

An anti-inflammatory role for a phosphoinositide 3-kinase inhibitor LY294002 in a mouse asthma model

Wei Duan^a, Ana M.K. Aguineldo Datiles^a, Bernard P. Leung^b,
Chris J. Vlahos^c, W.S. Fred Wong^{a,*}

^a*Department of Pharmacology, Faculty of Medicine, National University of Singapore, MD2 18 Medical Drive, 117597 Singapore*

^b*Department of Physiology, National University of Singapore, 117597 Singapore*

^c*Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, IN 46285, USA*

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Abstract

Phosphoinositide 3-kinase (PI3K) exhibits broad functional effects in immune cells. We investigated the role of PI3K in allergic airway inflammation using LY294002, a specific PI3K inhibitor, in a mouse asthma model. BALB/c mice were sensitized and challenged with ovalbumin (OVA), and developed airway eosinophilia, mucus hypersecretion, elevation in cytokine levels, and airway hyperresponsiveness. Intratracheal administration of LY294002 significantly inhibited OVA-induced increases in total cell counts, eosinophil counts, and IL-5, IL-13, and eotaxin levels in bronchoalveolar lavage fluid. Histological studies show that LY294002 dramatically inhibited OVA-induced lung tissue eosinophilia and airway mucus production. In addition, LY294002 significantly suppressed OVA-induced airway hyperresponsiveness to inhaled methacholine. Western blot analysis of whole lung lysates shows that LY294002 markedly attenuated OVA-induced serine phosphorylation of Akt, a direct downstream substrate of PI3K. Taken together, our findings suggest that inhibition of PI3K signaling pathway can suppress T-helper type 2 (Th2) cytokine production, eosinophil infiltration, mucus production, and airway hyperresponsiveness in a mouse asthma model and may have therapeutic potential for the treatment of allergic airway inflammation.

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1. Introduction

Allergic asthma is a complex chronic airway disorder characterized by airway inflammation, mucus hypersecretion, elevated serum IgE level, and airway hyperresponsiveness [1]. T-helper type

* Corresponding author. Tel.: +65 687 432 63; fax: +65 687 376 90.

E-mail address: phcwongf@nus.edu.sg (W.S.F. Wong).

2 cells (Th2 cells) together with mast cells, B cells, and eosinophils are proposed to play a critical role in the initiation and perpetuation of allergic asthma [1,2]. Upon activation, Th2 cells produce pleiotropic cytokines such as IL-4, IL-5, and IL-13, which lead to B cell maturation, IgE synthesis, airway eosinophilia, mucus secretion, and ultimately airway hyper-responsiveness [3]. The importance of these Th2 cytokines in the pathogenesis of allergic asthma has been demonstrated in either specific gene knockout or antibody neutralization studies [4–6]. In addition, chemokines such as RANTES and eotaxin are critical to the recruitment of eosinophils to the airways.

PI3K family plays a prominent role in various inflammatory cells by controlling cell growth, differentiation, survival, proliferation, and cytokine production through its downstream components [7]. PI3K is divided into four classes, namely, I_A, I_B, II, and III, on the basis of their structural characteristics and substrate specificity. Upon activation, PI3K converts the plasma membrane lipid phosphoinositide-4,5-bisphosphate (PI(4,5)P₂) to PI(3,4,5)P₃, which then acts as a targeting site for downstream signaling molecules with pleckstrin homology (PH) domains, such as Akt (also called protein kinase B) and phosphoinositide-dependent kinase 1 (PDK1). In turn, they can activate isoforms of protein kinase C (e.g., PKC ζ and PKC θ) and mitogen-activated protein kinase (MAPK) signaling pathway [8,9]. These signaling proteins are actively involved in the activation of T cells, B cells, and mast cells [10–12].

Our laboratory has recently demonstrated that LY294002, a PI3K inhibitor [13,14], significantly inhibited antigen-induced anaphylactic contraction of bronchial rings and release of histamine and peptidoleukotrienes from chopped lung preparations from sensitized guinea pigs [15], and thrombin-induced airway smooth muscle cell proliferation [16]. The purpose of the present study was to extend our recent in vitro findings of anti-inflammatory effects of LY294002 to an in vivo mouse asthma model. LY294002, given intratracheally, significantly reduced ovalbumin (OVA)-induced airway eosinophilia, Th2 cytokine release, mucus overproduction, and airway hyperresponsiveness to inhaled methacholine in OVA-sensitized mice. Our findings imply

that inhibition of PI3K could modulate allergic airway inflammation.

