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# Phosphoinositide 3-kinase δ inhibitor suppresses interleukin-17 expression in a murine asthma model

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**ABSTRACT: Phosphoinositide 3-kinases (PI3Ks) contribute to the pathogenesis of asthma by regulating the activation of inflammatory mediators, inflammatory cell recruitment and immune cell function. Recent findings have indicated that PI3Ks also regulate the expression of interleukin (IL)-17, which has been recognised as an important cytokine involved in airway inflammation.**

**In the present study, we investigated a role of PI3Kδ in the regulation of IL-17 expression in allergic airway disease using a murine model of asthma.**

**After ovalbumin inhalation, administration of a selective p110δ inhibitor, IC87114, significantly attenuated airway infiltration of total cells, lymphocytes, neutrophils and eosinophils, as well as airway hyperresponsiveness, and attenuated the increase in IL-17 protein and mRNA expression. Moreover, IC87114 reduced levels of IL-4, -5 and -13, expression of keratinocyte chemoattractant protein and mRNA, and nuclear factor (NF)-κB activity. In addition, a NF-κB inhibitor, BAY 11-7085 substantially reduced the increase in IL-17 protein levels. Our results also showed that inhibition of IL-17 activity with an anti-IL-17 antibody remarkably reduced airway inflammation and hyperresponsiveness.**

**These findings suggest that inhibition of the p110δ signalling pathway suppresses IL-17 expression through regulation of NF-κB activity and, thus, has therapeutic potential in asthma.**

**KEYWORDS: Asthma, interleukin-17, nuclear factor-κB, phosphoinositide 3-kinase, p110δ**

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Asthma is a chronic inflammatory disease of the airways characterised by reversible airway obstruction and airway hyperresponsiveness [1]. Airway inflammation in asthma increases expression of various components of the inflammatory cascade, which include cytokines, chemokines, adhesion molecules, growth factors and enzymes. Central to this inflammatory cascade is a complex interaction between receptor signalling, and downstream lipid and protein kinases. In particular, phosphoinositide 3-kinase (PI3K) is an important kinase involved in the inflammatory process.

The PI3K is a large family of signalling kinases that generate lipid second messengers responsible for regulating a number of cellular activities. PI3Ks mediate key signal transduction reactions during immune and inflammatory responses and, thus, represent an attractive target for therapeutic development in various inflammatory diseases [2, 3]. Research conducted over the past few years has highlighted the importance of PI3Ks in bronchial asthma. Our previous study with a murine model of asthma has shown that PI3K activity increases after ovalbumin (OVA) challenge compared with the pre-challenge period [4]. In addition, administration of PI3K inhibitor, wortmannin or LY-294002, reduces T-helper cell (Th) type 2 cytokine production, pulmonary eosinophilia, airway inflammation and bronchial hyperresponsiveness [4]. There is increasing evidence that PI3Ks contribute to the pathogenesis of asthma by regulating the expression and activation of inflammatory mediators, inflammatory cell recruitment and immune cell function [2, 4]. Recent work has begun to explore specific roles of different PI3K isoforms, and p110δ and p110γ have been suggested to be the dominant isoforms involved in PI3K-mediated immune responses [5]. Two commercially available PI3K inhibitors used to investigate the biological roles of PI3Ks and to analyse PI3K-driven pathways, wortmannin and LY-294002, are poorly selective among the four

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members of PI3K class I. Therefore, development of PI3K isoform-specific inhibitors has been an area of considerable interest. A selective inhibitor of p110δ catalytic activity, IC87114, has recently been synthesised and used to explore the role of PI3Kδ in neutrophil migration [6, 7]. In addition, previous studies using IC87114 have suggested that p110δ plays an important role in the pathogenesis of allergic asthma [8, 9].

The interleukin (IL)-17 family is a recently described group of cytokines that consists of six members, namely IL-17 (also called IL-17A), IL-17B, IL-17C, IL-17D, IL-17E and IL-17F [10]. IL-17, the most extensively investigated member of this family, has been implicated in many immune and inflammatory responses, primarily as a proinflammatory regulator, inducing expression of many inflammatory mediators, such as cytokines, chemokines, adhesion molecules and growth factors [11, 12]. IL-17 mRNA is upregulated in inflamed airways after allergen inhalation in a mouse model of allergic asthma [13]. Consistent with the animal study, IL-17 mRNA and/or protein expression is increased in sputum, bronchoalveolar lavage (BAL) fluids and peripheral blood in asthmatics [14–18]. Moreover, the levels of IL-17 expression in airways correlate with the severity of airway hypersensitivity in asthmatic patients [14]. These findings have indicated the potential role of IL-17 in the pathogenesis of asthma. Recent studies, including our own, have found that IL-17 production is mediated by the activation of PI3K/Akt pathway [12, 19–21]. However, the contributions of individual PI3K isoforms to the up-regulation of IL-17 expression have not been examined. In the present study, we investigated an involvement of PI3Kδ in IL-17 expression and its molecular mechanism using a murine model of asthma.

## MATERIALS AND METHODS

A more detailed methodology is available in the online supplementary material.

### Animals and experimental protocol

Female C57BL/6 mice, 8–10 weeks of age and free of murine-specific pathogens, were obtained from Orientbio Inc. (Seoungnam, South Korea). All animal experiments were approved by the Institutional Animal Care and Use Committee (Chonbuk National University, Jeonju, South Korea). Mice were sensitised on days 1 and 14 by intraperitoneal injection of 20 μg 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 μL, as described previously [8, 9]. On days 21, 22 and 23 after the initial sensitisation, the mice were challenged for 30 min with an aerosol of 3% (weight/volume) OVA in saline (or saline alone as a control) using an ultrasonic nebuliser (NE-U12; Omron, Tokyo, Japan). BAL was performed 48 h after the last challenge. At the time of lavage, mice were sacrificed with an overdose of sodium pentobarbital (100 mg per kg of body weight, administered intraperitoneally).

### Administration of IC87114, LY-294002, anti-IL-17 antibody, isotype-control monoclonal antibody, Akt inhibitor or BAY 11-7085

IC87114 (0.1 or 1 $mg \cdot kg^{-1} \cdot day^{-1}$), LY-294002 (a selective inhibitor of PI3K; 1.5 $mg \cdot kg^{-1} \cdot day^{-1}$; BIOMOL Research Laboratories Inc., Plymouth Meeting, PA, USA) or vehicle control (dimethylsulfoxide (DMSO)) diluted with 0.9% NaCl, was administered in a volume of 50 μL by intratracheal instillation to each animal, once on day 21 (1 h before the first OVA challenge) and again on day 23 (3 h after the last OVA challenge) [8, 9]. Anti-IL-17 antiobody (Ab) or isotype-control monoclonal (m)Ab (5 $mg \cdot kg^{-1} \cdot day^{-1}$; R&D Systems, Inc., Minneapolis, MN, USA) was administered intraperitoneally to each animal, once on day 21 and again on day 24 (24 h after the last OVA challenge). An Akt inhibitor, 1L6-hydroxymethyl-chiro-inositol-2-(R)-2-*O*-methyl-3-*O*-octadecyl-*sn*-glycerocarbonate (3 $mg \cdot kg^{-1} \cdot day^{-1}$; Calbiochem, Darmstadt, Germany), dissolved in DMSO and diluted with 0.9% NaCl, was administered by intraperitoneal injection to each animal, once on day 21 and again on day 24. An inhibitor of nuclear factor (NF)-κB activation, BAY 11-7085 (20 $mg \cdot kg^{-1} \cdot day^{-1}$; BIOMOL Research Laboratories Inc.), dissolved in DMSO and diluted with 0.9% NaCl, was administered by intraperitoneal injection to each animal, once on day 21 and again on day 24.

### Western blot analysis

Protein expression levels were analysed by means of Western blot analysis, as described previously [8]. The primary antibodies used were: anti-IL-17 Ab (R&D Systems, Inc.), anti-IL-4 Ab (Serotec Ltd, Oxford, UK), anti-IL-5 Ab (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-IL-13 Ab (R&D Systems, Inc.), biotinylated anti-mouse keratinocyte chemoattractant (KC) Ab (R&D Systems, Inc.), anti-Akt Ab (Cell Signaling Technology Inc., Beverly, MA, USA), anti-phosphorylated Akt (p-Akt) Ab (Cell Signaling Technology Inc.), and anti-inhibitor of κBα (I-κBα) Ab (Santa Cruz Biotechnology).

### RNA isolation and RT-PCR

Levels of mRNA expression were analysed by RT-PCR assay using total RNA isolated from lung tissues by a rapid extraction method (TRI-Reagent; Sigma–Aldrich) as previously described [12].

### Quantitative real-time RT-PCR

Quantitative real-time RT-PCR analysis was performed using the LightCycler® FastStart DNA Master SYBR Green I (Roche Diagnostics, Mannheim, Germany) as described previously [12].

### Measurement of Th2 cytokines

Levels of IL-4, IL-5 and IL-13 were quantified by enzyme immunoassays (R&D Systems, Inc.).

### Cytosolic or nuclear protein extractions for analysis of NF-κB p65

Cytosolic or nuclear extractions were performed as described previously [9].

### Measurement of PI3K enzyme activity in lung tissues

PI3K enzyme activity in lung tissues was measured as described previously [9]. Phosphatidylinositol (3,4,5)-trisphosphate ($PIP_3$) was quantified using a $PIP_3$ competition enzyme immunoassay (Echelon, Inc., Salt Lake City, UT, USA).

### Determination of airway responsiveness

Airway responsiveness was assessed as a change in airway function after challenge with aerosolised methacholine, as described previously [9].

### Densitometric analyses and statistics

All immunoreactive and phosphorylation signals were analysed by densitometric scanning (Gel Doc XR; Bio-Rad Laboratories Inc., Hercules, CA, USA). Data are presented as mean ± SEM. Statistical comparisons were performed using one-way ANOVA followed by Scheffe's test. Statistical significance was set at $p<0.05$.

## RESULTS

### Effect of IC87114 on IL-17 protein levels and mRNA expression in lung tissues of OVA-sensitised and OVA-challenged mice

Western blot analysis revealed that the levels of IL-17 protein in lung tissues were significantly increased 48 h after the last inhalation of OVA compared with those in the control mice (figs 1a and 1c). The increase in the IL-17 protein levels after OVA inhalation was attenuated significantly by the administration of IC87114. RT-PCR and real-time RT-PCR analysis showed that the increase in the IL-17 mRNA expression after OVA inhalation was significantly reduced by the administration of IC87114 (figs 1b and 1d).

### Effect of IC87114 on IL-17 protein levels in BAL fluids of OVA-sensitised and OVA-challenged mice

Western blot analysis revealed that the levels of IL-17 protein in BAL fluids were significantly increased 48 h after OVA inhalation compared with those in the control mice (fig. 2). The increase in the IL-17 protein levels after OVA inhalation was significantly inhibited by the administration of IC87114.

### Effect of IC87114 or anti-IL-17 Ab on cellular changes in BAL fluids

Numbers of total cells, lymphocytes, neutrophils and eosinophils in BAL fluids were increased significantly 48 h after OVA inhalation compared with the numbers after saline inhalation (fig. 3a). The increases in the numbers of total cells, lymphocytes, neutrophils and eosinophils were significantly reduced by the administration of IC87114. In addition, administration of anti-IL-17 Ab to OVA-sensitised and -challenged mice significantly decreased the increases in the numbers of total cells, lymphocytes, neutrophils and eosinophils compared with the numbers in OVA-nebulised mice administered drug vehicle or isotype-control mAb.

