[*Online data supplement*]

Airway epithelial phosphoinositide 3-kinase delta contributes to the modulation of fungi-induced innate immune response

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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*. In addition, p110δ knockout (KO) mice genetically deficient for catalytic subunit of phosphoinositide 3-kinase (PI3K)-δ (p110δ) were purchased from the Jackson Laboratory (Sacramento, CA, USA). PI3K-δ KO mice were generated using strain 129S5/SvEv-derived embryonic stem cells by Gene Trap method through retroviral insertion that disrupt the gene between coding exons 3 and 4 of mouse Pik3cd gene, the ortholog of human PIK3CD which encodes p110δ. Backcross to C57BL/6 was performed throughout the study. All animal experiments were approved by the Institutional Animal Care and Use Committee of the Chonbuk National University (CBU 2014-00030) and were performed in accordance with the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines. For the generation of an *Aspergillus fumigatus* (*Af*)-induced allergic lung inflammation model, all mice received mixture of a total of 10 μg of *Af* crude antigen extract (Greer Laboratories, Lenoir, NC, USA), in which the cellular fungal material was inactivated and lyophilized, and 0.2 ml of incomplete Freund's adjuvant (Sigma-Aldrich, St. Louis, MO, USA) 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. In the *Alternaria alternata* (*Aa*)-induced allergic lung inflammation model, all mice were treated with the mixture of a total of 25 μg of *Aa* crude antigen extract dissolved in normal saline via

intranasal route on days 0, 3, 6, and 9, as previously described elsewhere.[1]

Administration of drugs

A selective inhibitor of catalytic subunit of PI3K-δ, IC87114 (0.1 or 1.0 mg/kg body weight/day, Calbiochem, San Diego, CA, USA) was administered one time by intratracheal instillation to each animal 24 hours after the last challenge with *Af* or *Aa*. Dexamethasone (1 mg/kg body weight/day, Sigma-Aldrich) was administered two times by means of oral gavage to each animal, once 24 hours before and again at two hours before the last challenge with *Af* or *Aa*. A mitochondrial reactive oxygen species (mtROS) scavenger, NecroX-5 (30 mg/kg body weight/day, Enzo Life Sciences, Farmingdale, NY, USA) was administered two times by intraperitoneal injection to each animal 24 hours before and after the last challenge with *Af*. MCC 950 (50 mg/kg body weight/day, Cayman Chemical Co., Ann Arbor, MI, USA), a selective inhibitor of NLRP3 inflammasome,[2] was administered one time by intraperitoneal injection to each animal one hour before the last challenge with *Af* or *Aa*. Anti-IL-1β antibody (Ab) or isotype control Ab (100 μg/kg body weight/day, eBioscience, San Diego, CA, USA) was administered intravenously two times to each animal at 24 hours before and after the last challenge with *Af*.

Bronchoalveolar lavage (BAL)

BAL was performed at 72 hours after the last challenge with *Af* or *Aa*. At the time of lavage, the mice (5-7 mice in each group) were killed by means of cervical dislocation. The chest cavity was exposed to allow for expansion, after which the trachea was carefully intubated and the catheter was secured with ligatures. Prewarmed 0.9% NaCl solution was slowly instilled into the lung and withdrawn. The collected solutions were pooled and kept at 4 °C.

A part of each pool was then centrifuged, and the supernatants were kept at −70 °C until use. Total cell numbers were counted with a hemocytometer. Smears of BAL cells were prepared by cytospin (Thermo Fisher Scientific Inc., Waltham, MA, USA) and stained with Diff-Quik solution (Dade Diagnostics of Puerto Rico Inc., Aguada, Puerto Rico) in order to examine cell differentials. Level of IL-1β protein in BAL fluids was measured using IL-1β enzyme-linked immunosorbent assay (ELISA) kit (eBioscience), according to the manufacturer's protocol.

Cell viability assay

To assess the potential toxic effect of the IC87114, viability assay of BAL cells was performed after intratracheal administration of the drug in both normal and *Af*-exposed mice, using the automated NucleoCounter NC-100 (Chemometec, Denmark) according to the manufacturer's protocol.

Measurement of PI3K enzyme activity in lung tissue

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, Santa Clara, CA, USA). 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 phosphate buffered saline (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, Montgomery, TX, USA) 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, St. Paul, MN, USA) according to the manufacturer's protocol.