2. Materials and methods

2.1. Animals

Male BALB/c mice 6–8 weeks of age (Interfauna, East Yorkshire, UK) were sensitized by intraperitoneal injections of 20 μ g OVA and 4 mg Al(OH)₃ suspended in 0.1 ml saline on days 0 and 14. On days 18–20, animals were challenged with 1% OVA aerosol for 20 min. LY294002 (3.75 and 7.5 mg/kg, Lilly Research Laboratories, Indianapolis, IN) or vehicle (polyethylene glycol [PEG] Sigma, St. Louis, MO) was given intratracheally 2 h before each OVA aerosol challenge. Animal experiments were performed according to the Institutional Guidelines for Animal Care and Use of the National University of Singapore.

2.2. Bronchoalveolar lavage (BAL)

Twenty-four hours after the last aerosol OVA challenge, mice were anaesthetized by an intraperitoneal injection of 100 μ l anesthetic mixture (hypnorm/dormicum/H₂O=1:1:2, Janssen, Beerse, Belgium and Roche, Basel, Switzerland). Tracheotomy was performed, and a cannula was inserted into the trachea. Ice-cold PBS (0.5 ml) was instilled into the lungs, and BAL fluid was collected. Total cell counts were performed using a hemocytometer. Cytospin (Thermo Shandon, Pittsburgh, PA) slides were stained in a modified Wright stain. Differential cell count was performed on at least 500 cells in each slide.

2.3. ELISA

For BAL fluid cytokine and chemokine level determination, BAL fluid was collected 2 h after the last aerosol OVA challenge, as previously described [17]. Our preliminary time-course study revealed a major surge in cytokine levels in BAL fluid starting from 2 h after the last OVA challenge. The levels of cytokine and chemokine in the BAL fluid were measured using ELISA. IL-4 and IL-5 ELISA were

obtained from BD PharMingen (San Diego, CA). IL-13, eotaxin, and IFN γ ELISA were purchased from R&D Systems (Minneapolis, MN). The limits of detection were as follows: IL-4 and IL-5, 4 pg/ml; IL-13 and IFN γ , 15.6 pg/ml; and eotaxin, 2 pg/ml.

2.4. Histologic examination

Lung tissues were fixed in 10% neutral formalin, paraffin-embedded, cut in 6 μ m sections, and stained with H&E for examining cell infiltration and with periodic acid-Schiff (PAS) stain (Sigma) for measuring mucus production. Quantitative analysis of cell infiltration and mucus production was performed blinded, as previously described [17]. Briefly, the scoring system for cell infiltration was: 0, no cells; 1, a few cells; 2, a ring of cells 1 cell layer deep; 3, a ring of cells 2–4 cells deep; 4, a ring of cells >4 cells deep. Goblet cell hyperplasia in the airway epithelium was quantified based on a five-point system: 0, no goblet cells; 1, <25% of the epithelium; 2, 25–50% of the epithelium; 3, 50–75% of the epithelium; 4, >75% of the epithelium.

2.5. Measurement of airway hyperresponsiveness

Airway responsiveness to increasing concentrations of methacholine (5–80 mg/ml, Sigma) was measured using a single-chamber whole-body plethysmograph (Buxco, Sharon, CT) based on the method of Hamelmann et al. [18]. Mice were challenged with aerosolized methacholine for 3 min, and bronchoconstriction was recorded for an additional 5 min for each increasing dose of methacholine. The highest Penh value obtained during each methacholine challenge was expressed as percentage of basal Penh value in response to PBS challenge.

2.6. Western blot analysis

Analysis for phospho-Akt was conducted in lung tissues from mice with and without LY294002 pretreatment. Proteins were separated by 10% SDS-PAGE and transferred onto a PVDF membrane (Bio-Rad, Hercules, CA). The membrane was probed with anti-Akt or antiphospho-Akt antibody (Cell Signaling Technology, Beverly, MA), followed by AP-conjugated secondary antibody, and visualized using BCIP

and NBT (Life Technologies, Gaithersburg, MD). Blots were quantitated using Gel-Pro imaging software (Media Cybernetics, Silver Spring, MD).

2.7. Statistical analysis

Data are presented as means $\pm$ S.E.M. One-way ANOVA followed by Dunnett test was used to determine significant differences between treatment groups. The critical level for significance was set at $P < 0.05$.

