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PI3K γ -deficient mice have reduced levels of allergen-induced eosinophilic inflammation and airway remodeling

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Lim DH, Cho JY, Song DJ, Lee SY, Miller M, Broide DH. PI3K γ -deficient mice have reduced levels of allergen-induced eosinophilic inflammation and airway remodeling. *Am J Physiol Lung Cell Mol Physiol* 296: L210–L219, 2009. First published November 21, 2008; doi:10.1152/ajplung.90275.2008.—In this study, we have examined the role of phosphoinositide 3 kinase γ (PI3K γ), a class Ib PI3K, in contributing to airway remodeling utilizing PI3K γ -deficient mice exposed to chronic allergen challenge. Wild-type (WT) mice sensitized to ovalbumin (OVA) and chronically challenged with OVA for 1 mo developed significantly increased levels of eosinophilic inflammation and airway remodeling. In contrast, PI3K γ -deficient mice challenged with OVA had significantly reduced numbers of bronchoalveolar lavage and peribronchial eosinophils compared with WT mice. There was no significant difference in the number of bone marrow or circulating peripheral blood eosinophils when comparing WT mice and PI3K γ -deficient mice, suggesting that trafficking of eosinophils into the lung was reduced in PI3K γ -deficient mice. PI3K γ -deficient and WT mice had similar levels of IL-5 and eotaxin-1. The reduced eosinophil recruitment to the airway in PI3K γ -deficient mice challenged with OVA was associated with significantly reduced numbers of TGF- β 1+ peribronchial cells, reduced numbers of pSmad 2/3+ airway epithelial cells, and pSmad 2/3+ peribronchial cells, as well as significantly reduced levels of peribronchial fibrosis (quantitated by trichrome staining and image analysis as well as by lung collagen levels). In addition, the area of peribronchial α -smooth muscle staining was significantly reduced in PI3K γ -deficient compared with WT mice. Overall, this study demonstrates an important role for PI3K γ in mediating allergen-induced eosinophilic airway inflammation and airway remodeling, suggesting that PI3K γ may be a novel therapeutic target in asthma.

transforming growth factor- β 1; pSmad2/3; smooth muscle; mucus

AIRWAY HYPERRESPONSIVENESS and airway inflammation are cardinal features of asthma (2), with eosinophils being a prominent component of the inflammatory response in allergic asthmatics (28). In addition, asthma is also characterized in a subset of patients by characteristic structural changes to the airway termed airway remodeling (8). Features of airway remodeling include peribronchial fibrosis, smooth muscle hypertrophy/hyperplasia, mucus metaplasia, and angiogenesis (4). In mouse models of allergen-induced airway remodeling (7) as well as in human studies of asthmatics with airway remodeling (11), depletion of eosinophils expressing TGF- β 1 significantly reduces levels of airway remodeling. As phosphoinositide 3 kinases (PI3K) are key leukocyte intracellular enzymes that regulate a variety of cellular functions important to inflammation, including leukocyte migration and adhesion (6, 13, 27), we have examined whether targeting PI3K γ , a class Ib PI3K,

would reduce allergen-induced eosinophilic inflammation, airway responsiveness, and airway remodeling in a mouse model of chronic allergen challenge.

PI3K are divided into three main classes (class I, II, and III) based on their protein domain structure, associated regulatory subunits, and the particular type of phospholipids they can phosphorylate (27, 30). Class I PI3K have been the focus of many studies in inflammation, as well as in autoimmunity and cancer (5, 27, 30, 31). The enzyme component of class I PI3K are encoded by genes distinct from that of class II and III PI3K (27, 30). Class I PI3K are subdivided into class Ia (PI3K α , PI3K β , and PI3K δ isoforms) and class Ib (PI3K γ only) (27, 30) (Table 1). In this study, we have focused on investigating the role of PI3K γ , a class Ib PI3K, in asthma using PI3K γ -deficient mice (29). PI3K γ is a heterodimer composed of a catalytic subunit of 110 kDa and a tightly associated regulatory subunit (either p101 or p84) that controls expression, activation, and subcellular localization (27, 30). PI3K γ activation is induced by the activation of G protein-coupled receptors such as chemokine receptors (27, 30). After leukocyte receptor stimulation, intracellular PI3K are recruited to the inner surface of the cell surface membrane where they catalyze the generation of the lipid-based second messenger phosphatidylinositol-3,4,5-triphosphate by direct phosphorylation of phosphatidylinositol-4,5-bisphosphate (27, 30). The PI3K-induced second messenger phosphatidylinositol-3,4,5-triphosphate then serves as a docking platform for pleckstrin homology domain-containing proteins (such as AKT and others), which leads to a cascade of further intracellular signaling and biological responses important to inflammation (27, 30).

As PI3K may be activated by engagement of cytokine and/or chemokine receptors and are mainly expressed by leukocytes, the role of PI3Ks in inflammation in a variety of diseases including asthma have been investigated (24, 27). Class Ib PI3K, such as PI3K γ investigated in this study, differ from class Ia PI3K in their regulatory subunits (Table 1), which dictate to which upstream receptor signals the class of PI3K respond. For example, class Ib PI3K are activated by G protein-coupled receptors (such as chemokine receptors), whereas class Ia PI3K are activated by receptor tyrosine kinases such as cytokine receptors (27, 30). In the case of class Ib PI3K, they preferentially bind to G $\beta\gamma$ -subunits released from heterodimeric G proteins following activation of G protein-coupled receptors (27). In contrast, class Ia regulatory subunits associate with activated tyrosine kinase receptors resulting in activation of class Ia PI3K by many cytokines (27). Studies investigating the role of PI3K in airway inflammation in

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Table 1. Class Ia and class Ib phosphoinositide 3 kinases

Class Ia and Class Ib Phosphoinositide 3 Kinases			
	Enzyme	Regulatory Subunit	PI3K Activation Signal
Class Ia	p110 α , p110 β , p110 δ	p85 α , p85 β , p55 γ	Receptor tyrosine kinase (e.g., cytokine)
Class Ib	p110 γ	p101, p84	G protein-coupled receptor (e.g., chemokine)

asthma have identified an important role for class Ia PI3K (such as PI3K δ as well as the p85 regulatory subunit of class Ia PI3K) (25, 26), but have not investigated the role of the class Ib PI3K (i.e., PI3K γ) in airway inflammation in asthma. Therefore, in this study, we have investigated whether targeting PI3K γ (a class Ib PI3K activated by G protein-coupled receptors rather than receptor tyrosine cytokine receptors) would not only reduce airway inflammation and airway responsiveness (as has been demonstrated for class Ia PI3K activated by receptor tyrosine cytokine receptors) but also reduce levels of airway remodeling, which has not been reported in studies targeting any PI3K.

