[Revised online data supplement]

Phosphoinositide 3-kinase-δ regulates fungus-induced allergic lung inflammation through endoplasmic reticulum stress

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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 Orient Bio Inc. (Seoungnam, Korea), housed throughout the experiments in a laminar flow cabinet and 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 (CBU 2014-00030). All mice received mixture of a total of 10 µg of *Af* crude antigen (Greer Laboratories) and 0.2 ml of incomplete Freund's adjuvant (Sigma-Aldrich) dissolved in normal saline. One-half of this preparation was then deposited in the peritoneal cavity, and the remainder was delivered subcutaneously. Two weeks later, mice received a total of 20 µg of *Af* antigens dissolved in normal saline via the intranasal route. Four days after the intranasal challenge, mice received 20 µg of *Af* antigen dissolved in normal saline via the intratracheal route.

BAL was performed at 72 hours after the last challenge with *Af* in *Af*-exposed mice. At the time of lavage, the mice were sacrificed by cervical dislocation. The chest cavity was exposed to allow for expansion, after which the trachea was carefully intubated and the catheter secured with ligatures. Prewarmed 0.9% NaCl solution was slowly instilled into the lung and withdrawn. A part of each pool was then centrifuged. Total cell numbers were counted with a hemocytometer. Smears of BAL cells were prepared by cytospin (Thermo Electron, Waltham, MA, USA) and stained with Diff-Quik solution (Dade Diagnostics of Puerto Rico Inc., Aguada, Puerto Rico) in order to examine cell differentials. Two independent, blinded investigators counted the cells using a microscope. Approximately 400 cells were counted in each of four different random locations. Inter-investigators variation

was less than 5%. The mean number from the two investigators was used to estimate the cell differentials.

PI3K enzyme activity

Whole lung tissues were homogenized in the presence of protease inhibitors to obtain extracts of lung proteins. Protein concentrations were determined using the Bradford reagent (Bio-Rad Laboratories Inc., Hercules, CA, USA). The amount of PIP_3 produced was quantified by the PIP_3 competition enzyme immunoassay according to the manufacturer's protocol (Echelon Inc.). The enzyme activity was expressed as pmol PIP_3 produced in 1 ml of lung tissue extract containing equal amounts of total protein.

Serum total IgE and Af-specific IgE

For the *Af*-specific IgE assay, 96-well immunosorbent plates were coated with *Af* antigen (10 μg/ml, Greer Laboratories) in carbonate-bicarbonate buffer (Sigma-Aldrich). After blocking the plates with 1% bovine serum albumin (BSA) in PBS, serially diluted mouse serum was added. The plates were incubated for two hours at 37 °C. Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgE Ab (Bethyl Laboratories) was used to detect *Af*-bound IgE. The plates were developed with tetramethylbenzidine substrate (Bethyl Laboratories), and the reaction was stopped with H_2SO_4, and the absorbance was determined at 450 nm. Total serum IgE was measured using a mouse Total IgE ELISA Kit (MD Bioproducts) according to the manufacturer's protocol.

1 **Isolation/primary culture of TECs**

2 Murine TECs were isolated under sterile conditions. The epithelial cells were seeded onto
3 60-mm collagen-coated dishes for submerged culture. The growth medium, DMEM
4 (Invitrogen) containing 10% fetal bovine serum (FBS), penicillin, streptomycin, and
5 amphotericin B, was supplemented with insulin, transferrin, hydrocortisone,
6 phosphoethanolamine, cholera toxin, ethanolamine, bovine pituitary extract, and BSA. The
7 cells were maintained in a humidified 5% CO_2 incubator at 37 °C until they adhered. To
8 verify the role of PI3K-δ in the regulation of ER stress in *Af*-stimulated primary cultured
9 TECs, cells were treated with IC87114 (10 μmol/l) for two hours, then stimulated by *Af*
10 antigen (5 μg/ml) for additional 12 hours. After that, cells were harvested.

