


Astragaloside IV Protects from PM2.5-Induced Lung Injury by Regulating Autophagy via Inhibition of PI3K/Akt/mTOR Signaling in vivo and in vitro

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Introduction: Prolonged exposure to air polluted with airborne fine particulate matter (PM2.5) can increase respiratory disease risk. Astragaloside IV (AS-IV) is one of the main bioactive substances in the traditional Chinese medicinal herb, *Astragalus membranaceus* Bunge. AS-IV has numerous pharmacological properties; whereas there are few reports on the prevention of PM2.5-induced lung injury by AS-IV through modulation of the autophagic pathway. This study aimed to investigate the protective effects and the underlying mechanisms of AS-IV in PM2.5-induced lung injury rats and rat alveolar macrophages (NR8383 cells).

Methods: The pneumotoxicity model was established by intratracheal injection of PM2.5 in rats, and PM2.5 challenge in NR8383 cells. The severity of lung injury was evaluated by wet weight to dry weight ratio and McGuigan pathology scoring. Inflammatory factors and oxidative stress were detected through ELISA. The expressions of p-PI3K, p-Akt, and p-mTOR proteins were analyzed by immunohistochemistry. Immunofluorescence and transmission electron microscopy were used to detect autophagosomes. The expressions of autophagy marker protein (LC3B and p62), PI3K/Akt/mTOR signaling and NF- κ B translocation were detected by Western blot in lung tissue and NR8383 cells.

Results: After PM2.5 stimulation, rats showed severe inflammation and oxidative stress, along with inhibition of autophagy in lung tissue. AS-IV not only decreased pulmonary inflammation and oxidative stress by inhibiting nuclear factor kappa B translocation, but also regulated autophagy by inhibiting PI3K/Akt/mTOR signaling. After treatment with 3-methyladenine (a classic PI3K inhibitor, blocking the formation of autophagosomes), the protective effect of AS-IV on PM2.5-induced lung injury was further strengthened. In parallel, using Western blot, immunohistochemistry, and transmission electron microscopy, we demonstrated that AS-IV restore autophagic flux mainly through regulating the degradation of autophagosomes rather than suppressing the formation in vivo and in vitro.

Conclusion: Our data indicated that AS-IV protects from PM2.5-induced lung injury in vivo and in vitro by inhibiting the PI3K/Akt/mTOR pathway to regulate autophagy and inflammation.

Keywords: PM2.5, lung injury, astragaloside IV, autophagy, inflammation

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Introduction

Environmental pollution by fine particulate matter (PM2.5), which has an aerodynamic diameter of $\leq 2.5 \mu\text{m}$, has become a significant public health threat in China and other developing countries.^{1,2} Several epidemiological studies have shown that long-term exposure to high levels of PM2.5 increases the risk of

many respiratory diseases, such as influenza,^{3,4} asthma,⁵ bronchitis,⁶ chronic obstructive pulmonary disease (COPD),^{7,8} pulmonary fibrosis,⁹ and lung cancer.^{10,11} As a result, PM2.5-induced lung injury poses an enormous social and economic burden.^{12,13} Currently, respiratory diseases caused by PM2.5 have attracted widespread attention from scholars.^{14,15} However, most studies present epidemiological and environmental toxicological data. At present, there are no effective therapies for controlling the progression of PM2.5-induced lung injury. Therefore, there is a need to seek preventive and therapeutic approaches to prevent PM2.5-induced lung injury.

Several PM2.5 toxicology studies have shown that PM2.5-induced lung injury is closely associated with autophagy and inflammation.^{16,17} Autophagy, a type II programmed cell death modality, is usually considered a protective mechanism for cells, while excessive autophagy induces autophagic cell death.¹⁸ Normal autophagy helps balanced inflammatory, which in turn has a protective effect against disease. Altered autophagy can lead to maladaptive inflammatory responses, increased inflammatory diseases.^{19,20} Several recent studies have shown that autophagy is an anti-inflammatory mechanism that prevents endothelial damage triggered by various endogenous or infectious agents and prevents unnecessary or excessive inflammation.^{21,22} In contrast, some studies have suggested that over-activated autophagy exacerbates inflammatory damage, which in turn promotes lung disease.^{23,24} Alveolar macrophages are the most common immune cells residing in the alveoli, which plays pivotal regulatory roles in immune and inflammatory systems. Previous studies have shown that abnormal autophagy in PM2.5-induced alveolar macrophages are closely related to the pathophysiological progression of acute and chronic lung inflammation. Therefore, regulating the balance between autophagy and inflammation may be a potential mechanism to combat PM2.5-induced lung injury.^{25,26} Despite some interventions showing great potential, no therapies are available that specifically modulate autophagy in humans.

Astragaloside IV (AS-IV) is one of the main bioactive substances in the traditional Chinese medicinal herb, *Astragalus membranaceus* Bunge. Previous studies have shown that AS-IV has pharmacological effects as an anti-oxidant, anti-inflammatory, immunomodulatory, antiviral, and antitumor agent.²⁷ In recent years, AS-IV has received attention in the prevention of respiratory diseases, such as asthma,²⁸ pulmonary fibrosis²⁹ and

lung cancer.³⁰ However, there are few reports on the prevention of PM2.5-induced lung injury by AS-IV through modulation of the autophagic pathway.³¹ Therefore, in this study, we investigated the protective effect of AS-IV pre-treatment on PM2.5-induced lung injury and explored the potential mechanisms by which AS-IV protects against PM2.5-induced lung injury via the autophagy-inflammation pathway in vivo and in vitro.

Materials and Methods

Materials

AS-IV (HPLC \geq 98.81%) and fine particulate matter standards were respectively purchased from Chengdu Munster Company (China) and National Institute of Standards and Technology (USA). 3-Methyladenine (3-MA) was purchased from Selleck, and polyvinylidene fluoride membranes were purchased from Bio-Rad (USA). Antibodies against the following targets were obtained from Abcam (Cambridge, UK): LC3B (ab48394), p62 (ab56416), PI3K (ab182651), Akt (ab185633), p-Akt (ab38449), mTOR (ab2732), p-mTOR (ab137133), Lamin B (ab16048), and GAPDH (ab181602). An antibody against p-PI3K (AF3242) was obtained from Affinity Biosciences (USA). An antibody against p65 (8242S) was obtained from Cell Signaling Technology (Danvers, MA, USA). Horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG secondary antibody (GB23303) and 4°C tissue radioimmunoprecipitation assay lysates were obtained from Servicebio (Wuhan, China).

Ethics Statement

All animal procedures were approved by the Animal Studies Ethics Committee of Chengdu University of Traditional Chinese Medicine. Animal handling followed the dictates of the National Animal Welfare Law of China. Animal welfare conformed to the National Institutes of Health animal welfare guidelines.