METHODS

Mouse model of OVA-induced airway remodeling. Eight- to ten-week-old PI3K γ -deficient mice ($n = 16$ /group) kindly provided by Dr. J. Penninger (Amgen, Canada) (29) and wild-type (WT) ($n = 16$ /group) mice on a background of C57/B1 (The Jackson Laboratory, Bar Harbor, ME) were immunized subcutaneously on days 0, 7, 14, and 21 with 25 μ g of ovalbumin (OVA; grade V, Sigma) adsorbed to 1 mg of alum (Aldrich) in 200 μ l of normal saline as previously described (7, 18, 21). Intranasal OVA challenges (20 μ g/50 μ l in PBS) were administered on days 27, 29, and 31 under isoflurane (Vedco, St. Joseph, MO) anesthesia. Intranasal OVA challenges were then repeated twice a week for 4 wk. Age- and sex-matched control mice were sensitized but not challenged with OVA during the study. Mice were killed 24 h after the final OVA challenge, and bronchoalveolar lavage (BAL) fluid was collected by lavaging the lung with 1 ml of PBS via a tracheal catheter (7, 18). Lungs from the different experimental groups were processed as a batch for either histological staining or immunostaining under identical conditions. Stained and immunostained slides were all quantified under identical light microscope conditions, including magnification ($\times 20$), gain, camera position, and background illumination. All animal experimental protocols were approved by the University of California, San Diego Animal Subjects Committee.

Airway hyperreactivity to methacholine. Airway responsiveness to methacholine (MCh) was assessed 24 h after the final OVA challenge in intubated and ventilated mice (flexiVent ventilator; Scireq, Montreal, PQ, Canada) anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) intraperitoneally as previously described (18). The dynamic airway resistance was determined using Scireq software in mice exposed to nebulized PBS and MCh (3, 24, 48 mg/ml). The following ventilator settings were used: tidal volume (10 ml/kg), frequency (150/min), and positive end-expiratory pressure (3 cmH₂O).

Blood, bone marrow, BAL, and peribronchial eosinophils. Peripheral blood was collected from mice by cardiac puncture into EDTA-containing tubes (3, 33). Erythrocytes were lysed using a 1:10 solution of 100 mM potassium carbonate-1.5 M ammonium chloride. The remaining cells were resuspended in 1 ml of PBS. BAL was collected by lavaging the lung with 1 ml of PBS via a tracheal catheter (3, 33). Bone marrow cells were flushed from femurs with 1 ml of PBS, centrifuged, and resuspended in 1 ml of PBS. BAL was centrifuged, supernatant was collected and frozen at -80°C , and cells were resuspended in 1 ml of PBS. Total leukocytes were counted using a

hemocytometer. To perform differential cell counts, 200 μ l of resuspended BAL cells, peripheral-blood leukocytes, or 20 μ l of bone marrow cell suspensions was cytospun onto microscope slides and air-dried. Slides were stained with Wright-Giemsa, and differential cell counts were performed under a light microscope (3, 33).

Lung sections were processed for MBP immunohistochemistry as previously described (7) using an anti-mouse MBP Ab (kindly provided by Dr. James Lee, Mayo Clinic, Scottsdale, AZ). The number of individual cells staining positive for MBP in the peribronchial space was counted using a light microscope. Results are expressed as the number of peribronchial cells staining positive for MBP per bronchiole with 150–200 μ m of internal diameter. At least 10 bronchioles were counted in each slide.

IL-5 and eotaxin-1. IL-5 and eotaxin-1 levels were measured in lung tissue by ELISA. Lung tissue was homogenized in lysis buffer, and lung supernatants (obtained by centrifugation 10,000 g for 20 min) were passed through an 0.8- μ m pore size filter and frozen at -80°C in polypropylene tubes until used in assays (7, 33). IL-5 and eotaxin-1 levels were quantitated by ELISA (R&D Systems, Minneapolis, MN). The IL-5 assay has a sensitivity of 15.6 pg/ml, and the eotaxin-1 assay has a sensitivity of 7.8 pg/ml. Lung protein levels were quantitated using a BCA protein assay (Pierce, Rockford, IL). Results are expressed as picogram of eotaxin-1 per microgram of lung protein or picogram of IL-5 per microgram of lung protein.

Peribronchial trichrome staining. Lungs in the different groups of mice were equivalently inflated with an intratracheal injection of a similar volume of 4% paraformaldehyde solution (Sigma Chemical, St. Louis, MO) to preserve the pulmonary architecture. The area of peribronchial trichrome staining in paraffin-embedded lungs was outlined and quantified under a light microscope (Leica DMLS, Leica Microsystems) attached to an image analysis system (Image-Pro Plus, Media Cybernetics) as previously described (7). Results are expressed as the area of trichrome staining per micrometer length of basement membrane of bronchioles 150–200 μ m of internal diameter.

Lung collagen assay. The amount of lung collagen was measured as previously described in this laboratory (7, 18) with a collagen assay kit that uses a dye reagent that selectively binds to the [Gly-X-Y] $_n$ tripeptide sequence of mammalian collagens (Biocolor, Newton-abbey, Northern Ireland, UK). In all experiments, a collagen standard was used to calibrate the assay.

Peribronchial TGF- β 1+ cells. The number of peribronchial TGF- β 1+ cells was quantitated by immunohistochemistry using an anti-TGF- β 1 Ab as previously described in this laboratory (7).

pSmad 2/3+ cells in airway epithelium and peribronchial region. To determine whether reductions in the numbers of peribronchial TGF- β 1+ cells resulted in reduced activation of TGF- β -induced signaling through Smad 2/3, we quantitated the number of pSmad 2/3+ cells in the bronchial epithelium as well as in the peribronchial region in lungs immunostained with an anti-pSmad 2/3 Ab. Lung sections were processed for immunohistochemistry using an anti-pSmad 2/3 Ab (Santa Cruz) using methods as previously described in this laboratory (18). The pSmad 2/3 Abs detect nuclear phosphorylation of either Smad 2 or Smad 3. Species- and isotype-matched Abs were used as controls. The number of pSmad 2/3+ cells was quantitated in both the peribronchial region (excluding bronchial epithelium) and separately in the bronchial epithelium. Results are ex-

pressed as the number of peribronchial cells staining positive for pSmad 2/3 per bronchiole with a 150- to 200- μ m internal diameter, as well as the % of bronchial epithelial cells/bronchus staining positive for pSmad 2/3. Three bronchioles were counted in each slide stained with the pSmad 2/3 Ab.

Peribronchial smooth muscle layer thickness. Lung sections were also immunostained with an anti- α -smooth muscle actin primary antibody (Sigma-Aldrich) to detect peribronchial smooth muscle cells. Species- and isotype-matched Abs were used as controls in place of the primary Ab. The area of peribronchial α -smooth muscle actin staining in paraffin-embedded lungs was outlined and quantified under a light microscope (Leica DMLS) attached to an image analysis system (Image-Pro Plus) as previously described (7). Results are expressed as the area of peribronchial α -smooth muscle actin staining per micrometer length of basement membrane of bronchioles 150–200 μ m of internal diameter.

Airway mucus expression. To quantitate the level of mucus expression in the airway, the number of periodic acid-Schiff (PAS)-positive and PAS-negative epithelial cells in individual bronchioles were counted as previously described in this laboratory (7). At least 10 bronchioles were counted in each slide. Results are expressed as the percentage of PAS-positive cells per bronchiole, which is calculated from the number of PAS-positive epithelial cells per bronchus divided by the total number of epithelial cells of each bronchiole.