11

12 **PI3K-δ specific or scrambled siRNA transfection**

13 Primary cultured murine TECs were transfected with the PI3K-δ specific (PIK3CD) or
14 scrambled siRNA (Dharmacon). To perform transfection, Lipofectamine™ RNAiMAX
15 reagent (Invitrogen) was used according to the method described by the manufacturer. At 48
16 hours after siRNA transfection, culture medium was replaced with fresh medium and then
17 cells were stimulated by *Af* antigen (5 μg/ml) for 12 hours. Subsequently, cells were harvested
18 and protein or RNAs were isolated.

19

20 **RNA isolation and quantitative real-time reverse transcription polymerase chain**
21 **reaction (RT-PCR)**

22 To analyze the silencing effect of siRNA, RNA was extracted from cells using TRIzol

(Invitrogen) as previously described [1], and quantitative real-time RT-PCR analysis was performed using the LightCycler® FastStart DNA Master SYBR Green I (Roche Diagnostics, Mannheim, Germany). Real-time RT-PCR data were analyzed by the comparative cycle threshold method with the LightCycler® Software version 4.1 and normalized to internal controls (β-actin). The primers used were: PI3K-δ sense: 5'-CACAGGTCTCA-TCGAGGTGGTC-3', antisense: 5'-TGGACTTGAGCCAGTTGAGCA-3' and β-actin sense: 5'-CAGATCATGTTTGAGACCTTC-3', antisense: 5'-ACTTCATGATGGAATTGAATG-3'.

Immunofluorescence staining for GRP78, CHOP, and mtROS

Paraffin-embedded lung tissue sections were deparaffinized and hydrated. The sections or *Af*-stimulated primary cultured murine TECs were fixed with ice cold methanol and permeabilized in PBS containing 0.25% Triton X-100 for 10 minutes at room temperature and washed three times with PBS. Subsequently, after antigen retrieval for 15 minutes at 37 ℃ in proteinase K (Dako, Glostrup, Denmark), nonspecific bindings were blocked with 1% BSA (Sigma-Aldrich) in PBS containing 0.05% Tween 20 for one hour. Specimens were then incubated in a humidified chamber for two hours at room temperature with an antibody to GRP78 (Santa Cruz Biotechnology, Dallas, TX, USA) and antibody to CHOP (Santa Cruz Biotechnology). For the detection of each binding antibody to GRP78 or CHOP, Alexa Fluor 488 (green) labeled donkey anti-goat IgG and Alexa Fluor 594 (red) labeled goat anti-rabbit IgG (Invitrogen) in 1% BSA were loaded for one hour at room temperature in dark, respectively. After the specimens were washed, nuclei were stained using DAPI (Invitrogen). Stained cells were mounted on slides using fluorescent mounting medium (Golden Bridge International, Inc., Mukilteo, WA, USA), and then visualized using a confocal laser scanning

1 microscope (Zeiss LSM 510 Meta, Carl Zeiss) equipped with a C-Apochromat 63×/1.20W
2 Korr UV-VIS-IR M27 water immersion objective.

3 To demonstrate intensity of mtROS in *Af*-stimulated primary cultured murine TECs, cells
4 were stained with Mitotracker Red CM-H2ROS (Invitrogen) in dark at room temperature.
5 After 30 minutes, cells were washed with PBS and analyzed using a confocal laser scanning
6 microscope (Carl Zeiss). For visualization of mitochondrial ROS in BAL cells, smears of
7 BAL cells were prepared by cytospin (Thermo Electron) after staining with Mitotracker Red
8 CM-H2ROS (Invitrogen) in dark at room temperature.

