

# Ophiopogonin D attenuates PM2.5-induced inflammation via suppressing the AMPK/NF- $\kappa$ B pathway in mouse pulmonary epithelial cells

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**Abstract.** Exposure to fine particulate matter, such as particulate matter of  $\leq 2.5 \mu\text{m}$  in diameter (PM2.5), causes pulmonary inflammation and injury to other organs. It has been reported that Ophiopogonin D (OP-D) has anti-inflammatory activity. The aim of the present study was to investigate this anti-inflammatory activity of OP-D on PM2.5-induced acute airway inflammation and its underlying mechanisms. The viability of PM2.5-treated mouse lung epithelial (MLE-12) cells with or without OP-D treatment was determined using a Cell Counting Kit-8 assay. The corresponding levels of IL-1 $\beta$ , IL-6, IL-8 and TNF- $\alpha$  were examined via ELISA. Subcellular localization of NF- $\kappa$ Bp65 was detected using immunofluorescence staining. The expression levels of AMP-activated protein kinase (AMPK), phosphorylated (p)-AMPK, NF- $\kappa$ Bp65 and p-NF- $\kappa$ Bp65 were analyzed using western blotting. The selective AMPK inhibitor Compound C (CC) was utilized to investigate the involvement of AMPK in the protection against PM2.5-induced cell inflammation by OP-D treatment. The results demonstrated that OP-D significantly ameliorated the PM2.5-stimulated release of proinflammatory cytokines (TNF- $\alpha$ , IL-1 $\beta$ , IL-6 and IL-8) and inhibited the translocation of NF- $\kappa$ Bp65 from the cytoplasm to the nucleus in MLE-12 cells. Moreover, OP-D significantly prevented the PM2.5-triggered phosphorylation of NF- $\kappa$ Bp65 and upregulated AMPK activity. The anti-inflammatory activity of OP-D could also be attenuated by the AMPK-specific inhibitor CC. The present results suggested that the anti-inflammatory activity of OP-D was mediated via AMPK activation and NF- $\kappa$ B signaling pathway downregulation, which ameliorated the expression of proinflammatory cytokines. Therefore, OP-D

could be a candidate drug to treat PM2.5-induced airway inflammation.

## Introduction

Pollution with particulate matter of  $\leq 2.5 \mu\text{m}$  in diameter (PM2.5) is known to have significant deleterious effects on human health (1-3). The respiratory system exchanges gases with the ambient gas environment directly; therefore, PM2.5-containing air can cause airway lesions (4). The increase of inflammatory cytokines has been revealed to be positively correlated with the exposure dose and duration (5), and even a low dose of PM2.5 can cause inflammation and pulmonary injury (6). Moreover, PM2.5 has a cumulative effect on the respiratory system. For instance, the longer the exposure time, the stronger the adverse influence of PM2.5 to the respiratory system (7). Previous studies have reported that PM2.5 can activate the NF- $\kappa$ B pathway, as well as upregulate the transcription and secretion of proinflammatory cytokines, including IL-1 $\beta$ , TNF- $\alpha$ , IL-6 and IL-8, ultimately inducing widespread pulmonary inflammatory lesions (8,9). In addition, it has been observed that upregulation of AMP-activated protein kinase (AMPK) suppresses the inflammatory response via inhibition of inflammatory cytokines and NF- $\kappa$ B (10,11). Therefore, downregulation of the NF- $\kappa$ B signaling pathway or activation of AMPK may be a valid method for controlling PM2.5-induced respiratory inflammation and preventing or limiting lung injury.