Isolation and primary culture of murine tracheal epithelial cells (EpCs) and IC87114 treatment

Murine tracheal EpCs were isolated under sterile conditions as described previously.[3] Briefly, the EpCs were seeded onto 60-mm collagen-coated dishes for submerged culture. The growth medium, DMEM (Thermo Fisher Scientific Inc.) containing 10% fetal bovine serum (FBS), penicillin, streptomycin, and amphotericin B, was supplemented with insulin, transferrin, hydrocortisone, phosphoethanolamine, cholera toxin, ethanolamine, bovine pituitary extract, and BSA. The cells were maintained in a humidified 5% CO_2 incubator at 37 °C until they adhered. Cells were treated with IC87114 (10 μmol/l) for two hours, and then stimulated by *Af* antigen (5 μg/ml) for additional 12 hours.

Normal human bronchial epithelial (NHBE) cells culture and treatment

NHBE cells were purchased from Lonza (Walkersville, MD, USA). The cells were cultured in bronchial epithelial basal medium (BEBM, Lonza) supplemented with 10% (v/v) FBS (Lonza), bovine pituitary extract, gentamicin, amphotericin B, hydrocortisone, epidermal growth factor, epinephrine, insulin, triiodothyronine, transferrin, and retinoic acid, which were maintained in a humidified incubator of 5% CO_2 atmosphere at 37 °C. Cells were seeded in culture dishes and grown until 70% confluence. Cells were treated with IC87114 (10 μmol/l) for two hours, and then stimulated by *Af* antigen (5 μg/ml) for additional 12 hours.

PI3K-δ specific siRNA transfection in primary cultured tracheal EpCs and NHBE cells

To analyze the silencing effect of siRNA, RNA was extracted from cells using TRIzol (Thermo Fisher Scientific Inc.) as previously described,[4] and quantitative real-time reverse transcription polymerase chain reaction (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'-CACAGGTCTCATCGAGGTGGTC-3', antisense: 5'-TGGACTTGAGCCAGTTGAGCA-3' and β-actin sense: 5'-CAGATCATGTTTGAGACCTTC-3', antisense: 5'-ACTTCATGATGGAATTGAATG-3'. As for siRNA knockdown of NHBE cells, the cells were transfected using ON-TARGETplus siRNA against PI3K-δ (PIK3CD, catalog no. L-006775-00) (Thermo Fisher Scientific Inc., Waltham, MA, USA) or ON-TARGETplus Non-targeting siRNA at a final concentration of 10 nM. The transfection agent was DharmaFECT 4 from Thermo Scientific Inc., and Opti-MEM I reduced serum medium (Invitrogen, Carlasbad, CA, USA) was used to dilute siRNA and transfection agent,

according to the manufacturer's protocol. siRNAs (10 nM) were transfected 24 hours before the stimulation of NHBE cells with *Af*.

Immunofluorescence staining for NLRP3, Caspase-1, and ASC and cytoplasmic localization of mtROS

Paraffin-embedded lung tissue sections were deparaffinized and hydrated. The sections and *Af*-stimulated primary cultured tracheal EpCs 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 °C 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 Ab to NLRP3 (Adipogen International, San Diego, CA, USA), Ab to Caspase-1 (Santa Cruz Biotechnology, Dallas, TX, USA), and Ab to ASC (Santa Cruz Biotechnology). For the detection of primary Ab, Alexa Fluor 546 (red) labeled goat anti-mouse IgG (Thermo Fisher Scientific Inc.) for NLRP3 and Alexa Fluor 488 (green) labeled goat anti-rabbit IgG (Thermo Fisher Scientific Inc.) for caspase-1 or ASC, in 1% BSA were loaded for one hour at room temperature in dark, respectively. After the specimens were washed, nuclei were stained using 4',6-diamidino-2-phenylindole (DAPI) (Thermo Fisher Scientific Inc.). Stained specimens were mounted on slides using fluorescent mounting medium (Golden Bridge International, Inc., Mukilteo, WA, USA), and then visualized using a confocal laser scanning microscope (Zeiss LSM 510 Meta, Carl Zeiss, Jena, Germany) installed in the Center for University Research Facility (CURF) at Chonbuk National University. To demonstrate the immunofluorescence intensity of mtROS in BAL cells, collected cells were stained with MitoTracker Red CM-

H_2Xros (Thermo Fisher Scientific Inc.) and DAPI, then analyzed using a confocal laser scanning microscope.

Immunohistochemistry (IHC)

Human lung tissue sections came from regional bank of biospecimen in Chonbuk National University Hospital supported by the Korea Bank Project, Ministry for Health and Welfare, Republic of Korea. All samples were deidentified, and all experimental protocols regarding human tissues were approved by the Institutional Review Board of the Biomedical Research Institute of Chonbuk National University Hospital (IRB file No. 2013-11-007-001). For IHC of NLRP3, the deparaffinized 4-μm sections were incubated sequentially according to the instruction using the R. T. U. Vectastain Universal Quick kit from Vector Laboratories Inc. (Burlingame, CA, USA). Briefly, the slides were incubated in 3% H_2O_2 for 20 minutes and in pepsin for 10 minutes at 37 °C. Then, the slides were incubated in normal horse serum for 30 minutes at room temperature, probed with Ab to NLRP3 (Santa Cruz Biotechnology) for two hours at room temperature, and then incubated with prediluted biotinylated pan-specific IgG for 30 minutes. To visualize the Ab 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 Ab. After immunostaining, the slides were photomicrographed.