9

10 **Western blot analysis**

11 Lung tissues or *Af*-stimulated primary cultured TECs were homogenized in the presence of
12 protease inhibitor cocktail (Sigma-Aldrich), and protein concentrations were determined using
13 Bradford reagent (Bio-Rad Laboratories). Samples were loaded onto a SDS-PAGE gel. After
14 electrophoresis at 120 V for 90 minutes, proteins were transferred to PVDF membranes (Bio-
15 Rad Laboratories) at 250 mA for 90 minutes by a wet transfer method. Nonspecific sites were
16 blocked with 5% non-fat dry milk in Tris-buffered saline Tween 20 (TBST; 25 mmol/l Tris,
17 pH 7.5, 150 mmol/l NaCl, 0.1% Tween 20) for one hour, and the blots were then incubated
18 overnight at 4 °C with an antibody to IL-4 (AbD Serotec, Kidlington, near oxford, UK),
19 antibody to IL-5 (Santa Cruz Biotechnology), antibody to IL-13 (R&D Systems, Minneapolis,
20 MN, USA), antibody to IL-17 (R&D Systems), antibody to GRP78 (Cell Signaling
21 Technologies, Danvers, MA, USA), antibody to CHOP (Santa Cruz Biotechnology), antibody
22 to p-IRE1α (Novus biologicals, Littleton, CO, USA), antibody to IRE1α (Cell Signaling
23 Technologies), antibody to p-eIF2α (Ser51) (Cell Signaling Technologies), antibody to eIF2α

1 (Cell Signaling Technologies), antibody to p-Akt (R&D Systems), antibody to Akt (Cell
2 Signaling Technologies), and antibody to actin (Sigma-Aldrich). Anti-rabbit or anti-mouse
3 HRP-conjugated-IgG (Cell Signaling Technologies) was used to detect binding of antibodies.
4 The binding of the specific antibody was visualized by exposing to photographic film after
5 treating with enhanced chemiluminescence (ECL) system reagents (Promega Co., Madison,
6 WI, USA). The film was scanned (ImageScanner III, GE Healthcare, Little Chalfont,
7 Buckinghamshire, UK) and quantified using a quantification software (Gel Doc XR, Bio-Rad
8 Laboratories). For the quantification of specific bands, the square with same size was drawn
9 around each band to measure the density and then the value was adjusted by the density of the
10 background near that band. The results of densitometric analysis were expressed as a relative
11 ratio of the target protein to reference protein. The relative ratio of the target protein of control
12 group is arbitrarily presented as 1.

13

14 **Nuclear protein extractions**

15 Lungs were removed and homogenized in two volumes of buffer A (50 mmol/l Tris-HCl, pH
16 7.5, 1 mmol/l EDTA, 10% glycerol, 0.5 mmol/l DTT, 5 mmol/l $MgCl_2$, and 1 mmol/l PMSF)
17 containing protease inhibitor cocktails. The homogenates were centrifuged at 1,000 × *g* for 15
18 minutes at 4 °C. The pellets were washed twice in the buffer A, resuspended in buffer B (1.3
19 mol/l sucrose, 1.0 mmol/l $MgCl_2$, and 10 mmol/l potassium phosphate buffer, pH 6.8) and
20 then pelleted at 1,000 × *g* for 15 minutes. The pellets were suspended in the buffer B with a
21 final sucrose concentration of 2.2 mol/l and centrifuged at 100,000 × *g* for one hour. The
22 resulting pellets were washed once with a solution containing 0.25 mol/l sucrose, 0.5 mmol/l
23 $MgCl_2$, and 20 mmol/l Tris-HCl, pH 7.2, and centrifuged at 1,000 × *g* for 10 minutes. The

pellets were solubilized with a solution containing 50 mmol/l Tris-HCl, pH 7.2, 0.3 mol/l sucrose, 150 mmol/l NaCl, 2 mmol/l EDTA, 20% glycerol, 2% Triton X-100, 2 mmol/l PMSF, and protease inhibitor cocktails. The mixture was kept on ice for one hour with gentle stirring and centrifuged at 12,000 × *g* for 30 minutes. The resulting supernatant was used as soluble nuclear proteins for analysis of NF-κB p65, ATF-4, and XBP-1. The protein levels were analyzed by Western blotting using antibody to NF-κB p65 (Millipore, Billerica, MA, USA), antibody to ATF-4 (Santa Cruz Biotechnology), antibody to XBP-1 (Santa Cruz Biotechnology), and antibody to lamin B_1 (Santa Cruz Biotechnology) as described above.

Histology

At 72 hours after the last *Af* challenge, mice were euthanized for histological assessment. Lung and trachea were removed from the mice. For fixation, 10% (volume/volume) neutral buffered formalin was used. 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 Microsystem Nussloch GmbH, Wetzlar, Germany), placed on glass slides, deparaffinized, and stained sequentially with H&E (Richard-Allan Scientific, Kalamazoo, MI, USA). Stained tissue sections on slides were analyzed under identical light microscope (Axio Imager M1, Carl Zeiss) conditions, including magnification (× 20), gain, camera position, and background illumination.