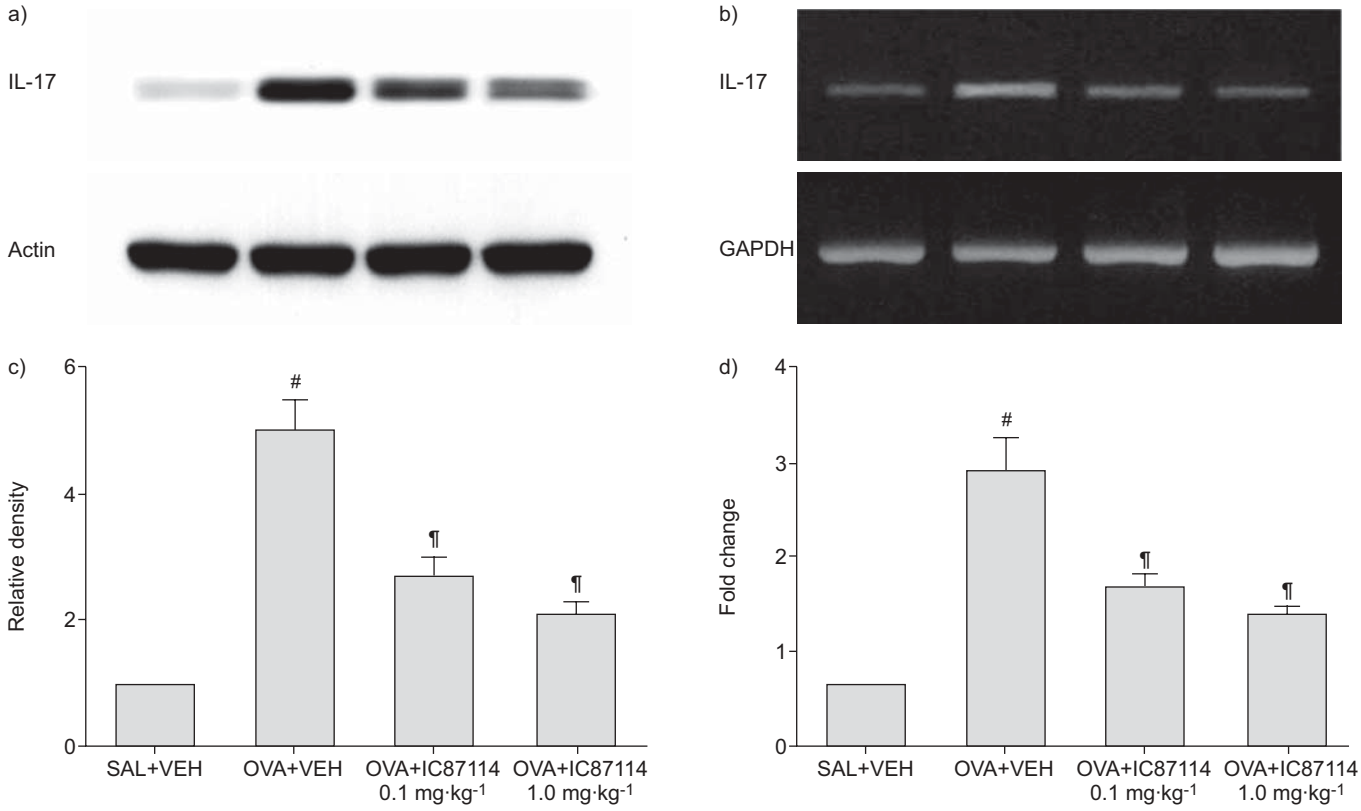


**FIGURE 1.** Effect of IC87114 on interleukin (IL)-17 protein levels and mRNA expression in lung tissues of ovalbumin (OVA)-sensitised and OVA-challenged mice. Sampling was performed 48 h after the last challenge in saline-nebulised mice administered drug vehicle (dimethylsulfoxide) (SAL+VEH), OVA-nebulised mice administered drug vehicle (OVA+VEH), OVA-nebulised mice administered 0.1 mg·kg$^{-1}$ IC87114 (OVA+IC87114 0.1 mg·kg$^{-1}$), and OVA-nebulised mice administered 1.0 mg·kg$^{-1}$ IC87114 (OVA+IC87114 1.0 mg·kg$^{-1}$). a) Western blot analyses of IL-17 protein. b) Representative RT-PCR analyses of IL-17 mRNA expression. c) Densitometric analyses are presented as the relative ratio of IL-17 in OVA+VEH, OVA+IC87114 1.0 mg·kg$^{-1}$ to those in SAL+VEH. The relative ratio of IL-17 in the lung tissues of SAL+VEH is arbitrarily presented as 1. d) Quantitative analyses of IL-17 mRNA expression by means of real-time RT-PCR. Data represent mean ± SEM from seven mice per group. GAPDH: glyceraldehyde-3-phosphate dehydrogenase. #: $p<0.05$ *versus* SAL+VEH; ¶: $p<0.05$ *versus* OVA+VEH.

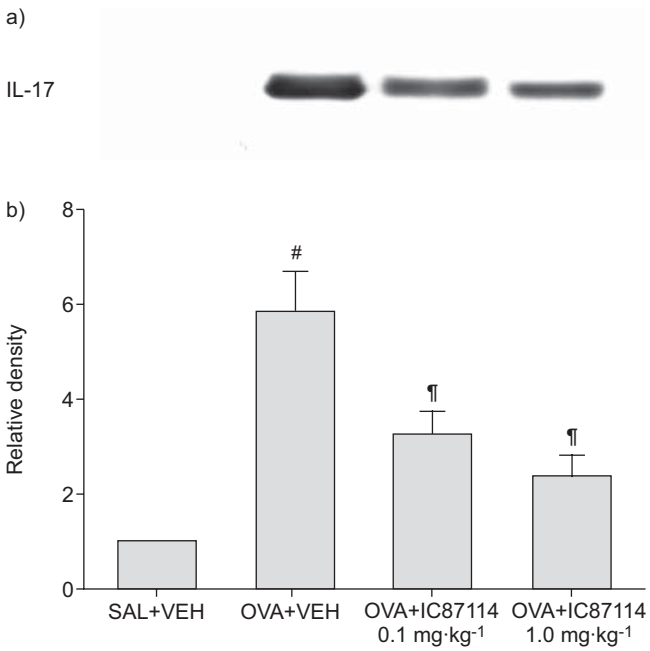


**FIGURE 2.** Effect of IC87114 on interleukin (IL)-17 protein levels in bronchoalveolar lavage fluids of ovalbumin (OVA)-sensitised and OVA-challenged mice. Sampling was performed 48 h after the last challenge in saline-nebulised mice administered drug vehicle (SAL+VEH), OVA-nebulised mice administered drug vehicle (OVA+VEH), OVA-nebulised mice administered 0.1 mg·kg$^{-1}$ IC87114 (OVA+IC87114 0.1 mg·kg$^{-1}$), and OVA-nebulised mice administered 1.0 mg·kg$^{-1}$ IC87114 (OVA+IC87114 1.0 mg·kg$^{-1}$). a) Western blot analyses of IL-17 protein. b) Densitometric analyses are presented as the relative ratio of IL-17 to actin. The relative ratio of IL-17 in the lung tissues of SAL+VEH is arbitrarily presented as 1. Data are presented as mean±SEM of seven mice per group. #: $p<0.05$ *versus* SAL+VEH; ¶: $p<0.05$ *versus* OVA+VEH.

### *Effect of IC87114 or anti-IL-17 Ab on pathological changes of OVA-induced asthma*

Histological analysis revealed the typical pathological features of asthma in the OVA-exposed mice. Numerous inflammatory cells infiltrated around the bronchioles, and mucus and debris had accumulated in the lumens of bronchioles (fig. 3c) compared with the control (fig. 3b). Mice treated with IC87114 (fig. 3d) or anti-IL-17 Ab (fig. 3e) showed marked reductions in the infiltration of inflammatory cells in the peribronchiolar region and in the amount of debris in the airway lumens.

### *Effect of IC87114 or anti-IL-17 Ab on airway hyperresponsiveness*

The airway responsiveness was assessed as a percentage increase in the resistance of the respiratory system ($R_{rs}$) in response to increasing doses of methacholine. In OVA-sensitised and -challenged mice, the dose–response curve of percentage $R_{rs}$ shifted to the left compared with that of the control mice (fig. 3f). In addition, the percentage $R_{rs}$ produced by methacholine administration (at a dose of 50 mg·mL$^{-1}$) increased significantly in the OVA-nebulised mice compared with the control mice. OVA-sensitised and -challenged mice treated with IC87114 showed a dose–response curve of percentage $R_{rs}$ was that shifted to the right and a significant reduction in the percentage $R_{rs}$ produced by 50 mg·mL$^{-1}$ methacholine compared with those of untreated mice. In addition, the administration of anti-IL-17 Ab to OVA-sensitised and -challenged mice also decreased significantly the percentage $R_{rs}$ produced by 50 mg·mL$^{-1}$ methacholine compared with that of OVA-nebulised mice administered drug vehicle or isotype-control mAb. These results indicate that treatment of IC87114 or anti-IL-17 Ab reduces OVA-induced airway hyperresponsiveness.

### *Effect of IC87114 on levels of IL-4, IL-5 and IL-13 in lung tissues and BAL fluids of OVA-sensitised and OVA-challenged mice*

Western blot analysis revealed that the protein levels of IL-4, IL-5 and IL-13 in lung tissues were increased 48 h after OVA inhalation compared with those in the control mice (fig. 1Sa in the online supplementary material). The increases in these cytokine levels were reduced by the administration of IC87114. Consistent with the Western blot analysis results, enzyme immunoassays of IL-4, IL-5 and IL-13 showed that the levels of these cytokines in BAL fluids were significantly reduced by the administration of IC87114 (fig. 1Sb in the online supplementary material).

### *Effect of IC87114 on KC protein levels and mRNA expression in lung tissues of OVA-sensitised and OVA-challenged mice*

Western blot analysis revealed that the levels of KC protein in lung tissues were significantly increased 48 h after OVA inhalation compared with those in the control mice (fig. 4a and c). The increase in the KC protein levels after OVA inhalation was decreased significantly by the administration of IC87114. RT-PCR and real-time RT-PCR analysis showed that the increase in the KC mRNA expression after OVA inhalation was significantly reduced by the administration of IC87114 (fig. 4e and g).

### *Effect of anti-IL-17 Ab on KC protein levels and mRNA expression in lung tissues of OVA-sensitised and OVA-challenged mice*

Western blot analysis revealed that administration of anti-IL-17 Ab to OVA-sensitised and -challenged mice significantly decreased the levels of KC protein in lung tissues compared with the levels in OVA-inhaled mice administered isotype control mAb (fig. 4b and d). In addition, RT-PCR and real-time RT-PCR analysis showed that the administration of anti-IL-17 Ab to OVA-sensitised and -challenged mice significantly decreased the KC mRNA expression compared with that in OVA-nebulised mice administered isotype-control mAb (fig. 4f and h).