Animals and Experimental Design

Male Sprague-Dawley rats (120–150g) were obtained from Chengdu Dashuo Experimental Animal Co., Ltd. All rats were raised in a standard environment (temperature, 25°C \pm 1°C; humidity, 55% \pm 5%; photoperiod, 12:12 hours). After adapting for 1 week, rats were randomly assigned into seven groups (n = 7 per group): (1) control; (2) AS-IV (100 mg/kg); (3) PM2.5 (7.5 mg/kg); (4) AS-IV 50 (AS-IV 50 mg/kg + PM2.5 7.5 mg/kg); (5) AS-IV 100

(AS-IV 100 mg/kg + PM2.5 7.5 mg/kg); (6) 3-MA (3-MA 15 mg/kg + PM2.5 7.5 mg/kg); (7) AS-IV 100 + 3-MA (AS-IV 100 mg/kg + 3-MA 15 mg/kg + PM2.5 7.5 mg/kg). Before PM2.5 administration, all groups were treated by intraperitoneal injection with AS-IV (dissolved in normal saline containing 0.1% dimethyl sulfoxide [DMSO]) once a day for 3 days consecutively, except for the control and PM2.5 groups. The AS-IV 100 + 3-MA group was first intraperitoneally injected with AS-IV (100 mg/kg), and 3-MA (15 mg/kg) was intraperitoneally injected 1 hour later once a day for 3 days consecutively.³² The control and AS-IV groups were administered an equivalent volume of sterile saline containing 1% DMSO by the same method. The rat model of lung injury induced by PM2.5 was established in accordance with previous literature.^{23,31,33} All groups except the control and AS-IV groups received an intratracheal injection of PM2.5 dust suspension (7.5 mg/kg dissolved in a certain volume of saline according to the animal's weight). Intratracheal instillation of PM2.5 suspension was repeated after 24 hours. The control and AS-IV groups received the same volume of saline. All rats were anesthetized and sacrificed with 0.4% pentobarbital after PM2.5 instillation for 12 hours.

Bronchoalveolar Lavage Fluid (BALF)

Acquisition

The whole left lung was for BALF collection. BALF was gathered via three intratracheal injections of 2 mL sterile saline. BALF was centrifuged at 5000 r/minute for 10 minutes at 4°C. The cell-free supernatant was maintained and stored at -80°C for cytokine detection.

Lung Wet Weight to Dry Weight (W/D) Ratio

After rats were sacrificed, the inferior lobe of the right lung was excised and weighed for wet weight. Then, lung tissue was placed in an incubator at 60°C for 48 hours, and dry weight was obtained. The lung W/D ratio was calculated.

Histological Analysis

The superior lobe of the right lung was extracted and immediately fixed in 4% paraformaldehyde for 24 hours, embedded in paraffin, sectioned to 4 µm, and dewaxed. Slices were stained with hematoxylin and eosin (H&E) and observed under an inverted light microscope. The

extent of lung tissue damage was assessed using the McGuigan pathology scoring method and was independently scored by two specialized pathologists.³⁴

Cytokines and Oxidative Stress

Tumor necrosis factor- α (TNF- α), interleukin (IL)-6, and C-reactive protein (CRP) in BALF were measured using enzyme-linked immunosorbent assay (ELISA) kits (MultiSciences Biotech, Hangzhou, China). The contents of malondialdehyde (MDA), catalase (CAT), and superoxide dismutase (SOD) in lung tissue homogenates were determined using available assay kits (Jiancheng Bioengineering Institute, Nanjing, China).

Immunofluorescence

Immunofluorescence sections were prepared in the same way as H&E-stained sections. Then, sections were incubated with anti-LC3B monoclonal antibodies prepared according to the manufacturer's instructions. Secondary antibodies were added to cover the tissue and incubated at room temperature for 50 minutes. Slices were observed using a confocal microscope (C2; Nikon, Tokyo, Japan), and images were acquired. The relative fluorescence intensity of LC3B was analyzed by Image J software.³⁵

Immunohistochemistry

p-PI3K, p-Akt, and p-mTOR were analyzed by immunohistochemistry. Paraffin sections were routinely dewaxed and antigenically repaired with 3% hydrogen peroxide to block peroxidase. Sections were incubated with primary antibodies against p-PI3K (1:100), p-Akt (1:250), and p-mTOR (1:100) at 4°C overnight. Subsequently, sections were incubated with HRP-labeled secondary antibodies at room temperature for 1 hour. Sections were stained with 3,3'-diaminobenzidine (DAB) and counterstained with hematoxylin. Under the microscope, each section was observed, and brown particles were considered to indicate positive results.

Cells Cultures and Treatments

Rat alveolar macrophages (NR8383 cells) were purchased from National Collection of Authenticated Cell Cultures (Shanghai, China) and cultured in Ham's F12K medium (Thermo) containing 20% heat-inactivated fetal bovine serum (FBS, PAA, Australia) and 1% penicillin-streptomycin (100 Units/mL) with a sub-confluent density at 37 °C in a 5% CO₂ atmosphere. Then, the NR8383s were divided into eight groups (control, DMSO, AS-IV

50, PM2.5, AS-IV 10+PM2.5, AS-IV 50+PM2.5, 3-MA+PM2.5, and 3-MA+AS-IV+PM2.5). The control group cultured with normal culture medium, the DMSO group was treated with 0.1% DMSO and the AS-IV group was treated with AS-IV (50 μ M). AS-IV 10+PM2.5 group and AS-IV 50+PM2.5 group were pre-treated with AS-IV (10 and 50 μ M) for 12 h, then the cells were exposure to PM2.5 (50 μ g/mL) for 24 h. In the last two groups, cells were pretreated with 3-MA (5mM) for 12 h followed by treatment with PM2.5 (50 μ g/mL),^{23,36} and treated with different drugs with or without AS-IV (50 μ M) for 12 h.

Cell Viability Assay

CCK-8 kit (Biosharp, Hefei, China) was used to analyzed the total number of live cells. Specific detailed CCK-8 testing steps were according to the guidelines provided by the supplier. After cell grouping and treatment, cells were incubated with 100 μ L of CCK8 reagent for 2 h at 37°C. The absorbance was measured at 450nm using a microplate spectrophotometer.

Western Blot

The left lung was homogenized in lysis buffer (containing phenylmethylsulfonyl fluoride (PMSF), protease, and phosphatase inhibitor). The homogenate was centrifuged at 12,000 \times g for 10 minutes at 4°C, and then the supernatant was collected as total protein. Similarly, the different groups of NR8383 cells were lysed in lysis buffer. The protein concentration was quantified using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific, USA). Equal protein concentrations were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis at 8–12% and then transferred onto polyvinylidene fluoride membranes. Membranes were blocked using 5% milk or 5% bovine serum albumin for 1 hour at room temperature. Subsequently, membranes were incubated with primary antibodies at 4°C overnight and incubated with HRP-labeled goat anti-rabbit secondary antibody for 2 hours at room temperature. Finally, protein bands were visualized by enhanced chemiluminescence and detected with a Bio-Rad image analysis system. The gray value of protein bands was analyzed with Image J software. Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime Biotechnology, Shanghai, China) was used to extract the cytoplasmic/nuclear proteins of p65 according to the manufacturer's protocol.

Quantitative GFP-LC3 Analysis

Cells were transfected with either GFP-LC3 (Hanbio, Shanghai, China) constructs 24 h prior to treatment. After drug treatment, the cellular localization of GFP-LC3 was visualized using a confocal microscope (C2; Nikon, Tokyo, Japan). GFP-LC3-positive or endogenous LC3-positive punctate structures were obtained by counting at least 100 positive cells in each working condition of three independent experiments.