Airway responsiveness to methacholine

Anesthesia was achieved through intraperitoneal injection of 45 mg/kg body weight of

1 sodium pentobarbital. The trachea was then exposed through midcervical incision,
2 tracheostomized, and an 18-gauge metal needle was inserted. Mice were connected to a
3 computer-controlled small animal ventilator (flexiVent, SCIREQ, Montreal, Canada). The
4 mouse was quasi-sinusoidally ventilated with nominal tidal volume of 10 ml/kg body weight
5 at a frequency of 150 breaths/minute and a positive end-expiratory pressure of 2 cm H_2O to
6 achieve a mean lung volume close to that during spontaneous breathing. This was achieved by
7 connecting the expiratory port of the ventilator to water column. Methacholine aerosol was
8 generated with an in-line nebulizer and administered directly through the ventilator. To
9 determine the differences in airway response to methacholine, each mouse was challenged
10 with methacholine aerosol in increasing concentrations (5.0 to 50 mg/ml in saline). After each
11 methacholine challenge, the data of calculated R_{rs} were continuously collected. Maximum
12 values of R_{rs} were selected to express changes in airway function, which was represented as a
13 percentage change from the baseline after saline aerosol.

14

15 **Immunohistochemistry**

16 Human lung tissue sections came from regional bank of biospecimen in Chonbuk National
17 University Hospital supported by the Korea Bank Project, Ministry for Health and Welfare,
18 Republic of Korea. All samples were deidentified, and all experimental protocols regarding
19 human tissues were approved by the Institutional Review Board of the Biomedical Research
20 Institute of Chonbuk National University Hospital (IRB file No. 2013-11-007-001). For
21 immunohistochemistry of GRP78, the deparaffinized 4-μm sections were incubated
22 sequentially according to the instruction using the R. T. U. Vectastain Universal Quick kit
23 from Vector Laboratories Inc. (Burlingame, CA, USA). Briefly, the slides were incubated in

Endo/Blocker for 15 minutes and in proteinase K (Dako) for 15 minutes at 37 °C. The slides were then incubated in normal horse serum for 30 minutes at room temperature, probed with antibody to GRP78 (Santa Cruz Biotechnology) for two hours at room temperature, and then incubated with prediluted biotinylated pan-specific IgG for 30 minutes. To visualize the antibody reactivity, the slides were incubated in streptavidin/peroxidase complex reagent for 15 minutes and then in 3-amino-9-ethylcarbazole substrate kit for 5 minutes. Controls consisted of sections of normal human lung tissues were incubated without the primary antibody. After immunostaining, the slides were photomicrographed.

Clinical information regarding lung tissues of healthy controls (3 persons) or patients with ABPA (6 patients) was evaluated through assessing previous medical records from the Chonbuk National University Hospital. Patients who met at least four of the classic diagnostic criteria for ABPA were considered to have ABPA [2, 3]. Briefly, 1) presence of bronchial asthma, 2) immediate cutaneous hyperreactivity on *Aspergillus* skin test (type I hypersensitivity reaction), 3) elevated serum IgE (>417 IU/ml), 4) elevated serum *Af*-specific IgE and/or IgG levels (>0.35 kUA/l), 5) precipitating antibodies (IgG) in serum against *Af*, 6) eosinophilia (>1000 cells/ml), 7) central bronchiectasis, and 8) transient or fixed pulmonary opacities on images. The *Aspergillus* skin test was performed using *Af* antigen (Bencard, Bradford, UK). The test was interpreted after 15 to 20 minutes. At least 3-mm diameter wheal with equivalent erythema more than diluent control done at the same time was considered as type I cutaneous hypersensitivity reaction. Levels of serum total IgE and *Af*-specific IgG were measured by commercially available kits using the fluorescent enzyme immunoassay. *Af*-specific IgE and precipitins for *Af* were not measured due to the limitation of our facilities.