### *Effect of LY-294002 or IC87114 on p-Akt and Akt protein levels, and PI3K enzyme activity in lung tissues of OVA-sensitised and OVA-challenged mice*

The levels of p-Akt protein in the lung tissues were significantly increased at 48 h after OVA inhalation compared with levels in the control mice (fig. 5a and c). However, no significant changes in the Akt protein levels were observed in any of the groups tested. The increase of p-Akt but not Akt protein levels in the lung tissues 48 h after OVA inhalation was

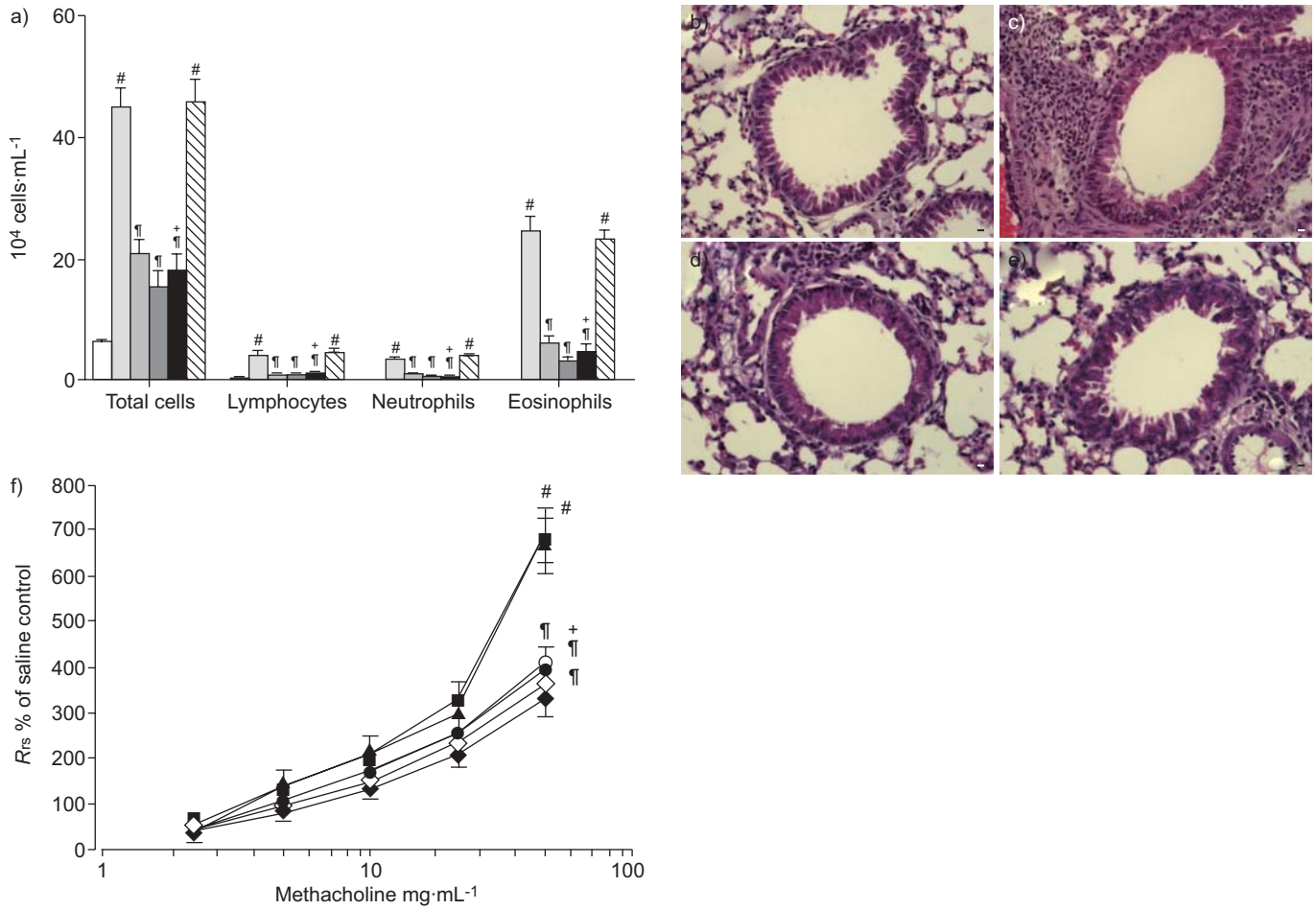


**FIGURE 3.** a) Effect of IC87114 or anti-interleukin-17 antibody (anti-IL-17 Ab) on total and differential cell counts in bronchoalveolar lavage (BAL) fluids of ovalbumin (OVA)-sensitised and OVA-challenged mice. Differential cell counts in BAL fluids from saline-nebulised mice administered drug vehicle (SAL+VEH; □), OVA-inhaled mice administered drug vehicle (OVA+VEH; ■), OVA-nebulised mice administered 0.1 mg·kg$^{-1}$ IC87114 (OVA+IC87114 0.1 mg·kg$^{-1}$; ■), OVA-nebulised mice administered 1.0 mg·kg$^{-1}$ IC87114 (OVA+IC87114 1.0 mg·kg$^{-1}$; ■), OVA-nebulised mice administered anti-IL-17 Ab (OVA+anti-IL-17 Ab; ■), and OVA-nebulised mice administered isotype-control monoclonal Ab (mAb) (OVA+control mAb; ▧). b–e) Effect of IC87114 or anti-IL-17 Ab in lung tissues of OVA-sensitised and OVA-challenged mice. Representative haematoxylin and eosin-stained sections of the lungs. Sampling was performed 48 h after the last challenge in b) SAL+VEH, c) OVA+VEH, d) OVA+IC87114 0.1 mg·kg$^{-1}$ and e) OVA+anti-IL-17 Ab mice. Scale bars=20 μm. Results were similar in seven mice per group. f) Effect of IC87114 or anti-IL-17 Ab on airway responsiveness in OVA-sensitised and OVA-challenged mice. Airway responsiveness was measured 48 h after the last challenge in SAL+VEH (◆), OVA+VEH (■), OVA+IC87114 0.1 mg·kg$^{-1}$ (○), OVA+IC87114 1.0 mg·kg$^{-1}$ (◇), OVA+anti-IL-17 Ab (●) and OVA+control mAb (▲). Data are presented as mean±SEM from seven mice per group. $R_{rs}$: resistance of respiratory system. #: $p<0.05$ *versus* SAL+VEH; ¶: $p<0.05$ *versus* OVA+VEH; +: $p<0.05$ *versus* OVA+control mAb.

significantly reduced by the administration of LY-294002 or IC87114. Consistent with these results, $PIP_3$ levels in the lung tissues were significantly increased 48 h after OVA inhalation compared with those in the control mice (fig. 5e). The increase of the $PIP_3$ levels in the lung tissues 48 h after OVA inhalation was significantly reduced by the administration of LY-294002 or IC87114.

### *Effect of LY-294002 or IC87114 on I-κBα protein levels in lung tissues of OVA-sensitised and OVA-challenged mice*

Western blot analysis revealed that the I-κBα protein levels in the lung tissues were significantly decreased 48 h after OVA inhalation compared with those in the control mice (fig. 5b and d). The decrease in the I-κBα protein levels after OVA inhalation was significantly inhibited by the administration of LY-294002 or IC87114.

### *Effect of Akt inhibitor on I-κBα and NF-κB p65 protein levels in lung tissues of OVA-sensitised and OVA-challenged mice*

The I-κBα protein levels decreased after OVA inhalation was significantly increased by the administration of Akt inhibitor (fig. 6a and c). Western blot analysis revealed that the levels of NF-κB p65 in nuclear protein extracts from the lung tissues were increased 48 h after OVA inhalation compared with the levels in the control mice administered saline (fig. 6b and 6d). The administration of Akt inhibitor significantly reduced the increase in the NF-κB p65 levels in nuclear protein extracts after OVA inhalation. In contrast, the levels of NF-κB p65 in cytosolic protein fractions from the lung tissues were decreased after OVA inhalation compared with the levels in the control mice administered saline. The decrease in the NF-κB p65 levels in cytosolic protein fractions was substantially inhibited by the administration of Akt inhibitor.

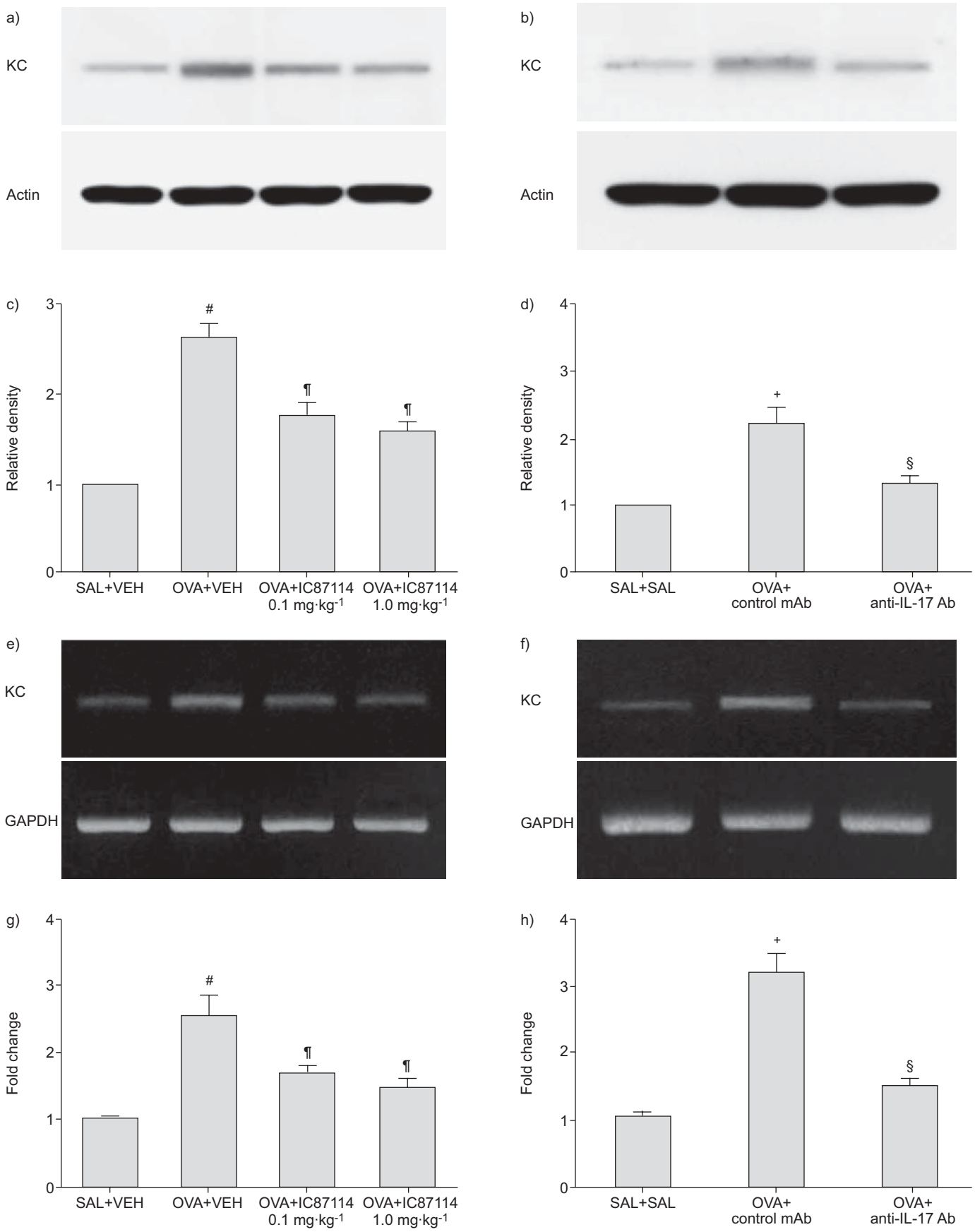
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**FIGURE 4.** Effect of IC87114 or anti-interleukin-17 antibody (anti-IL-17 Ab) on keratinocyte chemoattractant (KC) protein levels and mRNA expression in lung tissues of ovalbumin (OVA)-sensitised and OVA-challenged mice. a, c, e, g) Sampling was performed 48 h after the last challenge in saline-nebulised mice administered drug vehicle (SAL+VEH), OVA-nebulised mice administered drug vehicle (OVA+VEH), OVA-nebulised mice administered 0.1 mg·kg$^{-1}$ IC87114 (OVA+IC87114 0.1 mg·kg$^{-1}$) and OVA-inhaled mice administered 1.0 mg·kg$^{-1}$ IC87114 (OVA+IC87114 1.0 mg·kg$^{-1}$). b, d, f, h) Sampling was performed 48 h after the last challenge in saline-inhaled mice administered saline (SAL+SAL), OVA-nebulised mice administered isotype-control monoclonal Ab (OVA+control mAb) and OVA-nebulised mice administered anti-IL-17 Ab (OVA+anti-IL-17 Ab). a and b) Western blot analyses of KC protein. c and d) Densitometric analyses are presented as the relative ratio of KC to actin. The relative ratio of KC in the lung tissues of SAL+VEH (c) or SAL+SAL (d) is arbitrarily presented as 1. e and f) Representative RT-PCR analyses of KC mRNA expression. g and h) Quantitative analyses of KC mRNA expression by means of real-time RT-PCR. Data are presented as mean±SEM of seven mice per group. GAPDF: glyceraldehyde-3-phosphate dehydrogenase. #: $p<0.05$ *versus* SAL+VEH; ¶: $p<0.05$ *versus* OVA+VEH; +: $p<0.05$ *versus* SAL+SAL; §: $p<0.05$ *versus* OVA+control mAb.