Transmission Electron Microscopy (TEM)

Lung tissue from the upper lobe of the right lung was removed, quickly placed on a glass slide containing chloral fixative, and shaken to completely immerse the tissue in fixative for 24 hours. After dehydration with different concentrations of ethanol, tissues were immersed in acetone and 812 embedding agent, and samples were inserted into the embedding plate pre-placed with pure 812 embedding agent and placed in a 37°C oven overnight. Similarly, Cells were fixed in 0.1 M PBS with 2.5% glutaraldehyde, and then treated in 1% osmium tetroxide buffer. Fixed cells were post-fixed in 2% OsO₄, dehydrated in graded alcohol and flat embedded in Epon 812. Ultra-thin sections (50–60 nm) were imaged using TEM (HT7700, Hitachi, Tokyo, Japan), focusing on the autophagic vacuoles (autophagosomes, Avi; autolysosomes, Avd) of alveolar macrophages.³⁷

Statistical Analysis

Data are presented as mean \pm standard error. Data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test using GraphPad Prism 8.0, and a P value of <0.05 was considered statistically significant.

Results

Effects of as-IV on PM2.5-Induced Lung Injury in Rats

As shown in [Figure 1A](#), H & E staining of lung tissues showed that the lung tissues of the Con and AS-IV groups were intact without obvious inflammatory infiltration or damage. After PM2.5 stimulation, lung tissue showed alveolar congestion, alveolar wall thickening, and pulmonary interstitial neutrophil and lymphocyte infiltration. With AS-IV pre-treatment, there was a dose-dependent reduction in lung tissue damage. As shown in [Figure 1B](#), AS-IV distinctly suppressed the lung injury score of PM2.5-induced lung injury in rats. As depicted in [Figure 1C](#), compared with the control group, the W/D

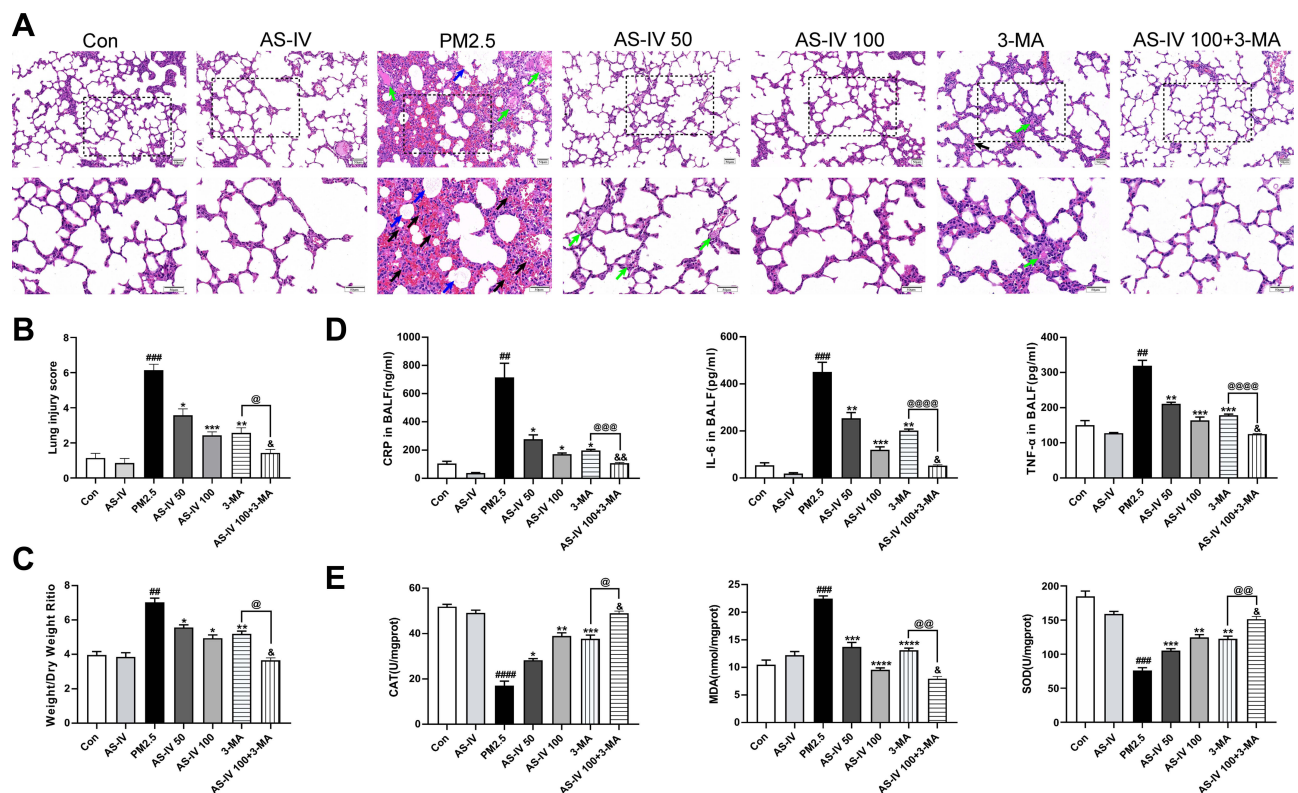


Figure 1 Effects of AS-IV on PM2.5-induced lung injury in rats. **(A)** HE staining of lung tissues from Con, AS-IV, PM2.5, AS-IV 50, AS-IV 100, 3-MA, and AS-IV 100+3-MA groups. (Black arrows represent hemorrhage, green arrows represent clear inflammatory cells, and blue arrows represent inflammatory exudate). (Magnification 200× for the upper row and 400× for the lower row. Scale bar = 50 μm). **(B)** Semiquantitative histopathological score of lung injury. **(C)** Effect of AS-IV on lung wet/dry ratio of PM2.5-induced lung injury. **(D)** Effect of AS-IV on CRP, IL-6, and TNF-α in the BALF in PM2.5-induced lung injury. **(E)** Effect of AS-IV on CAT, MDA, and SOD in the lung tissues in PM2.5-induced lung injury. Data are mean±SEM (n=6-7). ###P<0.01, ####P<0.001, and #####P<0.0001 compared with control group; *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001 compared with PM2.5 group; &P<0.05, and &&P<0.01 compared with AS-IV 100 group; @P<0.05, @@P<0.01, @@@P<0.001, and @@@@P<0.0001 compared with 3-MA group.

ratio was significantly higher after PM2.5 stimulation ($P < 0.05$), while the W/D ratio declined after pre-administration of AS-IV. No significant difference was observed between the AS-IV group and the control group in terms of W/D ratio ($P > 0.05$). To analyze the effects of AS-IV on the PM2.5-induced inflammatory response, secretion of TNF- α , IL-6, and CRP in BALF was measured by ELISA. As shown in Figure 1D, PM2.5 markedly increased the production of CRP, TNF- α , and IL-6 in BALF compared with the control and AS-IV groups, whereas pre-administration of AS-IV considerably inhibited the expression of inflammatory cytokines in BALF. As shown in Figure 1E, PM2.5 stimulation significantly up-regulated MDA activity in lung tissue. In contrast, SOD and CAT were markedly reduced in lung tissues after PM2.5 stimulation. AS-IV reversed the PM2.5-induced changes in SOD, CAT, and MDA. These results indicate that AS-IV decreases pulmonary inflammation and oxidative stress in the lung tissues of rats exposed to PM2.5. Interestingly, compared with AS-IV pre-treatment groups, 3-MA (a

classic PI3K inhibitor, blocking the formation of autophagosomes) has a similar protective effect in vivo. Furthermore, AS-IV combined with 3-MA could further improved PM2.5-induced lung injury in rats, suggesting that AS-IV might act by inhibiting PI3K pathway.

