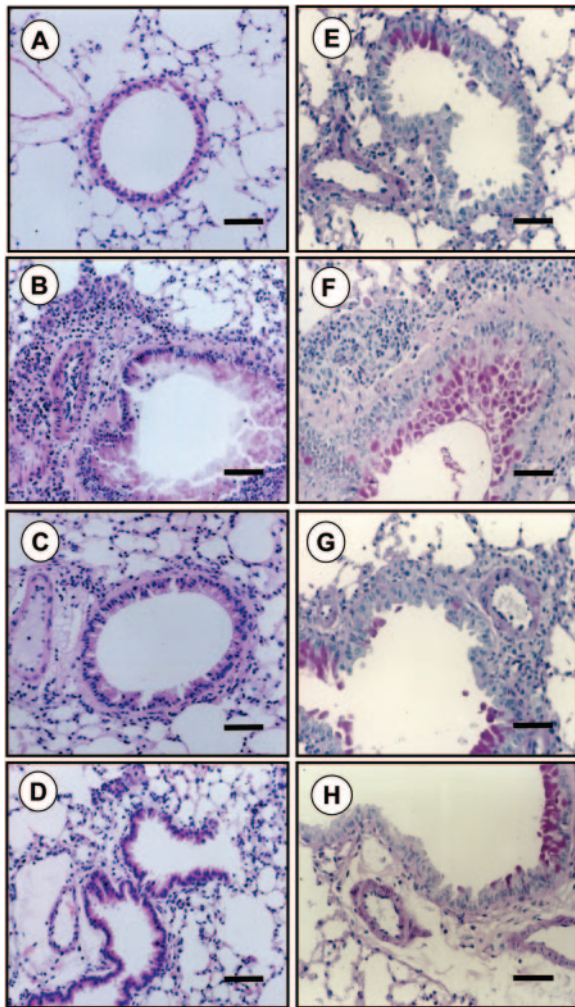


Figure 3. IC87114 reduces lung tissue eosinophilia and mucus secretion in OVA-sensitized/challenged mice. Lung tissue was fixed, sectioned at 4 μm thickness and stained with hematoxylin and eosin for tissue eosinophilia (A–D) or periodic acid-Schiff (PAS) for mucus production (E–H). Sampling was performed 72 h after the last challenge in saline-inhaled mice with administration of saline (A, E), OVA-inhaled mice with the administration of vehicle control (0.05% DMSO) (B, F), OVA-inhaled mice with the administration of 0.1 mg/kg of IC87114 (C, G), and OVA-inhaled mice with the administration of 1 mg/kg of IC87114 (D, H). PAS stained cells have purple cytoplasmic inclusions. Bars indicate scale of 50 μm .

acid-Schiff (PAS) for detection of goblet cells. Compared with the control (Fig. 3E), OVA-exposed mice (Fig. 3F) showed goblet cell hyperplasia in the airways, which was markedly reduced by IC87114-treatment (Fig. 3G, H). The inflammation scores of peribronchial, perivascular regions as well as total lung were increased significantly ($P < 0.05$) 72 h after OVA inhalation compared with scores after saline inhalation (Fig. 4). The increased lung inflammation observed after OVA inhalation was reduced by $>50\%$ by administration of the p110 δ inhibitor. Taken together, these results suggest that p110 δ significantly contributes to allergen-induced leukocyte influx, airway inflammation, and goblet cell hyperplasia.

P110 δ activity is required for production of cytokines and expression of cell adhesion molecules during allergen-induced airway inflammation

Given the essential role of Th2 cytokines in evoking allergic inflammatory responses, we measured the concentrations of IL-4, IL-5, and IL-13 in BAL fluid as well as in the lung tissue from OVA-challenged mice that received either p110 δ inhibitor or vehicle control (0.05% DMSO). OVA-challenge induced significant ($P < 0.05$) increases in the concentrations of all three cytokines detected by protein-blotting in lung tissues (Fig. 5A) as well as by ELISA of BAL fluids (Fig. 5B) compared with the levels detected after saline challenge. The increased levels of these cytokines in both lung tissue and in BAL fluids were significantly ($P < 0.05$) reduced by IC87114 in a dose-dependent manner.