### *Effect of IC87114 on NF-κB p65 protein levels in lung tissues of OVA-sensitised and OVA-challenged mice*

The increase in the NF-κB p65 levels in nuclear protein extracts of the lung tissues after OVA inhalation was significantly reduced by the administration of IC87114 (fig. 7). In contrast, the decrease in the NF-κB p65 levels in cytosolic protein fractions of the lung tissues was substantially inhibited by the administration of IC87114.

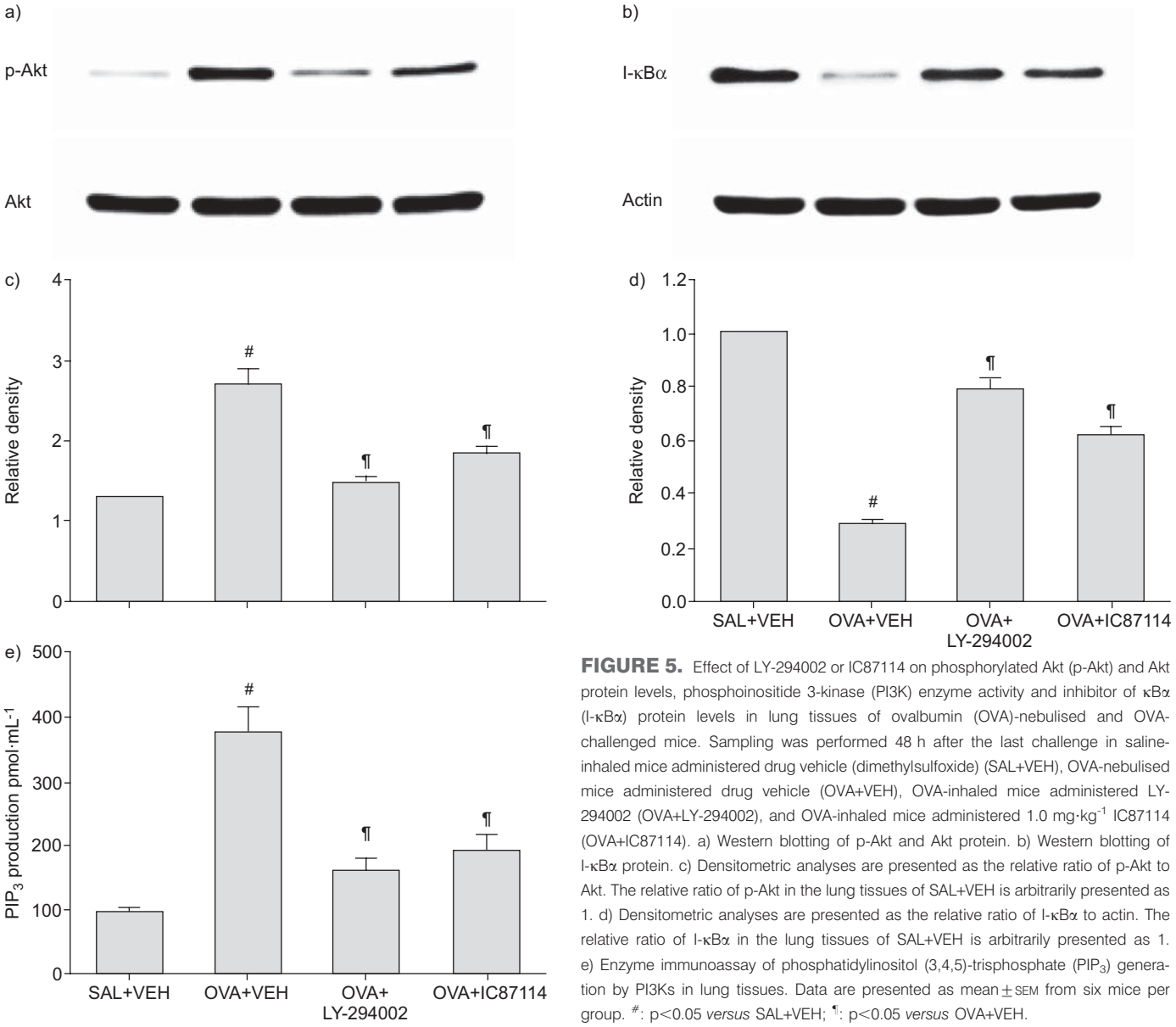


**FIGURE 5.** Effect of LY-294002 or IC87114 on phosphorylated Akt (p-Akt) and Akt protein levels, phosphoinositide 3-kinase (PI3K) enzyme activity and inhibitor of κBα (I-κBα) protein levels in lung tissues of ovalbumin (OVA)-nebulised and OVA-challenged mice. Sampling was performed 48 h after the last challenge in saline-inhaled mice administered drug vehicle (dimethylsulfoxide) (SAL+VEH), OVA-nebulised mice administered drug vehicle (OVA+VEH), OVA-inhaled mice administered LY-294002 (OVA+LY-294002), and OVA-inhaled mice administered 1.0 mg·kg$^{-1}$ IC87114 (OVA+IC87114). a) Western blotting of p-Akt and Akt protein. b) Western blotting of I-κBα protein. c) Densitometric analyses are presented as the relative ratio of p-Akt to Akt. The relative ratio of p-Akt in the lung tissues of SAL+VEH is arbitrarily presented as 1. d) Densitometric analyses are presented as the relative ratio of I-κBα to actin. The relative ratio of I-κBα in the lung tissues of SAL+VEH is arbitrarily presented as 1. e) Enzyme immunoassay of phosphatidylinositol (3,4,5)-trisphosphate ($PIP_3$) generation by PI3Ks in lung tissues. Data are presented as mean±SEM from six mice per group. #: $p<0.05$ *versus* SAL+VEH; ¶: $p<0.05$ *versus* OVA+VEH.

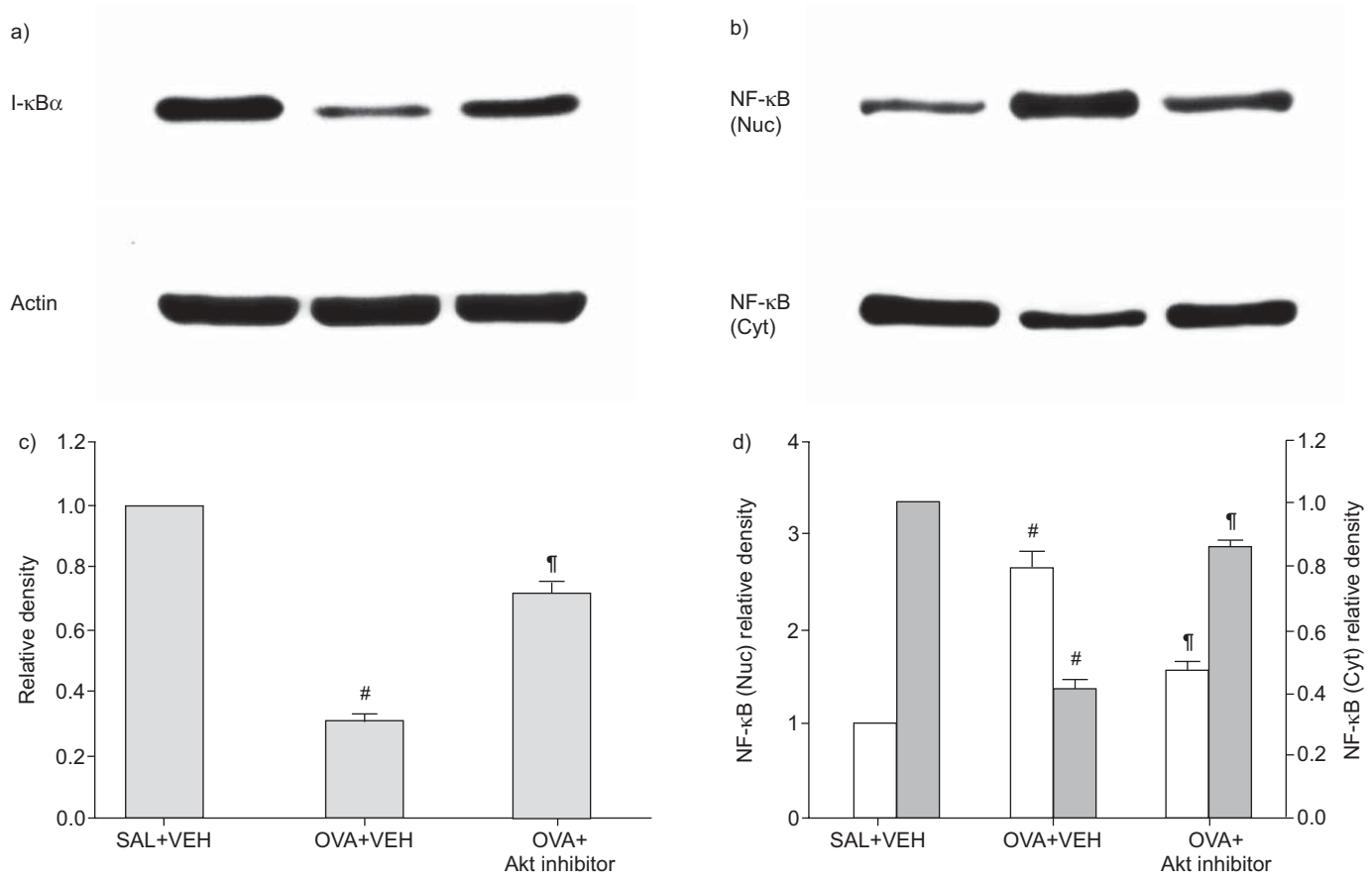


**FIGURE 6.** Effect of Akt inhibitor on inhibitor of κBα (I-κBα) and nuclear factor (NF)-κB p65 protein levels in lung tissues of ovalbumin (OVA)-sensitised and OVA-challenged mice. Sampling was performed 48 h after the last challenge in saline-nebulised mice administered drug vehicle (dimethylsulfoxide) (SAL+VEH), OVA-inhaled mice administered drug vehicle (OVA+VEH) and OVA-inhaled mice administered Akt inhibitor (OVA+Akt inhibitor). a) Western blotting of I-κBα protein. b) Western blot analyses of NF-κB p65 levels in nuclear (Nuc) and cytosolic (Cyt) protein extracts from lung tissues. c) Densitometric analyses are presented as the relative ratio of I-κBα to actin. The relative ratio of I-κBα in the lung tissues of SAL+VEH is arbitrarily presented as 1. d) Densitometric analyses are presented as the relative ratio of NF-κB p65 levels in OVA+VEH or OVA+Akt inhibitor to those in SAL+VEH. The relative ratio of NF-κB in nuclear protein extracts from the lung tissues of SAL+VEH is arbitrarily presented as 1. Data are presented as mean±SEM of six mice per group. □: Nuc; ■: Cyt. #: $p<0.05$ *versus* SAL+VEH; ¶: $p<0.05$ *versus* OVA+VEH.