AS-IV Restores Impaired Autophagy in Lung Tissues from Rats Exposed to PM2.5

To clarify the effect of AS-IV on autophagy in PM2.5-induced lung injury, we measured the levels of key autophagy molecules, including LC3B-II/I and p62, by Western blot. As shown in Figure 2A and B, the expression of LC3B-II/I in the PM2.5 group was significantly increased, meanwhile the expression of p62 was significantly enhanced ($P < 0.01$), suggestive of compromised autophagic degradation. AS-IV pre-treatment decreased the levels of LC3B-II/I and p62 in the lung tissues of rats exposed to PM2.5. To demonstrate that activation of autophagy is the primary mechanism by which AS-IV exert its

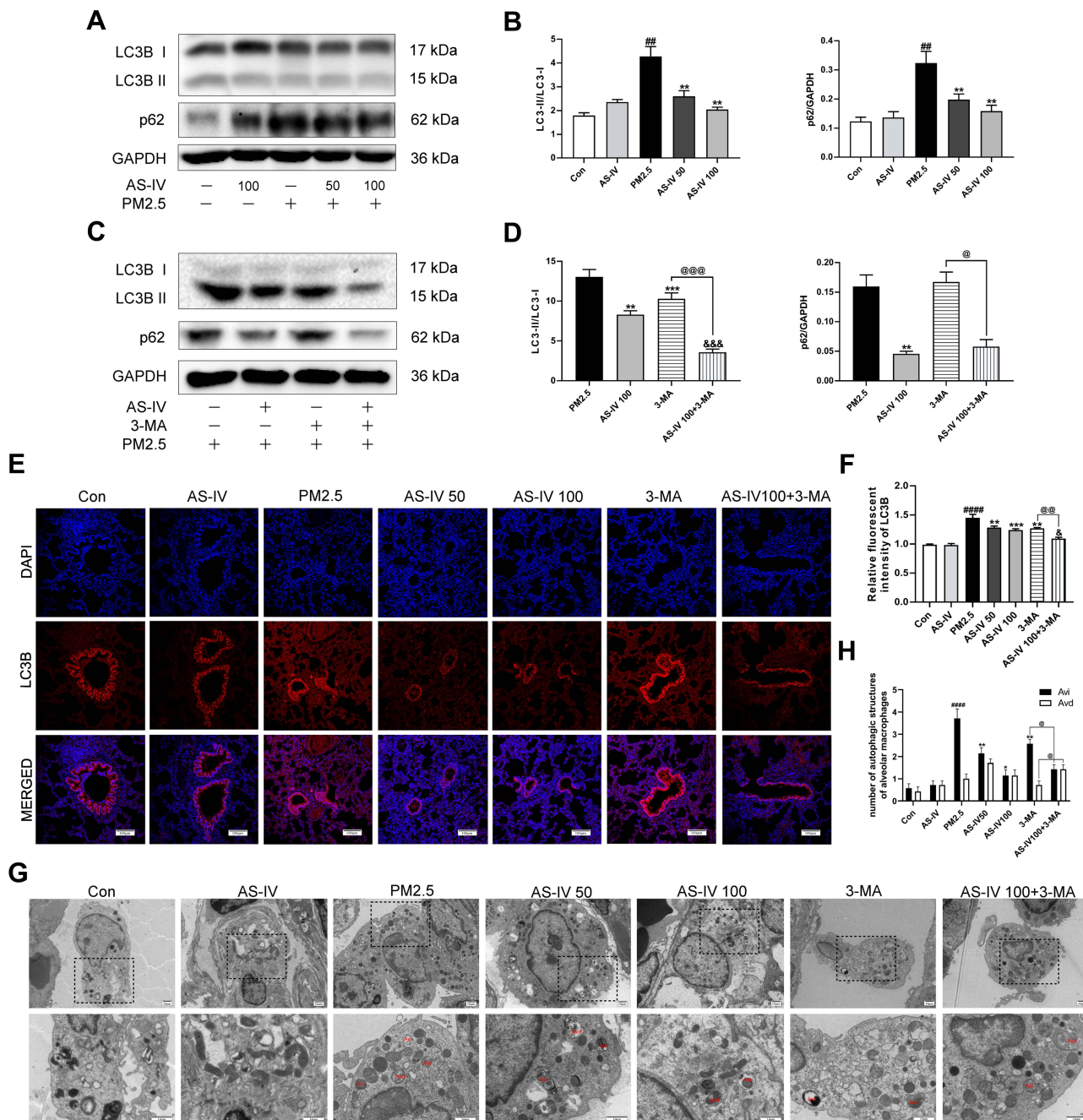


Figure 2 AS-IV restores impaired autophagy in lung tissues from rats exposed to PM2.5. (A–D) The levels of LC3B and p62 were determined by Western blot analysis (n=6-7). (E) Immunofluorescence staining of LC3B in lung tissues (Magnification 200×, Scale bar = 100 μm). (F) The relative fluorescence intensity of LC3B in lung tissues (n=6-7). (G) TEM was utilized to visualize autophagic vacuoles (autophagosomes, Avi; autolysosomes, Avd) in alveolar macrophages. (n =3, Magnification 2000× for the upper row and 5000× for the lower row, Scale bar = 10 μm). (H) Qualitative analysis of the number of autophagic vacuoles in alveolar macrophages under TEM. Data are mean±SEM. ###P<0.01, and #####P<0.0001 compared with control group; *P<0.05, **P<0.01, and ***P<0.001 compared with PM2.5 group; #P<0.05, and &&&P<0.001 compared with AS-IV 100 group; @P<0.05, @@P<0.01, and @@@P<0.001 compared with 3-MA group.

protective effects outlined above, we administered 3-MA (an inhibitor of autophagosome biogenesis) alone and co-administered 3MA with AS-IV, then evaluated outcomes. Compared with the AS-IV 100 group, AS-IV combined with 3-MA effectively suppressed LC3B-II/I (P<0.001) (Figure 2C and D). Immunofluorescence showed that AS-

IV significantly decreased the relative fluorescence intensity of LC3B (Figure 2E and F); meanwhile, the frequency of LC3II-positive cells in the lung tissues treated with AS-IV and 3-MA is decreased compared with AS-IV alone. Moreover, AS-IV decreased the levels of p62. Interestingly, there was not a statistically significant

difference between AS-IV 100 group and AS 100+3-MA group, indicating that AS-IV restored autophagic flux mainly through regulating the degradation of autophagosomes rather than suppressing the formation. It was challenging to detect autophagic vacuoles (autophagosomes, Avi; autolysosomes, Avd) in lung tissue in the control and AS-IV groups using TEM. Avi were increased in the lung exposed to PM2.5 without a corresponding increase in Avd. However, AS-IV pre-administration decreased the number of Avi with increasing the number of Avd. Compared with the 3-MA group, AS-IV balanced the number of Avi and Avd, suggesting that AS-IV activates autophagic flux by increasing autophagosome–lysosome fusion (Figure 2G and H). Together, these observations indicate that AS-IV pre-treatment might restore impaired autophagy in PM2.5-induced lung injury.