PI3K γ lung immunostaining. To determine whether cells in the lung expressed PI3K γ , lung sections from WT and PI3K γ -deficient mice were immunostained with a goat anti-mouse PI3K γ Ab directed against the p110 γ -component of PI3K γ (Santa Cruz Biotechnology, Santa Cruz, CA) using the immunoperoxidase method as previously described in this laboratory (7).

Statistical analysis. Results in the different groups of mice were compared by ANOVA using the non-parametric Kruskal-Wallis test followed by posttesting using the Dunn multiple comparison of means. All results are presented as means $\pm$ SE. A statistical software package (GraphPad Prism, San Diego, CA) was used for the analysis. $P < 0.05$ was considered statistically significant.

RESULTS

Chronic OVA challenged PI3K γ -deficient mice and airway hyperresponsiveness. Chronic OVA challenge in WT mice induced a statistically significant increase in airway responsiveness to MCh (WT OVA vs. WT PBS diluent, 48 mg/ml MCh) ($P = 0.001$) (Fig. 1). In contrast, chronic OVA challenge in PI3K γ -deficient mice did not induce any detectable increase in airway responsiveness from that noted in diluent-challenged PI3K γ -deficient mice (PI3K γ -deficient mice OVA vs. PI3K γ -deficient mice PBS diluent) ($P =$ not significant) (Fig. 1). PI3K γ -deficient mice challenged with OVA had a small but statistically significant reduction in airway responsiveness compared with WT mice challenged with OVA (PI3K γ -deficient mice OVA vs. WT mice OVA) ($P = 0.001$) (Fig. 1). There was no difference in compliance or elastance between WT and PI3K γ -deficient mice (data not shown).

Chronic OVA-challenged PI3K γ -deficient mice have reduced levels of BAL eosinophils and peribronchial eosinophils. Chronic OVA challenge in WT mice induced a significant increase in the number of BAL eosinophils ($P = 0.001$) (WT OVA vs. WT no OVA) (Fig. 2A) as well as a significant increase in the number of peribronchial eosinophils ($P = 0.001$) (WT OVA vs. WT no OVA) (Fig. 2B) compared with non-OVA-challenged mice. The number of BAL eosinophils in chronic OVA-challenged PI3K γ -deficient mice was significantly less than that in chronic OVA-challenged WT mice (283 ± 43 vs. 613 ± 32 BAL eosinophils $\times 10^2$) (PI3K γ -

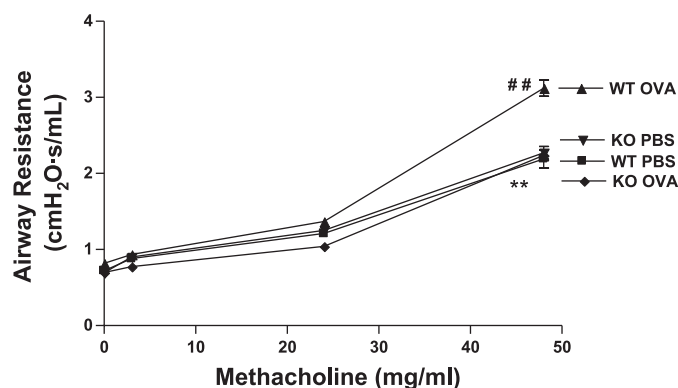


Fig. 1. Levels of airway responsiveness in phosphoinositide 3 kinase γ (PI3K γ)-deficient vs. wild-type (WT) mice. Airway resistance was measured (cmH₂O·s/ml) in different groups of intubated and ventilated PI3K γ -deficient or WT mice following nebulization of either PBS diluent or methacholine (MCh) (3, 24, 48 mg/ml). Chronic ovalbumin (OVA) challenge in WT mice induced a significant increase in airway resistance (WT no OVA vs. WT OVA; $###P = 0.001$, 48 mg/ml MCh). PI3K γ -deficient mice challenged with OVA had significantly reduced airway responsiveness compared with WT mice (PI3K γ -deficient OVA vs. WT OVA; $**P = 0.001$, 48 mg/ml MCh) ($n = 16$ mice/group). KO, knockout.

deficient OVA vs. WT OVA) ($P = 0.002$) (Fig. 2A). Similarly, the number of peribronchial eosinophils in chronic OVA-challenged PI3K γ -deficient mice were significantly less than that in chronic OVA-challenged WT mice (32 ± 2 vs. 52 ± 4 MBP+ eosinophils/bronchus) (PI3K γ -deficient OVA vs. WT OVA) ($P = 0.001$) (Fig. 2B).

Chronic OVA-challenged PI3K γ -deficient mice do not have reduced levels of bone marrow eosinophils and peripheral blood eosinophils. Chronic OVA challenge in WT mice induced a significant increase in the number of bone marrow eosinophils ($P = 0.01$) (WT OVA vs. WT no OVA) (Fig. 2D). The number of bone marrow eosinophils in OVA-challenged PI3K γ -deficient mice was not significantly different from that of OVA-challenged WT mice (PI3K γ -deficient OVA vs. WT OVA) ($P = 0.16$) (Fig. 2D). Similarly, the percentage of peripheral blood eosinophils in OVA-challenged PI3K γ -deficient mice was not significantly different from that of OVA-challenged WT mice (6.8 ± 1.8 vs. $5.5 \pm 1.7\%$ eosinophils) (PI3K γ -deficient OVA vs. WT OVA) ($P = 0.33$) (Fig. 2C).

Effect of PI3K γ deficiency on IL-5 and eotaxin-1 levels. The level of IL-5 expression in the lung of OVA-challenged WT mice was significantly increased compared with non-OVA-challenged WT mice (25.0 ± 3.8 vs. 15.7 ± 1.9 pg of IL-5/ μ g lung protein; $P = 0.003$; Fig. 3A). There was no significant difference in levels of lung IL-5 in OVA-challenged WT mice compared with OVA-challenged PI3K γ -deficient mice ($P =$ ns) (Fig. 3A).

The level of eotaxin-1 expression in the lung of OVA-challenged WT mice was also significantly increased compared with non-OVA-challenged WT mice (542.5 ± 132.6 vs. 230.9 ± 16.3 pg of eotaxin-1/ μ g lung protein; $P = 0.001$; Fig. 3B). There was no significant difference in levels of lung eotaxin-1 in OVA-challenged WT mice compared with OVA-challenged PI3K γ -deficient mice ($P =$ not significant) (Fig. 3B).

