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Thorax Online First, published on November 5, 2015 as 10.1136/thoraxjnl-2015-207096

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ORIGINAL ARTICLE

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

Kyung Sun Lee,[1] Jae Seok Jeong,[2] So Ri Kim,[1,3] Seong Ho Cho,[4] Narasaiah Kolliputi,[4] Yun Hee Ko,[1,3] Kyung Bae Lee,[1,3] Suk Chul Park,[1,3] Hae Jin Park,[1,3] Yong Chul Lee[1,3]

▶ Additional material is published online only. To view please visit the journal online (http://dx.doi.org/10.1136/thoraxjnl-2015-207096).

[1]Department of Internal Medicine, Research Center for Pulmonary Disorders, Chonbuk National University Medical School, Jeonju, South Korea
[2]Department of Immunology, Chonbuk National University Medical School, Jeonju, South Korea
[3]Research Institute of Clinical Medicine of Chonbuk National University–Biomedical Research Institute of Chonbuk National University Hospital, Jeonju, South Korea
[4]Division of Allergy and Immunology, Internal Medicine, Morsani College of Medicine, University of South Florida, Tampa, Florida, USA

Correspondence to
Professor Yong Chul Lee, Department of Internal Medicine, Chonbuk National University Medical School, san 2-20, Geumam-dong, Deokjin-gu, Jeonju 561-180, South Korea; leeyc@jbnu.ac.kr

KSL, JSJ and SRK contributed equally.

Received 24 March 2015
Revised 7 September 2015
Accepted 9 October 2015

ABSTRACT

Background Sensitisation with *Aspergillus fumigatus* (*Af*) is known to be associated with severe allergic lung inflammation, but the mechanism remains to be clarified. Phosphoinositide 3-kinase (PI3K)-δ and endoplasmic reticulum (ER) stress are suggested to be involved in steroid-resistant lung inflammation. We aimed to elucidate the role of PI3K-δ and its relationship with ER stress in fungus-induced allergic lung inflammation.

Methods Using *Af*-exposed in vivo and in vitro experimental systems, we examined whether PI3K-δ regulates ER stress, thereby contributing to steroid resistance in fungus-induced allergic lung inflammation. Moreover, we checked expression of an ER stress marker in lung tissues isolated from patients with allergic bronchopulmonary aspergillosis.

Results *Af*-exposed mice showed that ER stress markers, unfolded protein response (UPR)-related proteins, phosphorylated Akt, generation of mitochondrial reactive oxygen species (mtROS), eosinophilic allergic inflammation, and airway hyperresponsiveness (AHR) were increased in the lung. Similarly, glucose-regulated protein 78 was increased in lung tissues of patients with ABPA. A PI3K-δ inhibitor reduced *Af*-induced increases in ER stress markers, UPR-related proteins, allergic inflammation and AHR in mice. However, dexamethasone failed to reduce *Af*-induced allergic inflammation, AHR and elevation of ER stress. Administration of an ER stress inhibitor or a mtROS scavenger improved *Af*-induced allergic inflammation. The PI3K-δ inhibitor reduced *Af*-induced mtROS generation and the mtROS scavenger ameliorated ER stress. In primary cultured tracheal epithelial cells, *Af*-induced ER stress was inhibited by blockade of PI3K-δ.

Conclusions These findings suggest that PI3K-δ regulates *Af*-induced steroid-resistant eosinophilic allergic lung inflammation through ER stress.

Key messages

What is the key question?
- Does phosphoinositide 3-kinase (PI3K)-δ play a crucial role in fungus-induced severe allergic lung inflammation?

What is the bottom line?
- PI3K-δ regulates *Aspergillus fumigatus*-induced steroid-resistant allergic lung inflammation through modulation of endoplasmic reticulum stress.

Why read on?
- Inhibition of PI3K-δ is efficacious in preclinical models of fungus-induced severe allergic lung disease and may have potential for treating patients with steroid-resistant allergic lung disorders.

INTRODUCTION

Aspergillus fumigatus (*Af*) is ubiquitous in normal air and the human respiratory system is constantly exposed to airborne spores of *Af*. Various lung disorders including invasive aspergillosis, aspergilloma, bronchial asthma and allergic bronchopulmonary aspergillosis (ABPA) are commonly associated with *Af*.[1] Among them, ABPA and bronchial asthma are representative allergic lung inflammatory disorders related to *Af*. ABPA is one of the complex hypersensitivity reactions to *Aspergillus* species usually colonised in bronchi and it occurs in patients with asthma.[2] Although many patients with ABPA respond well to treatment with systemic steroids, the condition of some patients is poorly controlled by conventional management.[3] Additionally, in patients with bronchial asthma, sensitisation with *Af* is known to cause severe phenotypes of the disease.[4 5] However, the mechanisms by which *Af* is involved in allergic lung inflammation remain poorly understood.

Class I phosphoinositide 3-kinases (PI3Ks) are involved in the induction of T helper type 2 cell (T_H2)-related immune responses.[6] They exist as heterodimeric complexes in which a catalytic p110 subunit (designated as α, β, γ or δ) is associated with a particular regulatory subunit (designated as p85, p55, p50 or p101). These proteins are a family of lipid signalling kinases that generate phosphatidylinositol-3,4,5-trisphosphate (PIP_3) and phosphorylate effector proteins such as Akt.[7] The p110-α and p110-β isoforms are ubiquitously expressed. However, expression of p110-δ isoform is restricted to circulating haematogenous cells and endothelial cells.[8] PI3K-δ plays a role in a variety of immune processes involving B-cell and T-cell activation,[9] mast cell degranulation,[10] and trafficking and activation of neutrophils and eosinophils.[11 12]

To cite: Lee KS, Jeong JS, Kim SR, *et al*. *Thorax* Published Online First: [*please include* Day Month Year] doi:10.1136/thoraxjnl-2015-207096

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In particular, PI3K-δ mediates steroid resistance in chronic respiratory disorders in which neutrophilic inflammation predominates.[13] Therefore, inhibition of this pathway may serve as a critical target for treating steroid-resistant severe asthma and non-severe asthma.[14 15] Unfortunately, there is a lack of information on whether PI3K-δ contributes to fungus-induced steroid-resistant eosinophilic allergic lung inflammation.

The endoplasmic reticulum (ER) serves as a protein-folding factory essential for production of functional proteins and trafficking of secretory and biological membrane proteins in cells.[16] ER stress causes accumulation of unfolded and/or misfolded proteins in ER, interferes with protein synthesis and secretion, induces reactive oxygen species (ROS) generation, and increases inflammation partly via nuclear factor (NF)-κB activation.[17] As a result, ER stress is implicated in the pathogenesis of diverse inflammatory processes.[18] Recently, our group demonstrated that ER stress influences the pathogenesis of bronchial asthma, especially the steroid-resistant neutrophilic asthma model.[19] Although various signalling pathways are involved in ER stress,[20 21] the relationship between PI3K-δ and ER stress in allergic lung inflammation is not clearly understood. In addition, there are few data about the role of ER stress in fungus-induced allergic lung inflammation.

In this study, we examined whether PI3K-δ regulates ER stress, thereby contributing to steroid resistance in eosinophil-dominant allergic lung inflammation using *Af*-exposed mice and primary cultured tracheal epithelial cells (TECs). In addition, we analysed the expression of a representative ER stress marker, glucose-regulated protein 78 (GRP78), in lung tissues from patients with ABPA to evaluate the association of ER stress with the pathogenesis of *Af*-related lung inflammation in humans.

METHODS

Animals and experimental protocol

Female C57BL/6 mice, 8–10 weeks of age and free of murine specific pathogens, were used. 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). For the generation of an *Af*-induced allergic lung inflammation model, mice were sensitised and challenged with *Af* antigen, in which the cellular fungal material was inactivated and lyophilised, as previously described.[22] Bronchoalveolar lavage (BAL) was performed at 72 h after the last challenge with *Af* in *Af* sensitised and challenged mice.

Administration of drugs

A selective p110-δ inhibitor, IC87114 (0.1 or 1 mg/kg body weight/day, Calbiochem, San Diego, California, USA) or vehicle control (0.05% dimethyl sulfoxide, DMSO) diluted with 0.9% NaCl was administered once by intratracheal instillation to each animal 24 h after the last challenge with *Af*. A chemical chaperone, 4-phenylbutyric acid (4-PBA) (80 mg/kg body weight/day, Calbiochem) diluted with phosphate-buffered saline, was administered twice by intraperitoneal injection to each animal 24 h before and after the last challenge with *Af*. An inhibitor of NF-κB activation, BAY 11-7085 (20 mg/kg body weight/day, BIOMOL International L.P., Plymouth Meeting, Pennsylvania, USA) dissolved in DMSO and diluted with 0.9% NaCl was administered twice by intraperitoneal injection to each animal 24 h before and after the last challenge with *Af*. Dexamethasone (1 mg/kg body weight/day, Sigma-Aldrich, St Louis, Missouri, USA) diluted with saline was administered twice by means of oral gavage to each animal, once 24 h before and again at 2 h before the last challenge with *Af*.[19 23] A mitochondrial ROS (mtROS) scavenger, NecroX-5 (30 mg/kg body weight/day, Enzo Life Sciences, Farmingdale, New York, USA) diluted with distilled water was administered twice by intraperitoneal injection to each animal 24 h before and after the last challenge with *Af*.