Clinical information regarding lung tissues of healthy controls (3 persons), patients with idiopathic pulmonary fibrosis (IPF) (4 persons) or patients with allergic bronchopulmonary aspergillosis (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.[5] 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 Abs (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.

Western blot analysis

Lung tissues or *Af*-stimulated primary cultured tracheal EpCs were homogenized in the presence of protease inhibitor cocktail (Sigma-Aldrich), and protein concentrations were determined using Bradford reagent (Bio-Rad Laboratories). For Western blot analysis, samples were loaded onto a SDS-PAGE gel. After electrophoresis at 120 V for 90 minutes, proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories) at 250 mA for 90 minutes by a wet transfer method. Nonspecific sites were blocked with 5% non-fat dry milk in Tris-buffered saline Tween 20 (TBST; 25 mmol/l Tris pH 7.5, 150 mmol/l NaCl, 0.1% Tween 20) for one hour, and the blots were then incubated overnight at 4 °C with an Ab to IL-4 (AbD Serotec, Kidlington, UK), Ab to IL-5 (Santa Cruz Biotechnology), Ab to IL-13 (R&D Systems, Minneapolis, MN, USA), Ab to NLRP3 (Adipogen international), Ab to Caspase-1 (Santa Cruz Bitotechnology), Ab to IL-1β

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

Immunoprecipitation (IP)

Lung tissue homogenates were obtained in the presence of protease inhibitor cocktail (Sigma-Aldrich) and protein concentrations were determined using Bradford reagent (Bio-Rad Laboratories). Co-IP of NLRP3 and interacting proteins was performed using Dynabeads protein G (ThermoFisher Scientific) according to the manufacturer's instructions. Briefly, anti-mouse NLRP3 Ab (Adipogen international) diluted in PBS with 0.02% Tween 20 was incubated with Dynabeads protein G for 10 minutes at room temperature and beads-Ab complex was isolated. Then, lung tissue homogenates were added and incubated for 10 minutes at room temperature to produce antigen-Ab-beads complex. Finally, protein components containing NLRP3 were eluted with Elution buffer (50 mM glycine pH 2.8) and were analyzed by Western blotting.

Trichloroacetic acid (TCA)-mediated protein precipitation of BAL fluids

TCA-mediated protein precipitation of BAL fluids was performed according to the method described elsewhere with some modifications.[6] In this study, saline of 1 ml was instilled for all lavages and 700 μl of BAL fluids were obtained from each mouse. Thereafter, we performed TCA-mediated protein precipitation of BAL fluids and determined the levels of secreted mature IL-1β in BAL fluids. In short, BAL fluids were centrifuged at 3000 × *g* for 2 minutes to remove cell debris. 100% TCA solution (Sigma-Aldrich) was added to the supernatants to a final concentration of 20% TCA and incubated for 10 minutes at 4 °C. Then, the samples were centrifuged at 16000 × *g* for 5 minutes and the pellets were washed with cold acetone. After centrifugation at 16000 × *g* for two minutes, the pellets were resuspended in protein lysis buffer in the presence of protease inhibitor and analyzed by Western blotting.

ASC oligomerization assay

ASC oligomerization assay was performed according to the method described elsewhere with some modifications.[7] Briefly, primary cultured tracheal EpCs were harvested two hours after treatment with IC87114, and cytosolic fractions were isolated through resuspending cells in 0.3 ml buffer containing 20 mM HEPES (pH 7.5), 150 mM KCL, 1.5mM $MgCl_2$, 1m M EGTA, 1mM EDTA, 320 mM sucrose, and protease inhibitor mixture. Cell lysates were centrifuged at 520 × *g* for 10 minutes. Supernatants were diluted with equal volume of 3-[(3-Cholamidopropyl)-dimethylammonio]-1-propane sulfonate (CHAPS) buffer [20 mM HEPES (pH 7.5), 5 mM $MgCl_2$, 0.5 mM EGTA, 0.1% CHAPS]. After the centrifugation at 4000 × *g* for 10 minutes, pelletes containing ASC oligomers were isolated. And, cross-linking of ASC

oligomers was performed through resuspending the pellets in CHAPS buffer containing 2 mM disuccinimidyl suberate (DSS) cross-linker. The cross-linked pellets were then collected by centrifugation at 4000 × *g* for 10 minutes and ASC oligomerization was analyzed by immunoblotting with Ab to ASC (Santa Cruz Biotechnology).