Chronic OVA-challenged PI3K γ -deficient mice have reduced levels of peribronchial fibrosis. Chronic OVA challenge in WT mice induced a significant increase in levels of peri-

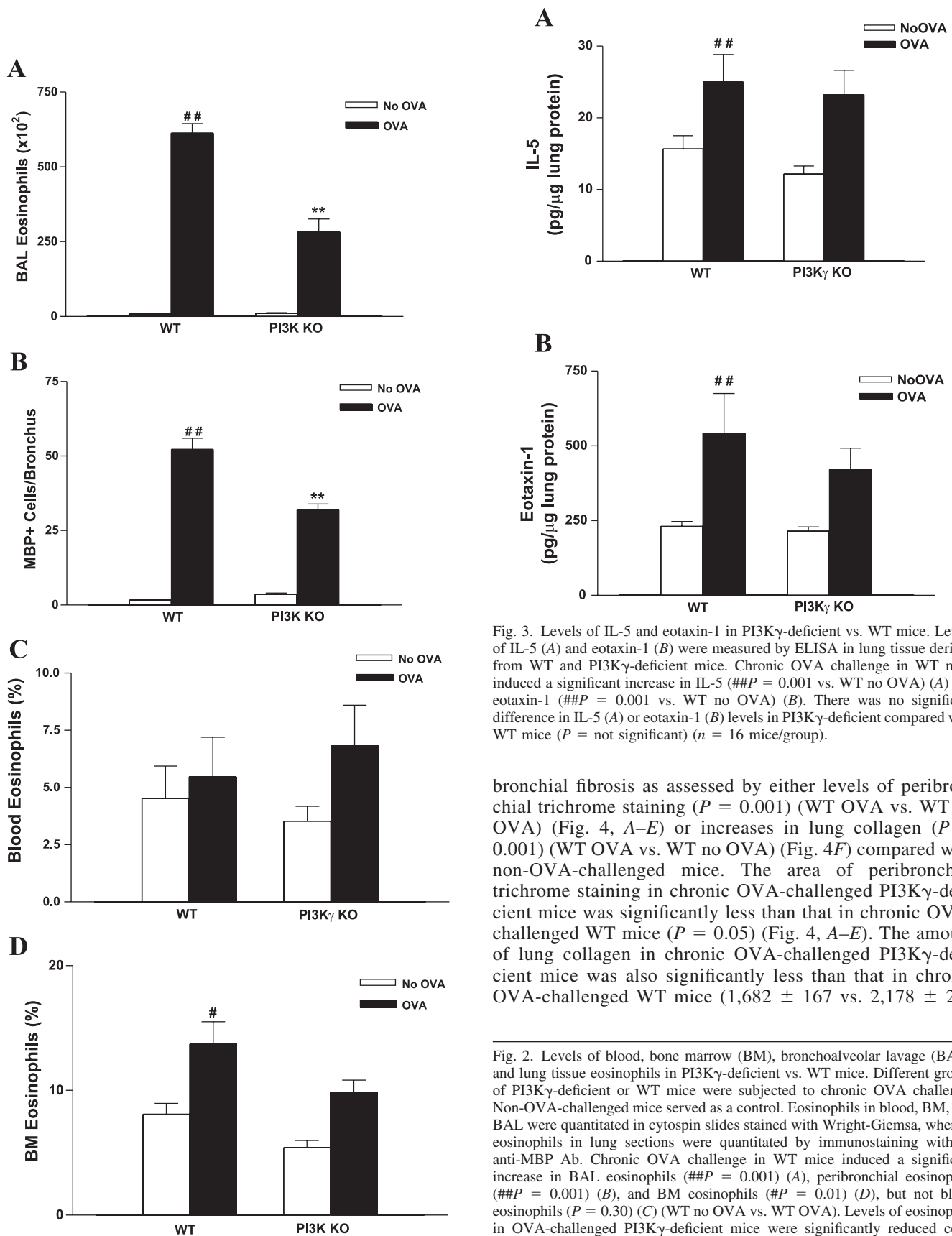


Fig. 3. Levels of IL-5 and eotaxin-1 in PI3K γ -deficient vs. WT mice. Levels of IL-5 (A) and eotaxin-1 (B) were measured by ELISA in lung tissue derived from WT and PI3K γ -deficient mice. Chronic OVA challenge in WT mice induced a significant increase in IL-5 ($##P = 0.001$ vs. WT no OVA) (A) and eotaxin-1 ($##P = 0.001$ vs. WT no OVA) (B). There was no significant difference in IL-5 (A) or eotaxin-1 (B) levels in PI3K γ -deficient compared with WT mice ($P =$ not significant) ($n = 16$ mice/group).

bronchial fibrosis as assessed by either levels of peribronchial trichrome staining ($P = 0.001$) (WT OVA vs. WT no OVA) (Fig. 4, A–E) or increases in lung collagen ($P = 0.001$) (WT OVA vs. WT no OVA) (Fig. 4F) compared with non-OVA-challenged mice. The area of peribronchial trichrome staining in chronic OVA-challenged PI3K γ -deficient mice was significantly less than that in chronic OVA-challenged WT mice ($P = 0.05$) (Fig. 4, A–E). The amount of lung collagen in chronic OVA-challenged PI3K γ -deficient mice was also significantly less than that in chronic OVA-challenged WT mice ($1,682 \pm 167$ vs. $2,178 \pm 234$

Fig. 2. Levels of blood, bone marrow (BM), bronchoalveolar lavage (BAL), and lung tissue eosinophils in PI3K γ -deficient vs. WT mice. Different groups of PI3K γ -deficient or WT mice were subjected to chronic OVA challenge. Non-OVA-challenged mice served as a control. Eosinophils in blood, BM, and BAL were quantitated in cytospin slides stained with Wright-Giemsa, whereas eosinophils in lung sections were quantitated by immunostaining with an anti-MBP Ab. Chronic OVA challenge in WT mice induced a significant increase in BAL eosinophils ($##P = 0.001$) (A), peribronchial eosinophils ($##P = 0.001$) (B), and BM eosinophils ($#P = 0.01$) (D), but not blood eosinophils ($P = 0.30$) (C) (WT no OVA vs. WT OVA). Levels of eosinophils in OVA-challenged PI3K γ -deficient mice were significantly reduced compared with WT mice challenged with OVA in the BAL (A) ($**P = 0.002$) and lung (B) ($**P = 0.001$), but not in the blood (C) ($P = 0.33$) or BM (D) ($P = 0.16$) ($n = 16$ mice/group).