Measurement of PI3K enzyme activity in lung tissue

The amount of PIP_3 produced was quantified by the PIP_3 competition enzyme immunoassay (Echelon, Inc., Salt Lake City, Utah, USA).

Measurement of glutathione and glutathione disulfide in lung tissues

Total glutathione (GSH) and glutathione disulfide (GSSG) levels were determined using a GSH Assay Kit (Cayman Chemical Co., Ann Arbor, Michigan, USA) according to the manufacturer's protocol.

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, Lenoir, North Carolina, USA). Horseradish peroxidase-conjugated goat anti-mouse IgE antibody (Bethyl Laboratories, Montgomery, Texas, USA) was used to detect *Af*-bound IgE. Total serum IgE was measured using a mouse Total IgE ELISA Kit (MD Bioproducts, St Paul, Minnesota, USA) according to the manufacturer's protocol.

Isolation and primary culture of murine TECs and IC87114 treatment

Murine TECs were isolated under sterile conditions as described previously.[6] To verify the role of PI3K-δ in the regulation of ER stress in *Af*-stimulated primary cultured TECs, cells were treated with IC87114 (10 μmol/L) for 2 h, and then stimulated by *Af* antigen (5 μg/mL) for an additional 12 h.

PI3K-δ specific or scrambled small interfering RNA transfection in primary cultured TECs

Primary cultured murine TECs were transfected with the PI3K-δ specific (PIK3CD) or scrambled small interfering RNA (siRNA) (Dharmacon, Lafayette, Louisiana, USA) by using Lipofectamine RNAiMAX reagent (Invitrogen, Carlsbad, California, USA).

Immunofluorescence staining for GRP78, CCAAT/enhancer-binding protein-homologous protein (CHOP) and mtROS

Immunofluorescence staining for GRP78 and CHOP was performed as previously described.[19] To demonstrate the intensity of mtROS in *Af*-stimulated primary cultured TECs and BAL cells, cells were stained with Mitotracker Red CM-H2ROS (Invitrogen) and analysed using a confocal laser scanning microscope (Carl Zeiss, Jena, Germany).

Western blot analysis

Protein levels were determined by Western blotting as previously described.[19]

Nuclear protein extractions

Extraction of nuclear proteins was performed as previously described.[19]

Histology

Histologic assessment was performed as previously described.[19]

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Determination of airway responsiveness to methacholine

Invasive measurement of airway responsiveness was performed as described elsewhere.[19]

Statistics

We used SPSS statistical software (V.18.0, SPSS, Chicago, Illinois, USA). Data are expressed as mean±SEM. Statistical comparisons were performed using one-way ANOVA followed by Scheffe's test. The Mann–Whitney U test was used to compare the two groups of human subjects. A value of $p<0.05$ was considered statistically significant.

RESULTS

PI3K-δ regulates ER stress in vivo

To examine whether p110-δ contributes to regulation of ER stress in *Af*-exposed mice, we evaluated the effects of IC87114, a selective p110-δ inhibitor, on levels of ER stress markers, GRP78 and CHOP, and unfolded-protein response (UPR)-related markers in lung tissues. Levels of GRP78 and CHOP were substantially increased in *Af*-exposed mice compared with those in control mice (figure 1A–D). *Af*-induced increases of GRP78 and CHOP were significantly reduced by administration of IC87114.

Moreover, *Af*-induced increases in UPR-related markers including p-IRE1α, p-eIF2α, XBP-1 and ATF-4 in *Af*-exposed mice were significantly reduced by administration of IC87114 (figure 1E–L).

To demonstrate the involvement of ER stress in *Af*-related allergic lung disorder in humans, we performed immunofluorescence and immunohistochemical analyses with lung tissues obtained from patients with ABPA and healthy controls. The results showed that GRP78 was remarkably increased in patients with ABPA compared with that in healthy controls (see online supplementary figure S1A–C).

PI3K-δ regulates ER stress in vitro

Epithelial cells are central in allergic lung inflammation through regulation of T_H2-related mediators.[24] In particular, we have previously shown that PI3K-δ is involved in the pathogenesis of allergic lung disorders through modulating the release of mediators in the lung epithelial cells.[25] Therefore, to further assess the above interrelationship between PI3K-δ and ER stress in *Af*-induced allergic lung inflammation, primary cultured TECs were used. Levels of GRP78 and CHOP were significantly increased at 12 h after *Af* stimulation compared with the levels in control cells (figure 2A–D). Treatment with IC87114 significantly reduced *Af*-induced increases in GRP78 and CHOP. Supporting the data, treatment with IC87114 limited *Af*-induced increases in immunofluorescence intensities of GRP78 and CHOP in these cells (figure 2E).

To support the link of PI3K-δ to ER stress, PI3K-δ was knocked down in TECs using PI3K-δ-specific siRNA. The level of PI3K-δ mRNA was remarkably increased at 12 h after *Af* stimulation compared with control cells, suggesting that *Af* regulates PI3K-δ expression at gene level (see online supplementary figure S2). In addition, *Af*-induced increase in PI3K-δ mRNA was significantly decreased by transfection of PI3K-δ-specific siRNA. Moreover, *Af*-induced increases in levels of GRP78 and CHOP were significantly decreased by PI3K-δ-specific siRNA (figure 2F–I). Similarly, *Af*-induced increases in immunofluorescence intensities of GRP78 and CHOP were decreased by PI3K-δ-specific siRNA (figure 2J). However, no significant changes were observed by scrambled siRNA.

IC87114 inhibits PI3K activity

To ensure an involvement of PI3K-δ in *Af*-induced allergic lung inflammation, we measured levels of p-Akt and PI3K activity in the lung. The level of p-Akt protein was increased at 72 h after *Af* challenge compared with the level in control mice (figure 3A, B). Consistent with these findings, PI3K activity was significantly increased after *Af* challenge compared with that in control mice (figure 3C). Intratracheal administration of IC87114 reduced *Af*-induced increases of p-Akt and PI3K activity. However, no significant changes in total Akt levels were observed in any of the groups tested.

Effect of IC87114 on *Af*-induced allergic lung inflammation

An increase in the numbers of cells, especially eosinophils, in BAL fluids of *Af*-exposed mice was significantly reduced by administration of IC87114 (figure 3D).

Airway hyperresponsiveness (AHR) was assessed as a change in respiratory system resistance (R_{rs}). In *Af*-exposed mice, the dose–response curve of R_{rs} shifted to the left compared with that in control mice (figure 3E). Administration of IC87114 at 1 mg/kg substantially reduced R_{rs} at 25 and 50 mg/mL of methacholine.

Histological analysis revealed infiltration of various inflammatory cells into bronchioles of *Af*-exposed mice (figure 3G) compared with those in control mice (figure 3F). In addition, *Af*-exposed mice treated with 0.1 mg/kg (figure 3H) or 1.0 mg/kg (figure 3I) of IC87114 showed marked reduction in infiltration of inflammatory cells.

We determined levels of T_H2 cytokines including IL-4, IL-5, and IL-13 and IL-17 in the lung. Western blot analyses showed that *Af*-induced increase in these cytokines was significantly reduced by administration of IC87114 (figure 3J–Q).

Lastly, we determined whether IC87114 modifies *Af*-specific T_H2 response by analysing IgE levels. Treatment with IC87114 significantly lowered *Af*-induced increases in circulating total and *Af*-specific IgE level in a dose-dependent manner (figure 3R).

Effects of dexamethasone on pathophysiologic features and ER stress in *Af*-induced allergic lung inflammation

Steroid resistance is a common feature in severe allergic inflammation.[15] Therefore, we investigated whether dexamethasone improves pathophysiologic features of *Af*-induced allergic lung inflammation. Increases in the numbers of cells including eosinophils in BAL fluids from *Af*-exposed mice were not significantly changed by treatment with dexamethasone (figure 4A). In addition, treatment with dexamethasone did not improve the increase in R_{rs} induced by methacholine (figure 4B). In *Af*-exposed mice administered dexamethasone, the grade of inflammatory cell infiltration into bronchioles was similar to that of *Af*-exposed mice administered the drug vehicle only (figure 4C–E). Moreover, increases in levels of IL-4, IL-5 and IL-13 in the lung were not significantly reduced by dexamethasone (figure 4F–K). These results suggest that the current fungus-induced allergic inflammation model represents a steroid-resistant severe phenotype.

We then examined whether treatment with dexamethasone reduces *Af*-induced increases in ER stress markers. Interestingly, treatment with dexamethasone failed to reduce *Af*-induced elevation of GRP78 and CHOP in the lung of *Af*-exposed mice (figure 4L–O).