Histology

At 72 hours after the last *Af* or *Aa* challenge, mice were euthanized for histological assessment. Lung and trachea of mice 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.

Determination of airway responsiveness to methacholine

Anesthesia was performed through intraperitoneal injection of 45 mg/kg body weight of sodium pentobarbital. 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/kg body weight at a frequency of 150 breaths/minutes 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 the differences in airway response to methacholine, each mouse was challenged with methacholine aerosol in increasing concentrations (5.0 to 50 mg/ml in saline). After each methacholine challenge, the data of calculated R_{rs} were continuously collected. Maximum values of R_{rs} were selected to express changes in airway function, which was represented as a percentage change from the baseline after saline aerosol.

Statistics

We used SPSS statistical software (version 18.0, SPSS, Chicago, IL, USA). Data are expressed as mean ± SEM. Statistical comparisons were performed using one-way ANOVA followed by the Scheffe's test. Significant differences between two groups (analyses between control and *Af*-exposed groups and analyses between *Af*-exposed wild type and *Af*-exposed p110δ KO mice) were determined using unpaired t-test. A value of $P < 0.05$ was considered statistically significant.

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SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure 1. NLRP3 inflammasome is implicated in *Aspergillus fumigatus* (*Af*)-induced allergic lung inflammation. (A-C) Quantifications of the immunofluorescence intensities for NLRP3 (A) and caspase-1 (Casp-1; B) and their cytoplasmic co-localization (C) in bronchoalveolar lavage (BAL) cells from saline-exposed (Control) or *Aspergillus fumigatus*-exposed (*Af*) mice. (D-F) Quantifications of the immunofluorescence intensities for ASC (D) and NLRP3 (E) and their cytoplasmic co-localization (F) in BAL cells from Control or *Af* mice. (G-I) Quantifications of the immunofluorescence intensities for NLRP3 (G) and Casp-1 (H) and their co-localization (I) in lung tissues of Control or *Af* mice. Bars represent mean ± SEM from six mice per group. $^{\#}P < 0.05$ versus control. (J) Quantifications of the immunofluorescence intensities for NLRP3 in lung tissues from healthy controls, patients with idiopathic pulmonary fibrosis (IPF; disease controls), and patients with allergic bronchopulmonary aspergillosis (ABPA), respectively. (K) Quantifications of the immunohistochemical staining score for NLRP3 in lung tissues from healthy controls, patients with IPF, and patients with ABPA, respectively. Bars represent mean ± SEM from 3 persons in healthy control group, 4 persons in IPF patient group, and 6 persons in ABPA patient group. $^{\#}P < 0.05$ versus healthy control group; $^{*}P < 0.05$ versus IPF patient group.

Supplementary Figure 2. *Aspergillus fumigatus* (*Af*) stimulation leads to NLRP3 inflammasome assembly in tracheal epithelial cells (EpCs). (A) Representative confocal images of tracheal EpCs show the localization of NLRP3 (red) and caspase-1 (Casp-1, green) in normal (Control) or *Af*-stimulated (*Af*) cells under low power field of view. Bars indicate 50 μm. (B-D) Quantifications of the immunofluorescence intensities for NLRP3 (B) and Casp-1 (C) and their co-localization (D) in Control or *Af* cells. (E) Representative confocal

images of tracheal EpCs show the localization of ASC (green) and NLRP3 (red) in Control or *Af* cells under low power field of view. Bars indicate 50 μm. (F-H) Quantifications of the immunofluorescence intensities for ASC (F) and NLRP3 (G) and their co-localization (H) in Control or *Af* cells. Bars represent mean ± SEM from six independent experiments. $^{\#}P < 0.05$ versus control.