Enzyme immunoassays revealed that levels of IL-1 β and TNF α in BAL fluids were also increased significantly ($P < 0.05$) 72 h after OVA inhalation compared with the levels after saline inhalation (Fig. 5C). IC87114 (0.01, 0.1, and 1 mg/kg) significantly ($P < 0.05$) lowered the increased levels of these proinflammatory cytokines in a dose-dependent manner with $>50\%$ reduction of both cytokines at 1 mg/kg dose of IC87114 (Fig. 5C). One of the responses to these cytokines is the induction of the expression of leukocyte-endothelial adhesion molecules on endothelium. Indeed, levels of ICAM-1 and VCAM-1 proteins in the lung tissue were increased significantly ($P < 0.05$) 72 h after OVA inhalation (Fig. 6A, B), and these levels were substantially reduced by the administration of IC87114.

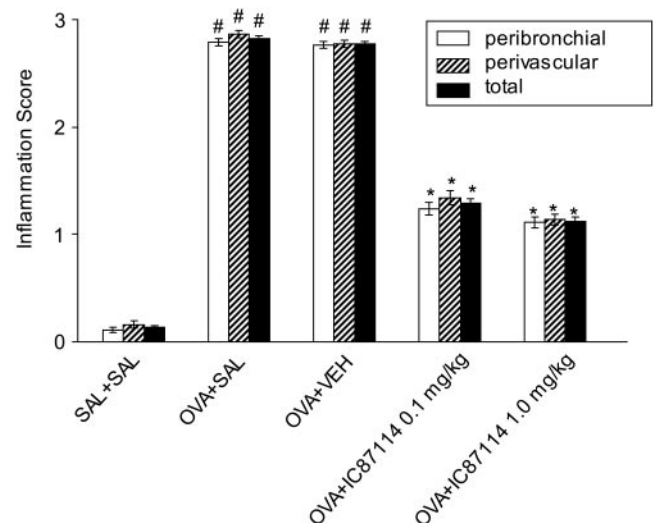


Figure 4. IC87114 attenuates lung inflammation in OVA-sensitized/challenged mice. Peribronchial, perivascular, and total lung inflammation in mice treated as described in Fig. 2 legend was measured 72 h after the last challenge. Total lung inflammation was defined as the average of the peribronchial and perivascular inflammation scores. Bars represent mean $\pm$ SE from 6 independent experiments. # $P < 0.05$ vs. SAL+SAL; * $P < 0.05$ vs. OVA+SAL.