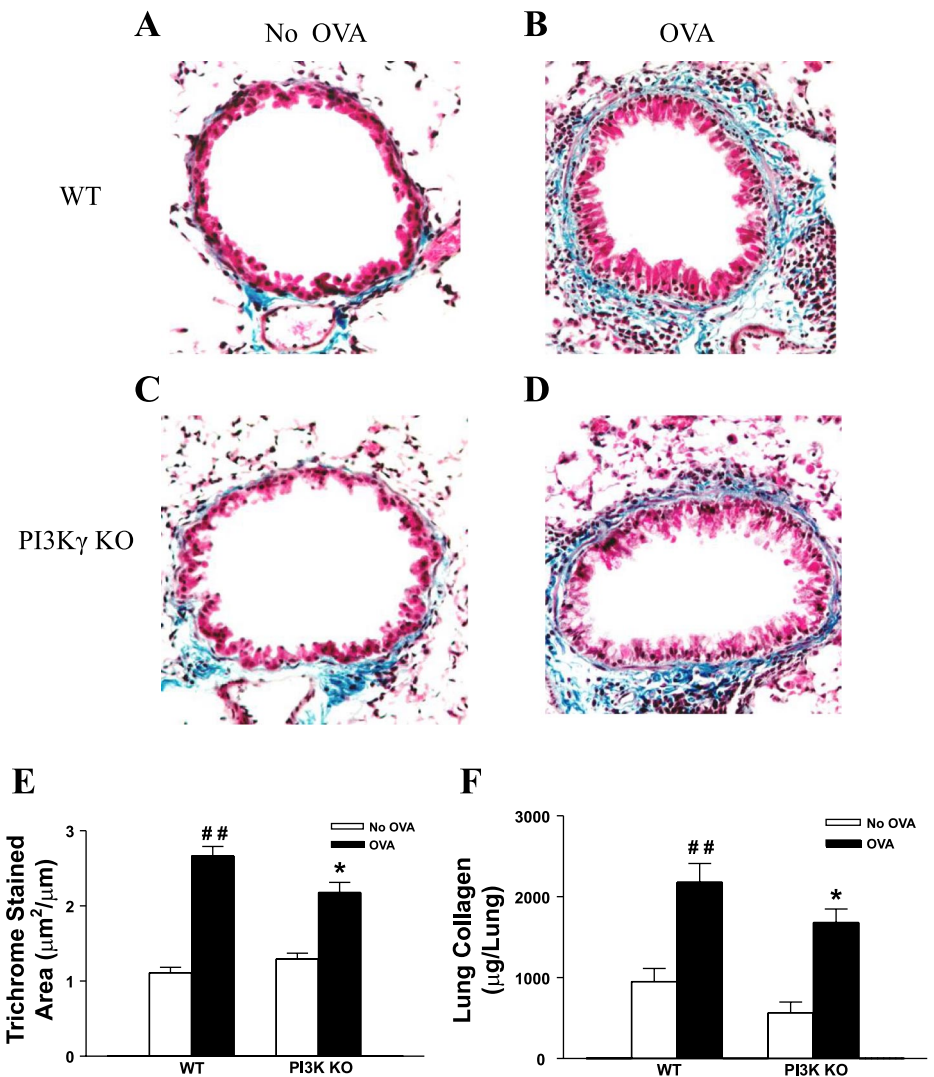


Fig. 4. Levels of peribronchial fibrosis in PI3K γ -deficient vs. WT mice. Different groups of PI3K γ -deficient or WT mice were subjected to chronic OVA challenge. Non-OVA-challenged mice served as a control. Levels of peribronchial fibrosis were quantitated in lung sections stained with trichrome by image analysis (A–D) as well as by assaying collagen levels in lungs (F). Chronic OVA challenge in WT mice induced a significant increase in the area of peribronchial trichrome staining ($##P = 0.001$) (E) and lung collagen ($##P = 0.001$) (F) (WT no OVA vs. WT OVA). Levels of peribronchial fibrosis in OVA-challenged PI3K γ -deficient mice were significantly reduced compared with WT mice challenged with OVA as assessed by trichrome staining and image analysis (E) ($*P = 0.05$) and by lung collagen levels (F) ($*P = 0.04$) ($n = 16$ mice/group).

μg collagen/lung) (PI3K γ -deficient OVA vs. WT OVA) ($P = 0.04$) (Fig. 4F).

Chronic OVA-challenged PI3K γ -deficient mice have reduced levels of peribronchial TGF- β 1+ cells as well as reduced numbers of peribronchial pSmad 2/3+ cells. As TGF- β 1 has been implicated in peribronchial fibrosis in asthma (18, 23), we examined whether PI3K γ -deficient mice had reduced numbers of TGF- β + peribronchial cells. Chronic OVA challenge in WT mice induced a significant increase in the number of TGF- β + peribronchial cells ($P = 0.001$) (WT OVA vs. WT no OVA) (Fig. 5, A–C) compared with non-OVA-challenged mice. The number of TGF- β + peribronchial cells in chronic OVA-challenged PI3K γ -deficient mice was significantly less than that in chronic OVA-challenged WT mice (39.5 ± 2.3 vs. 52.0 ± 2.3 TGF- β + cells/bronchus) (PI3K γ -deficient OVA vs. WT OVA) ($P = 0.04$) (Fig. 5, A–E). As previously reported in our laboratory (7), the airway epithelium in non-OVA-challenged WT mice exhibits TGF- β 1 immunoreactivity that increases in intensity with OVA challenge in WT mice (Fig. 5, A and B). There was no significant difference in epithelial TGF- β 1 immunoreactivity when comparing WT and PI3K γ -deficient mice (Fig. 5, A–F).

As TGF- β signals through Smad 2/3, we examined whether the reduced number of peribronchial TGF- β 1+ cells was associated with reduced numbers of pSmad 2/3+ cells in the epithelium as well as in the peribronchial area. Chronic OVA challenge in WT mice induced a significant increase in the number of pSmad 2/3+ epithelial cells ($P = 0.001$) (WT OVA vs. WT no OVA) (Fig. 5H) as well as a significant increase in the number of pSmad 2/3+ peribronchial cells ($P = 0.001$) (WT OVA vs. WT no OVA) (Fig. 5I) compared with non-OVA-challenged mice. The number of pSmad 2/3+ epithelial cells in chronic OVA-challenged PI3K γ -deficient mice was significantly less than that observed in chronic OVA-challenged WT mice (35 ± 2 vs. 51 ± 2 pSmad 2/3+ epithelial cells/bronchus) (PI3K γ -deficient OVA vs. WT OVA) ($P = 0.001$) (Fig. 5H). In addition, the number of pSmad 2/3+ peribronchial cells in chronic OVA-challenged PI3K γ -deficient mice was significantly less than that observed in chronic OVA-challenged WT mice (53 ± 4 vs. 75 ± 5 pSmad 2/3+ peribronchial cells/bronchus) (PI3K γ -deficient OVA vs. WT OVA) ($P = 0.003$) (Fig. 5I).

Chronic OVA-challenged PI3K γ -deficient mice have reduced levels of smooth muscle thickness. To determine whether changes in smooth muscle could have contributed to reduced