AS-IV Inhibits the PI3K/Akt/mTOR Pathway in Lung Tissues from Rats Exposed to PM2.5

To further elucidate the mechanism of the in-vivo findings, the PI3K/Akt/mTOR pathway was examined (Figure 3A–D). Western blot revealed that AS-IV significantly inhibited the relative contents of p-PI3K, p-Akt, and p-mTOR protein ($P<0.01$), which were further decreased after adding 3-MA ($P<0.05$). In addition, we examined the expression of p-PI3K, p-Akt, and p-mTOR in lung tissue using immunohistochemistry. As shown in Figure 3E and F, PM2.5 stimulation elevated the positive expression of p-PI3K, p-Akt, and p-mTOR in lung tissues, while AS-IV suppressed expression in a dose-dependent manner. As a PI3K inhibitor, compared with AS-IV pre-treatment, 3-MA further inhibited the expression of p-PI3K, p-Akt, and p-mTOR to some extent in Western blot analysis and immunohistochemistry. These results suggest that AS-IV inhibits the PI3K/Akt/mTOR pathway in lung tissues of rats with PM2.5-induced lung injury.

AS-IV Inhibits the NF- κ B Translocation in Lung Tissues from Rats Exposed to PM2.5

In order to study whether NF- κ B is activated in lung tissues induced by PM2.5, we measured the protein the expression level of NF- κ B (Figure 4A–D). Compared with the control group, the nuclear expression of p65 levels was significantly promoted by PM2.5 ($P<0.01$), and cytoplasmic p65 significantly decreased ($P<0.01$). However, AS-IV dose-dependently reduced p65 protein translocating

into the nuclear. Meanwhile, the above trend was further suppressed by 3-MA ($P<0.01$). Collectively, these findings suggest that AS-IV inhibits the NF- κ B translocation through induction of PI3K-dependent autophagy.

AS-IV Restores Impaired Autophagy in PM2.5-Induced NR8383 Cells

To verify AS-IV can regulate autophagy, we conducted experiments with NR8383 rat alveolar macrophages to AS-IV in vitro. As shown in Figure 5A, compared with non-treated controls, the viability of NR8383 cells was reduced by PM2.5, which indicated that 50 μ g/mL PM2.5 induced apparent cellular damage. Concurrently, cell viability increased significantly after AS-IV treatment at concentrations between 1 and 100 μ M. According to the above results, 10 μ M and 50 μ M of AS-IV were used for further research. As illustrated in Figure 5B and C, the protein expression of LC3B-II/I and p62 was elevated by PM2.5, suggesting that PM2.5 led to defective autophagy. In parallel, AS-IV decreased the levels of LC3B-II/I and p62, while 3-MA only effectively suppressed LC3B-II/I. Interestingly, there was no significant difference in the level of p62 between AS-IV 50 μ M group and AS 50+3-MA group, which was consistent with that observed in vivo. Consistent with the protein levels, PM2.5 significantly increased the accumulation of autophagosomes in these cells transfected with GFP-LC3 (Figure 5D and E). AS-IV significantly suppressed the amount of GFP-LC3-labeled puncta formation, suggesting that AS-IV decreased the accumulation of autophagosomes. Additionally, AS-IV and 3-MA cotreatment dramatically reduced the accumulation of GFP-LC3-labeled puncta compared with a single treatment. Similar to the in vivo studies, TEM showed that PM2.5 promoted the accumulation of Avi, but AS-IV balanced the number of Avi and Avd, further emphasizing the ability of AS-IV to restore impaired autophagic flux. (Figure 5F). Collectively, these data suggested that AS-IV restored autophagic flux mainly through regulating the degradation of autophagosomes rather than suppressing the formation.

AS-IV Inhibits the PI3K/Akt/mTOR Pathway and NF- κ B Translocation in PM2.5-Induced NR8383 Cells

As demonstrated in Figure 6A and B, similar to the results in vivo, PM2.5 significantly increased the levels of p-PI3K, p-Akt and p-mTOR in PM2.5-induced NR8383

