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# 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 Prewarmed 0.9% NaCl solution was slowly infused into the lungs and withdrawn.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·kg$^{-1}$ body weight·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·kg$^{-1}$ body weight·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 $U \cdot \mu L^{-1}$ 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 $U \cdot \mu L^{-1}$ *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**


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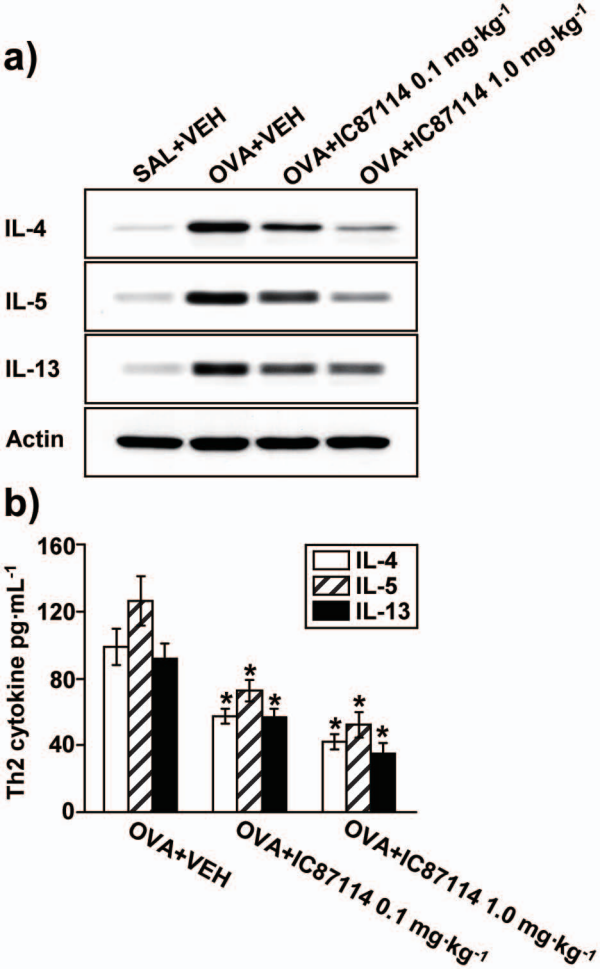
## SUPPLEMENTARY FIGURE LEGENDS

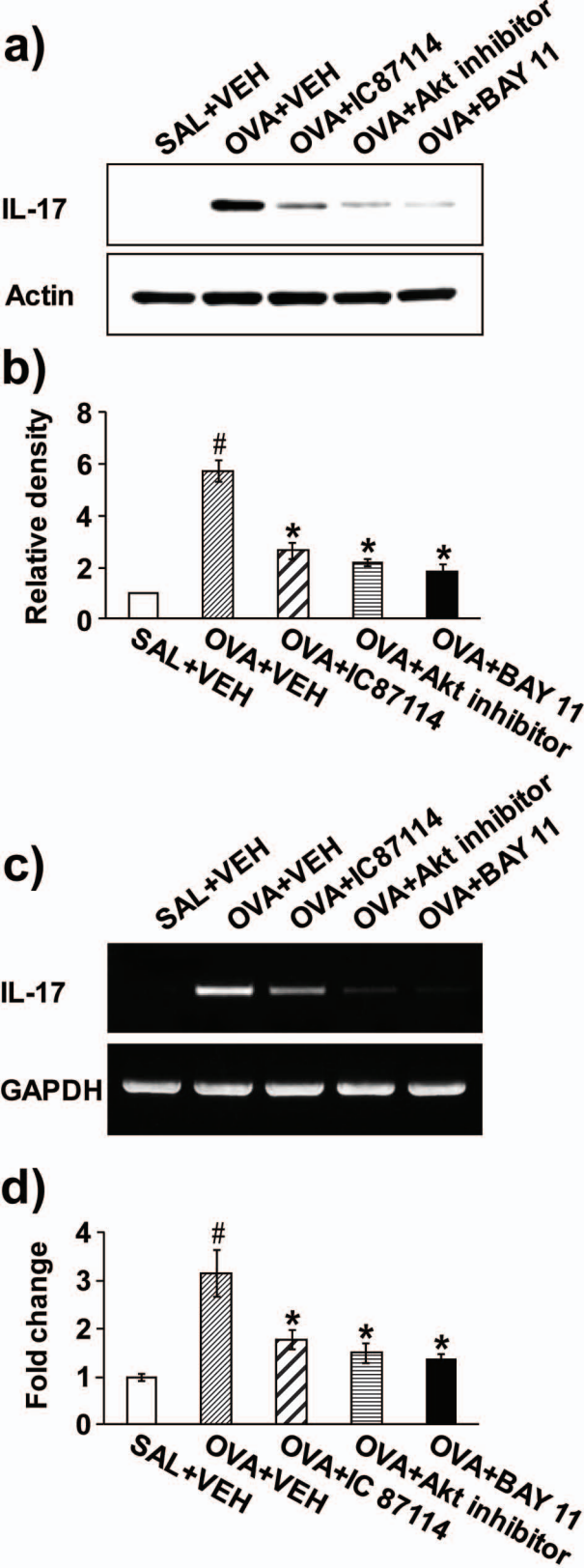
**FIGURE 1S.** Effect of IC87114 on levels of T-helper 2 (Th2) cytokines in lung tissues and bronchoalveolar lavage (BAL) fluids of ovalbumin (OVA)-sensitized and -challenged mice. Levels of interleukin (IL)-4, IL-5, or IL-13 were measured at 48 h after the last challenge in saline-inhaled mice administered 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
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*
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SAL+VEH
OVA+VEH
OVA+IC 87114
OVA+Akt inhibitor
OVA+BAY 11

FIGURE 2S