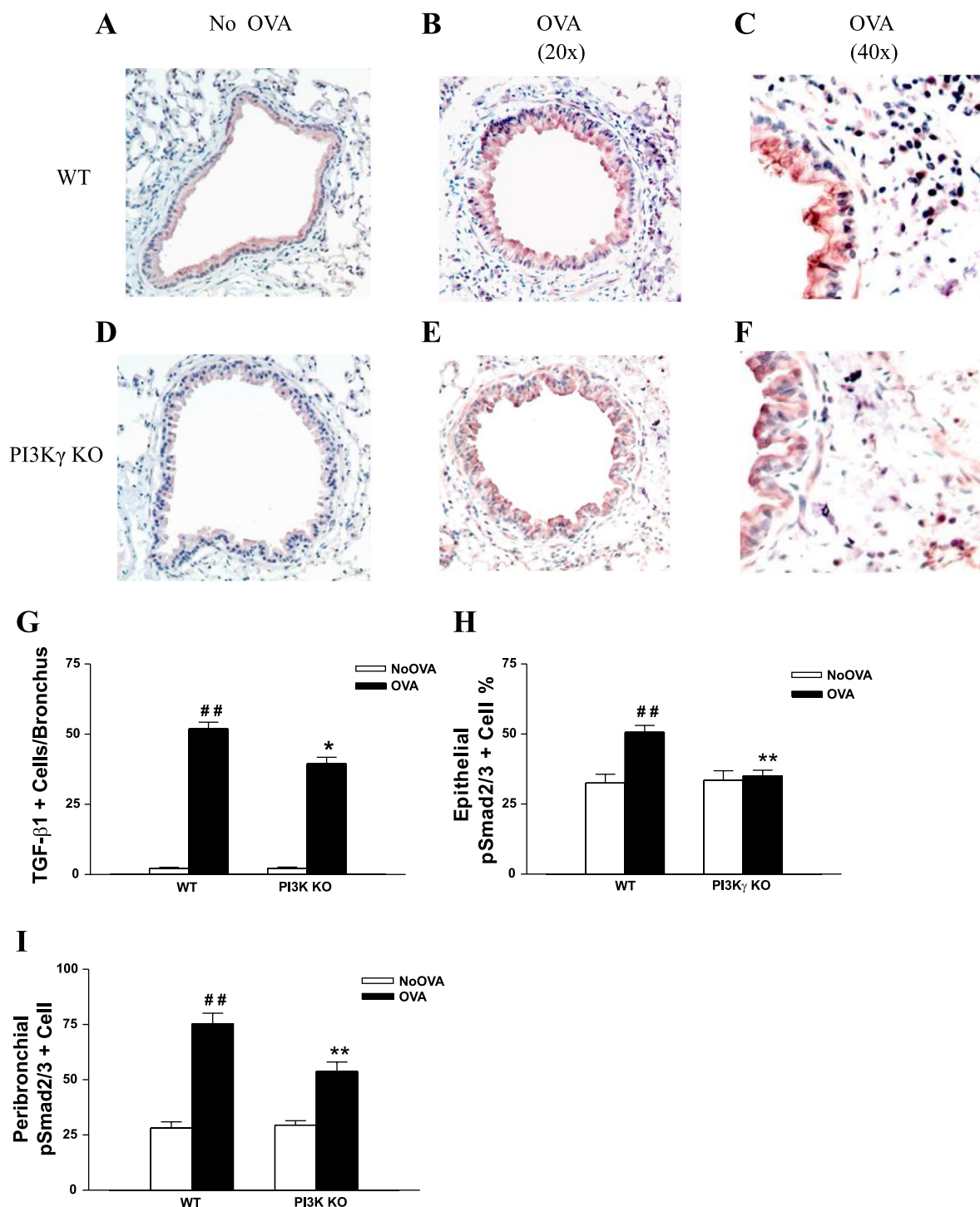


Fig. 5. Levels of peribronchial TGF- β + and pSmad 2/3+ cells in PI3K γ -deficient vs. WT mice. Different groups of PI3K γ -deficient or WT mice were subjected to chronic OVA challenge. Non-OVA-challenged mice served as a control. Levels of TGF- β + and pSmad 2/3+ cells were quantitated in lung sections by immunohistochemistry and image analysis (A–F). Chronic OVA challenge in WT mice induced a significant increase in the number of peribronchial TGF- β + cells ($###P = 0.001$) (G), epithelial pSmad 2/3+ cells ($###P = 0.001$) (H), and peribronchial pSmad 2/3+ cells ($###P = 0.001$) (I) (WT no OVA vs. WT OVA). Levels of peribronchial TGF- β + cells ($*P = 0.04$) (G), airway epithelial pSmad 2/3+ cells ($**P = 0.001$) (H), and peribronchial pSmad 2/3+ cells ($**P = 0.003$) (I) in OVA-challenged PI3K γ -deficient mice were all significantly reduced compared with WT mice challenged with OVA ($n = 16$ mice/group).

airway responsiveness in PI3K γ -deficient mice, we measured the area of peribronchial smooth muscle in PI3K γ -deficient and WT mice. Chronic OVA challenge in WT mice induced a significant increase in the area of peribronchial α -smooth muscle actin staining (1.67 ± 0.13 vs. $0.55 \pm 0.07 \mu\text{m}^2$

peribronchial α -smooth muscle actin staining per micrometer length of basement membrane) ($P = 0.001$) (WT OVA vs. WT no OVA) (Fig. 6A) compared with non-OVA-challenged WT mice. PI3K γ -deficient mice challenged with OVA had a significant reduction in the area of peribronchial α -smooth muscle

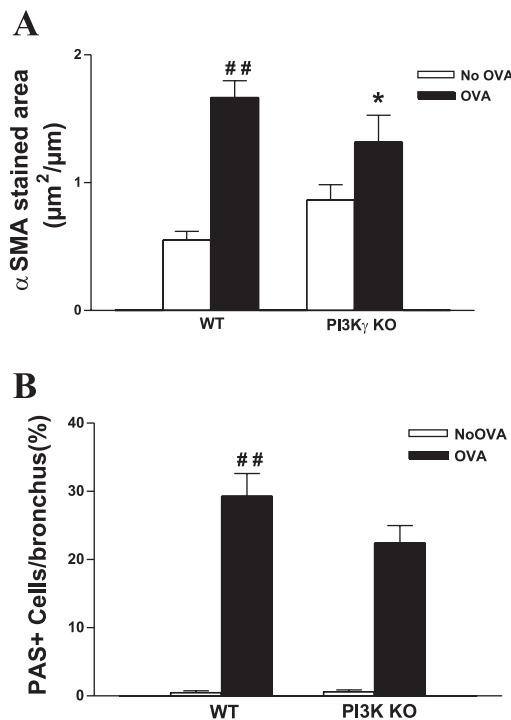


Fig. 6. Area of the peribronchial smooth muscle layer and levels of mucus expression in PI3K γ -deficient vs. WT mice. Different groups of PI3K γ -deficient or WT mice were subjected to chronic OVA challenge. Non-OVA-challenged mice served as a control. The area of the smooth muscle layer was quantitated in lung sections by immunohistochemistry (A) and levels of mucus expression by periodic acid-Schiff (PAS) staining (B). Chronic OVA challenge in WT mice induced a significant increase in the area of peribronchial α -smooth muscle actin immunostaining ($##P = 0.001$) (A) and the number of PAS+ mucus cells ($##P = 0.001$) (B) (WT no OVA vs. WT OVA). The area of peribronchial α -smooth muscle actin immunostaining in OVA-challenged PI3K γ -deficient mice was significantly reduced compared with WT mice challenged with OVA ($*P = 0.03$) (A). Levels of mucus expression were not different in OVA-challenged WT and OVA-challenged PI3K γ -deficient mice ($P = 0.20$) (B) ($n = 16$ mice/group).

actin staining compared with OVA-challenged WT mice (PI3K γ -deficient mice OVA vs. WT OVA) ($P = 0.03$) (Fig. 6A).

Chronic OVA-challenged PI3K γ -deficient mice have similar levels of airway mucus as WT mice. Chronic OVA challenge in WT mice induced a significant increase in the number of PAS+ mucus cells ($P = 0.001$) (WT OVA vs. WT no OVA) (Fig. 6B) compared with non-OVA-challenged mice. PI3K γ -deficient mice challenged with OVA did not have a statistically significant reduction in PAS+ mucus cells compared with OVA-challenged WT mice (22.4 ± 2.5 vs. $29.2 \pm 3.3\%$ PAS+ cells/bronchus) (PI3K γ -deficient OVA vs. WT OVA) ($P = 0.20$) (Fig. 6B).