Inhibition of ER stress alleviates *Af*-induced allergic lung inflammation

To ascertain the association of ER stress with pathophysiologic features of *Af*-induced allergic lung inflammation, we evaluated

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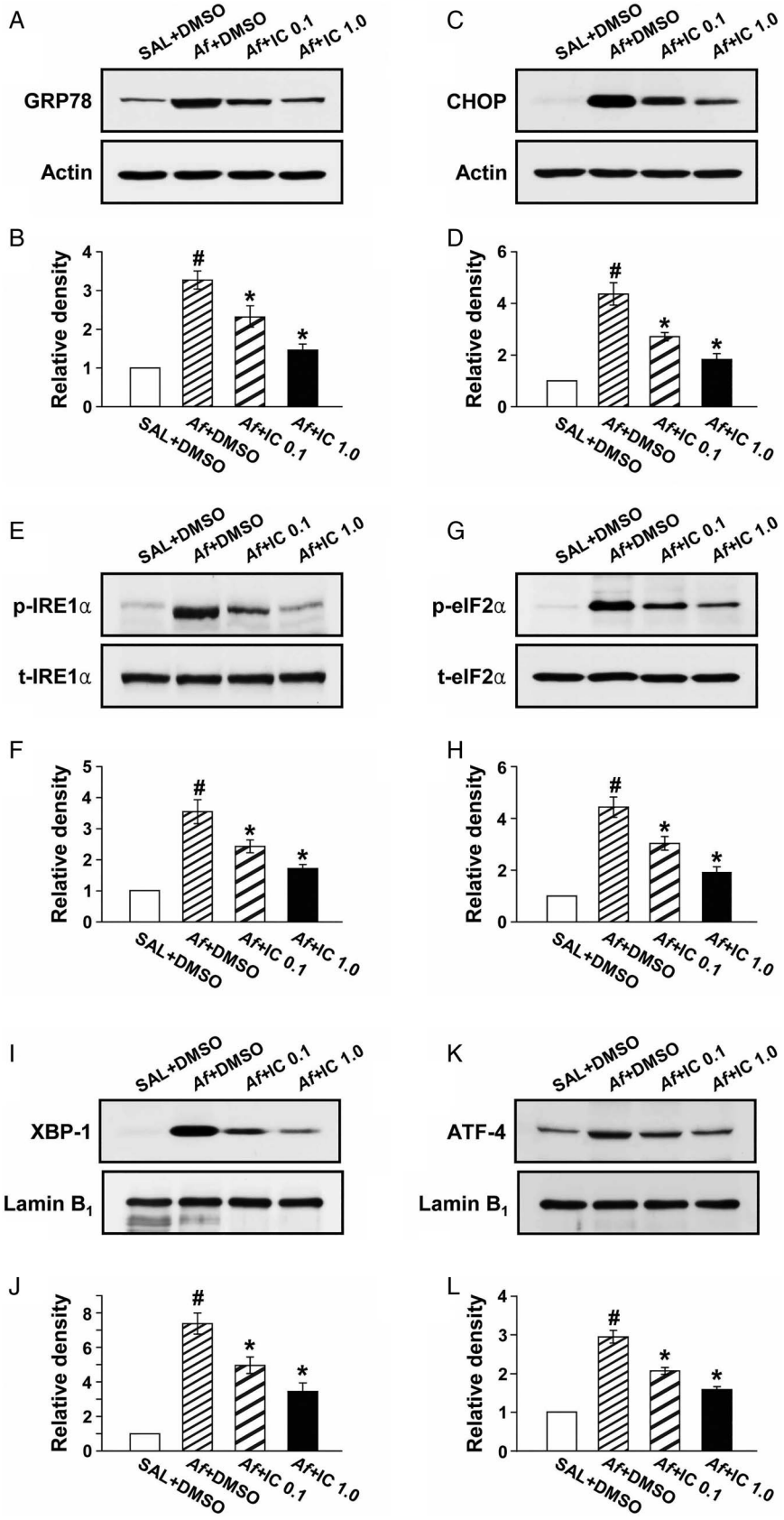

Figure 1 Inhibition of phosphoinositide 3-kinase (PI3K)-δ reduces endoplasmic reticulum (ER) stress and unfolded protein response (UPR) in *Aspergillus fumigatus* (*Af*)-exposed mice. Representative immunoblots of GRP78 (A), CHOP (C), phospho (p)-IRE1α (E), p-eIF2α (G), XBP-1 (I) and ATF-4 (K) in lung tissues and densitometric analyses of GRP78 (B), CHOP (D), p-IRE1α (F), p-eIF2α (H), XBP-1 (J) and ATF-4 (L) from saline sensitised and challenged mice administered drug vehicle (dimethyl sulfoxide, DMSO) (SAL+DMSO), *Af* sensitised and challenged mice administered drug vehicle (*Af*+DMSO), *Af* sensitised and challenged mice administered 0.1 mg/kg IC87114 (*Af*+IC 0.1) or *Af* sensitised and challenged mice administered 1.0 mg/kg IC87114 (*Af*+IC 1.0). Bars represent mean±SEM from six mice per group (five mice per group for p-eIF2α). #p<0.05 versus SAL+DMSO; *p<0.05 versus *Af*+DMSO.

the therapeutic effects of a potent ER stress inhibitor, 4-PBA. Increased numbers of cells, especially eosinophils, in BAL fluids of *Af*-exposed mice were reduced by administration of 4-PBA (figure 5A). Treatment with 4-PBA also reduced the *Af*-induced increase in R_{rs} by methacholine (figure 5B). In addition, *Af*-exposed mice treated with 4-PBA manifested marked

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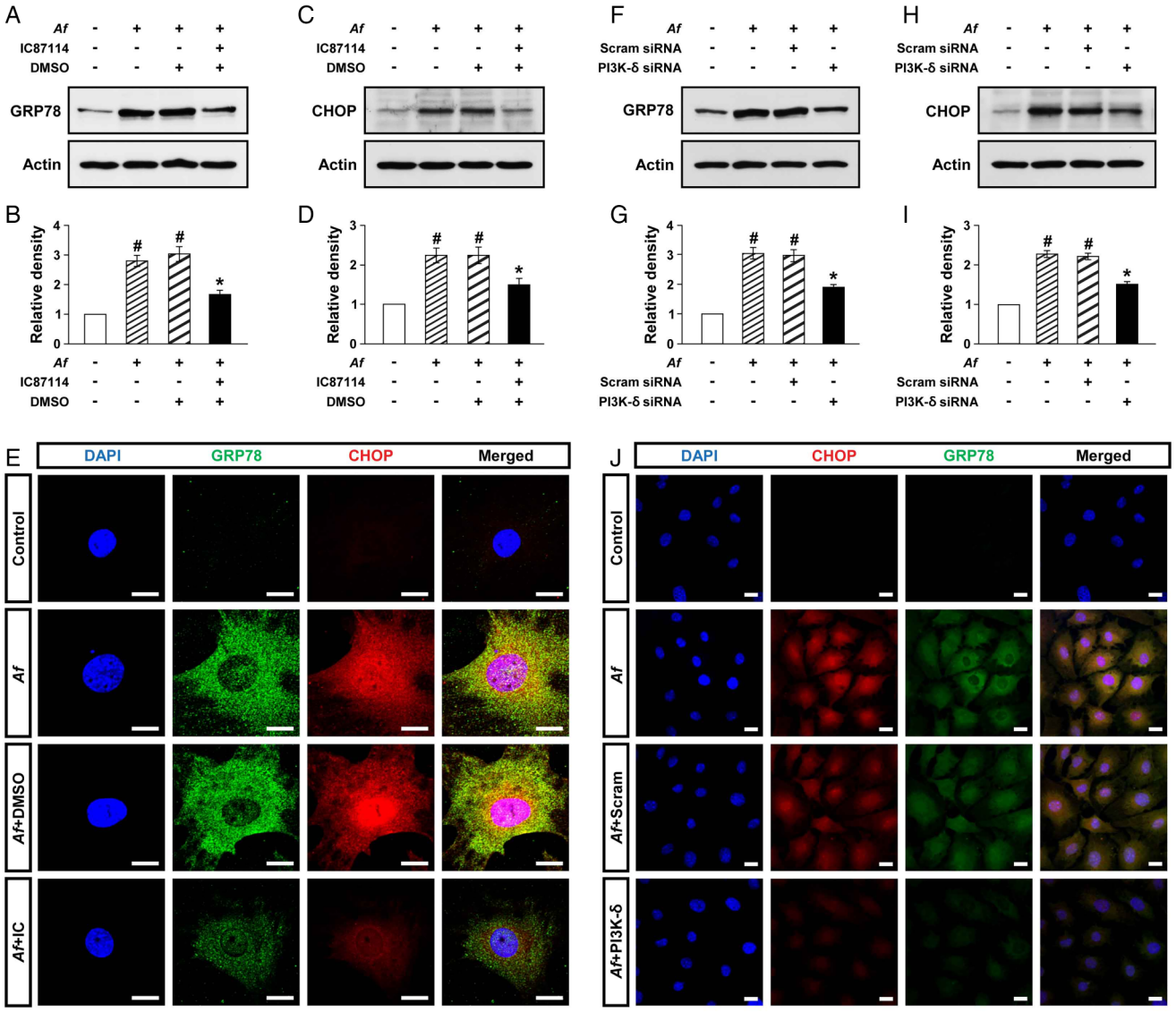