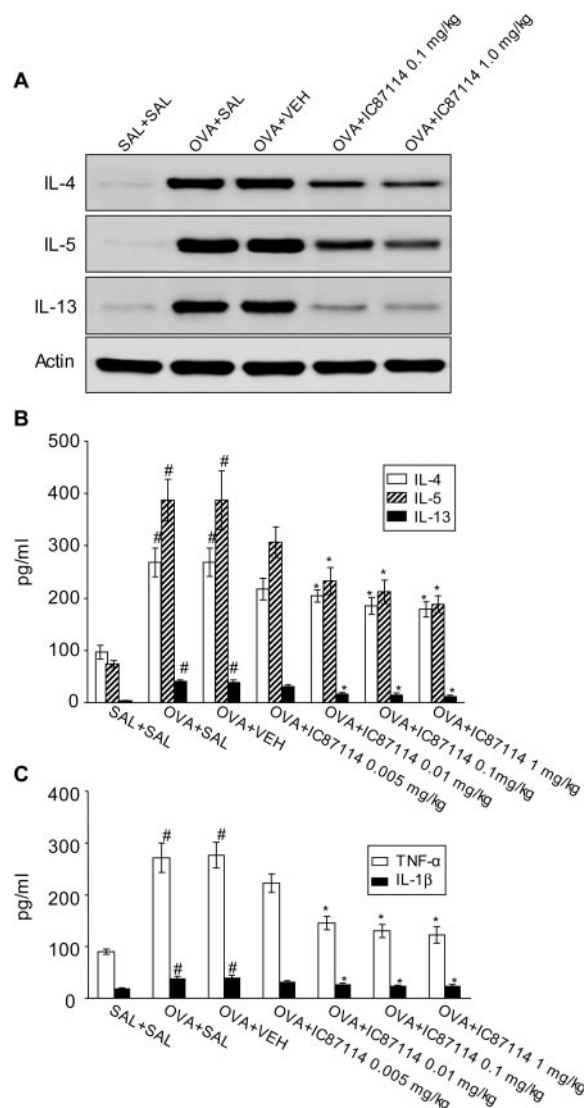


Figure 5. IC87114 reduces cytokine protein expression in lung tissues and BAL fluids in OVA-inflamed lung. A) Western blot analysis of IL-4, IL-5, and IL-13 in the lung tissue extracts from mice treated as described in Fig. 2 legend was performed 72 h after the last challenge. B) Enzyme immunoassay of IL-4, IL-5, and IL-13 in BAL fluids of animals as in panel A). C) Enzyme immunoassay of TNF α and IL-1 β in BAL fluids of animals as in A). Bars represent mean $\pm$ SE from 6 independent experiments. # P < 0.05 vs. SAL+SAL; * P < 0.05 vs. OVA+SAL.

Effects of IC87114 on eotaxin and RANTES protein levels in lung tissues and BAL fluids of OVA-sensitized and -challenged mice

Chemokines such as RANTES and eotaxin are central to the recruitment of eosinophils to the airways. Western blot analysis revealed that protein levels of eotaxin and RANTES in the lung tissue were increased significantly (P < 0.05) 72 h after OVA inhalation compared with the saline control (Fig. 7A–D). IC87114 reduced the increased levels of these chemokines by >50%. In addition, enzyme immunoassays revealed that increased levels of RANTES in BAL fluids 72 h after OVA

inhalation were also significantly (P < 0.05) reduced by IC87114 (0.01, 0.1, and 1 mg/kg) treatment in a dose-dependent manner, with ~65% reduction of this chemokine at the maximal dose (1 mg/kg) of IC87114 (Fig. 7E).

Effect of IC87114 on serum IgE levels and LTC₄ release in BAL fluid

We next determined whether IC87114 could modify OVA-specific Th2 response in vivo by analyzing circulating IgE Ab levels 72 h after OVA challenge. Substantial elevation in total IgE and OVA-specific IgE was observed in serum from OVA-challenged mice compared with untreated mice (Fig. 8A). IC87114 significantly (P < 0.05) lowered total circulat-