Chronic OVA-challenged WT mice have increased numbers of peribronchial cells expressing immunoreactive PI3K γ . In non-OVA-challenged WT mice, we did not detect significant PI3K γ staining in the peribronchial region or in the airway epithelium, whereas in OVA-challenged WT mice, we detected strong PI3K γ staining in peribronchial inflammatory cells (Fig. 7). The airway epithelium in OVA-challenged WT mice had faint staining that was significantly less than the intense red staining of PI3K γ -positive peribronchial leukocytes (Fig. 7). The PI3K γ -deficient mice (no

OVA as well as OVA challenged) did not have detectable PI3K γ immunostaining in lung sections.

DISCUSSION

In this study, we have utilized PI3K γ -deficient mice to demonstrate an important role for PI3K γ in mediating allergen-induced eosinophilic inflammation and features of remodeling including subepithelial fibrosis and smooth muscle changes. While PI3K γ -deficient mice also had statistically significant reductions in airway responsiveness, the magnitude of the change in airway responsiveness was small. The mechanism by which inactivating PI3K γ contributes to reduced airway responsiveness is likely through its effect on reducing eosinophilic airway inflammation, as well as potential effects of PI3K γ in peribronchial smooth muscle. Evidence to support this is derived from our studies demonstrating that PI3K γ -deficient mice challenged with allergen have significantly reduced levels of BAL eosinophils and peribronchial eosinophils. As PI3K γ is known to be expressed particularly in leukocytes (27), and is also known to be activated by G protein-coupled chemokine receptors to induce leukocyte migration (27), the reduced recruitment of eosinophils into the airway in PI3K γ -deficient mice is likely due to reduced chemokine-induced migration of eosinophils from the circulation to account for the reduced numbers of eosinophils recruited to the airway. In addition, PI3K γ -deficient mice had similar levels of IL-5 and eotaxin-1 compared with WT mice. Thus, reduced chemokine-induced migration of eosinophils from the circulation into the airway due to an inability of PI3K γ -deficient eosinophils to respond to chemokine signals is likely to account for reduced numbers of eosinophils in BAL and the peribronchial region. The contribution of eosinophils to increased airway hyperreactivity is suggested from studies in IL-5-deficient mice (12), as well as in PHIL mice (19), which lack eosinophils. Although the reduced eosinophil influx may have contributed to the reduced airway hyperreactivity noted in PI3K γ -deficient mice, other explanations are also possible. For example, the reduced amount of peribronchial smooth muscle as assessed by α -smooth muscle actin staining may have also contributed to reduced airway responsiveness. Interestingly, studies of human airway smooth muscle have shown that TGF- β -induced mitogenesis of airway smooth muscle can be inhibited by pharmacological inhibitors of PI3K such as LY-294002 (32). PI3K has been implicated in induction of cell growth and regulation of cyclin-dependent kinase activity (32).

In addition to having reduced levels of eosinophilic airway inflammation, PI3K γ -deficient mice also had reduced levels of peribronchial fibrosis as assessed by trichrome staining and lung collagen levels. As previous studies have demonstrated that TGF- β 1 and the Smad 2/3 signaling pathway play an important role in mediating allergen-induced peribronchial fibrosis (18, 23), we examined whether the reduced levels of peribronchial fibrosis in PI3K γ -deficient mice were associated with reduced numbers of peribronchial TGF- β 1+ cells and reduced Smad 2/3 signaling. PI3K γ -deficient mice had significantly reduced numbers of peribronchial TGF- β 1+ cells, which is likely a reflection of the reduced number of eosino-

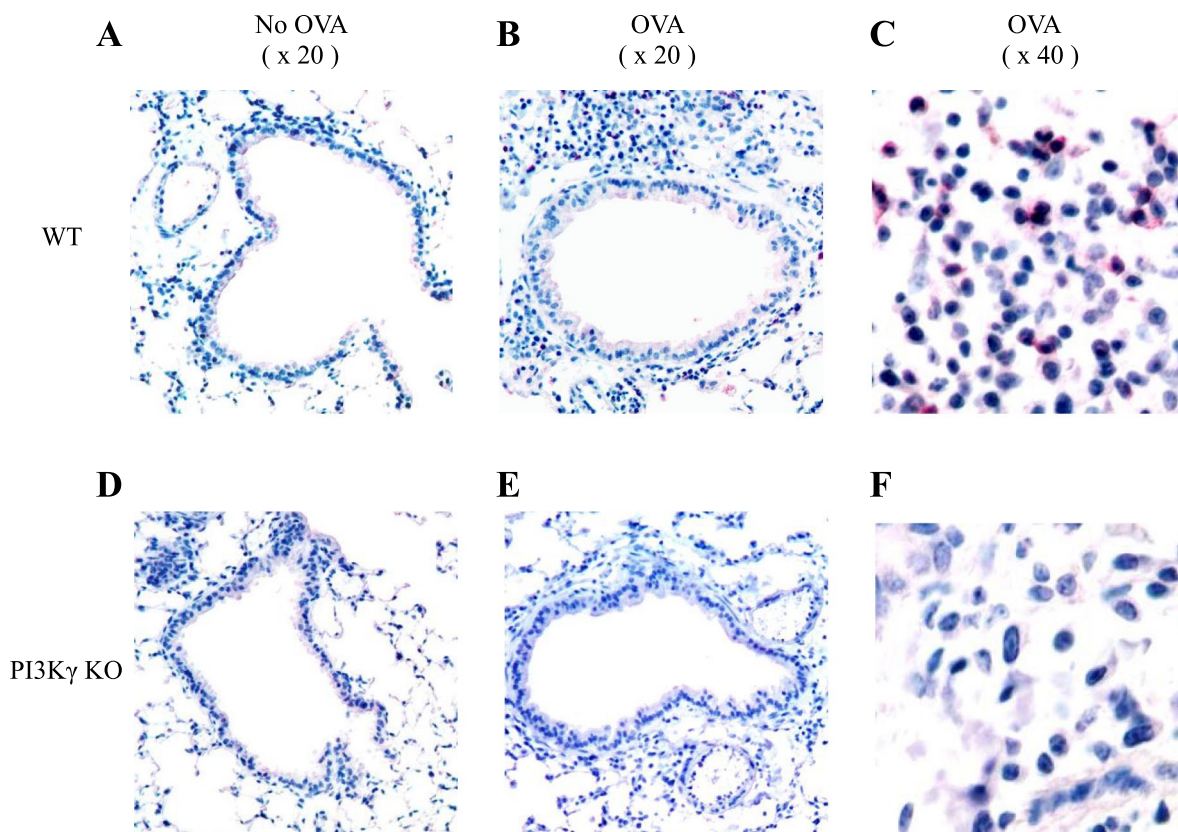


Fig. 7. Immunostaining of lung sections with an anti-PI3K γ Ab. Lungs from WT mice (A–C) or PI3K γ -deficient mice (D–F) were immunostained with an anti-PI3K γ Ab directed against the p110 γ -component of PI3K γ using the immunoperoxidase method. OVA-challenged WT mice (C) had peribronchial cells that immunostained positive for PI3K γ .

phils expressing TGF- β 1 recruited to the airway. Both murine (7) and human studies (11, 14) of airway remodeling in asthma have demonstrated that airway remodeling is associated with an increased number of eosinophils in the airway that express TGF- β 1. Moreover, studies in which eosinophils have been depleted using IL-5-deficient mice (7) or anti-IL-5 therapy in human asthmatics (11) have both demonstrated that reducing levels of airway eosinophils is associated with reduced levels of TGF- β 1 and reduced levels of airway remodeling. In addition to exhibiting reduced numbers of peribronchial TGF- β 1+ cells, PI3K γ -deficient mice had significantly reduced signaling through Smad 2/3 as quantitated by the number of pSmad 2/3+ cells in the airway epithelium and peribronchial region. The importance of Smad 2/3 signaling to airway remodeling is suggested from studies of allergen-challenged Smad 2/3-deficient mice, which have significantly reduced levels of airway remodeling (18). Interestingly, Smad 2/3-deficient mice do not have the reduced levels of airway responsiveness exhibited by PI3K γ -deficient mice, suggesting that PI3K γ contributes to airway responsiveness through a Smad 2/3-independent pathway.