3. Results

3.1. Effects of LY294002 on OVA-induced eosinophil recruitment in BAL fluid

BAL fluid was collected 24 h after the last OVA aerosol challenge, and total and differential cell counts were performed. LY294002 (3.75 and 7.5 mg/kg) substantially reduced the total cell number recovered in BAL fluid as compared to vehicle control, which was mainly due to a significant ($P < 0.05$) reduction in eosinophils and neutrophils in the LY294002-treated mice (Fig. 1). The numbers of macrophages and lymphocytes were not affected by LY294002.

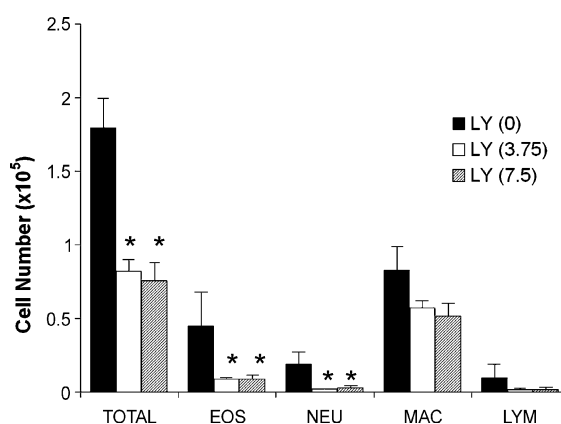


Fig. 1. Effects of LY294002 on BAL fluid cell infiltration. BAL fluids were collected 24 h after the last OVA challenge. Differential cell count was performed on a minimum of 500 cells to identify eosinophil (EOS), neutrophil (NEU), macrophage (MAC), and lymphocyte (LYM). Each point represents mean $\pm$ S.E.M. of four to six experiments. LY—LY294002 (mg/kg). *Significant difference from PEG control, $P < 0.05$.

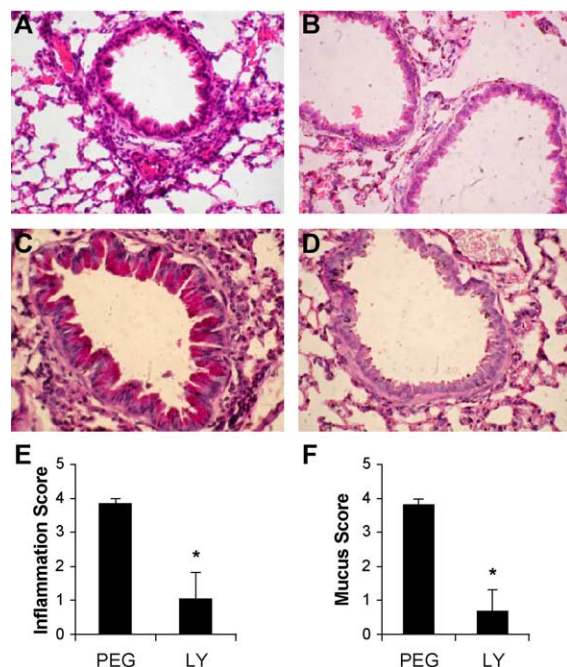


Fig. 2. Effects of LY294002 on lung tissue eosinophilia and mucus production. Histological examination of lung tissue eosinophilia (A, B; magnification $\times 400$) and mucus secretion (C, D; magnification $\times 400$) 24 h after the last OVA challenge without (A, C) and with 7.5 mg/kg LY294002 (B, D). Quantitative analyses of inflammatory cell infiltration (E) and mucus production (F) in lung sections were performed, as described previously [17]. Lung tissues were fixed, sectioned at 6 μ m thickness and stained with H&E for tissue eosinophilia or PAS for mucus production. Data represent mean $\pm$ S.E.M. of four experiments. *Significant difference from PEG control, $P < 0.05$.