### *Effect of BAY 11-7085 on IL-17 protein levels in lung tissues of OVA-sensitised and OVA-challenged mice*

Western blot analysis showed that the IL-17 protein levels increase after OVA inhalation was significantly reduced by the administration of BAY 11-7085, an inhibitor of NF-κB activation (fig. 8).

## DISCUSSION

In this study, we demonstrated that inhibition of the p110δ signalling pathway suppressed IL-17 protein and mRNA expression in the lungs, and attenuated allergen-induced airway inflammation and airway hyperresponsiveness. The OVA-induced model of asthma used in this study revealed the following typical pathological features of asthma: increased numbers of inflammatory cells in airways, airway hyperresponsiveness, and increased levels of Th2 cytokines (IL-4, IL-5 and IL-13), chemokine KC and nuclear NF-κB. In addition, the expression of IL-17 protein and mRNA in the lungs was increased after OVA inhalation. The administration of a selective PI3Kδ inhibitor, IC87114, significantly attenuated airway infiltration of total cells, lymphocytes, neutrophils and eosinophils, as well as airway hyperresponsiveness, and also decreased IL-17 protein and mRNA expression. Moreover, IC87114 reduced levels of Th2 cytokines, expression of KC protein and mRNA, Akt phosphorylation, I-κBα degradation and NF-κB activity. Supporting these results, an Akt inhibitor also increased I-κBα protein levels while reducing NF-κB activity, and an NF-κB inhibitor, BAY 11-7085, substantially reduced the increase in IL-17 protein levels after OVA inhalation. In addition, the inhibition of IL-17 activity with an anti-IL-17 Ab remarkably reduced bronchial inflammation and airway hyperresponsiveness. These results suggest that the therapeutic effect of a PI3Kδ inhibitor in asthma is partly mediated by suppressing IL-17 expression *via* modulation of Akt-mediated NF-κB activation. To the best of our knowledge, this is the first study clarifying a role of PI3Kδ in regulation of IL-17 expression.

The PI3K family is divided into class I, II and III, and the class I PI3Ks are further subdivided into class IA (PI3Kα, PI3Kβ and PI3Kδ isoforms) and class IB (PI3Kγ isoform) on the basis of structure and substrate specificities [22]. Structurally, the class IA PI3K consists of a heterodimer composed of a 110-kd (p110α, β or δ)

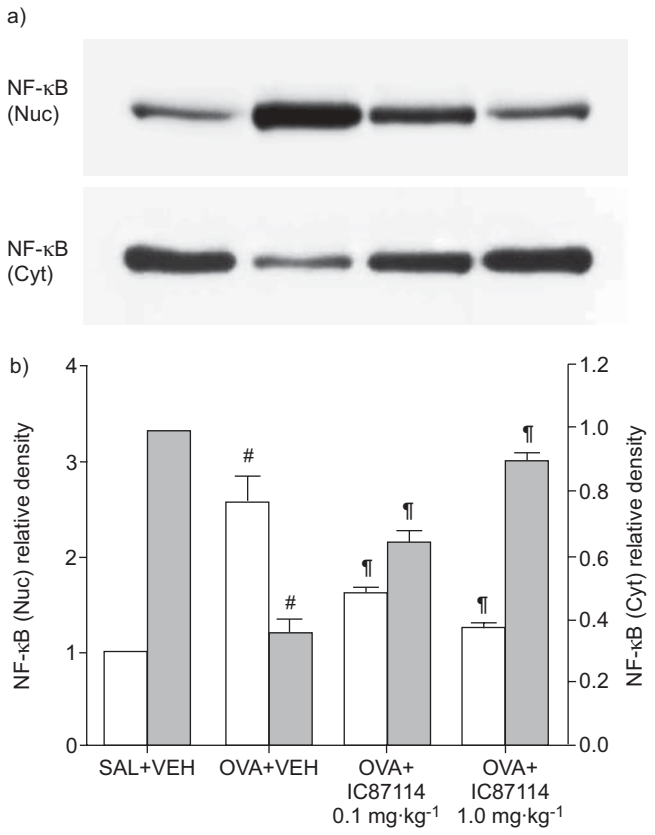


**FIGURE 7.** Effect of IC87114 on nuclear factor (NF)-κB p65 protein levels in lung tissues of ovalbumin (OVA)-sensitised and OVA-challenged mice. Levels of NF-κB p65 were measured 48 h after the last challenge in saline-nebulised mice administered drug vehicle (SAL+VEH), OVA-nebulised mice administered drug vehicle (OVA+VEH), OVA-inhaled mice administered 0.1 mg·kg$^{-1}$ IC87114 (OVA+IC87114 0.1 mg·kg$^{-1}$) and OVA-inhaled mice administered 1.0 mg·kg$^{-1}$ IC87114 (OVA+IC87114 1.0 mg·kg$^{-1}$). a) Western blot analyses of NF-κB p65 levels in nuclear (Nuc) and cytosolic (Cyt) protein extracts from lung tissues. b) Densitometric analyses are presented as the relative ratio of NF-κB p65 levels in OVA+VEH, OVA+IC87114 0.1 mg·kg$^{-1}$ or OVA+IC87114 1.0 mg·kg$^{-1}$ to those in SAL+VEH. The relative ratio of NF-κB in nuclear protein extracts from the lung tissues of SAL+VEH is arbitrarily presented as 1. Data are presented as mean±SEM of seven mice per group. □: Nuc; ■: Cyt. #: $p<0.05$ *versus* SAL+VEH; ¶: $p<0.05$ *versus* OVA+VEH.

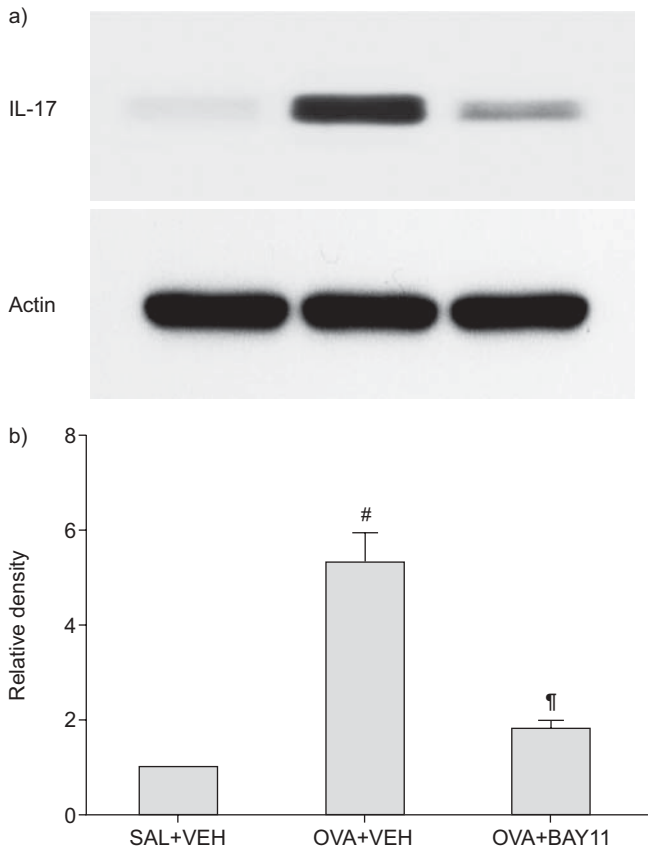


**FIGURE 8.** Effect of BAY11-7085 on interleukin (IL)-17 protein levels in lung tissues of ovalbumin (OVA)-sensitised and OVA-challenged mice. Sampling was performed 48 h after the last challenge in saline-nebulised mice administered drug vehicle (dimethylsulfoxide) (SAL+VEH), OVA-inhaled mice administered drug vehicle (OVA+VEH) and OVA-inhaled mice administered BAY11-7085 (OVA+BAY 11). a) Western blot analyses of IL-17. b) Densitometric analyses are presented as the relative ratio of IL-17 to actin. The relative ratio of IL-17 in the lung tissues of SAL+VEH is arbitrarily presented as 1. Data are presented as mean±SEM of seven mice per group. #: $p<0.05$ *versus* SAL+VEH; ¶: $p<0.05$ *versus* OVA+VEH.

catalytic subunit and a regulatory subunit (p85α, p85β, p55α, p55γ or p50α) [23]. These differences allow PI3K isoforms to mediate distinct functions. Of these isoforms of PI3K, p110δ has been implicated in immune responses and inflammation [24]. Recent studies have demonstrated a novel biological role of p110δ signalling in allergic airway inflammation and emphasised the importance of PI3Kδ isoform as a potential therapeutic target in asthma [8, 9]. The genetic inactivation of p110δ has resulted in reduction of antigen receptor function, Th2 cytokine production and Th2 cell-mediated inflammatory reactions [25]. In the present study, the results showed that administration of a specific inhibitor of PI3Kδ substantially reduced the pathological features of asthma, including airway inflammation, airway hyperresponsiveness and the increases in Th2 cytokine levels. These results are consistent with the findings of previous studies [8, 9, 25], confirming the therapeutic potential of the PI3Kδ inhibitor in asthma.

IL-17 is a new cytokine that has recently captured the attention of many scientists due to its involvement in immune and inflammatory responses [26]. IL-17 in airways is most likely produced by a unique Th lineage called Th17 cells [27]. Recent studies have indicated that eosinophils, neutrophils and monocytes are also sources of IL-17 [15, 26]. The biological function of IL-17 appears to be associated with neutrophil-dominated inflammation as a promoter of granulopoiesis, neutrophil accumulation and neutrophil activation in the lung [11, 26]. Intratracheal administration of IL-17 has been shown to increase the absolute number of neutrophils in BAL fluids [28]. In addition, IL-17 increases the expression of IL-8 mRNA in bronchial epithelial cells and the neutrophil chemotactic effect of IL-17 is blocked by an anti-IL-8 Ab [29]. Therefore, it is likely that IL-17 is able to recruit neutrophils selectively into airways by inducing the release of the potent neutrophil-directed

chemokine IL-8 [29, 30]. In this study, we evaluated an effect of an anti-IL-17 Ab on the expression of KC, which is a functional murine homologue of IL-8. Consistent with previous studies, we found that inhibiting IL-17 activity with the anti-IL-17 Ab remarkably reduced the increase in airway infiltration of neutrophils, and KC protein and mRNA expression after allergen inhalation. These results suggest that IL-17 orchestrates neutrophilic influx into airways by inducing the release of IL-8 in our model of allergic airway inflammation. It is well established that the pathognomonic features of asthma are mediated by eosinophils, mast cells and Th2 cells, as well as their cytokines [31]. However, it has been reported that the numbers of neutrophils in airways are increased in severe asthma [32]. In asthma, neutrophils potentially contribute to airway gland hypersecretion, airway obstruction and airway remodelling [17, 33]. Data from the present study support the notion that IL-17-induced neutrophil recruitment into airways is implicated in the pathogenesis of asthma.

Interestingly, a recent study has demonstrated that IL-17 receptor gene-deficient mice show a reduced recruitment of not only neutrophils but also eosinophils into airways upon antigen challenge [34]. In addition, eosinophil peroxidase activity in lung tissues and OVA-specific serum immunoglobulin E concentrations are reduced in the absence of IL-17 receptor signalling [35]. Furthermore, an involvement of IL-17 in the activation of allergen-specific T-cells has also been reported [36]. IL-17-deficient or IL-17 receptor-deficient mice show decreases in Th2 cytokine levels, which are associated with reduced airway hypersensitivity [34, 35]. A more recent study has shown that IL-17 mRNA levels correlate positively with IL-5 mRNA levels in sputum from asthmatic patients [18]. Therefore, IL-17 seems to contribute to Th2 cell-mediated and eosinophilic inflammation in asthma. Consistent with these observations, our present data showed that inhibition of IL-17 activity with an anti-IL-17 Ab remarkably reduced antigen-induced airway infiltration of eosinophils and airway hyperresponsiveness. We also observed that inhibition of IL-17 activity significantly reduced the increases in Th2 cytokine levels in BAL fluids. Taken together, these findings indicate that IL-17 plays a crucial role in the pathogenesis of asthma, contributing to neutrophilic inflammation as well as Th2 cell-mediated eosinophilic inflammation.