Figure 2 Phosphoinositide 3-kinase (PI3K)-δ modulates endoplasmic reticulum (ER) stress in vitro. Representative immunoblots of GRP78 (A) and CHOP (C) after stimulation with *Aspergillus fumigatus* (*Af*) in primary cultured murine tracheal epithelial cells (TECs) in the presence and absence of IC87114 and densitometric analyses of GRP78 (B) or CHOP (D). Bars represent mean±SEM from five independent experiments. #p<0.05 versus control; *p<0.05 versus cells stimulated with *Af* alone. (E) Representative confocal laser immunofluorescence photomicrographs of primary cultured TECs showed the localisation of GRP78 and CHOP in controls (no treatment), *Af*-stimulated cells (*Af*), *Af*-stimulated cells administered drug vehicle (dimethyl sulfoxide, DMSO) (*Af*+DMSO), or *Af*-stimulated cells administered IC87114 (*Af*+IC). 4′,6-diamidino-2-phenylindole (DAPI) stain was used for nuclear localisation. The right panels show the merger views. Bars indicate 20 μm. Representative immunoblots of GRP78 (F) and CHOP (H) after stimulation with *Af* in primary cultured murine TECs transfected with either scrambled small interfering RNA (Scram siRNA) or PI3K-δ-specific siRNA and densitometric analyses of GRP78 (G) or CHOP (I). 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) Representative confocal laser immunofluorescence photomicrographs of primary cultured TECs showed the localisation of CHOP and GRP78 in controls (no treatment), *Af*-stimulated cells (*Af*), *Af*-stimulated cells administered scrambled siRNA (*Af*+Scram), or *Af*-stimulated cells administered PI3K-δ-specific siRNA (*Af*+PI3K-δ). Bars indicate 20 μm.

reduction of the *Af*-induced infiltration of inflammatory cells into peribronchiolar regions (figure 5C–E).

4-PBA reduces nuclear translocation of NF-κB p65

To understand the molecular basis upon which inhibition of ER stress improves *Af*-induced allergic lung inflammation, we measured NF-κB p65 protein, a well known molecule involved in inflammation, in *Af*-exposed mice. The level of NF-κB p65 in nuclear protein extracts was significantly increased at 72 h after the last *Af* challenge compared with the control (figure 5F, G). The *Af*-induced increase in NF-κB p65 was decreased by administration of 4-PBA.

Effect of NF-κB inhibition on pathophysiologic features of *Af*-induced allergic lung inflammation

To further demonstrate the involvement of NF-κB, we evaluated the effects of a NF-κB inhibitor, BAY 11-7085, on *Af*-induced allergic lung inflammation.

Af-induced increases in numbers of total cells and eosinophils in BAL fluids were significantly reduced by administration of BAY 11-7085 (figure 5H). Treatment with BAY 11-7085 also attenuated the R_{rs} produced at 10 and 50 mg/mL of methacholine in *Af*-exposed mice (figure 5I). In addition, increases in numbers of infiltrated cells around bronchioles after the last *Af* challenge were markedly reduced by BAY 11-7085

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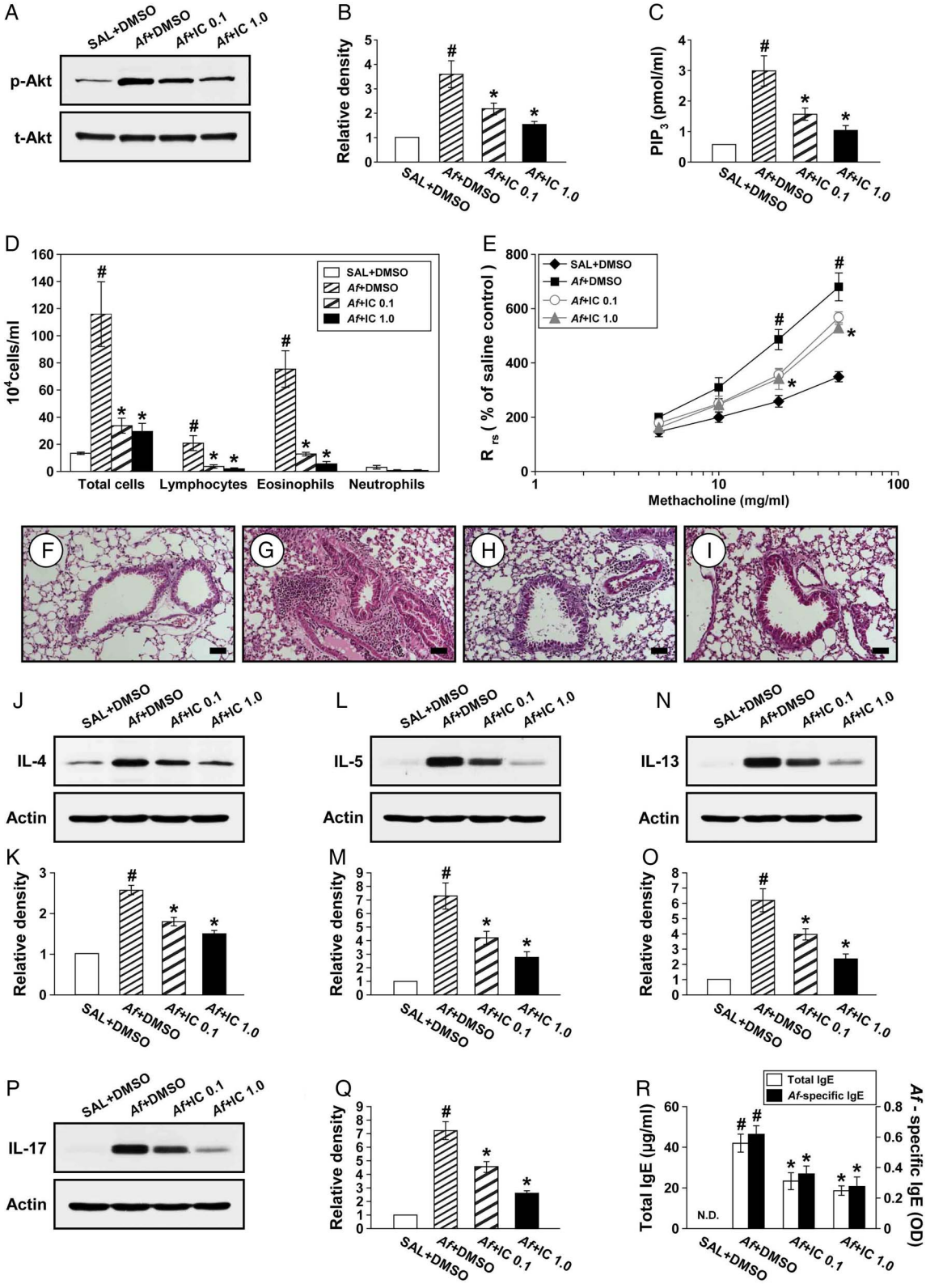

Figure 3 Inhibition of phosphoinositide 3-kinase (PI3K)-δ ameliorates *Aspergillus fumigatus* (*Af*)-induced allergic lung inflammation. Representative immunoblots of p-Akt and total (t)-Akt in lung tissues (A) from SAL+dimethyl sulfoxide (DMSO), *Af*+DMSO, *Af*+IC 0.1 or *Af*+IC 1.0, and densitometric analyses of p-Akt (B). Bars represent mean±SEM from six mice per group. (C) Enzyme immunoassays of PIP_3 in lung tissues. Bars represent mean±SEM from six mice per group. (D) Cellular changes in bronchoalveolar lavage (BAL) fluids. Bars represent mean±SEM from six mice per group. (E) Airway responsiveness assessed by invasive (R_{rs}) measurements. Bars represent mean±SEM from five mice per group. Representative H&E stained sections of the lung from SAL+DMSO (F), *Af*+DMSO (G), *Af*+IC 0.1 (H) or *Af*+IC 1.0 (I). Bars indicate scale of 50 μm. Representative immunoblots of IL-4 (J), IL-5 (L), IL-13 (N) and IL-17 (P) in lung tissues and densitometric analyses of IL-4 (K), IL-5 (M), IL-13 (O) and IL-17 (Q). Bars represent mean±SEM from six mice per group. (R) Levels of total IgE and *Af*-specific IgE in the serum. Bars represent mean±SEM from five mice per group. #p<0.05 versus SAL+DMSO; *p<0.05 versus *Af*+DMSO.