In addition to studies demonstrating that eosinophils express TGF- β 1 (7, 11), there are also studies in mouse models demonstrating that epithelial cells (1, 7), as well as other cell types (macrophages, mast cells) (7), may contribute to the levels of TGF- β 1 detected. While several studies suggest an important role for TGF- β 1 and Smad 2/3 in mediating airway remodeling (18, 23), there are also studies that demonstrate that an anti-TGF- β 1 Ab does not reduce remodeling in mice (10),

and that an anti-TGF- β 1 Ab increase airway hyperreactivity in mice (1). The differences in the results of these studies may be due to differences in the mouse models used in terms of antigen dose, route of antigen delivery (intranasal vs. nebulized), duration of antigen administration (weeks vs. months), and type of antigen used (OVA vs. house dust mite extract) in the different studies (1, 7, 10, 18, 23).

Potential explanations for incomplete inhibition of eosinophil recruitment to the lung in PI3K γ -deficient mice include the possibility that different isoforms of PI3K distinct from the class Ib PI3K γ (i.e., class Ia PI3K such as PI3K α , PI3K β , and PI3K δ isoforms) mediate residual effects in PI3K γ -deficient mice. In addition, it is possible that not all chemokine signaling is dependent on PI3K and that PI3K-independent pathways exist for eosinophil recruitment. Similar explanations may also explain incomplete inhibition of remodeling in PI3K γ -deficient mice. The majority of class Ia PI3K is widely expressed with PI3K α , PI3K β being ubiquitously expressed (27), whereas PI3K δ is detected in leukocytes, breast tissue, and melanocytes (27). Genetic ablation in mice of either PI3K α or PI3K β results in embryonic lethality, whereas PI3K δ -deficient mice are viable (27). As PI3K γ is also expressed by other leukocytes, it is also possible that PI3K γ effects on cell types other than eosinophils and/or smooth muscle could have contributed to the reduced airway remodeling we have observed in PI3K γ -deficient mice. For example, although mast cell Fc ϵ RI do not directly activate PI3K γ , PI3K γ can amplify mast cell function through autocrine and

paracrine loops involving activation of G protein-coupled mast cell receptors by adenosine and/or chemokines (17). In addition, PI3K γ -deficient neutrophils and macrophages exhibit reduced chemotaxis (27). Thus, our studies are able demonstrate that PI3K γ is important to the development of airway responsiveness, eosinophilic inflammation, and airway remodeling, but are not able to determine which cell type(s) expressing PI3K γ are mediating this effect, as such studies would require selective inactivation of PI3K γ in individual leukocyte subtypes.

While this study has demonstrated an important role for PI3K γ (a class Ib PI3K) in airway remodeling and eosinophil recruitment to the airway, previous studies have examined the importance of class Ia PI3K (such as PI3K δ as well as the p85 regulatory subunit of class Ia PI3K) (9, 15, 20, 25, 26) in models of asthma. Studies of PI3K δ -deficient mice acutely challenged with OVA exhibit reduced eosinophilic inflammation and mucus expression (26). In addition to studies using PI3K-deficient mice, studies have also targeted PI3K using pharmacological or adenoviral construct inhibitors of PI3K in animal models of asthma (16, 25). For example, intratracheal administration of pharmacological inhibitors of PI3K (wortmannin or LY-294002) or adenoviruses carrying PTEN cDNA (AdPTEN) inhibit allergen-induced airway inflammation in mouse models (16). Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) is part of a complex signaling system that blocks the action of PI3K by dephosphorylating the signaling lipid phosphatidylinositol 3,4,5-triphosphate (16). A limitation of these studies in determining the role of class Ia compared with class Ib PI3K is that pharmacological inhibitors and adenoviruses carrying PTEN cDNA are not specific for individual PI3K (i.e., PI3K γ , PI3K δ , etc.), and pharmacological inhibitors of PI3K are also known to inhibit signaling pathways other than PI3K (22). The role of the p85 regulatory subunit of the class Ia PI3K in antigen-induced airway inflammation has been studied in mice using a dominant negative form of p85 fused to HIV-TAT (TAT-p85) (25). These studies have demonstrated that administration of TAT-p85 targeting the class Ia PI3K reduced eosinophilic inflammation and airway responsiveness (25). Our study extends these observations to demonstrate that not only class Ia PI3K (i.e., the p85 regulatory subunit) (25) but also a structurally distinct class Ib PI3K, such as PI3K γ , is a potential target for reducing not only airway responsiveness and eosinophilic inflammation but also significantly reducing airway remodeling, an observation not previously reported with inhibiting any PI3K.

A limitation of all studies in which genetically manipulated mice are used, including this study, is the unknown effect of the gene deletion on development. The PI3K γ -deficient mice we used had morphologically normal lungs, but this does not rule out a compensatory enzyme or other effect induced during development in PI3K γ -deficient mice. Alternative strategies to target PI3K γ , such as using neutralizing Abs, would not be effective in targeting an intracellular enzyme such as PI3K γ , while pharmacological inhibitors of PI3K inhibit signaling pathways other than PI3K (22) and are also not specific for individual PI3K isoforms.

In summary, in this study we have utilized PI3K γ -deficient mice to demonstrate an important role for this class Ib PI3K in mediating allergen-induced eosinophilic airway inflammation and airway remodeling. Furthermore, we demonstrate that

PI3K γ -deficient mice have reduced eosinophil influx from the blood stream into the airway as PI3K γ -deficient mice have similar peripheral blood and bone marrow eosinophil responses to allergen challenge as do WT mice. This reduced eosinophil trafficking to the airway was associated with reduced number of TGF- β 1+ peribronchial cells and reduced Smad 2/3 signaling, suggesting that this may have contributed to the reduced peribronchial fibrosis noted. In addition, PI3K γ -deficient mice have significantly reduced levels of peribronchial smooth muscle in response to chronic allergen challenge suggesting that PI3K γ , through effects on reducing inflammation and/or direct effects on smooth muscle, may contribute to reduced airway responsiveness. The reduction in eosinophilic inflammation and airway remodeling noted in PI3K γ -deficient mice suggests that PI3K γ may be a novel therapeutic target in asthma.

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