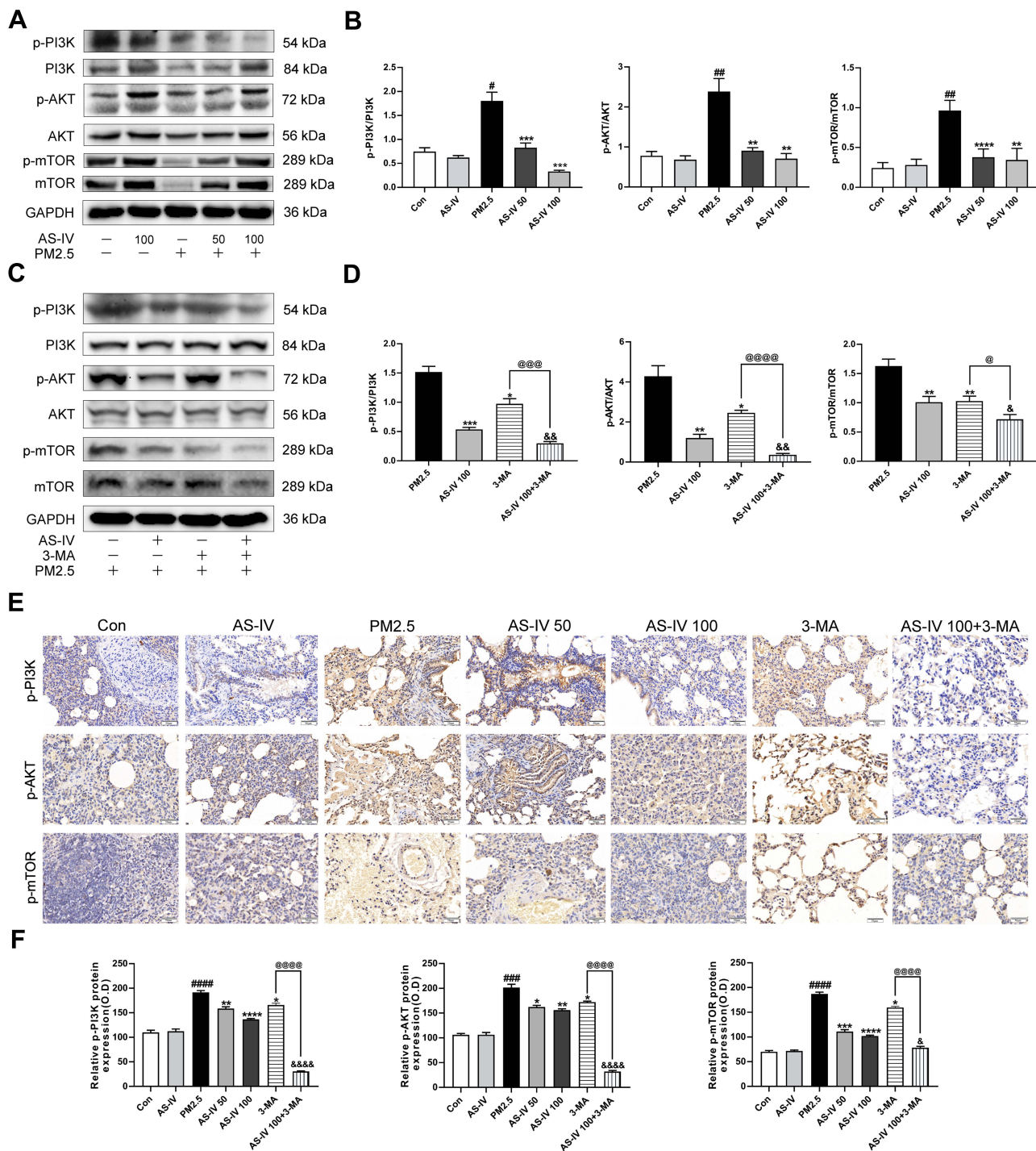


Figure 3 AS-IV inhibits the PI3K/Akt/mTOR pathway in lung tissues from rats exposed to PM2.5. (A–D) The levels of p-PI3K, PI3K, p-AKT, AKT, p-mTOR, and mTOR were determined by Western blot analysis. (E) Effects of p-PI3K, p-AKT, and p-mTOR expression in lung tissues by immunohistochemistry staining. (Magnification 200×, Scale bar = 50 μm). (F) Statistical chart of positive expression of p-PI3K, p-AKT, and p-mTOR proteins. Graphs represent mean±SEM (n=6-7). ###P<0.01, ####P<0.001, and #####P<0.0001 compared with control group; *P<0.05, **P<0.01, and ***P<0.001 compared with PM2.5 group; &P<0.05, &&P<0.01, and &&&P<0.001 compared with AS-IV 100 group; @P<0.05, @@@P<0.001, and @@@@P<0.0001 compared with 3-MA group.

cells. AS-IV treatment decreased the phosphorylation proteins expression of PI3K, Akt and mTOR. In addition, PM2.5 stimulated NF-κB p65 translocation significantly, while AS-IV treatment markedly inhibited p65 protein

translocating into the nuclear in a dose-dependent manner (Figure 6C and D). Furthermore, 3-MA promoted the effects caused by AS-IV. These results suggest that PI3K/Akt/mTOR pathway and NF-κB translocation

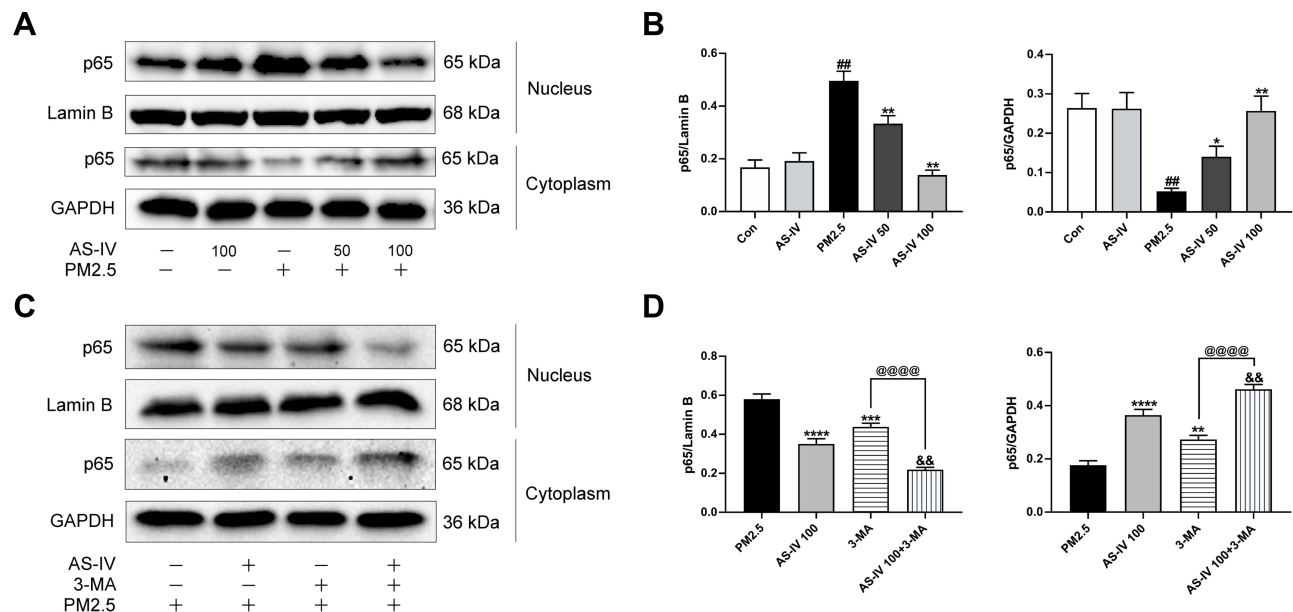


Figure 4 AS-IV inhibits the NF- κ B translocation in lung tissues from rats exposed to PM2.5. (A–D) The levels of cytoplasmic and nuclear p65 were determined by Western blot analysis. Graphs represent mean \pm SEM (n=6-7). ^{##}P<0.01 compared with control group; *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001 compared with PM2.5 group; &&P<0.01 compared with AS-IV 100 group; @@@@P<0.0001 compared with 3-MA group.

might deeply involve in the process of autophagy of AS-IV in PM2.5-induced rat alveolar macrophages.

Discussion

Currently, there is no effective strategy to prevent and treat PM2.5-induced lung injury. Inflammatory damage and oxidative stress induced by an imbalance in autophagy are closely related to PM2.5-induced lung injury. Therefore, regulating the relationship between autophagy and inflammation may be a potential prevention and control strategy.^{19,26} This study revealed that AS-IV protects from PM2.5-induced lung injury by inhibiting the PI3K/Akt/mTOR signaling pathway to restore impaired autophagy in vivo and in vitro. To explore this possibility, 3-MA was used to block autophagy through inhibiting PI3K. In addition, this study provides a new understanding of the preventive effect of AS-IV on PM2.5-induced lung injury.

PM2.5 damages lung tissue, triggers the release of inflammatory mediators that participate in airway inflammation and lung tissue damage, and ultimately increases morbidity and mortality from respiratory disease.³⁸ However, the pathogenesis of PM2.5-induced lung injury is complicated.³⁹ AS-IV is widely used to prevent respiratory diseases, but its protective effect on PM2.5-induced lung injury is rarely reported. Although many advances have been made in understanding the molecular mechanisms of PM2.5-induced lung injury,⁴⁰⁻⁴² there is still a lack

of preventive and therapeutic strategies. In our study, we found that AS-IV significantly ameliorated pulmonary edema in rats with PM2.5-induced lung injury. Compared with the PM2.5 group, the AS-IV 50 and AS-IV 100 groups showed clear and intact lung tissue structures with no bleeding, neutrophil infiltration, alveolar wall thickening, or other classic pathological changes associated with lung injury.

The inflammatory response is accompanied by degranulation and activation of topical mast cells and macrophages, as well as release of various proinflammatory mediators.⁴³ Previous studies have shown that a variety of proinflammatory cytokines, such as IL-6, IL-8, and IL-10, are elevated in PM2.5-induced lung injury.^{44,45} Among them, TNF- α and IL-6, which are key components of the cytokine network, are essential inflammatory mediators for lung injury initiation.^{46,47} CRP is a protein that rises sharply in plasma during tissue damage and plays a vital role in the innate immune process.^{48,49} After AS-IV pretreatment, proinflammatory cytokines (IL-6 and TNF- α) and CRP were significantly reduced in BALF. These results preliminarily indicate that AS-IV may attenuate PM2.5-induced lung injury by inhibiting inflammation.