Supplementary Figure 3. Phosphoinositide 3-kinase (PI3K)-δ inhibition improves *Aspergillus fumigatus* (*Af*)-induced allergic lung inflammation. (A) Schematic diagram of the experimental protocol involving IC87114. i.p: intraperitoneal, s.c: subcutaneous, i.n: intranasal, i.t: intratracheal. (B and C) Representative immunoblots of phosphorylated (p)-AKT and total (t)-AKT (B) and densitometric analyses of p-AKT (C) in lung tissues from saline-sensitized/challenged mice administered drug vehicle (SAL+VEH), *Af*-sensitized/challenged mice administered drug vehicle (*Af*+VEH), *Af*-sensitized/challenged mice administered 0.1 mg/kg IC87114 (*Af*+IC 0.1) or *Af*-sensitized/challenged mice administered 1.0 mg/kg IC87114 (*Af*+IC 1.0). Bars represent mean ± SEM from 6 mice per group. (D) Enzyme immunoassays of PIP3 in the lung. Bars represent mean ± SEM from 6 mice per group. (E) Levels of total IgE and *Af*-specific IgE in the serum. Bars represent mean ± SEM from 6 mice per group. ND: not detected. (F-I) Representative H&E stained sections of the lung from SAL+VEH (F), *Af*+VEH (G), *Af*+IC 0.1 (H) or *Af*+IC 1.0 (I). Bars indicate scale of 50 μm. (J) Airway responsiveness assessed by invasive (R_{rs}) measurements. Bars represent mean ± SEM from 6 mice per group. (K) Cellular changes in bronchoalveolar lavage fluids. Bars represent mean ± SEM from 6 mice per group. (L-Q) Representative immunoblots of IL-4 (L), IL-5 (N), and IL-13 (P) in lung tissues and densitometric analyses of IL-4 (M), IL-5 (O), and IL-13 (Q). Bars represent mean ± SEM from 6 mice per group. $^{\#}P$

< 0.05 versus SAL+VEH; $^{*}P < 0.05$ versus *Af*+VEH.

Supplementary Figure 4. Quantifications for the protein expressions of NLRP3, caspase-1 (Casp-1), and ASC and their cytoplasmic co-localizations in bronchoalveolar lavage (BAL) cells. (A-C) Quantifications for the protein expressions of NLRP3 (A) and Casp-1 (B) and their cytoplasmic co-localization (C) in BAL cells from saline-exposed mice administered drug vehicle (SAL+VEH), *Af*-exposed mice administered drug vehicle (*Af*+VEH), *Af*-exposed mice administered 0.1 mg/kg IC87114 (*Af*+IC 0.1) or *Af*-exposed mice administered 1.0 mg/kg IC87114 (*Af*+IC 1.0). (D-F) Quantifications for the protein expressions of ASC (D) and NLRP3 (E) and their cytoplasmic co-localization (F) in BAL cells from SAL+VEH, *Af*+VEH, *Af*+IC 0.1 or *Af*+IC 1.0. Bars represent mean ± SEM from 6 mice per group. $^{\#}P <$ 0.05 versus SAL+VEH; $^{*}P < 0.05$ versus *Af*+VEH.

Supplementary Figure 5. Effects of intratracheal administration of IC87114 on the cell viability. (A) Percentage of viable cell numbers in bronchoalveolar lavage (BAL) fluids from normal mice administered drug vehicle (CONT+VEH), normal mice administered 0.1 mg/kg IC87114 (CONT+IC 0.1), or normal mice administered 1.0 mg/kg IC87114 (CONT+IC 1.0). Bars represent mean ± SEM from six mice per group. (B) Percentage of viable cell numbers in BAL fluids from *Aspergillus fumigatus* (*Af*)-exposed mice administered drug vehicle (*Af*+VEH), *Af*-exposed mice administered 0.1 mg/kg IC87114 (*Af*+IC 0.1), or *Af*-exposed mice administered 1.0 mg/kg IC87114 (*Af*+IC 1.0). Bars represent mean ± SEM from six mice per group.

Supplementary Figure 6. Quantifications for the protein expressions of NLRP3, caspase-1 (Casp-1), and ASC and their cytoplasmic co-localizations and mitochondrial reactive oxygen species (mtROS) in *Aspergillus fumigatus* (*Af*)-exposed experimental systems. (A-C) Quantifications of the immunofluorescence intensities for NLRP3 (A) and Casp-1 (B) and their cytoplasmic co-localization (C) in murine tracheal epithelial cells (EpCs) in the control (no treatment), *Af*-stimulated cells (*Af*), *Af*-stimulated cells administered drug vehicle (*Af*+VEH), or *Af*-stimulated cells administered IC87114 (*Af*+IC). (D-F) Quantifications of the immunofluorescence intensities for ASC (D) and NLRP3 (E) and their cytoplasmic co-localization (F) in murine tracheal EpCs in the control, *Af*, *Af*+VEH, or *Af*+IC. Bars represent mean ± SEM from six independent experiments. $^{\#}P < 0.05$ versus control; $^{*}P < 0.05$ versus cells stimulated with *Af* alone. (G-I) Quantifications of the immunofluorescence intensities for NLRP3 (G) and Casp-1 (H) and their cytoplasmic co-localization (I) in murine tracheal EpCs in the control (no treatment), *Af*-stimulated cells (*Af*), *Af*-stimulated cells administered scrambled siRNA (*Af*+Scram), or *Af*-stimulated cells administered PI3K-δ siRNA (*Af*+PI3K-δ). Bars represent mean ± SEM from six independent experiments. $^{\#}P < 0.05$ versus control; $^{*}P < 0.05$ versus cells stimulated with *Af* transfected with scrambled siRNA. (J and K) Representative immunoblots and densitometric analyses of NLRP3 (J) and IL-1β (K) after stimulation with *Af* in EpCs in the presence or absence of IC87114. Bars represent mean ± SEM from six independent experiments. $^{\#}P < 0.05$ versus control; $^{*}P < 0.05$ versus cells stimulated with *Af* alone. (L and M) Representative immunoblots and densitometric analyses of NLRP3 (L) and IL-1β (M) after stimulation with *Af* in EpCs transfected with either scrambled siRNA (Scram siRNA) or PI3K-δ siRNA. Bars represent mean ± SEM from six independent experiments. $^{\#}P < 0.05$ versus control; $^{*}P < 0.05$ versus cells transfected with Scram siRNA. (N) Quantifications of the fluorescence intensity for mtROS in bronchoalveolar lavage (BAL) cells from saline-exposed mice administered drug vehicle