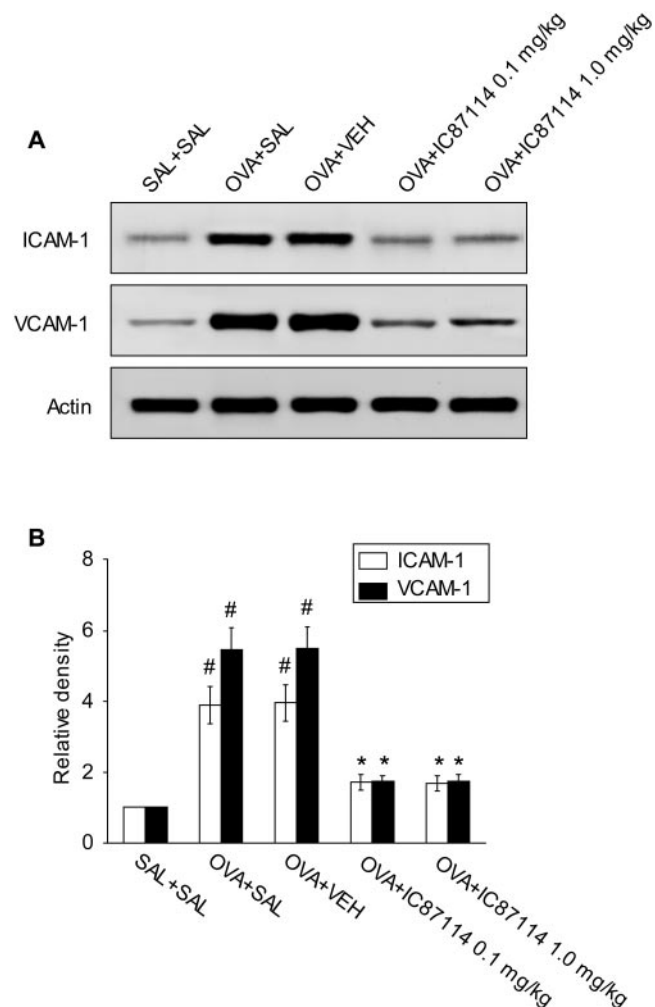


Figure 6. IC87114 reduces leukocyte adhesion molecule expression in OVA-inflamed lung. A) Western blot analysis of ICAM-1 and VCAM-1 in the lung tissue extracts from mice treated as described in Fig. 2 legend was performed 72 h after the last challenge. The membranes were stripped and reblotted with anti-actin antibody to verify equal loading of protein in each lane. B) Densitometric analyses are presented as relative ratio of ICAM-1 or VCAM-1 to actin. Relative ratio of ICAM-1 or VCAM-1 in lung tissues of SAL+SAL is arbitrarily presented as 1.

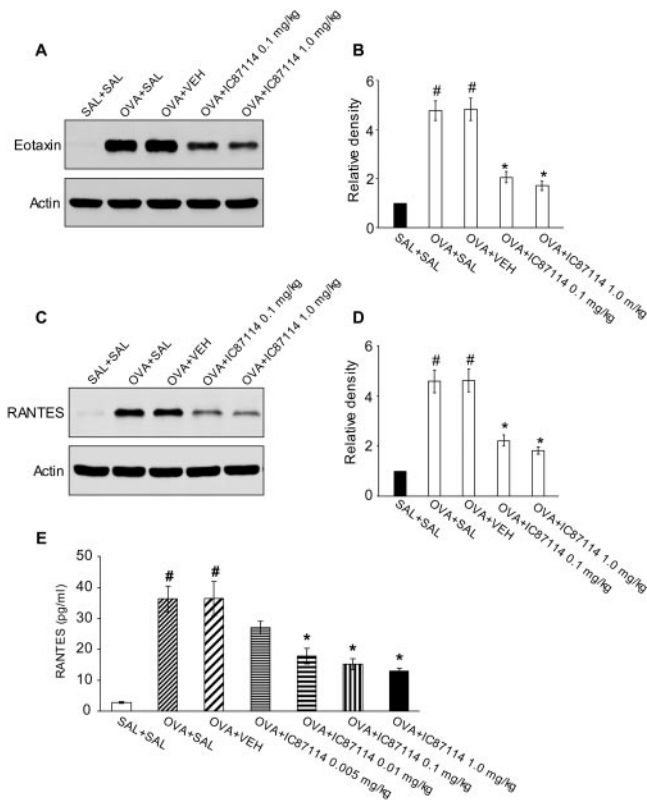


Figure 7. Effect of IC87114 on eotaxin, and RANTES expression in lung tissues and BAL fluids of OVA-sensitized and -challenged mice. Protein expression was measured 72 h after the last challenge in mice treated as described in Fig. 2 legend. *A*) Western blot analysis of eotaxin in lung tissue extracts. *B*) Densitometric analyses are presented as relative ratio of eotaxin to actin. Relative ratio of eotaxin in lung tissues of SAL+Sal is arbitrarily presented as 1. *C*) Western blot analysis of RANTES in lung tissue extracts. *D*) Densitometric analyses are presented as relative ratio of RANTES to actin. Relative ratio of RANTES in lung tissues of SAL+Sal is arbitrarily presented as 1. *E*) Enzyme immunoassay of RANTES in BAL fluids. Bars represent mean $\pm$ SE from 6 independent experiments. [#] $P < 0.05$ vs. SAL+Sal; ^{*} $P < 0.05$ vs. OVA+Sal.