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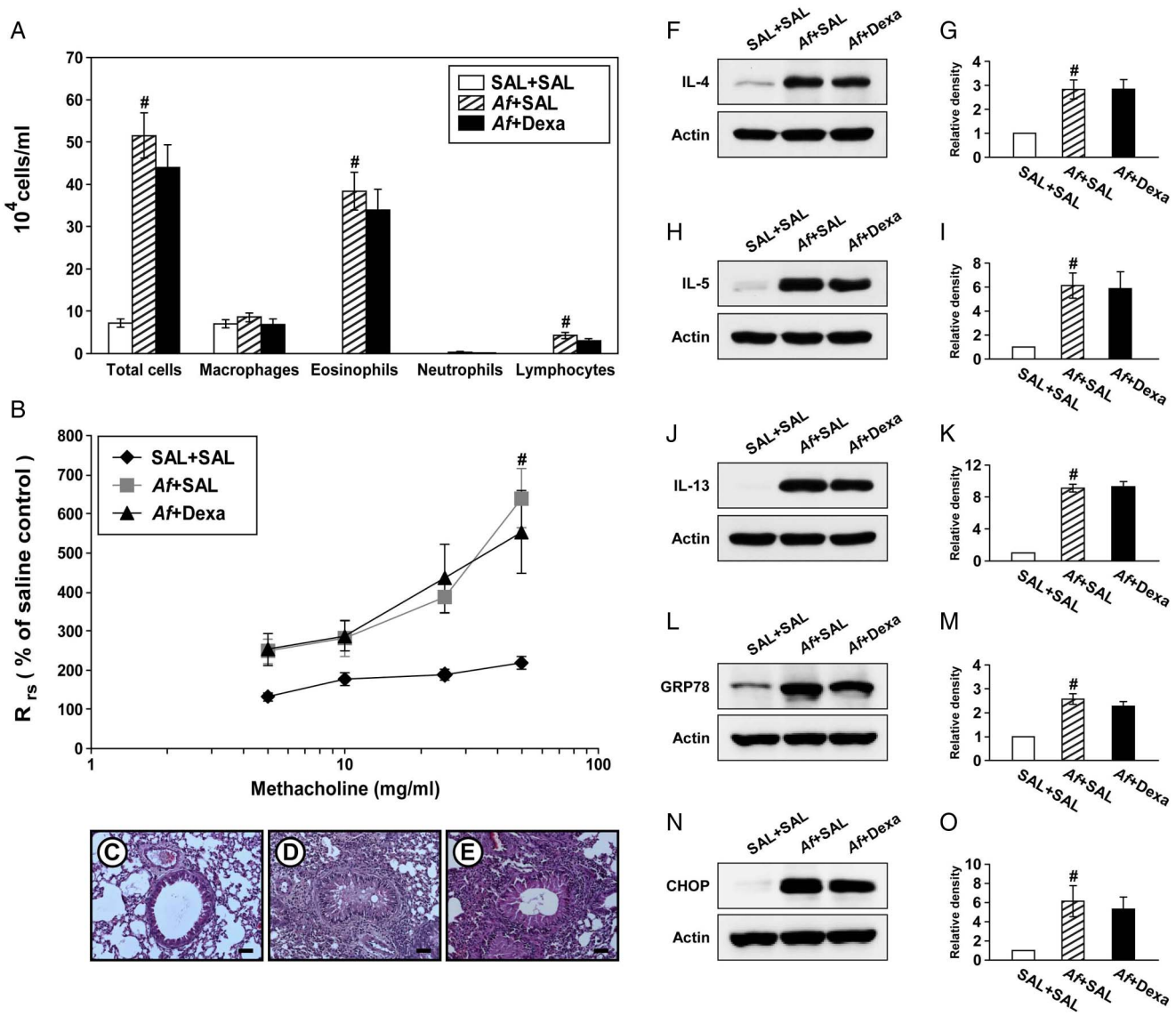

Figure 4 Dexamethasone treatment does not improve *Aspergillus fumigatus* (*Af*)-induced allergic inflammation and endoplasmic reticulum (ER) stress. (A) Cellular changes in bronchoalveolar lavage (BAL) fluids from saline sensitised and challenged mice administered drug vehicle (saline) (SAL+SAL), *Af* sensitised and challenged mice administered drug vehicle (*Af*+SAL) or *Af* sensitised and challenged mice administered dexamethasone (*Af*+Dexa). Bars represent mean±SEM from 10 mice per group. (B) Airway responsiveness. Bars represent mean±SEM from six mice per group. Representative H&E stained sections of the lung from SAL+SAL (C), *Af*+SAL (D) or *Af*+Dexa (E). Bars indicate scale of 50 μm. Representative immunoblots of IL-4 (F), IL-5 (H), IL-13 (J), GRP78 (L) and CHOP (N) in lung tissues and densitometric analyses of IL-4 (G), IL-5 (I), IL-13 (K), GRP78 (M) and CHOP (O) from SAL+SAL, *Af*+SAL and *Af*+Dexa. Bars represent mean±SEM from nine mice per group (five and six mice per group for GRP78 and CHOP, respectively). #p<0.05 versus SAL+SAL.

(figure 5J–L). Furthermore, *Af*-induced increases in IL-4, IL-5 and IL-13 protein were substantially reduced by BAY 11-7085 (figure 5M, N).

IC87114 restores GSH

Oxidative stress is one of the most important features in allergic lung inflammation.[26] Therefore, we measured GSH and GSSG in lung tissues. *Af*-exposed mice showed significantly lower GSH level than control mice (figure 6A). In contrast, GSSG level was increased after *Af* challenge (figure 6B). *Af*-induced decrease in GSH and increase in GSSG were substantially restored by administration of IC87114.

IC87114 reduces *Af*-induced mtROS generation

Mitochondrion has been recognised as a major source of intracellular ROS.[27] We therefore examined the relationship between PI3K-δ and mtROS generation in our experimental systems. Immunofluorescence intensity of mtROS in BAL cells was markedly increased in *Af*-exposed mice compared with the intensity in control mice (figure 6C). Administration of IC87114 reduced *Af*-induced increase in immunofluorescence intensity of mtROS. Consistent with in vivo data, *Af*-induced increase in fluorescence intensity of mtROS in TECs was markedly inhibited by administration of IC87114 (figure 6D). These results indicate that PI3K-δ may induce mtROS generation.

NecroX-5 ameliorates inflammatory cell infiltration and AHR

To further investigate the role of mtROS in *Af*-induced allergic lung inflammation, we evaluated the treatment effects of a potent mtROS scavenger, NecroX-5.[28] Increased numbers of cells, particularly eosinophils, in BAL fluids from *Af*-exposed mice were remarkably reduced by administration of NecroX-5

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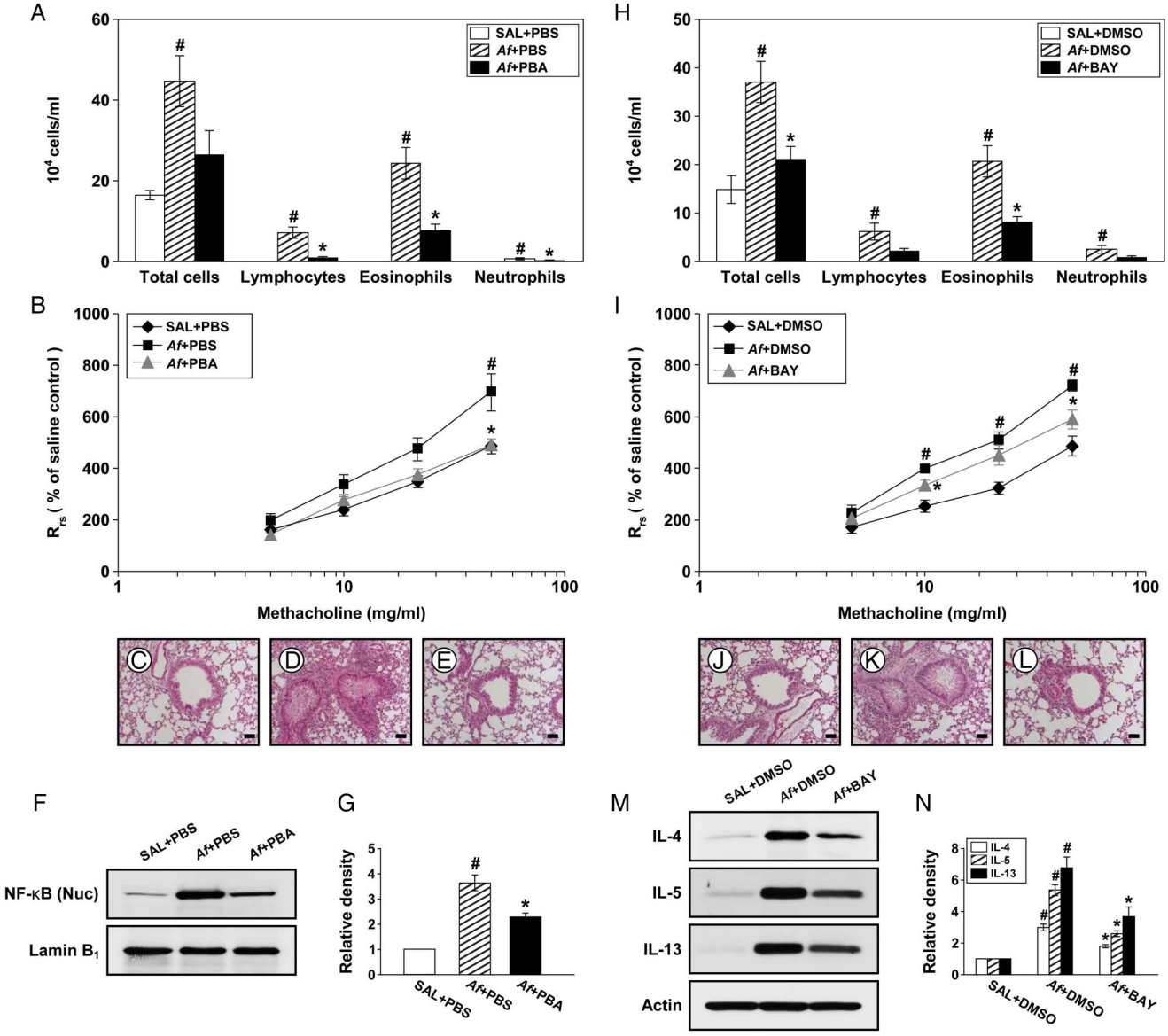