Disruption of the homeostasis between oxidation or antioxidants in cells may be a critical mechanism in PM2.5-induced lung injury.⁵⁰⁻⁵² PM2.5 mediates cellular oxidative stress through different pathways, resulting in

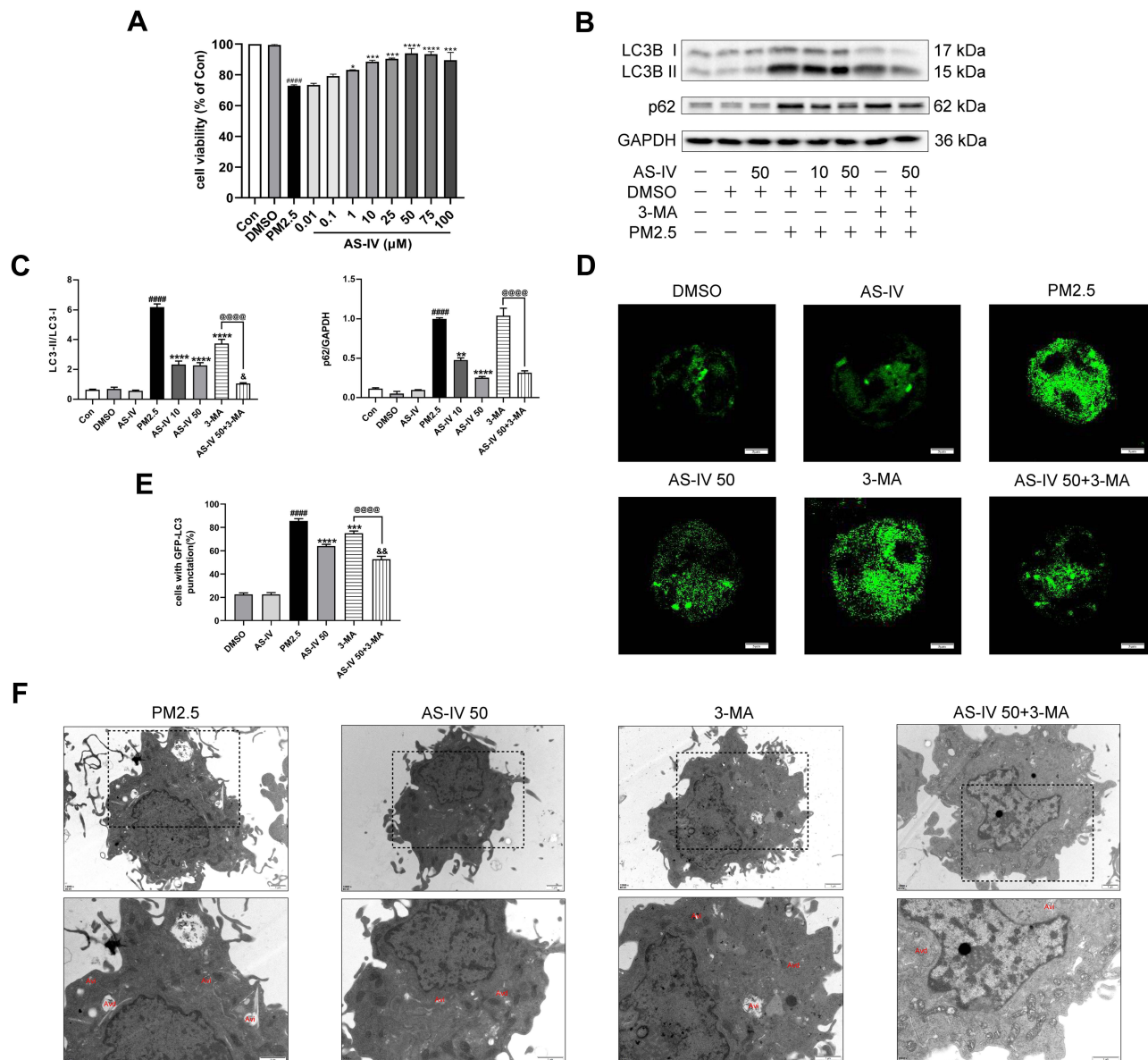


Figure 5 AS-IV restores impaired autophagy in PM2.5-induced NR8383 cells. **(A)** Cell viability was measured in AS-IV-treated (0.01, 0.1, 1, 10, 25, 50, 75, or 100 μM; 12 h) NR8383 cells by CCK-8 assays (n=3). **(B and C)** The levels of LC3B and p62 were determined by Western blot analysis (n=3). **(D and E)** NR8383 cells were treated with AS-IV (50 μM), 3-Ma (5 mM), or PM2.5 (50 μg/mL), or in combination for 24 h after transfection with GFP-LC3 plasmid (Scale bar = 20 μm). LC3 punctate-positive cells were observed by fluorescence microscopy and quantitation of the percentage of cells with punctate GFP-LC3 fluorescence per total GFP-LC3-positive cells. Data represent mean SEM calculated from three experiments of 100 transfected cells each. **(F)** TEM was utilized to visualize autophagic vacuoles (autophagosomes, Avi; autolysosomes, Avd) in NR8383 cells (n = 3, Magnification 12,000× for the upper row and 20,000× for the lower row, Scale bar=1 μm). Graphs represent mean±SEM. #####P<0.0001 compared with DMSO group; *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001 compared with PM2.5 group; &P<0.05 and &&P<0.01 compared with AS-IV 50 group; @@@@P<0.0001 compared with 3-MA group.

lung injury under the combined action of proinflammatory cytokines.^{53–55} Previous studies have shown that reactive oxygen species (ROS) play an indispensable role in the AS-IV-induced anti-inflammatory effect, including in endothelial,⁵⁶ epithelial,⁵⁷ and mesenchymal stem cell lines.⁵⁸ Our results show that excessive ROS caused by PM2.5 induced nuclear transcription factor activation factor NF-κB and further released proinflammatory factors

and cytokines, including TNF-α and IL-6, leading to lung inflammation. AS-IV increased CAT and SOD in lung tissue, inhibited MDA activity, and reduced ROS. However, further studies are needed to evaluate the role of AS-IV in regulating inflammation and oxidative stress to protect from PM2.5-induced lung injury.

Recently, some studies have shown that autophagy, as an important mechanism that maintains the dynamic balance of