ing IgE levels in a dose-dependent manner (Fig. 8A). In agreement with the inhibitory effect on total IgE level, IC87114 at 0.1 and 1 mg/kg significantly ($P < 0.05$) reduced OVA-specific IgE levels by 63 and 72%, respectively (Fig. 8A).

Leukotriene release into the airspace in response to allergen intranasal challenge in OVA-sensitized mice contributes to the airway mucus release and infiltration by eosinophils. The LTC₄ levels in BAL fluid 72 h after the last challenge were 3.1-fold ($P < 0.05$ compared with saline) higher in the OVA-sensitized/challenged mice than in mice receiving saline only. IC87114 (0.1 and 1 mg/kg) significantly ($P < 0.05$) inhibited LTC₄ levels by 37 and 50%, respectively (Fig. 8B). The amounts of LTC₄ in the BAL fluid of OVA-sensitized/challenged mice treated with vehicle control (0.05% DMSO) were not significantly different from those of the saline control group (Fig. 8B).

Inhibition of p110 δ attenuated airway hyperresponsiveness

Airway hyperresponsiveness was assessed as a change in airway resistance (R_L) (31, 32). In OVA-sensitized/challenged mice, the R_L in response to methacholine (from 2.5 mg/mL to 50 mg/mL) inhalation was substantially increased by at all methacholine levels compared with the saline-challenged group (Fig. 9). Administration of IC87114 to OVA-sensitized mice prior to OVA challenge showed a significant ($P < 0.05$) attenuation in R_L measured at all methacholine levels tested, suggesting a role for p110 δ in immune-mediated events leading to airway hyperresponsiveness in vivo.

DISCUSSION

PI3Ks are thought to contribute to the pathogenesis of asthma, but the contribution of the individual PI3K isoforms in airway inflammation and hyperresponsiveness has not been reported. Our present study with the