*Ophiopogon japonicus* (*O. japonicus*; commonly known as Maidong in China) was first recorded in Shen Nong's Materia Medica written during the Han dynasty and has been widely applied in traditional Chinese medicine (12). Currently, *O. japonicus* is often used in compound prescriptions as the main medicinal ingredient, such as in YiQiFuMai injection, Sheng Mai Yin and Xuanmai granule (13). According to the Chinese Pharmacopoeia, *O. japonicus* has been applied for 1,000s of years for the treatment of inflammatory diseases, such as pharyngitis, bronchitis, pneumonia and cough (14). Ophiopogonin D (OP-D) is a vital bioactive steroidal glycoside extracted from the root of *O. japonicus* (15). Accumulating evidence has indicated that OP-D possesses a broad range of pharmacological properties, including anti-inflammatory,

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antioxidative and antitussive effects, as well as inhibition of venous thrombosis (16-18). However, it remains unknown whether OP-D is able to protect alveolar epithelial cells from PM<sub>2.5</sub>-induced toxicity via its anti-inflammatory effects. Therefore, it was hypothesized that OP-D may be potentially useful for preventing PM<sub>2.5</sub>-induced pulmonary inflammation.

The aim of the present study was to investigate the protective effects of OP-D on PM<sub>2.5</sub>-induced pulmonary inflammation. In addition, the molecular mechanisms of the anti-inflammatory effects of OP-D were evaluated.

## Materials and methods

**Materials.** OP-D, extracted from *O. japonicus*, was obtained from Beijing Biotop Biotechnology Development (cat. no. 41753-55-3), dissolved in DMSO (Sigma-Aldrich; Merck KGaA), and diluted with basal DMEM (Beijing Transgen Biotech) (19). The final concentration of DMSO in the culture medium was 0.1% (v/v). The use of 0.1% DMSO alone in DMEM was used as a negative control in the corresponding experiments.

The AMPK-specific inhibitor Compound C (CC) was purchased from Selleck Chemicals (cat. no. S7306). Streptomycin, penicillin, FBS and trypsin were obtained from Gibco (Thermo Fisher Scientific, Inc.). A BCA Protein assay kit (cat. no. 23250) and an Enhanced Chemiluminescence western blotting Detection reagents kit were obtained from Thermo Fisher Scientific. The CellLytic™ NuCLEAR™ Extraction kit was purchased from Sigma-Aldrich; Merck KGaA (cat. no. NXTRACT-1KT). ELISA kits for IL-1 $\beta$  (cat. no. BPE20083), IL-6 (cat. no. BPE20012), IL-8 (cat. no. BPE20459) and TNF- $\alpha$  (cat. no. BPE20220) were obtained from Shanghai Lengton Bioscience Co., Ltd. The Cell Counting Kit-8 (CCK-8) was from Beijing Transgen Biotech (cat. no. FC101-04).

Antibodies against AMPK (1:1,000; cat. no. A1229), phosphorylated (p)-AMPK $\beta$ 1-S108 (1:800; cat. no. AP0597), p-NF- $\kappa$ Bp65 (1:1,000; cat. no. AP0417), NF- $\kappa$ Bp65 (1:1,000; cat. no. A14754), lamin B (1:2,000; cat. no. A1910) and GAPDH (1:2,000; cat. no. AC033), as well as HRP goat anti-rabbit antibody (1:5,000; cat. no. AS014), HRP goat anti-mouse antibody (1:5,000; cat. no. AS003) and FITC goat anti-rabbit antibody (1:80; cat. no. AS011), were obtained from ABclonal Biotech. All other reagents were obtained from Sigma-Aldrich; Merck KGaA.

**Cell culture.** Mouse lung epithelial cells (MLE-12; The Cell Bank of Type Culture Collection of the Chinese Academy of Sciences) were cultured in complete DMEM containing 10% (v/v) FBS, 100 U/ml penicillin, 100 g/ml streptomycin and 50 g/ml amphotericin B at 37°C in an incubator with 5% atmospheric CO<sub>2</sub>. The media was replaced every 2-3 days, and the cells were subcultured weekly (1,500-3,000 cells/cm<sup>2</sup>).