IL-17 production is mediated by activation of the PI3K/Akt pathway [23, 19–21]. It has been reported that LY-294002 inhibits IL-17 production and mRNA expression in CD4+ T cells [20]. Consistent with these *in vitro* data, we have previously reported that administration of wortmannin or LY-294002 reduces the increase in IL-17 levels after allergen inhalation in a murine model of asthma [12]. However, these inhibitors do not differentiate the four isoforms of class I PI3K. In the present study, pharmacological blockade of p110δ activity with IC87114 substantially decreased the expression of IL-17 protein and mRNA in the OVA-inflamed lungs. In addition, IC87114 was able to reduce the levels of Th2 cytokines as well as the expression of KC protein and mRNA, which are associated with IL-17 expression. PI3Kδ has been shown to play a prominent role in the accumulation and activation of neutrophils in inflamed tissues [6, 7]. However, the signalling pathways for the contribution of PI3Kδ to neutrophil chemotaxis have not been well defined. The results of the present study suggest that IL-17 is a key regulator underlying neutrophilic inflammation mediated by PI3Kδ. Taken together, these findings suggest that the therapeutic effect of the PI3Kδ inhibitor in the asthma is exerted by down-regulating IL-17 expression, thereby attenuating Th2 cell-mediated eosinophilic inflammation and neutrophilic inflammation.

A transcription factor, NF-κB, plays an essential role in immune and inflammatory responses and is, consequently, associated with the pathogenesis of asthma. As expected, nuclear NF-κB protein levels were substantially increased in our OVA-induced asthma model, indicating that NF-κB is activated. Activation of this transcription factor induces many inflammatory genes encoding cytokines (tumour necrosis factor-α, IL-4, IL-5, IL-6, IL-9 and IL-13), chemokines (RANTES (regulated on activation, normal T-cell expressed and secreted), eotaxin and IL-8), adhesion molecules (intracellular cell adhesion molecule-1 and vascular cell adhesion molecule-1), growth factors and receptors that are potentially relevant to the pathogenesis of asthma [37]. Furthermore, *in vitro* and *in vivo* studies have shown that inhibition of NF-κB activation reduces IL-17 production [13, 19–21]. These findings suggest that NF-κB is an important regulator of IL-17 expression. Activation of the PI3K/Akt pathway leads to a pronounced augmentation of NF-κB activity through I-κBα degradation [37, 38]. However, no data are available on the role of PI3K catalytic subunit p110δ in the activation of NF-κB. The present study showed that administration of a PI3Kδ inhibitor substantially decreased allergen-induced Akt phosphorylation with a significant reduction of I-κBα degradation and NF-κB activity in lung tissues. Consistent with the published data [38], we also found that an Akt inhibitor increased I-κBα protein levels while reducing NF-κB activity in lung tissues of OVA-challenged mice. In addition, the increase in IL-17 protein and mRNA expression after OVA inhalation was decreased by administration of an inhibitor of NF-κB activation, BAY 11-7085, or an Akt inhibitor (fig. 2S in the supplementary material). These findings suggest that p110δ activates Akt and, thereby, enhances I-κBα degradation and subsequent NF-κB activation, which regulates IL-17 expression in allergic airway inflammation.

In conclusion, our results have demonstrated that a specific inhibitor of PI3Kδ, IC87114, reverses all pathological features of asthma, including airway infiltration of inflammatory cells, Th2 cytokine levels and KC expression. In addition, IC87114 decreased the expression of IL-17 protein and mRNA in lungs increased by OVA challenge. These results suggest that IC87114 down-regulates IL-17 expression, and attenuates Th2 cell-mediated eosinophilic inflammation and neutrophilic inflammation. In addition, we have also found that this PI3Kδ inhibitor inhibits NF-κB activity and that NF-κB inhibition reduces the increase in IL-17 expression. Based on these observations, we suggest that inhibition of the p110δ signalling pathway has therapeutic potential in asthma by down-regulating the NF-κB pathway and the resultant suppressing IL-17 expression. Accordingly, the present study provides an important mechanism for the use of PI3Kδ inhibitor to prevent and/or treat asthma and other allergic airway diseases.

## SUPPORT STATEMENT

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## STATEMENT OF INTEREST

A statement of interest for K.D. Puri can be found at www.erj.ersjournals.com/site/misc/statements.xhtml

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## REFERENCES

1 Bousquet J, Jeffery PK, Busse WW, *et al.* Asthma. From bronchoconstriction to airways inflammation and remodeling. *Am J Respir Crit Care Med* 2000; 161: 1720–1745.
2 Park SJ, Min KH, Lee YC. Phosphoinositide 3-kinase delta inhibitor as a novel therapeutic agent in asthma. *Respirology* 2008; 13: 764–771.
3 Rommel C, Camps M, Ji H. PI3Kδ and PI3Kγ: partners in crime in inflammation in rheumatoid arthritis and beyond? *Nat Rev Immunol* 2007; 7: 191–201.
4 Kwak YG, Song CH, Yi HK, *et al.* Involvement of PTEN in airway hyperresponsiveness and inflammation in bronchial asthma. *J Clin Invest* 2003; 111: 1083–1092.
5 Chantry D, Vojtek A, Kashishian A, *et al.* p110δ, a novel phosphatidylinositol 3-kinase catalytic subunit that associates with p85 and is expressed predominantly in leukocytes. *J Biol Chem* 1997; 272: 19236–19241.
6 Sadhu C, Masinovsky B, Dick K, *et al.* Essential role of phosphoinositide 3-kinase δ in neutrophil directional movement. *J Immunol* 2003; 170: 2647–2654.
7 Puri KD, Doggett TA, Douangpanya J, *et al.* Mechanisms and implications of phosphoinositide 3-kinase δ in promoting neutrophil trafficking into inflamed tissue. *Blood* 2004; 103: 3448–3456.
8 Lee KS, Lee HK, Hayflick JS, *et al.* Inhibition of phosphoinositide 3-kinase δ attenuates allergic airway inflammation and hyperresponsiveness in murine asthma model. *FASEB J* 2006; 20: 455–465.
9 Lee KS, Park SJ, Kim SR, *et al.* Phosphoinositide 3-kinase-δ inhibitor reduces vascular permeability in a murine model of asthma. *J Allergy Clin Immunol* 2006; 118: 403–409.
10 Pappu BP, Angkasekwinai P, Dong C. Regulatory mechanisms of helper T cell differentiation: new lessons learned from interleukin 17 family cytokines. *Pharmacol Ther* 2008; 117: 374–384.
11 Park H, Li Z, Yang XO, *et al.* A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nat Immunol* 2005; 6: 1133–1141.
12 Kim SR, Lee KS, Park SJ, *et al.* PTEN down-regulates IL-17 expression in a murine model of toluene diisocyanate-induced airway disease. *J Immunol* 2007; 179: 6820–6829.
13 Hellings PW, Kasran A, Liu Z, *et al.* Interleukin-17 orchestrates the granulocyte influx into airways after allergen inhalation in a mouse model of allergic asthma. *Am J Respir Cell Mol Biol* 2003; 28: 42–50.
14 Barczyk A, Pierzchala W, Sozanska E. Interleukin-17 in sputum correlates with airway hyperresponsiveness to methacholine. *Respir Med* 2003; 97: 726–733.
15 Molet S, Hamid Q, Davoine F, *et al.* IL-17 is increased in asthmatic airways and induces human bronchial fibroblasts to produce cytokines. *J Allergy Clin Immunol* 2001; 108: 430–438.
16 Wong CK, Ho CY, Ko FW, *et al.* Proinflammatory cytokines (IL-17, IL-6, IL-18 and IL-12) and Th cytokines (IFN-γ, IL-4, IL-10 and IL-13) in patients with allergic asthma. *Clin Exp Immunol* 2001; 125: 177–183.
17 Sun YC, Zhou QT, Yao WZ. Sputum interleukin-17 is increased and associated with airway neutrophilia in patients with severe asthma. *Chin Med J* 2005; 118: 953–956.
18 Bullens DM, Truyen E, Coteur L, *et al.* IL-17 mRNA in sputum of asthmatic patients: linking T cell driven inflammation and granulocytic influx? *Respir Res* 2006; 7: 135.
19 Kim KW, Cho ML, Park MK, *et al.* Increased interleukin-17 production *via* a phosphoinositide 3-kinase/Akt and nuclear factor κB-dependent pathway in patients with rheumatoid arthritis. *Arthritis Res Ther* 2005; 7: R139–R148.
20 Cho ML, Ju JH, Kim KW, *et al.* Cyclosporine A inhibits IL-15-induced IL-17 production in $CD4^+$ T cells *via* down-regulation of PI3K/Akt and NF-κB. *Immunol Lett* 2007; 108: 88–96.
21 Cho ML, Kang JW, Moon YM, *et al.* STAT3 and NF-κB signal pathway is required for IL-23-mediated IL-17 production in spontaneous arthritis animal model IL-1 receptor antagonist-deficient mice. *J Immunol* 2006; 176: 5652–5661.
22 Hennessy BT, Smith DL, Ram PT, *et al.* Exploiting the PI3K/AKT pathway for cancer drug discovery. *Nat Rev Drug Discov* 2005; 4: 988–1004.
23 Dhand R, Hara K, Hiles I, *et al.* PI 3-kinase: structural and functional analysis of intersubunit interactions. *EMBO J* 1994; 13: 511–521.
24 Ward SG, Finan P. Isoform-specific phosphoinositide 3-kinase inhibitors as therapeutic agents. *Curr Opin Pharmacol* 2003; 3: 426–434.
25 Ali K, Bilancio A, Thomas M, *et al.* Essential role for the p110δ phosphoinositide 3-kinase in the allergic response. *Nature* 2004; 431: 1007–1011.
26 Kolls JK, Lindén A. Interleukin-17 family members and inflammation. *Immunity* 2004; 21: 467–476.
27 Harrington LE, Hatton RD, Mangan PR, *et al.* Interleukin 17-producing $CD4^+$ effector T cells develop *via* a lineage distinct from the T helper type 1 and 2 lineages. *Nat Immunol* 2005; 6: 1123–1132.
28 Hoshino H, Lötvall J, Skoogh BE, *et al.* Neutrophil recruitment by interleukin-17 into rat airways *in vivo*. Role of tachykinins. *Am J Respir Crit Care Med* 1999; 159: 1423–1428.
29 Laan M, Cui ZH, Hoshino H, *et al.* Neutrophil recruitment by human IL-17 *via* C-X-C chemokine release in the airways. *J Immunol* 1999; 162: 2347–2352.
30 Ye P, Rodriguez FH, Kanaly S, *et al.* Requirement of interleukin 17 receptor signaling for lung CXC chemokine and granulocyte colony-stimulating factor expression, neutrophil recruitment, and host defense. *J Exp Med* 2001; 194: 519–527.
31 Larché M, Robinson DS, Kay AB. The role of T lymphocytes in the pathogenesis of asthma. *J Allergy Clin Immunol* 2003; 111: 450–463.
32 Jatakanon A, Uasuf C, Maziak W, *et al.* Neutrophilic inflammation in severe persistent asthma. *Am J Respir Crit Care Med* 1999; 160: 1532–1539.
33 Woodruff PG, Khashayar R, Lazarus SC, *et al.* Relationship between airway inflammation, hyperresponsiveness, and obstruction in asthma. *J Allergy Clin Immunol* 2001; 108: 753–758.
34 Schnyder-Candrian S, Togbe D, Couillin I, *et al.* Interleukin-17 is a negative regulator of established allergic asthma. *J Exp Med* 2006; 203: 2715–2725.
35 Nakae S, Komiyama Y, Nambu A, *et al.* Antigen-specific T cell sensitization is impaired in IL-17-deficient mice, causing suppression of allergic cellular and humoral responses. *Immunity* 2002; 17: 375–387.
36 Kumar A, Takada Y, Boriek AM, *et al.* Nuclear factor-κB: its role in health and disease. *J Mol Med* 2004; 82: 434–448.