3.2. Effects of LY294002 on OVA-induced eosinophil infiltration and mucus production

Lung tissues were collected 24 h after the last OVA challenge. OVA aerosol challenge induced marked infiltration of inflammatory cells into the peribronchiolar and perivascular connective tissues. The majority of the infiltrated inflammatory cells was eosinophils. LY294002 (7.5 mg/kg) significantly attenuated the eosinophil-rich leukocyte infiltration

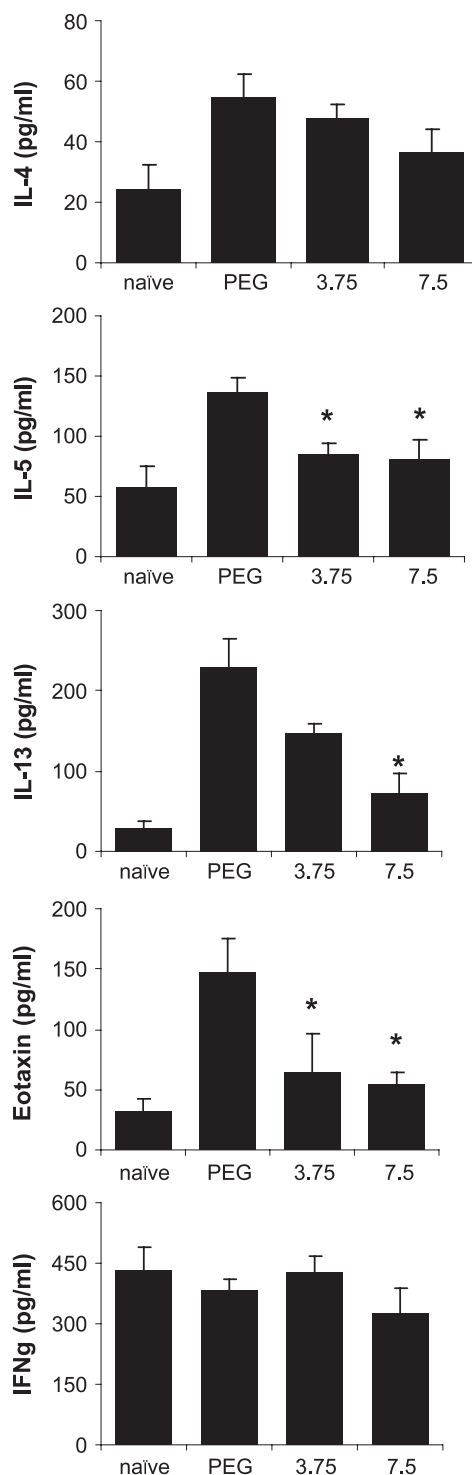


Fig. 3. Effects of LY294002 on cytokine and chemokine levels in BAL fluid. BAL fluids were collected 2 h after the last OVA challenge. Levels of IL-4, IL-5, IL-13, eotaxin, and IFN γ were analyzed using ELISA. Values shown are the mean $\pm$ S.E.M. of four separate experiments. *Significant difference from PEG control, $P < 0.05$.

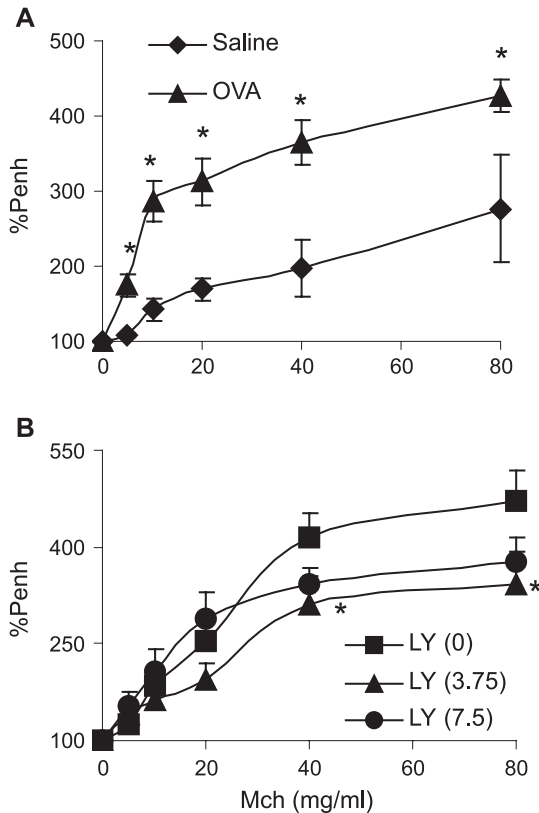


Fig. 4. Effects of LY294002 on airway hyperresponsiveness. (A) Airway responsiveness of sensitized mice challenged with aerosolized saline or 1% OVA for 3 consecutive days to increasing doses of inhaled methacholine. (B) Effects of LY294002 on OVA-induced airway responsiveness to inhaled methacholine. Data represent mean $\pm$ S.E.M. of four to seven experiments. LY—LY294002 (mg/kg). *Significant difference from PEG control, $P < 0.05$.

as compared to PEG control (Fig. 2A, B and E). On the other hand, OVA-induced mucus production was substantially attenuated by LY294002 (7.5 mg/kg) as compared to the PEG control (Fig. 2C, D and F).