SUPPLEMENTARY REFERENCES

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4 Natural history and classification of early disease by serologic and roentgenographic
5 studies. *Arch Intern Med* 1986;146:916-8.

6 3. Rosenberg M, Patterson R, Mintzer R, *et al*. Clinical and immunologic criteria for the
7 diagnosis of allergic bronchopulmonary aspergillosis. *Ann Intern Med* 1977;86:405-14.

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19 **FIGURE LEGENDS FOR SUPPLEMENTARY FIGURES**

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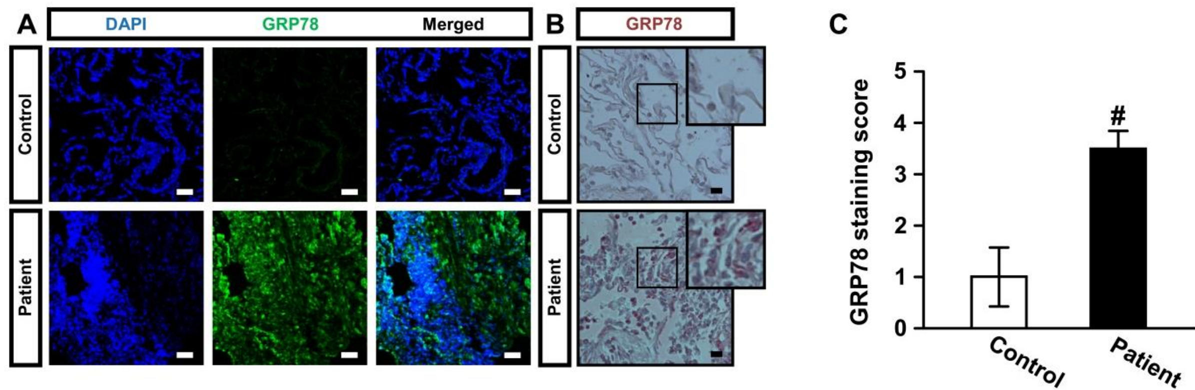

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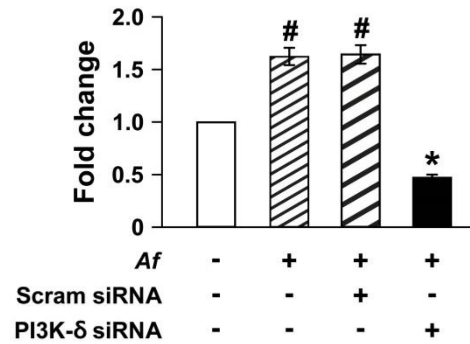
4 **Supplementary figure S1**

5 Expression of GRP78 is increased in the lung of patients with ABPA. Data related figure 1.
6 (A) Representative confocal laser immunofluorescence photomicrograph for GRP78 in lung
7 tissues from a healthy control and a patient with ABPA, respectively. DAPI stain was used
8 for nuclear localization. The right panels presented the merger views. Bars indicate scale of
9 50 μm. (B) Representative immunohistochemical staining of GRP78 in lung tissues of a
10 healthy person and a patient with ABPA, respectively. Brown-stained cells were considered
11 to express the GRP78 protein. The right upper inset box shows the magnification view of
12 positive reaction with GRP78 (twice enlarged photo from the original image). Bars indicate
13 20 μm. (C) Quantification of immunohistochemical staining scores for GRP78 in human lung
14 tissues. Bars represent mean ± SEM from 3 persons in healthy control group and 6 persons in
15 ABPA patient group. $^{\#}P < 0.05$ versus control.

16



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5 **Supplementary figure S2**

6 Effect of RNA interference on PI3K-δ mRNA level in *Af*-stimulated TECs. Data related
7 figures 2F-J. Quantitative real-time RT-PCR data of PI3K-δ mRNA after stimulation with *Af*
8 in primary cultured TECs transfected with either scrambled siRNA or PI3K-δ specific siRNA.
9 Bars represent mean ± SEM from 3 independent experiments. $^{\#}P < 0.05$ versus control; $^{*}P <$
10 0.05 versus cells stimulated with *Af* transfected with scrambled siRNA.

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