Figure 5 Endoplasmic reticulum (ER) stress is implicated in *Aspergillus fumigatus* (*Af*)-induced allergic lung inflammation through activating nuclear factor (NF)-κB. (A) Cellular changes in bronchoalveolar lavage (BAL) fluids from saline sensitised and challenged mice administered drug vehicle (phosphate-buffered saline, PBS) (SAL+PBS), *Af* sensitised and challenged mice administered drug vehicle (*Af*+PBS) or *Af* sensitised and challenged mice administered 4-phenylbutyric acid (PBA) (*Af*+PBA). Bars represent mean±SEM from six mice per group. (B) Airway responsiveness. Bars represent mean±SEM from five mice per group. Representative H&E stained sections of the lung from SAL+PBS (C), *Af*+PBS (D) or *Af*+PBA (E). Bars indicate scale of 50 μm. Representative immunoblot of NF-κB p65 in nuclear extracts from lung tissues (F) and densitometric analysis of NF-κB p65 (G). Bars represent mean±SEM from six mice per group. #p<0.05 versus SAL+PBS; *p<0.05 versus *Af*+PBS. (H) Cellular changes in BAL fluids from saline sensitised and challenged mice administered drug vehicle (dimethyl sulfoxide, DMSO) (SAL+DMSO), *Af* sensitised and challenged mice administered drug vehicle (*Af*+DMSO) or *Af* sensitised and challenged mice administered with BAY 11-7085 (*Af*+BAY). Bars represent mean±SEM from six mice per group. (I) Airway responsiveness. Bars represent mean±SEM from five mice per group. Representative H&E stained sections of the lung from SAL+DMSO (J), *Af*+DMSO (K) or *Af*+BAY (L). Bars indicate scale of 50 μm. Representative immunoblots of IL-4, IL-5 and IL-13 (M) in lung tissues and densitometric analyses of IL-4, IL-5 and IL-13 (N). Bars represent mean±SEM from six mice per group. #p<0.05 versus SAL+DMSO; *p<0.05 versus *Af*+DMSO.

(figure 7A). In addition, *Af*-exposed mice treated with NecroX-5 showed marked reduction in infiltration of inflammatory cells into the peribronchiolar region compared with that in control mice (figure 7B–D). Moreover, administration of NecroX-5 resulted in a significant attenuation in R_{rs} at 50 mg/mL of methacholine (figure 7E).

NecroX-5 reduces GRP78 and CHOP

To investigate whether mtROS modulates ER stress, we evaluated the interrelationship between them using NecroX-5. Levels of GRP78 and CHOP were significantly elevated in *Af*-exposed mice compared with those in control mice (figure 7F–I). Interestingly, administration of NecroX-5 markedly lowered *Af*-induced increases in these proteins, suggesting that mtROS generation induces ER stress.

Effect of NecroX-5 on *Af*-induced mtROS generation in BAL fluids

Immunofluorescence intensity of mtROS in BAL cells was markedly increased in *Af*-exposed mice compared with that in

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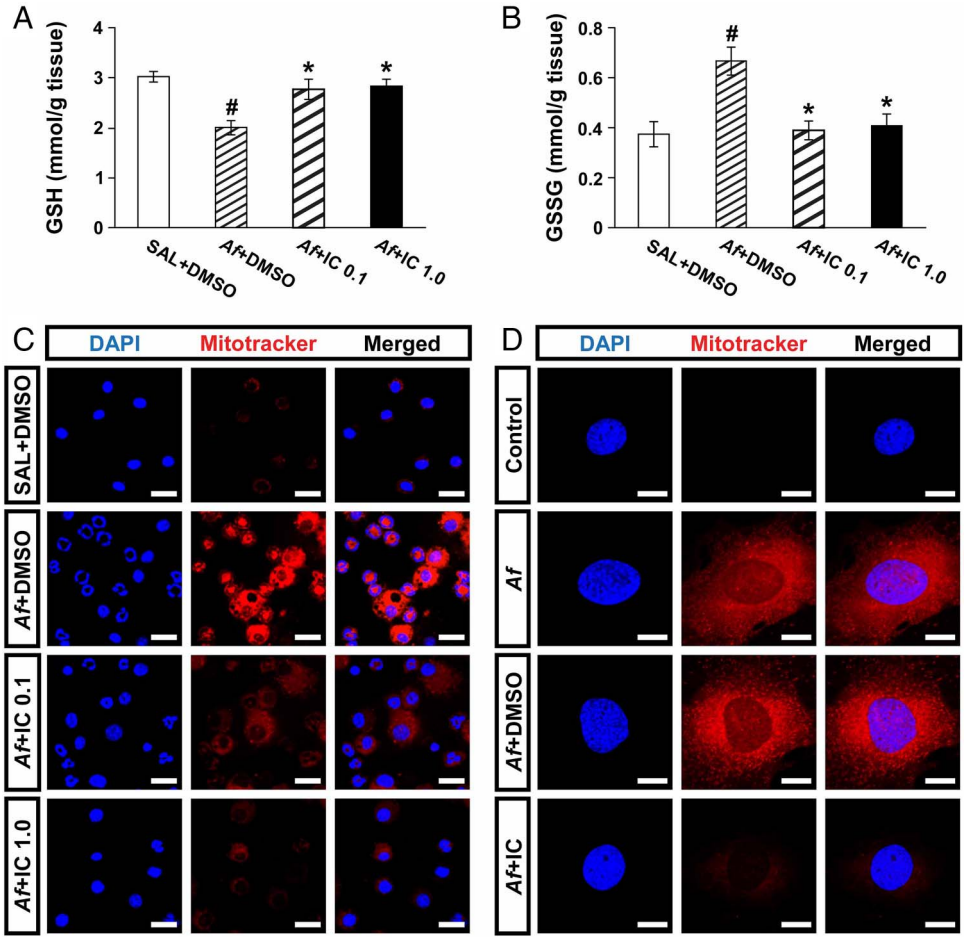

Figure 6 Phosphoinositide 3-kinase (PI3K)-δ regulates *Aspergillus fumigatus* (*Af*)-induced generation of mitochondrial reactive oxygen species (mtROS). Levels of glutathione (GSH) (A) and glutathione disulfide (GSSG) (B) in lung tissues from SAL+dimethyl sulfoxide (DMSO), *Af*+DMSO, *Af*+IC 0.1 or *Af*+IC 1.0. Bars represent mean±SEM from seven mice per group. #p<0.05 versus SAL+DMSO; *p<0.05 versus *Af*+DMSO. (C) Representative confocal laser immunofluorescence photomicrographs showed the localisation of mtROS in bronchoalveolar lavage (BAL) cells. Bars indicate a scale of 20 μm. (D) Representative confocal laser immunofluorescence photomicrographs of primary cultured TECs showed the localisation of mtROS in the control (no treatment), *Af*, *Af*+DMSO or *Af*+IC. Bars indicate a scale of 20 μm.

control mice (figure 7J). Administration of NecroX-5 substantially reduced *Af*-induced increase in immunofluorescence intensity of mtROS.

DISCUSSION

In this study, ER stress was increased in the lung of *Af*-exposed mice. Expression of GRP78 was also elevated in lung tissue from patients with ABPA, a hypersensitivity disorder induced by *Af*, compared with that in healthy controls. These results suggest that ER stress may be involved in the pathogenesis of *Af*-related allergic lung disorders. Additionally, we found a novel mechanism of action of PI3K-δ as an upstream regulator of ER stress through mtROS generation in *Af*-induced allergic lung inflammation. Also, blockade of PI3K-δ remarkably ameliorated *Af*-induced steroid-resistant allergic lung inflammation. These findings suggest that PI3K-δ plays an essential role in the pathogenesis of fungus-induced severe allergic lung inflammation.

Sensitivity to fungi has been known as one of the crucial determinants complicating clinical course of bronchial asthma.[4] Among various species, *Af* is a major indoor and outdoor fungi.[1] Allergic sensitisation to *Af* in patients with bronchial asthma is often associated with reduced lung function and immunologically mediated structural lung damage.[5] Furthermore, the conventional anti-inflammatory agents including corticosteroids may be insufficient for treating severe asthma associated with fungal sensitisation.[29] In this study, treatment with dexamethasone failed to alleviate various pathophysiologic features of *Af*-induced allergic lung inflammation, including eosinophil-dominant inflammatory cell infiltration into the lung, AHR, and elevation of T_H2-dominant pro-inflammatory cytokines. In fact, we revealed that under an identical protocol to this study, the same dose of dexamethasone attenuates the typical features of allergic asthma induced by ovalbumin which is a well established classic asthma model.[19] These results suggest that *Af*-induced lung inflammation in the current murine experimental model represents a unique endotype for steroid-resistant eosinophil-dominant allergic lung inflammation.