(SAL+VEH), *Af*-exposed mice administered drug vehicle (*Af*+VEH), or *Af*-exposed mice administered NecroX-5 (*Af*+NX5). Bars represent mean ± SEM from six mice per group. $^{\#}P < 0.05$ versus SAL+VEH; $^{*}P < 0.05$ versus *Af*+VEH. (O) Quantifications of the fluorescence intensity for mtROS in BAL cells from saline-exposed mice administered drug vehicle (SAL+VEH), *Af*-exposed mice administered drug vehicle (*Af*+VEH), *Af*-exposed mice administered 0.1 mg/kg IC87114 (*Af*+IC 0.1), or *Af*-exposed mice administered 1.0 mg/kg IC87114 (*Af*+IC 1.0). Bars represent mean ± SEM from six mice per group. $^{\#}P < 0.05$ versus SAL+VEH; $^{*}P < 0.05$ versus *Af*+VEH. (P) Quantifications of the fluorescence intensity for mtROS in murine tracheal EpCs in the control, *Af*, *Af*+VEH, or *Af*+IC. Bars represent mean ± SEM from six independent experiments. $^{\#}P < 0.05$ versus control; $^{*}P < 0.05$ versus cells stimulated with *Af* alone.

Supplementary Figure 7. Quantifications for the protein expressions of NLRP3, caspase-1 (Casp-1), and ASC and their cytoplasmic co-localizations and mitochondrial reactive oxygen species (mtROS) in *Aspergillus fumigatus* (*Af*)-stimulated normal human bronchial epithelial (NHBE) cells. (A-C) Quantifications of the immunofluorescence intensities for NLRP3 (A) and Casp-1 (B) and their cytoplasmic co-localization (C) in the control (no treatment), *Af*-stimulated cells (*Af*), *Af*-stimulated cells administered drug vehicle (*Af*+VEH), or *Af*-stimulated cells administered IC87114 (*Af*+IC). (D-F) Quantifications of the immunofluorescence intensities for ASC (D) and NLRP3 (E) and their cytoplasmic co-localization (F) in the control, *Af*, *Af*+VEH, or *Af*+IC. Bars represent mean ± SEM from six independent experiments. $^{\#}P < 0.05$ versus control; $^{*}P < 0.05$ versus cells stimulated with *Af* alone. (G-I) Quantifications of the immunofluorescence intensities for NLRP3 (G) and Casp-1 (H) and their cytoplasmic co-localization (I) in the control (no treatment), *Af*-stimulated

cells (*Af*), *Af*-stimulated cells administered Non-targeting siRNA (*Af*+NT), or *Af*-stimulated cells administered PI3K-δ siRNA (*Af*+PI3K-δ). Bars represent mean ± SEM from six independent experiments. $^{\#}P < 0.05$ versus control; $^{*}P < 0.05$ versus cells stimulated with *Af* transfected with Non-targeting siRNA. (J) Quantifications of the fluorescence intensity for mtROS in the control, *Af*, *Af*+VEH, or *Af*+IC. Bars represent mean ± SEM from six independent experiments. $^{\#}P < 0.05$ versus control; $^{*}P < 0.05$ versus cells stimulated with *Af* alone.