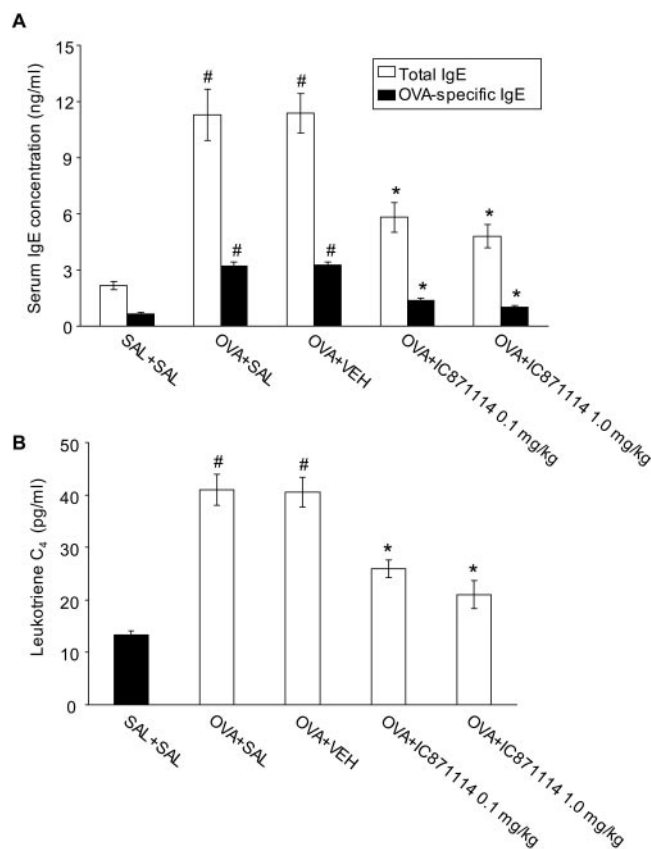


Figure 8. Effects of IC87114 on OVA-specific IgE levels in serum and leukotriene C₄ levels in BAL fluids of OVA-sensitized and -challenged mice. Mouse serum and BAL fluid samples from mice treated as described in Fig. 2 legend were collected 72 h after the last challenge. The levels of OVA-specific IgE in serum (*A*) and Leukotriene C₄ levels (*B*) in BAL fluids were analyzed using ELISA. Bars represent mean $\pm$ SE from 6 independent experiments. [#] $P < 0.05$ vs. SAL+Sal; ^{*} $P < 0.05$ vs. OVA+Sal.

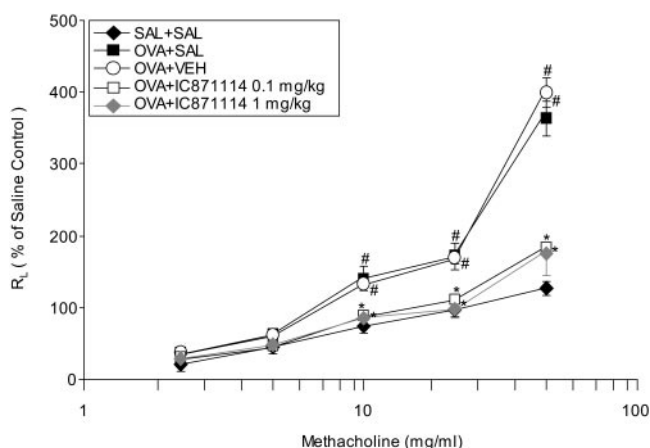


Figure 9. IC87114-treatment attenuates development of airway hyperresponsiveness to methacholine in OVA-sensitized/challenged mice. Airway responsiveness was measured 72 h after the last challenge in saline-inhaled mice administered saline (SAL+SAL), OVA-inhaled mice administered saline (OVA+SAL), OVA-inhaled mice with the administration of drug vehicle (OVA+VEH), OVA-inhaled mice administered IC87114 0.1 mg/kg (OVA+IC87114 0.1 mg/kg), and OVA-inhaled mice administered IC87114 1 mg/kg (OVA+IC87114 1 mg/kg). Airway resistance (R_L) values were obtained in response to increasing doses of methacholine as described in Materials and Methods. Data represent mean $\pm$ SE from 6 independent experiments. # $P < 0.05$ vs. SAL+SAL; * $P < 0.05$ vs. OVA+SAL.

OVA-induced model of asthma for the first time revealed that p110 δ was the main component of class I PI3K-dependent, allergen-induced Akt activation in the lung and pharmacological blockade of p110 δ activity by IC87114 attenuated OVA-induced bronchiolar inflammation, release of Th2 cytokines and chemokines into the airway, the number of mucus-producing cells in the airway, serum IgE levels, and airway hyperresponsiveness in sensitized mice.

Several cytokines, including IL-4, IL-5, IL-13, TNF α , and IL-1 β are known to be present in elevated levels in asthmatic lungs and play critical roles in the development and maintenance of the asthmatic IgE-driven condition. IL-4 regulates allergic inflammation by promoting Th2 cell differentiation, IgE synthesis and its receptor up-regulation, VCAM-1 expression, and mucus hypersecretion (34–37). IL-5 promotes eosinophilic inflammation and infiltration into airways (8). IL-13 promotes B cell differentiation and is capable of inducing isotype-switching in B cells to produce IgE (38). Cytokine production during allergic airway inflammation is believed to be regulated at least partly by class I PI3Ks (13, 24). PI3K activity is stimulated after allergen challenge in a murine model of asthma, and administration of wortmannin or LY294002, two broad-spectrum inhibitors of PI3Ks, reduced levels of Th2 cytokines in BAL fluids (24, 39). However, these inhibitors do not distinguish among the four class I PI3Ks and also inhibit protein kinases (25). In contrast, IC87114 is a selective inhibitor of p110 δ catalytic activity with >50-fold selectivity over the other class I PI3K isoforms (26). Recent publications report a >100-fold