ER stress and UPR activation are known to be closely involved in pathogen immunity by influencing innate and adaptive immune responses and being interconnected with diverse intracellular inflammatory platforms.[30] Previous studies have demonstrated that ER stress is implicated in the pathogenesis of various pulmonary disorders, including bronchial asthma, COPD, idiopathic pulmonary fibrosis and lipopolysaccharide-related acute lung injury.[19 31–33] Particularly, we recently reported that ER stress contributes to corticosteroid resistance in a murine model of neutrophil-dominant allergic lung

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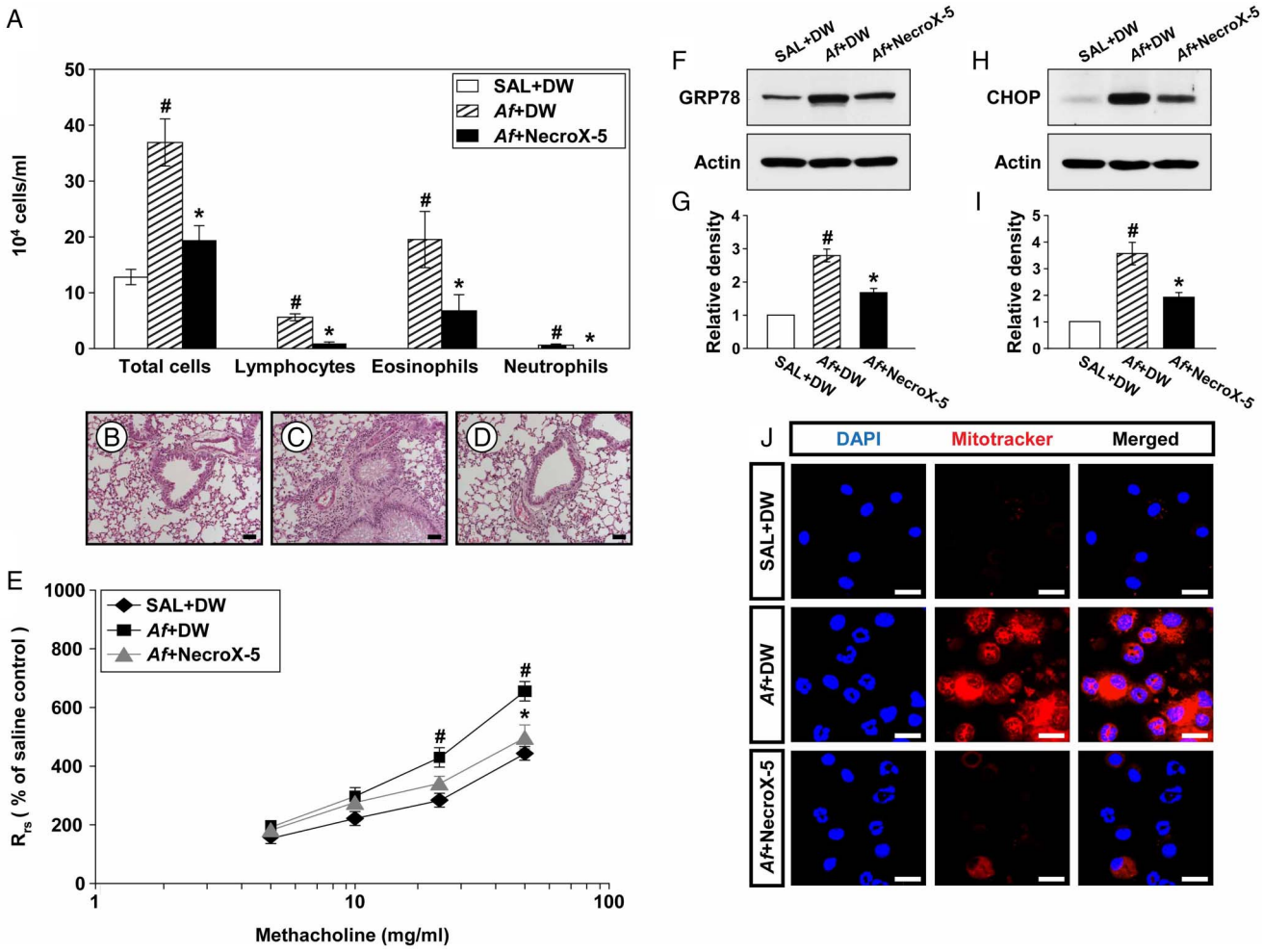

Figure 7 Depletion of mitochondrial reactive oxygen species (mtROS) improves *Aspergillus fumigatus* (*Af*)-induced allergic inflammation through modulating endoplasmic reticulum (ER) stress. (A) Cellular changes in bronchoalveolar lavage (BAL) fluids from saline sensitised and challenged mice administered drug vehicle (distilled water, DW) (SAL+DW), *Af* sensitised andchallenged mice administered drug vehicle (*Af*+DW), or *Af* sensitised and challenged mice administered NecroX-5 (*Af*+NecroX-5). Bars represent mean±SEM from six mice per group. Representative H&E stained sections of the lung from SAL+DW (B), *Af*+DW (C) and *Af*+NecroX-5 (D). Bars indicate scale of 50 μm. (E) Airway responsiveness. Bars represent mean±SEM from five mice per group. Representative immunoblots of GRP78 (F) and CHOP (H) in lung tissues and densitometric analyses of GRP78 (G) and CHOP (I). Bars represent mean±SEM from six mice per group. #p<0.05 versus SAL+DW; *p<0.05 versus *Af*+DW. (J) Representative confocal laser immunofluorescence photomicrographs showed the localisation of mtROS in BAL cells from SAL+DW, *Af*+DW or *Af*+NecroX-5. Bars indicate a scale of 20 μm.

inflammation.[19] In the current study, sensitisation/challenge with *Af* increased expression of ER stress markers and UPR-related proteins in the lung. Expression of ER stress markers was also elevated in *Af*-stimulated TECs. Moreover, in lung tissues from patients with ABPA, expression of GRP78 was also increased. In addition, blockade of ER stress improved *Af*-induced eosinophilic allergic lung inflammation and AHR. However, *Af*-induced increases in the levels of GRP78 and CHOP were not reduced by treatment with dexamethasone. These findings suggest that ER stress is involved in the pathogenesis of *Af*-induced allergic lung inflammation.

Activation of transcription factor NF-κB serves as an important molecular platform interconnecting ER stress and inflammatory process.[17] Recently, the NF-κB signalling pathway has been reported to be essential in ER stress-related lung inflammation.[19 33] In the present study, the level of nuclear NF-κB p65 in the lung was significantly increased after *Af* challenge. *Af*-induced increase in NF-κB p65 was decreased by administration of an ER stress inhibitor. Additionally, treatment with a NF-κB inhibitor substantially reduced increases in pro-inflammatory mediators, inflammatory cell infiltrations specifically eosinophils, and AHR in *Af*-exposed mice. Taken together, these findings suggest that NF-κB is a pivotal player in ER stress-related allergic lung inflammation.

The PI3K pathway mediates a number of critical cellular events, such as cell survival, proliferation and migration.[7] As for *Af*-related inflammatory disorders, to date, it remains unclear about the roles of PI3K and its isotypes. In fact, the PI3K pathway has been known to participate not only in host defence against *Af*[34 35] but also in the pathogenesis of *Af*-induced inflammatory disorders or aspergillosis.[36] In addition, there is little available information on the PI3K-δ subtype in *Af*-related pulmonary disorders. Previous studies have reported that PI3K-δ is crucially involved in the pathogenesis of a number of pulmonary inflammatory diseases, including bronchial asthma and COPD.[13 37] In particular, PI3K-δ is known to mediate steroid resistance in relation to neutrophil-dominant inflammatory processes in the lung. The basic mechanisms through which PI3K-δ modulates neutrophilic inflammation include trafficking and activation of neutrophils, and decrease in histone deacetylase-2

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expression.[11 13 15] However, severe allergic lung inflammation is heterogeneous and may arise from diverse pathophysiological processes involving various cell types. Among them, eosinophilic inflammation affects the majority of patients with severe asthma.[38] In our study, *Af* challenge in mice increased levels of PIP_3 and p-Akt in the lung. Stimulation with *Af* also upregulates PI3K-δ mRNA expression in TECs. In *Af*-exposed mice, PI3K-δ inhibition with a selective inhibitor significantly lowered *Af*-induced increases in PIP_3 and p-Akt and reduced infiltration of airway inflammatory cells, especially eosinophils, production of pro-inflammatory mediators, total and *Af*-specific IgE, and AHR. Interestingly, PI3K-δ inhibition significantly limited *Af*-induced increases in ER stress markers and UPR-related proteins in the lung. In addition, the PI3K-δ inhibitor significantly limited *Af*-induced increases in ER stress markers in TECs. Transfection of PI3K-δ siRNA also significantly limited *Af*-induced increases in GRP78 and CHOP in cells. In agreement with these findings, blockade of PI3K-δ using the PI3K-δ inhibitor or PI3K-δ siRNA in cells substantially limited *Af*-induced increases in immunofluorescence intensities of GRP78 and CHOP. These observations indicate that PI3K-δ may play an important role in *Af*-induced steroid-resistant eosinophil-dominant allergic lung inflammation. Moreover, PI3K-δ seems to contribute to steroid resistance in the current model through induction of ER stress in the lung.