**37** Uhlig U, Fehrenbach H, Lachmann RA, *et al.* Phosphoinositide 3-OH kinase inhibition prevents ventilation-induced lung cell activation. *Am J Respir Crit Care Med* 2004; 169: 201–208.

**38** Bai D, Ueno L, Vogt PK. Akt-mediated regulation of NFκB and the essentialness of NFκB for the oncogenicity of PI3K and Akt. *Int J Cancer* 2009; 125: 2863–2870.

**Marked Online Supplement**

# Phosphoinositide 3-kinase δ inhibitor suppresses IL-17 expression in a murine asthma model

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## SUPPLEMENTARY METHODS

### *Animals and experimental protocol*

Female C57BL/6 mice, 8 to 10 weeks of age and free of murine specific pathogens, were obtained from ~~the~~ Orientbio Inc. (Seoungnam, Korea), 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. Standard guidelines for laboratory animal care were followed [1]. Mice were sensitized on days 1 and 14 by intraperitoneal injection of 20 μg 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 μL, as described previously [2, 3]. On days 21, 22, and 23 after the initial sensitization, the mice were challenged for 30 min with an aerosol of 3% (weight/volume) OVA in saline (or with saline as a control) using an ultrasonic nebulizer (NE-U12; Omron, Tokyo, Japan). Bronchoalveolar lavage (BAL) was performed at 48 h after the last challenge. At the time of lavage, mice were sacrificed with an overdose of pentobarbital sodium (100 $mg \cdot kg^{-1}$ of body weight, administered intraperitoneally). 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. The aliquots were pooled and then stored at 4°C. A part of each

pool was then centrifuged and the supernatant was kept at -70°C until use. Total cell numbers were counted using a hemocytometer. Smears of BAL cells were prepared with a cytospin (Thermo Electron, Waltham, MA, USA). The smears were stained with Diff-Quik solution (Dade Diagnostics of P. R. Inc., Aguada, Puerto Rico) in order to determine differential cell counts. Two independent, blinded investigators counted the cells under a microscope. Approximately 400 cells were counted in each of four different random locations. Inter-investigator variation was less than 5%. Numbers counted by two investigators were averaged and these values were used to calculate differential cell counts.

***Administration of IC87114, LY-294002, anti-interleukin (IL)-17 antibody (Ab), isotype control monoclonal Ab (mAb), Akt inhibitor, or BAY 11-7085***

IC87114 (0.1 or 1 $mg \cdot kg^{-1}$ body $weight \cdot day^{-1}$), LY-294002 (a selective inhibitor of phosphoinositide 3-kinase (PI3K); 1.5 $mg \cdot kg^{-1}$ body $weight \cdot day^{-1}$; BIOMOL Research Laboratories Inc., Plymouth Meeting, PA, USA), or vehicle control (dimethyl sulfoxide (DMSO)) diluted with 0.9% NaCl, was administered in a volume of 50 μL by intratracheal instillation two times 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) [2, 3]. Anti-IL-17 Ab or isotype control mAb (5 $mg \cdot kg^{-1}$ body $weight \cdot day^{-1}$; R&D Systems, Inc., Minneapolis, MN, USA) was administered intraperitoneally two times to each animal, once

on day 21 (1 h before the first airway challenge with OVA) and the second time on day 24 (24 h after the last airway challenge with OVA). An Akt inhibitor, 1L6-hydroxymethyl-chiro-inositol-2-(R)-2-*O*-methyl-3-*O*-octadecyl-*sn*-glycerocarbonate (3 $mg \cdot kg^{-1}$ body $weight \cdot day^{-1}$; Calbiochem, Darmstadt, Germany) dissolved in DMSO and diluted with 0.9% NaCl, was administered by intraperitoneal injection two times to each animal, once on day 21 (1 h before the first airway challenge with OVA) and the second time on day 24 (24 h after the last airway challenge with OVA). An inhibitor of nuclear factor-κB (NF-κB) activation, BAY 11-7085 (20 $mg \cdot kg^{-1}$ body $weight \cdot day^{-1}$; BIOMOL Research Laboratories Inc.) dissolved in DMSO and diluted with 0.9% NaCl, was administered by intraperitoneal injection two times to each animal, once on day 21 (1 h before the first airway challenge with OVA) and the second time on day 24 (24 h after the last airway challenge with OVA).

***Western blot analysis***

Lung tissues were homogenized in the presence of protease inhibitors to obtain extracts of proteins. BAL fluids obtained from the tracheas of mice were centrifuged at 4000 × *g* for 1 min and each supernatant was recovered. Protein concentrations were determined using the Bradford reagent (Bio-Rad Laboratories Inc., Hercules, CA, USA). Samples were loaded on a SDS-PAGE gel. After electrophoresis at 120 V for 90 min, separated proteins were transferred to polyvinylidene difluoride membranes (GE Healthcare, Little Chalfont,

Buckinghamshire, UK) by the wet transfer method (250 mA, 90 min). Nonspecific sites were blocked with 5% non-fat dry milk in Tris-buffered saline containing Tween 20 (25 mM Tris, pH 7.5, 150 mM NaCl, and 0.1% Tween 20) for 1 h, and the blots were then incubated with an anti-IL-17 Ab (R&D Systems, Inc.), anti-IL-4 Ab (Serotec Ltd, Oxford, UK), anti-IL-5 Ab (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-IL-13 Ab (R&D Systems, Inc.), biotinylated anti-mouse keratinocyte chemoattractant (KC) Ab (R&D Systems, Inc.), anti-Akt Ab (Cell Signaling Technology Inc., Beverly, MA, USA), anti-phosphorylated Akt Ab (Cell Signaling Technology Inc.), or anti-inhibitor of κBα Ab (Santa Cruz Biotechnology) overnight at 4°C. Anti-mouse, anti-rabbit, or anti-avian horseradish peroxidase conjugated IgG was used to detect binding of the Abs. The membranes were stripped and reblotted with anti-actin Ab (Sigma-Aldrich) to verify equal loading of protein in each lane. The binding of the specific Abs was visualized by exposing to photographic film after treating with enhanced chemiluminescence system reagents (GE Healthcare).

***RNA isolation and RT-PCR***

Total RNA was isolated from lung tissues using a rapid extraction method (TRI-Reagent; Sigma-Aldrich) as described previously [4]. RNA was quantified by measuring absorption at 260 nm and was stored at -80°C until use. Total RNA was reverse-transcribed to cDNA in a buffer containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 5 mM $MgCl_2$, 10 mM

dithiothreitol; 0.5 μg random hexanucleotide primers, 2.5 mM dNTP, 40 U RNase inhibitor, and 50 U·μL$^{-1}$ SuperScript II RT (Invitrogen, Carlsbad, CA, USA), in a final volume of 20 μL. This mixture was incubated for 50 min at 42°C and was finally digested with 2 U·μL$^{-1}$ *E. coli* RNase H for 20 min at 37°C. The first-strand cDNAs were used for PCR amplification of IL-17, KC, or the housekeeping gene, GAPDH. PCR amplification was performed by mixing 3 μL of the RT reaction mixture with 47 μL of buffer containing 2.5 U of Taq DNA polymerase (Promega, Madison, WI, USA) and 30 pmol of specific primer pairs for mouse cDNA of IL-17, KC, or GAPDH, designed from published mouse gene sequences. The primers used were as follows: IL-17 sense: 5'-TCTCATCCAGCAAGAGATCC-3'; antisense: 5'-AGTTTGGGACCCCTTTACAC-3', KC sense: 5'-GGCCCCACTGCACCCAAACC-3'; antisense: 5'-CCGAGCGAGACGAGACCAGGAGA-3', and GAPDH sense: 5'-GCCATCAACGACCCCTTCATTGAC-3', antisense: 5'-ACGGAAGGCCATGCCAGTGAGCTT-3'. PCR reactions were performed in a thermocycler (GeneAmp® PCR System 2400; Applied Biosystems, Foster City, CA, USA) under the following reaction conditions; after an initial incubation for 2 min at 95°C, samples were subjected to 35 cycles of 1 min at 94°C, 2 min at 54°C (GAPDH), 55°C (IL-17), or 58°C (KC), and 1 min at 72°C. A final extension step was performed at 72°C for 10 min. The RT-PCR products were electrophoretically fractionated on 2% agarose gels stained with ethidium bromide. DNA bands were visualized under UV light.

***Quantitative real-time RT-PCR***

Quantitative RT-PCR analysis was performed using the LightCycler® FastStart DNA Master SYBR Green I (Roche Diagnostics, Mannheim, Germany). The sequences of primers used were as follows: IL-17 sense: 5'-TCTCATCCAGCAAGAGATCC-3', antisense: 5'-AGTTTGGGACCCCTTTACAC-3', KC sense: 5'-GGCCCCACTGCACCCAAACC-3'; antisense: 5'-CCGAGCGAGACGAGACCAGGAGA-3', and β-actin sense: 5'-CAGATCATGTTTGAGACCTTC -3', antisense: 5'-ACTTCATGATGGAATTGAATG-3'. Calculation of the relative mRNA levels of each sample was performed according to the manufacturer's protocol. The data have been normalized to the expression of β-actin.

***Measurement of T-helper 2 cytokines***

Levels of IL-4, IL-5, and IL-13 were quantified in the supernatants of BAL fluids by enzyme immunoassays according to the manufacturer's protocol (R&D Systems, Inc.). Sensitivities for IL-4, IL-5, and IL-13 assays were 2, 7, and 1.5 $pg \cdot mL^{-1}$, respectively.

***Histology***

Mice were sacrificed at 48 h after the last challenge, 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% (volume/volume) neutral buffered formalin. The specimens were dehydrated and embedded in paraffin. For histological examination, 4-μm 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 sequentially with hematoxylin 2 and eosin-Y (Richard-Allan Scientific, Kalamazoo, MI, USA).

***Cytosolic or nuclear protein extractions for analysis of NF-κB p65***

Lungs were removed and homogenized in 2 volumes of a buffer A (50 mM Tris-HCl, pH 7.5, 1 mM ethylene diamine tetraacetic acid, 10% glycerol, 0.5 mM dithiothreitol, 5 mM $MgCl_2$, and 1 mM phenylmethylsulfonyl fluoride) containing protease inhibitor cocktails. The homogenates were centrifuged at 1,000 × *g* for 15 min at 4 . The supernatant fraction was incubated on ice for 10 min and centrifuged at 100,000 × *g* for 1 h at 4 to obtain cytosolic proteins for analysis of NF-κB p65. The pellets were washed twice with the buffer A and resuspended in a buffer B (1.3 M sucrose, 1.0 mM $MgCl_2$, and 10 mM potassium phosphate buffer, pH 6.8) and pelleted at 1,000 × *g* for 15 min. The pellets were suspended in the buffer B with a final sucrose concentration of 2.2 M and centrifuged at 100,000 × *g* for 1 h. The resulting pellets were washed once with a solution containing 0.25 M sucrose, 0.5 mM $MgCl_2$, and 20 mM Tris-HCl, pH 7.2, and centrifuged at 1,000 × *g* for 10 min. The pellets were

solubilized with a solution containing 50 mM Tris-HCl (pH 7.2), 0.3 M sucrose, 150 mM NaCl, 2 mM ethylene diamine tetraacetic acid, 20% glycerol, 2% Triton X-100, 2 mM phenylmethylsulfonyl fluoride, and protease inhibitor cocktails. The mixture was kept on ice for 1 h with gentle stirring and centrifuged at 12,000 × *g* for 30 min. The resulting supernatant was used as soluble nuclear proteins for determination of NF-κB p65 levels. The levels of NF-κB p65 were analyzed by Western blotting using NF-κB p65 Ab (Upstate Biotech, Lake Placid, NY, USA).