lower inhibitory activity of this p110 δ inhibitor against a variety of kinases, including ATM, ATR, and DNA-PK and phosphatases (22, 26, 27). Puri et al. evaluated the effect of IC87114 on neutrophil trafficking in wild-type and p110 δ KO mice and validated the *in vivo* selectivity of this inhibitor toward the p110 δ signal transduction pathway (22). Our present data show that pharmacological blockade of p110 δ activity with IC87114 significantly reduced the levels of IL-4, IL-5, and IL-13 in the OVA-inflamed lungs. In addition, the elevation of eotaxin, RANTES, and LTC $_4$ levels in the lung of OVA-challenged mice were also dependent in part on p110 δ activity. Various resident lung cells, such as bronchial epithelial cells, tissue mast cells, and alveolar macrophages as well as infiltrated leukocytes such as T cells and eosinophils, contribute to cytokine production. P110 δ has been shown to play a potential role in T cell receptor- and costimulatory receptor CD28-mediated T cell differentiation and activation (20, 21, 40). In addition, p110 δ is also expressed in other leukocytes and plays an important role in the development and differentiation of B cells (20, 40), allergen-IgE-induced cytokine release from mast cells (28), recruitment (22) and activation of neutrophils (27). The observed reduction of cytokine levels in BAL fluid and lung tissue from IC87114-treated mice may be due to inhibition of p110 δ in those inflammatory and airway resident cells. Indeed, in the present study specific p110 δ inhibition by IC87114 in the mouse asthma model blocked phosphorylation at Ser-473 of Akt in lung tissue suggesting that p110 δ is the main component of PI3K-dependent, allergen-induced Akt activation leading to cytokine release in lung. Additional studies will be required to define the contribution of p110 δ in particular cellular population(s) in the lung responses observed here.

Cytokine-inducible, leukocyte-endothelial adhesion molecules are important in the recruitment and migration of leukocytes to the sites of inflammation (41, 42). The expression of ICAM-1 and VCAM-1 is modulated by cytokines such as IL-1 β , IL-4, IL-13, and TNF α . TNF α stimulates translocation of the transcription factor NF- κ B into the nucleus, where it can activate gene expression. Our data demonstrate that p110 δ inhibition dampened the levels of these inflammatory cytokines in OVA-challenged mice. P110 δ activity, however, was not required for TNF α -induced nuclear translocation of NF- κ B and surface expression of E-selectin and ICAM-1 on endothelial cells (29) (K. Puri, unpublished observations). Hence, these results suggest that paracrine suppression of cytokine levels confers reduced cell adhesion molecule levels rather than suppression of cytokine receptor-mediated gene expression.

Eosinophil accumulation and subsequent activation in bronchial tissues is known to play a critical role in the pathogenesis of allergic airway inflammation (2, 3). Eosinophil transmigration into the airways is a multi-step process orchestrated by Th2 cytokines (IL-4, IL-5, and IL-13) and coordinated by specific chemokines such as eotaxin in combination with adhesion mole-