ROS production, which leads to oxidative stress, is one of critical features in chronic airway disorders.[26] In addition, oxidative stress contributes to diverse pathologic processes through modulation of the PI3K signalling pathway.[39] Emerging evidence has indicated that mitochondrion is an important source in the production of most intracellular ROS and that considerable interplay between mitochondria and ER in several respiratory disorders has been demonstrated.[27 40] Moreover, PI3K signalling is closely associated with mitochondrial physiology.[41]

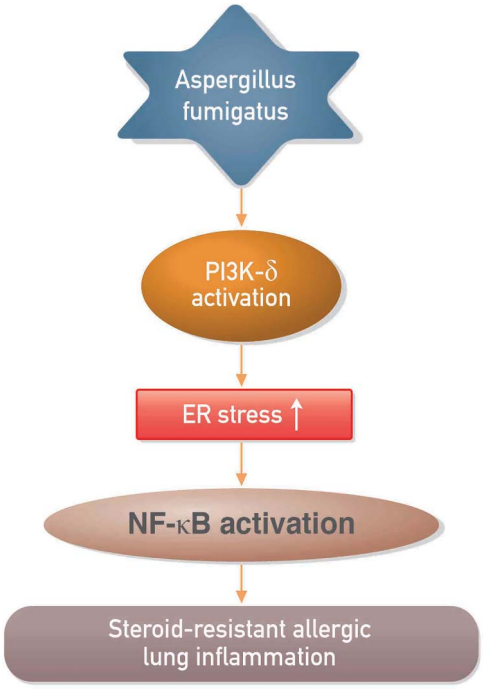

Figure 8 Proposed mechanism for the involvement of phosphoinositide 3-kinase (PI3K)-δ in the regulation of endoplasmic reticulum (ER) stress in *Aspergillus fumigatus* (*Af*)-related steroid-resistant allergic lung inflammation. NF-κB, nuclear factor κB.

In the present study, BAL cells of *Af*-exposed mice and *Af*-stimulated TECs showed increased production of mtROS. In addition, blockade of PI3K-δ reduced *Af*-induced increases in mtROS generation in these cells. PI3K-δ inhibition also blocked *Af*-induced decreases in GSH level in the lung of *Af*-exposed mice. Moreover, treatment with NecroX-5 effectively attenuated increases in mtROS in BAL cells, reduced increases in GRP78 and CHOP in *Af*-exposed mice, and ameliorated pathophysiologic features of *Af*-induced allergic airway inflammation. Taken together, PI3K-δ may activate the ER stress pathway at least in part through regulation of mtROS generation in the current model.

In summary, we have shown that PI3K-δ and ER stress are involved in the pathogenesis of *Af*-induced allergic lung inflammation and that PI3K-δ may regulate *Af*-induced steroid-resistant lung inflammation through ER stress (figure 8). Our results suggest that inhibition of PI3K-δ is efficacious in preclinical models of fungus-induced severe allergic lung disease and may have potential for treating patients with steroid-resistant eosinophil-dominant allergic lung disorders.

Acknowledgements We thank Professor Mie-Jae Im and Peter Lee for critical readings of the manuscript.

Contributors KSL performed experiments and analysed data; JSJ interpreted data and wrote the manuscript; SRK designed research, interpreted data, and edited the manuscript; SHC reviewed and edited the manuscript; NK reviewed and edited the manuscript; YHK performed experiments and analysed data; KBL analysed data and reviewed the manuscript; SCP analysed data and reviewed the manuscript; HJP analysed data and reviewed the manuscript; YCL designed research, interpreted data, and edited the manuscript.

Funding This work was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT and Future Planning (2014R1A2A1A01002823).

Competing interests None declared.

Ethics approval Institutional Review Board of the Biomedical Research Institute of Chonbuk National University Hospital (IRB file No. 2013-11-007-001).

Provenance and peer review Not commissioned; externally peer reviewed.

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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.

Isolation/primary culture of TECs

Murine TECs were isolated under sterile conditions. The epithelial cells were seeded onto 60-mm collagen-coated dishes for submerged culture. The growth medium, DMEM (Invitrogen) 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. To verify the role of PI3K-δ in the regulation of ER stress in *Af*-stimulated primary cultured TECs, cells were treated with IC87114 (10 μmol/l) for two hours, then stimulated by *Af* antigen (5 μg/ml) for additional 12 hours. After that, cells were harvested.

PI3K-δ specific or scrambled siRNA transfection

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

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

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 ºC 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

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

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

Western blot analysis

Lung tissues or *Af*-stimulated primary cultured TECs were homogenized in the presence of protease inhibitor cocktail (Sigma-Aldrich), and protein concentrations were determined using Bradford reagent (Bio-Rad Laboratories). Samples were loaded onto a SDS-PAGE gel. After electrophoresis at 120 V for 90 minutes, proteins were transferred to 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 antibody to IL-4 (AbD Serotec, Kidlington, near oxford, UK), antibody to IL-5 (Santa Cruz Biotechnology), antibody to IL-13 (R&D Systems, Minneapolis, MN, USA), antibody to IL-17 (R&D Systems), antibody to GRP78 (Cell Signaling Technologies, Danvers, MA, USA), antibody to CHOP (Santa Cruz Biotechnology), antibody to p-IRE1α (Novus biologicals, Littleton, CO, USA), antibody to IRE1α (Cell Signaling Technologies), antibody to p-eIF2α (Ser51) (Cell Signaling Technologies), antibody to eIF2α

(Cell Signaling Technologies), antibody to p-Akt (R&D Systems), antibody to Akt (Cell Signaling Technologies), and antibody to actin (Sigma-Aldrich). Anti-rabbit or anti-mouse HRP-conjugated-IgG (Cell Signaling Technologies) was used to detect binding of antibodies. The binding of the specific antibody was visualized by exposing to photographic film after treating with enhanced chemiluminescence (ECL) 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.

Nuclear protein extractions

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

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/minute 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.

Immunohistochemistry

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 immunohistochemistry of GRP78, 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

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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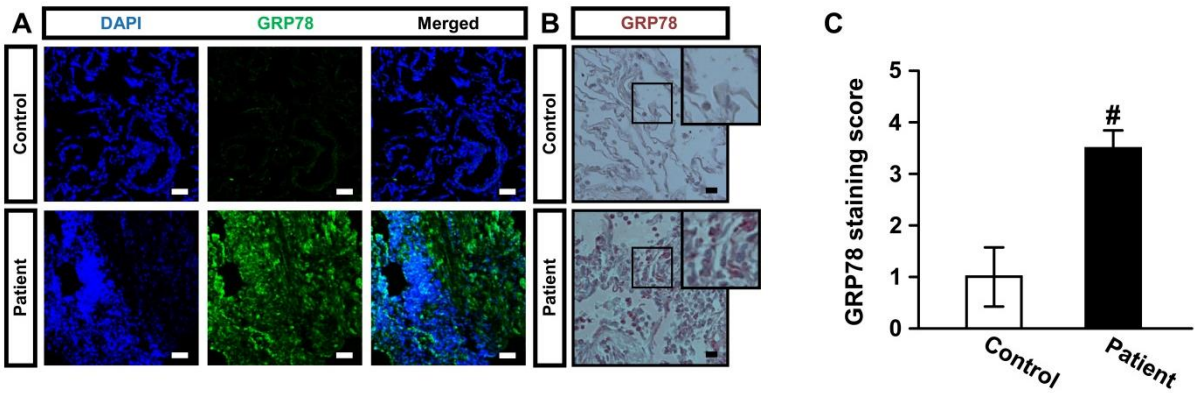
2. Patterson R, Greenberger PA, Halwig JM, *et al*. Allergic bronchopulmonary aspergillosis. Natural history and classification of early disease by serologic and roentgenographic studies. *Arch Intern Med* 1986;146:916-8.

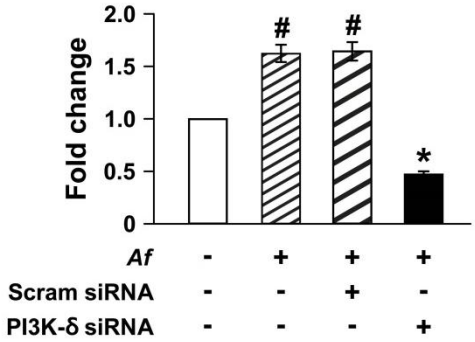
3. Rosenberg M, Patterson R, Mintzer R, *et al*. Clinical and immunologic criteria for the diagnosis of allergic bronchopulmonary aspergillosis. *Ann Intern Med* 1977;86:405-14.

FIGURE LEGENDS FOR SUPPLEMENTARY FIGURES



Supplementary figure S1

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



Supplementary figure S2

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