3.3. Effect of LY294002 on cytokine levels in BAL fluid

To determine the levels of cytokines in vivo, BAL fluid samples were collected 2 h after the last OVA challenge. IL-4, IL-5, IL-13, eotaxin, and IFN γ levels were measured using ELISA. As shown in Fig. 3, LY294002 significantly ($P < 0.05$) reduced IL-5, IL-13, and eotaxin levels in BAL fluid as compared to vehicle control. LY294002 had lesser effects on IL-4 level.

3.4. Effect of LY294002 on airway hyperresponsiveness

Sensitized mice challenged with 1% OVA aerosol for 20 min daily for 3 consecutive days developed airway hyperresponsiveness to inhaled methacholine as compared to the saline-challenged group (Fig. 4A). LY294002 suppressed airway hyperresponsiveness to inhaled methacholine, and, at 3.75 mg/kg, the inhibition was statistically significant ($P < 0.05$; Fig. 4B).

3.5. Phospho-Akt immunoblot analysis

To verify that LY294002 caused inhibition of PI3K in the mouse asthma model, we examined the effects of LY294002 on OVA-induced Akt phosphor-

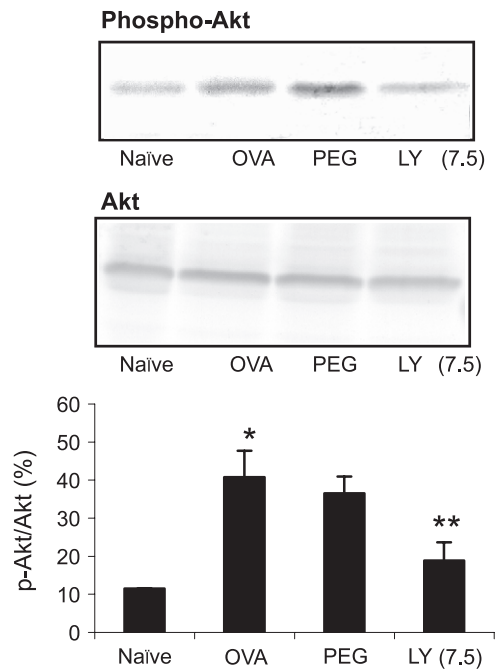


Fig. 5. Effects of LY294002 on Akt phosphorylation in lung tissue. Western blot analysis of OVA-induced Akt phosphorylation in lung fragments isolated from mice treated with PEG or 7.5 mg/kg LY294002. Lung fragments were homogenized in ice-cold lysis buffer. Proteins (30 μ g per lane) were separated by SDS-PAGE and probed with anti-Akt and antiphospho-Akt antibodies. The blot was developed by BCIP and NBT and was analyzed using Gel-Pro imaging software. Results are expressed as % phospho-Akt as compared to total Akt. Values shown are the mean $\pm$ S.E.M. of three separate experiments. *Significant difference from naive and **significant difference from PEG control, $P < 0.05$.

ylation in lungs isolated from mice treated with PEG or 7.5 mg/kg LY294002. Fig. 5 shows that Akt phosphorylation was markedly up-regulated in lungs from OVA-challenged mice, and LY294002 significantly ($P<0.05$) blocked the OVA-induced Akt phosphorylation.

4. Discussion

LY294002, a PI3K inhibitor, has been shown to inhibit the kinase activity by competing with the ATP binding with no effect on other protein kinases such as protein kinase A, protein kinase C, mitogen-activated protein kinase, PtdIns 4-kinase, and diacylglycerol kinase [13]. Walker et al. [14] described the crystal structure of PI3K γ with LY294002, showing that the morpholino group of LY294002 is absolutely required for binding and selectivity. Our present findings confirm our previous *in vitro* anti-inflammatory data [15,16] that inhibition of PI3K by LY294002 could attenuate OVA-induced pulmonary inflammation, release of Th2 cytokines into the airway, airway mucus production, and AHR in sensitized mice.