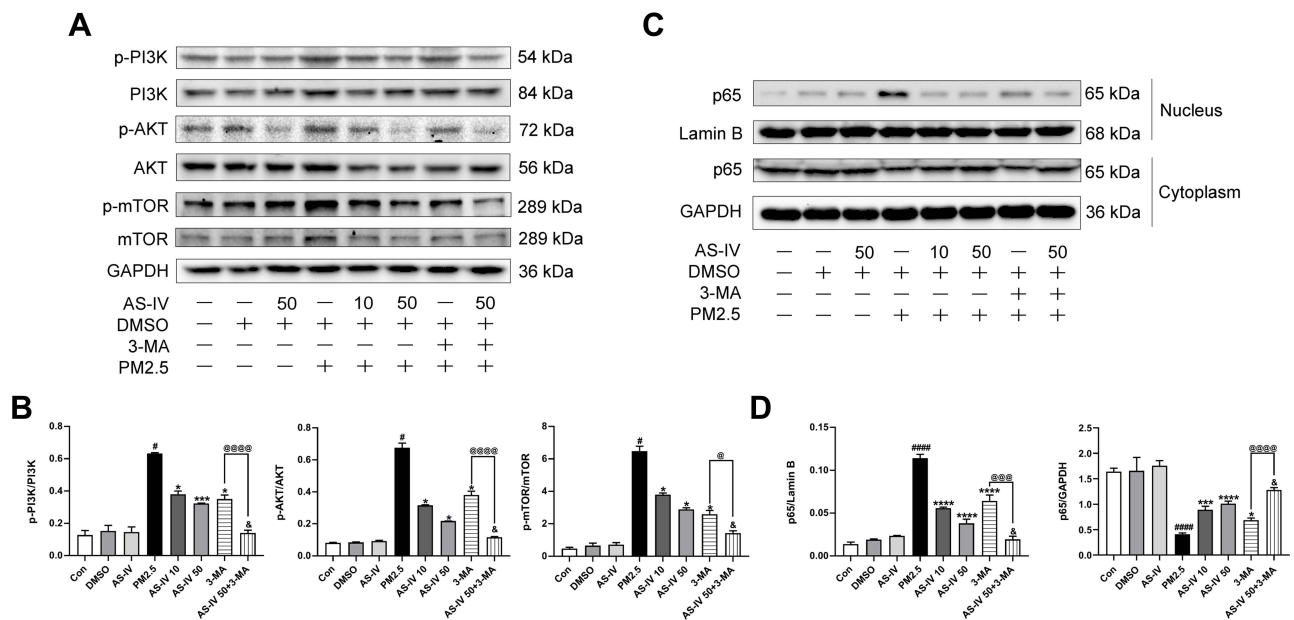


Figure 6 AS-IV inhibits the PI3K/Akt/mTOR pathway and NF- κ B translocation in PM2.5-induced NR8383 cells. **(A and B)** The levels of p-PI3K, PI3K, p-AKT, AKT, p-mTOR, and mTOR were determined by Western blot analysis. **(C and D)** The levels of cytoplasmic and nuclear p65 were determined by Western blot analysis. Graphs represent mean \pm SEM (n=3). #P<0.05, and #####P<0.0001 compared with DMSO group; *P<0.05, ***P<0.001, and ****P<0.0001 compared with PM2.5 group; &P<0.05 compared with AS-IV 50 group; @P<0.05, @@P<0.001, and @@@P<0.0001 compared with 3-MA group.

the cell microenvironment and cell self-renewal, is involved in the progression of respiratory diseases caused by PM2.5.^{17,59,60} During acute pulmonary inflammation, activation of autophagy appears to be a protective mechanism for the host to respond to bacterial and viral infections.⁶¹ Meanwhile, regulating autophagy has also become a potential mechanism to alleviate PM2.5-induced lung injury.⁶² The results showed that autophagosomes were increased in the lung exposed to PM2.5, but AS-IV pre-administration balanced the number of autophagosomes and autolysosomes in vivo and in vitro. The increase in the number of autophagosomes reflects either increased autophagosome formation due to enhanced autophagic activity or an accumulation of autophagosomes due to impaired autophagic degradation.⁶³ We then tested whether autophagy was induced in vivo and in vitro by analyzing commonly used autophagy markers including LC3 and p62. LC3 conversion (LC3-I to LC3-II) reflects the development of autophagic flux;⁶⁴ p62 directly binds to LC3 and is degraded by autophagy activation in autolysosome,⁶⁵ therefore, its accumulation always suggests a block in autophagosome degradation. The simultaneous increase of LC3 conversion and p62 expression level could indicate that the late degradation stage of autophagy is blocked.^{66,67} Our findings reveal that the levels of LC3B-II/LC3B-I and p62 in lung tissue increased simultaneously after modeling, which implies

that PM2.5 induces autophagy impairment via inhibiting autophagy degradation resulting in autophagosome accumulation in vivo and in vitro. As a previously reported,⁶⁸ dramatic increase in p62 and LC3-II expression, together with accumulation of autophagosomes, suggested that autophagic flux was impaired in PM2.5-induced blood-testis barrier. Furthermore, AS-IV restored impaired autophagy by decreasing LC3B-II/LC3B-I ratio, and inhibiting p62 protein expression in vivo and in vitro. Interestingly, 3-MA (a classic PI3K inhibitor, blocking the formation of autophagosomes) further reduced the levels of LC3B-II/LC3B-I, but not p62. The above results indicated that AS-IV restored autophagic flux mainly through regulating the degradation of autophagosomes rather than suppressing the formation.^{69,70} It has been reported that AS-IV can inhibit autophagy in LPS-induced pulmonary epithelial cell injury.⁷¹ In fact, for the regulation of autophagy, our results showed exactly the opposite outcome in vivo and in vitro. Basing on the paradoxical role of AS-IV in regulating autophagy, we tentatively suggest that different injured models, injury degrees, and administration times may differentially affect autophagy regulation, leading to varied outcomes.⁷² Therefore, further research would be needed to explore the regulation of autophagy by AS-IV in different disease models.

Further, we explored the possible mechanism of activation of autophagy by AS-IV. Autophagy is an

important mechanism to maintain cellular homeostasis, and multiple signaling pathways are involved in this process.⁷³ Since the PI3K/Akt/mTOR pathway is one of the most common factors related to the progression of inflammation and oxidative stress, the PI3K/Akt/mTOR pathway and its related molecules are regarded as prevention and treatment targets for lung injury.^{74,75} Therefore, we hypothesized that AS-IV enhances autophagy through the PI3K/Akt/mTOR signaling pathway in PM2.5-induced lung injury. We used 3-MA to illustrate whether AS-IV protects from PM2.5-induced NR8383 cells and rats through PI3K targets.⁷⁶ The results demonstrate that AS-IV suppresses the PI3K/Akt/mTOR signaling pathway in vivo and in vitro, and 3-MA significantly promotes this trend. More importantly, there was no significant differences in protective effect between AS-IV 100 group and 3-MA group. AS-IV and 3-MA cotreatment further reduced histopathological damage, decreased the lung injury score, reduced the lung dry/wet weight ratio, and decreased pulmonary inflammation and oxidative stress in the lung tissues. Also, under the action of 3-MA, AS-IV was effective at reducing NF- κ B translocation in vivo and in vitro. The above results suggest that AS-IV protects from PM2.5-induced lung injury by regulating autophagy via inhibition of PI3K/Akt/mTOR pathway. As an important downstream target of PI3K, mTOR is a convergent target of autophagy modulation and has a negative regulatory effect on autophagy.⁷⁷ Similar to our results, a previous study showed that rapamycin, an inhibitor of mTOR, protects from PM2.5-induced pulmonary inflammation in rat by activating autophagy.⁷⁸ These findings indicate that activation of autophagy may be a potential strategy to prevent PM2.5-induced lung injury. However, the mechanism of AS-IV-activated autophagy to attenuate PM2.5-induced lung injury requires further investigation.

Conclusion

The present study demonstrated that AS-IV protects from PM2.5-induced lung injury by inhibiting the PI3K/Akt/mTOR signaling pathway to restore impaired autophagy in vivo and in vitro. Meanwhile, our results show that AS-IV may be useful clinically to prevent and treat pulmonary diseases caused by PM2.5.

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Author Contributions

All authors contributed to data analysis, drafting and revising the article, have agreed on the journal to which the article will be submitted, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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