Supplementary Figure 8. Blockade of NLRP3 inflammasome improves *Alternaria alternata* (*Aa*)-induced allergic lung inflammation. (A) Cellular changes in bronchoalveolar lavage (BAL) fluids from saline-exposed mice administered drug vehicle (SAL+VEH), *Aa*-exposed mice administered drug vehicle (*Aa*+VEH), or *Aa*-exposed mice administered 50 mg/kg MCC 950 (*Aa*+MCC). Bars represent mean ± SEM from six mice per group. $^{\#}P < 0.05$ versus SAL+VEH; $^{*}P < 0.05$ versus *Aa*+VEH. (B-G) Representative H&E stained lung sections and their low power field view from SAL+VEH (B and E), *Aa*+VEH (C and F), and *Aa*+MCC (D and G), respectively. Bars indicate 50 μm (200 μm in low power field view). (H) Histopathologic quantifications of the inflammation scores of peri-bronchial, peri-vascular regions as well as total lung in SAL+VEH, *Aa*+VEH, or *Aa*+MCC. Bars represent mean ± SEM from six mice per group. $^{\#}P < 0.05$ versus SAL+VEH; $^{*}P < 0.05$ versus *Aa*+VEH. (I-M) Representative immunoblots and densitometric analyses of IL-4 (I), IL-5 (J), IL-13 (K), and IL-1β (L) in lung tissues and IL-1β in BAL fluids (M). Bars represent mean ± SEM from six mice per group. $^{\#}P < 0.05$ versus SAL+VEH; $^{*}P < 0.05$ versus *Aa*+VEH.

Supplementary Figure 9. Blockade of phosphoinositide 3-kinase (PI3K)-δ improves *Alternaria alternata* (*Aa*)-induced allergic lung inflammation through regulation of NLRP3 inflammasome. (A) Cellular changes in BAL fluids from saline-exposed mice administered drug vehicle (SAL+VEH), *Aa*-exposed mice administered drug vehicle (*Aa*+VEH), or *Aa*-exposed mice administered 0.1 mg/kg IC87114 (*Aa*+IC 0.1) or *Aa*-exposed mice administered 1.0 mg/kg IC87114 (*Aa*+IC 1.0). Bars represent mean ± SEM from six mice per group. $^{\#}P < 0.05$ versus SAL+VEH; $^{*}P < 0.05$ versus *Aa*+VEH. (B-I) Representative H&E stained lung sections and their low power field view from SAL+VEH (B and F), *Aa*+VEH (C and G), *Aa*+IC 0.1 (D and H), and *Aa*+IC 1.0 (E and I), respectively. Bars indicate 50 μm (200 μm in low power field view). (J) Histopathologic quantifications of the inflammation scores of peri-bronchial, peri-vascular regions as well as total lung in SAL+VEH, *Aa*+VEH, *Aa*+IC 0.1, or *Aa*+IC 1.0. Bars represent mean ± SEM from six mice per group. $^{\#}P < 0.05$ versus SAL+VEH; $^{*}P < 0.05$ versus *Aa*+VEH. (K-O) Representative immunoblots and densitometric analyses of IL-4 (K), IL-5 (L), IL-13 (M), and IL-1β (N) in lung tissues and IL-1β in BAL fluids (O). Bars represent mean ± SEM from six mice per group. $^{\#}P < 0.05$ versus SAL+VEH; $^{*}P < 0.05$ versus *Aa*+VEH.

Supplementary Figure 10. Different responses to dexamethasone between two murine models of fungal allergic lung inflammation. (A) Airway responsiveness assessed by invasive measurements in saline-exposed mice administered drug vehicle (SAL+VEH), *Aspergillus fumigatus* (*Af*)-exposed mice administered drug vehicle (*Af*+VEH), and *Af*-exposed mice administered dexamethasone (*Af*+Dexa). Bars represent mean ± SEM from six mice per group. $^{\#}P < 0.05$ versus SAL+VEH. (B) Cellular changes in bronchoalveolar lavage (BAL) fluids. Bars represent mean ± SEM from six mice per group. $^{\#}P < 0.05$ versus SAL+VEH. To

= total cells; Mac = macrophages; Eo = eosinophils; Neut = Neutrophils; Lym = lymphocytes. (C-E) Representative H&E stained sections of the lung from SAL+VEH (C), *Af*+VEH (D), and *Af*+Dexa (E). Bars indicate 50 μm. (F) Quantifications for histopathologic features through using threshold particle analysis in lung tissues from SAL+VEH, *Af*+VEH, or *Af*+Dexa. (G-I) Representative immunoblots and densitometric analyses of IL-4 (G), IL-5 (H), and IL-13 (I) in lung tissues. Bars represent mean ± SEM from six mice per group. $^{\#}P <$ 0.05 versus SAL+VEH. (J) Airway responsiveness assessed by invasive measurements in saline-exposed mice administered drug vehicle (SAL+VEH), *Alternaria alternata* (*Aa*)-exposed mice administered drug vehicle (*Aa*+VEH), and *Aa*-exposed mice administered dexamethasone (*Aa*+Dexa). Bars represent mean ± SEM from six mice per group. $^{\#}P < 0.05$ versus SAL+VEH; $^{*}P < 0.05$ versus *Aa*+VEH. (K) Cellular changes in BAL fluids. Bars represent mean ± SEM from six mice per group. $^{\#}P < 0.05$ versus SAL+VEH; $^{*}P < 0.05$ versus *Aa*+VEH. (L-N) Representative H&E stained sections of the lung from SAL+VEH (L), *Aa*+VEH (M), and *Aa*+Dexa (N). Bars indicate 50 μm. (O-Q) Representative immunoblots and densitometric analyses of IL-4 (O), IL-5 (P), and IL-13 (Q) in lung tissues. Bars represent mean ± SEM from six mice per group. $^{\#}P < 0.05$ versus SAL+VEH; $^{*}P < 0.05$ versus *Aa*+VEH.