It is believed that Th2 cytokines play an essential role in the pathogenesis of the allergic airway inflammation [2,19]. Th2 cytokines can be produced by various resident cells such as bronchial epithelial cells, tissue mast cells, and alveolar macrophages as well as infiltrated inflammatory cells such as lymphocytes and eosinophils. A recent *in vitro* imaging study [20] using mouse T cell line directly revealed antigen presentation-mediated activation of PI3K and of Akt. The PI3K–Akt pathway has been shown to be critical for T cell receptor- and costimulatory receptor CD28-mediated T cell differentiation, survival, activation, and cytokine production [7,10,11]. In different mouse models of asthma, dominant-negative class IA PI3K and LY294002 significantly reduced IL-4 and IL-5 levels in BAL fluid [21,22]. In contrast, our present data show that intratracheally administered LY294002 markedly reduced the levels of IL-5 and IL-13 as well as the level of eotaxin in BAL fluids, with no effect on the levels of IL-4 and IFN γ , a Th1 cytokine. Our observations are in line with a recent *in vitro* mechanistic study that wortmannin, the other PI3K inhibitor, was able to block CD28-stimulated T cell production of IL-5 and IL-13 but not IL-4 [23].

The present study also verified PI3K inhibition by LY294002 in the mouse asthma model by blocking (Ser⁴⁷³) phosphorylation of Akt in lung tissues from LY294002-treated mice as compared to PEG controls.

Eosinophil is the principal effector cell for the pathogenesis of allergic inflammation. Eosinophil transmigration into the airways is a multistep process that is orchestrated by Th2 cytokines such as IL-5 and IL-13, and coordinated by specific chemokines such as eotaxin in combination with adhesion molecules such as VCAM-1 and VLA-4 [2,24]. IL-13 has been shown by far as the most potent inducer of eotaxin expression by airway epithelial cells [25]. Our results demonstrated that LY294002 ameliorated airway eosinophilia, as shown by a reduction in eosinophil counts in BAL fluid and in lung tissues. This observation may be related to the reduction in airway eotaxin and IL-13 levels in these mice. In addition, LY294002 and wortmannin, two PI3K inhibitors, have been shown to inhibit chemokinesis of guinea pig bone marrow eosinophils stimulated by IL-5 [26]. PI3K has also been shown to be a critical signaling pathway upon binding of chemoattractants and Th2 cytokines to their receptors in eosinophils [27,28]. Furthermore, IL-13 can selectively induce VCAM-1 expression in human endothelial cells [29]. Taken together, the observed reduction in airway eosinophilia by LY294002 may be a result of composite inhibition of Th2 cytokine production and eotaxin release leading to reduced VCAM-1 expression via PI3K pathway inhibition.

Our findings demonstrated a dramatic reduction in mucus production with less goblet cell hyperplasia in LY294002-treated mice as compared with untreated control. Studies using undifferentiated tracheobronchial epithelial cells or cancer cell lines showed that Th2 cells and cytokines [30–32] are linked to mucus hypersecretion by their effects on mucin gene expression in the airway epithelium. Aside from infiltrated CD4⁺ cells, eosinophils are also required to achieve the airway mucus accumulation and goblet cell degranulation [33,34]. As such, the observed reduction in mucus production in LY294002-treated lung tissue may be attributed by the substantial drop in Th2 cytokine levels as well as reduction in eosinophilia in asthmatic mice.

It is believed that inflammatory mediators released during the allergic inflammation play a critical role in

AHR development [1,35,36]. Our data showed that PI3K blockade inhibited OVA-induced AHR to inhaled methacholine. It has been established that IL-5-mediated eosinophilia contributes to AHR by generating cytotoxic products such as major basic protein (MBP), eosinophilic cationic protein (ECP), and other lipid mediators such as platelet activated factor (PAF) and cysteinyl-leukotrienes leading to tissue damage [37]. In addition, IL-13 has been shown to induce AHR in mouse asthma models in which cysteinyl-leukotrienes have been implicated to play a major role in AHR [38]. As such, the observed inhibition of AHR by LY294002 may be associated with the reduction in IL-5 and IL-13 production and in tissue eosinophilia by LY294002.

Allergic airway inflammation and AHR development involve multiple inflammatory cells and a wide array of mediators. We report here that PI3K inhibition effectively reduced OVA-induced Th2 cytokine production, pulmonary eosinophilia, mucus hypersecretion, and AHR in a mouse asthma model. These findings support a potential role for PI3K inhibitor in the treatment of asthma.

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