cules such as VCAM-1 and VLA-4 (10, 11). IL-13 is a potent inducer of eotaxin expression in airway epithelial cells (43). Our results suggest that p110 δ is crucial for airway eosinophilia, as shown by a reduction in eosinophil counts in BAL fluid and lung tissues of IC87114-treated mice. This observation may be explained by the reduction in Th2 cytokines, chemokines, and expression of adhesion molecules observed in IC87114-treated mice. Many inflammatory mediators attract and activate eosinophils via PI3K-Akt signal transduction pathways (23, 44, 45). In addition, Palframan et al. reported that wortmannin inhibited IL-5-induced release of eosinophils from perfused bone marrow, as well as selective eosinophil chemokinesis in vitro (16). Human eosinophils express p110 δ and treatment of cells with IC87114 diminished their migration toward eotaxin (K. Puri, unpublished observations). Taken together, the observed reduction in airway eosinophilia by IC87114 may be a result of composite effects produced by multiple mechanisms, such as reduction in Th2 cytokines and eotaxin production, inhibition of VCAM-1 expression, decreased release of eosinophils from bone marrow, and direct inhibition of eosinophil migration. Overall, these observations suggest that p110 δ is a key player in the induction and maintenance of the eosinophil component of asthma.

Our findings demonstrated a dramatic reduction in goblet cell hyperplasia in IC87114-treated mice. Th2 cytokines, T cells, and eosinophils are required to produce airway mucus accumulation and goblet cell degranulation (3, 46, 47). Although a direct role of p110 δ in these cells cannot be ruled out, the observed decrease in mucus production in IC87114-treated lung tissue may be attributed to an indirect effect on goblet cells resulting from the combined effects of a substantial drop in Th2 cytokine levels as well as reduction in eosinophilia in OVA-sensitized/challenged mice.

In OVA-sensitized mice, repeated OVA challenge has been shown to induce a significant increase in total serum IgE that peaks 3 days after the last challenge (48). Our data showed that serum levels of total IgE and OVA-specific IgE were substantially reduced by IC87114. P110 δ has been shown to play a crucial role in development, differentiation, and antigen receptor-induced proliferation of mature B cells (20, 21). In addition, IL-4 and IL-13 are important in directing B cell growth, differentiation, and secretion of IgE (49). Therefore, the observed reduction in serum IgE in our asthma model by IC87114 may be the result of inhibitory effects on B cell activation and reduced Th2 cytokine release. The biological activities of IgE are mediated through high-affinity IgE receptors (Fc ϵ RI) on mast cells and basophils. Cross-linking of Fc ϵ RI initiates multiple signaling cascades leading to cellular degranulation and activation (50, 51). Recently, p110 δ activity was reported to be critical for allergen-IgE-induced mast cell degranulation and release of cytokines (28). Inhibition of p110 δ therefore would attenuate not only production of IgE but also allergen-IgE-induced mast cell activation during allergic

inflammation. p110 δ null mice were not used as an alternate approach to establish the role of p110 δ in IgE-driven allergic airway inflammation as these animals show substantial reduction in the number of mast cells (28) as well as lack certain population of B lymphocytes (20, 21).

Inflammatory mediators released during allergic airway inflammation play a role in the development of AHR (1, 12). IL-5 mobilizes and activates eosinophils, leading to the release of major basic protein, cysteinyl-leukotrienes, and eosinophil peroxidase that contributes to the tissue damage and AHR (8, 52). In addition, IL-4 and IL-13 have been shown to induce AHR in mouse asthma models (7, 52). IgE-triggered mast cell activation may contribute to AHR by producing a wide array of inflammatory mediators and cytokines (50, 53). Our data showed that IC87114 treatment significantly ($P < 0.05$) inhibited OVA-induced AHR to inhaled methacholine. As such, this observation may be associated with reduction in Th2 cytokine production, tissue eosinophilia, and mast cell activation, after p110 δ inhibition.

Allergic airway inflammation and AHR development involve multiple inflammatory cell types and a wide array of mediators. We report in this work for the first time that inhibition of p110 δ signal transduction pathway effectively reduced OVA-induced Th2 cytokine production, pulmonary eosinophilia, serum IgE levels, goblet cell hyperplasia, and AHR in a mouse asthma model. These findings support the conclusion that p110 δ plays an important role in the pathogenesis of allergic asthma. FJ

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