***Measurement of PI3K enzyme activity in lung tissues***

The measurement of PI3K enzyme activity in lung tissues was performed as described previously [2, 3]. ~~The a~~Amounts of phosphatidylinositol (3,4,5)-trisphosphate (PIP3) ~~was~~were quantified by a PIP3 competition enzyme immunoassay 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 extracts containing equal amounts of total protein.

***Determination of airway responsiveness***

Airway responsiveness was assessed as a change in airway function after challenge with aerosolized methacholine via airways, as described elsewhere [5]. Anesthesia was achieved

with 80 $mg \cdot kg^{-1}$ of pentobarbital sodium injected intraperitoneally. The trachea was then exposed through midcervical incision, tracheostomized, and an 18-gauge metal needle was inserted. Mice were connected to a computer-controlled small animal ventilator (flexiVent; SCIREQ, Montreal, Canada). The mouse was quasi-sinusoidally ventilated with nominal tidal volume of 10 $mL \cdot kg^{-1}$ at a frequency of 150 $breaths \cdot min^{-1}$ and a positive end-expiratory pressure of 2 cm $H_2O$ 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 differences in airway response to methacholine, each mouse was challenged with methacholine aerosol in increasing concentrations (2.5 to 50 $mg \cdot mL^{-1}$ in saline). After each methacholine challenge, the data of respiratory system resistance (Rrs) was continuously collected. Maximum values of Rrs were selected to express changes in airway function which was represented as a percentage change from baseline after administering saline aerosol.

## SUPPLEMENTARY RESULTS

### *Effect of IC87114, Akt inhibitor, or BAY 11-7085 on IL-17 protein levels and mRNA expression in lung tissues of OVA-sensitized and -challenged mice*

Western blot analysis revealed that the increase in the IL-17 protein levels after OVA inhalation was significantly decreased ~~significantly~~ by the administration of IC87114, Akt inhibitor, or BAY 11-7085 (figs 2Sa and 2Sb). RT-PCR and real-time RT-PCR analysis showed that the increase in the IL-17 mRNA expression after OVA inhalation was significantly reduced by the administration of IC87114, Akt inhibitor, or BAY 11-7085 (figs 2Sc and 2Sd).

## REFERENCES

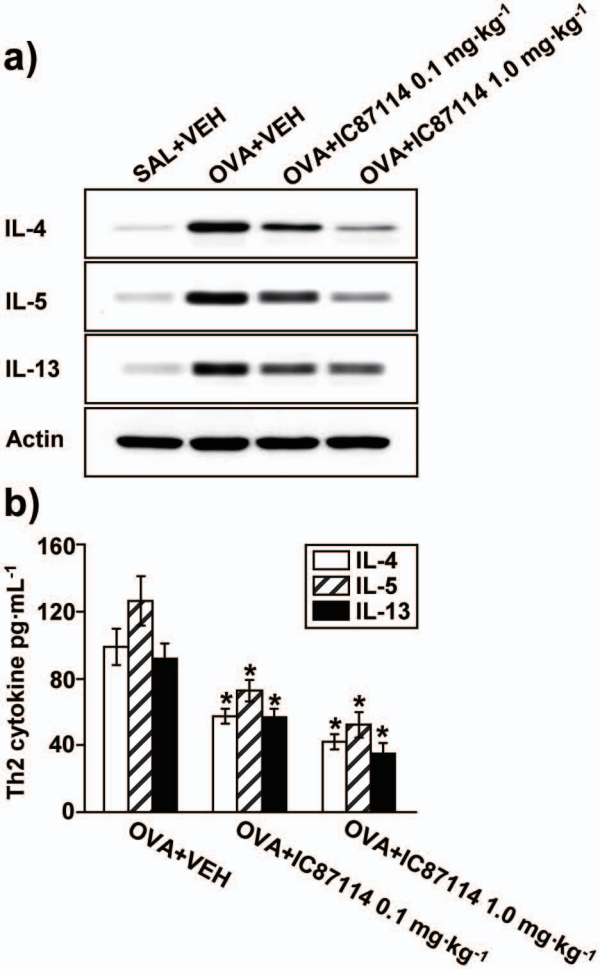

1. Institute of Laboratory Animal Research, Commission of Life Sciences, National Research Council. Guide for the care and use of laboratory animals. National Academy Press, Washington, DC, USA, 1996.
2. Lee KS, Lee HK, Hayflick JS, Lee YC, Puri KD. Inhibition of phosphoinositide 3-kinase delta attenuates allergic airway inflammation and hyperresponsiveness in murine asthma model. *FASEB J* 2006; 20: 455-465.
3. Lee KS, Park SJ, Kim SR, Min KH, Jin SM, Puri KD, Lee YC. Phosphoinositide 3-kinase-delta inhibitor reduces vascular permeability in a murine model of asthma. *J Allergy Clin Immunol* 2006; 118: 403-409.
4. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 1987; 162: 156-159.
5. Takeda K, Hamelmann E, Joetham A, Shultz LD, Larsen GL, Irvin CG, Gelfand EW. Development of eosinophilic airway inflammation and airway hyperresponsiveness in mast cell-deficient mice. *J Exp Med* 1997; 186: 449-454.

## SUPPLEMENTARY FIGURE LEGENDS

**FIGURE 1S.** Effect of IC87114 on levels of T-helper 2 (Th2) cytokines in lung tissues and bronchoalveolar lavage (BAL) fluids of ovalbumin (OVA)-sensitized and -challenged mice. Levels of interleukin (IL)-4, IL-5, or IL-13 were measured at 48 h after the last challenge in saline-inhaled mice administered drug vehicle (dimethylsulfoxide) (SAL+VEH), OVA-inhaled mice administered drug vehicle (OVA+VEH), OVA-inhaled mice administered IC87114 0.1 $mg{\cdot}kg^{-1}$ (OVA+IC87114 0.1 $mg{\cdot}kg^{-1}$), and OVA-inhaled mice administered IC87114 1.0 $mg{\cdot}kg^{-1}$ (OVA+IC87114 1.0 $mg{\cdot}kg^{-1}$). a) Representative Western blot analyses of IL-4, IL-5, and IL-13 in lung tissues. b) Enzyme immunoassay of IL-4, IL-5, and IL-13 in BAL fluids. Data represent mean ± SEM from 7 mice per group. *, $p<0.05$ versus OVA+VEH.

**FIGURE 2S.** Effect of IC87114, Akt inhibitor, or BAY 11-7085 on interleukin (IL)-17 protein levels and mRNA expression in lung tissues of ovalbumin (OVA)-sensitized and -challenged mice. Sampling was performed at 48 h after the last challenge in saline-inhaled mice administered drug vehicle (dimethylsulfoxide) (SAL+VEH), OVA-inhaled mice administered drug vehicle (OVA+VEH), OVA-inhaled mice administered IC87114 1.0 $mg{\cdot}kg^{-1}$ (OVA+IC87114), OVA-inhaled mice administered Akt inhibitor (OVA+Akt inhibitor), and OVA-inhaled mice administered BAY 11-7085 (OVA+BAY 11). a) Western blotting of IL-17. b) Densitometric analyses are presented as the relative ratio of IL-17 to actin. The relative

ratio of IL-17 in the lung tissues of SAL+VEH is arbitrarily presented as 1. c) Representative RT-PCR analyses of IL-17 mRNA expression. d) Quantitative analyses of IL-17 mRNA expression by means of real-time RT-PCR. Data represent mean ± SEM from 6 mice per group. #, $p<0.05$ versus SAL+VEH; *, $p<0.05$ versus OVA+VEH.


a)
SAL+VEH
OVA+VEH
OVA+IC87114 0.1 mg·kg-1
OVA+IC87114 1.0 mg·kg-1
IL-4
IL-5
IL-13
Actin
b)
Th2 cytokine pg·mL-1
160
120
80
40
0
IL-4
IL-5
IL-13
OVA+VEH
OVA+IC87114 0.1 mg·kg-1
OVA+IC87114 1.0 mg·kg-1

# FIGURE 1S

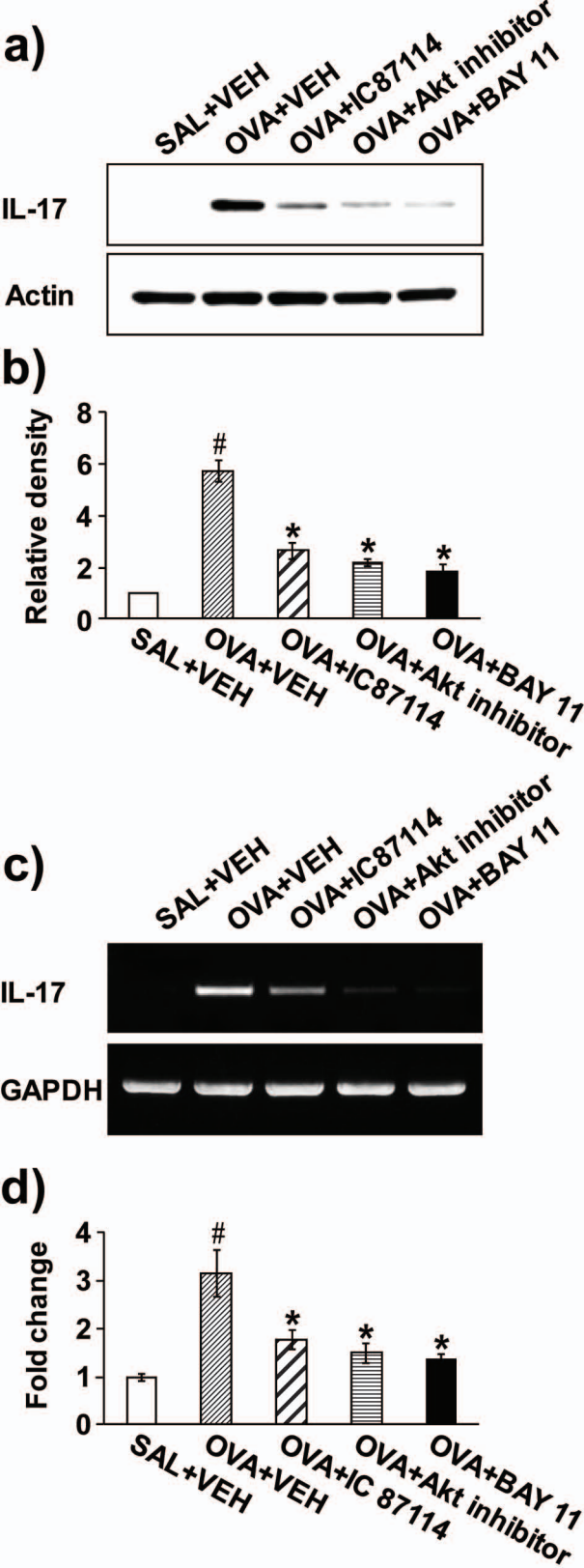

a)
SAL+VEH
OVA+VEH
OVA+IC87114
OVA+Akt inhibitor
OVA+BAY 11
IL-17
Actin
b)
Relative density
8
6
4
2
0
#
*
*
*
SAL+VEH
OVA+VEH
OVA+IC87114
OVA+Akt inhibitor
OVA+BAY 11
c)
SAL+VEH
OVA+VEH
OVA+IC87114
OVA+Akt inhibitor
OVA+BAY 11
IL-17
GAPDH
d)
Fold change
4
3
2
1
0
#
*
*
*
SAL+VEH
OVA+VEH
OVA+IC 87114
OVA+Akt inhibitor
OVA+BAY 11

FIGURE 2S