Supplementary Figure 11. Corticosteroid (CS)-resistant NLRP3 inflammasome contributes to *Aspergillus fumigatus* (*Af*)-induced allergic lung inflammation. (A and B) Representative immunoblots and densitometric analyses of IL-1β in lung tissues (A) and BAL fluids (B) from saline-exposed mice administered drug vehicle (SAL+VEH), *Aspergillus fumigatus* (*Af*)-exposed mice administered drug vehicle (*Af*+VEH), and *Af*-exposed mice administered dexamethasone (*Af*+Dexa). Bars represent mean ± SEM from six mice per group. $^{\#}P < 0.05$

versus SAL+VEH. (C and D) Representative immunoblots and densitometric analyses of IL-1β in lung tissues (C) and BAL fluids (D) from saline-exposed mice administered drug vehicle (SAL+VEH), *Alternaria alternata* (*Aa*)-exposed mice administered drug vehicle (*Aa*+VEH), and *Aa*-exposed mice administered dexamethasone (*Aa*+Dexa). Bars represent mean ± SEM from six mice per group. $^{\#}P < 0.05$ versus SAL+VEH; $^{*}P < 0.05$ versus *Aa*+VEH. (E and F) Representative immunoblots and densitometric analysis of NLRP3 (E) and cleaved caspase-1 (F) in lung tissues from SAL+VEH, *Af*+VEH, or *Af*+Dexa. Bars represent mean ± SEM from six mice per group. $^{\#}P < 0.05$ versus SAL+VEH. (G and H) Representative immunoblots and densitometric analysis of NLRP3 (G) and cleaved caspase-1 (H) in lung tissues from SAL+VEH, *Aa*+VEH, or *Aa*+Dexa. Bars represent mean ± SEM from five mice per group. $^{\#}P < 0.05$ versus SAL+VEH; $^{*}P < 0.05$ versus *Aa*+VEH.

Supplementary Figure 12. Effects of dexamethasone on mitochondrial reactive oxygen species (mtROS) generation and nuclear translocation of nuclear factor (NF)-κB in lungs of two different murine models. (A) Representative confocal images show the localization of mtROS in bronchoalveolar lavage (BAL) cells from saline-exposed mice administered drug vehicle (SAL+VEH), *Aspergillus fumigatus* (*Af*)-exposed mice administered drug vehicle (*Af*+VEH), or *Af*-exposed mice administered dexamethasone (*Af*+Dexa) and their quantifications. Bars represent mean ± SEM from six mice per group. $^{\#}P < 0.05$ versus SAL+VEH. Bars indicate 20 μm. (B) Representative confocal images show the localization of mtROS in BAL cells from saline-exposed mice administered drug vehicle (SAL+VEH), *Alternaria alternata* (*Aa*)-exposed mice administered drug vehicle (*Aa*+VEH), or *Aa*-exposed mice administered dexamethasone (*Aa*+Dexa) and their quantifications. Bars represent mean

± SEM from six mice per group. $^{\#}P < 0.05$ versus SAL+VEH; $^{*}P < 0.05$ versus *Aa*+VEH. Bars indicate 20 μm. (C and D) Representative immunoblots and densitometric analyses of NF-κB p65 in nuclear (Nuc) (C) and cytosolic (Cyt) (D) extracts from lung tissues of SAL+VEH, *Af*+VEH, or *Af*+Dexa. Bars represent mean ± SEM from five mice per group. $^{\#}P < 0.05$ versus SAL+VEH. (E and F) Representative immunoblots and densitometric analyses of NF-κB p65 in nuclear (Nuc) (E) and cytosolic (Cyt) (F) extracts from lung tissues of SAL+VEH, *Aa*+VEH, or *Aa*+Dexa. Bars represent mean ± SEM from five mice per group. $^{\#}P < 0.05$ versus SAL+VEH; $^{*}P < 0.05$ versus *Aa*+VEH.