**PM<sub>2.5</sub> collection and preparation.** PM<sub>2.5</sub> was collected between November 2016 and March 2017 in an urban area of Changchun, Jilin. The collection and preparation procedures of PM<sub>2.5</sub> were the same as those reported previously (20). Briefly, concentrated PM<sub>2.5</sub> was gathered using a multistage particle counter (100 l/min; total suspended

particulate/PM<sub>10</sub>/PM<sub>5</sub>/PM<sub>2.5</sub>; Laoshan Electronic Instrument Co., Ltd.). Daily PM<sub>2.5</sub> samples were collected on Teflon filters (90 mm; Whatman plc; Cytiva). The PM-loaded filters were put into a 50-ml centrifuge tube, followed by probe-sonication (700 W; 40 kHz; 25°C) for 1 h in 40 ml Milli-Q water. The suspension was freeze-dried in a vacuum for 12 h to obtain PM<sub>2.5</sub> powder, which was then weighed and stored at -80°C. Before the experiments, the PM<sub>2.5</sub> powder was dissolved in PBS and sonicated (700 W; 40 kHz; 25°C) for 30 min to avoid aggregation of particles.

**Cell viability assay.** Cell viability was assessed using a CCK-8 assay, according to the manufacturer's protocol. A total of 4x10<sup>3</sup> MLE-12 cells per well in 100  $\mu$ l media were cultivated in 96-well plates and grown at 37°C for 24 h. The cells were treated with OP-D (0-320  $\mu$ M) for 1 h at 37°C and then reacted with or without 15  $\mu$ g/cm<sup>2</sup> PM<sub>2.5</sub> at 37°C in an incubator for 24 h. CCK-8 solution (10  $\mu$ l) was added into each well, and the cells were incubated for another 4 h at 37°C. Finally, the absorbance of each well was measured at a wavelength of 450 nm using a microplate reader. Each sample assay was repeated three times.

**Immunofluorescence staining.** MLE-12 cells (1x10<sup>5</sup> cells/well) on a glass coverslip cultured in 24-well plates were treated with 15  $\mu$ g/cm<sup>2</sup> PM<sub>2.5</sub> and 80  $\mu$ M OP-D, cells were then fixed with 4% paraformaldehyde, blocked with 5% BSA (cat. no. A1933-25G; Sigma-Aldrich; Merck KGaA) for 30 min at room temperature (RT) and incubated with anti-NF- $\kappa$ Bp65 (1:200) overnight at 4°C. After washing with PBS, the coverslip was treated with secondary antibody (1:80) for 1 h at RT. Following washing with PBS, the nuclei were stained with DAPI (Invitrogen; Thermo Fisher Scientific, Inc.) for 20 min at RT. The stained cells were then observed under an FV-1,000 fluorescence microscope (Olympus Corporation) under x400 magnification. Semi-quantitative analysis was performed using ImageJ 1.51t software (National Institutes of Health).

**ELISA.** Based on the experimental groups, the MLE-12 cells (7x10<sup>5</sup> cells/well) were cultivated in six-well plates, pretreated with OP-D at 10, 20, 40 or 80  $\mu$ M with 10  $\mu$ M CC for 1 h, and then stimulated with 15  $\mu$ g/cm<sup>2</sup> PM<sub>2.5</sub> for 24 h at 37°C. The PM<sub>2.5</sub> group was stimulated with the corresponding dose of PM<sub>2.5</sub>. The OP-D 80  $\mu$ M group was only treated with 80  $\mu$ M OP-D. After incubation, the cultured media samples were collected to determine the levels of IL-1 $\beta$ , IL-6, IL-8 and TNF- $\alpha$ , using Mouse Quantikine ELISA kits, according to the manufacturer's instructions. The cells were harvested for western blot analysis.

**Western blotting.** Cell lysates were extracted via homogenization with RIPA buffer (Beyotime Institute of Biotechnology) containing phenylmethanesulfonyl fluoride, protease inhibitors and phosphatase inhibitors. The nuclear fraction was isolated using a Nuclear and Cytoplasmic extraction kit (cat. no. NXTRACT-1KT; Sigma-Aldrich; Merck KGaA). The concentration of each supernatant was determined using a BCA protein assay kit. Aliquoted proteins (60  $\mu$ g/well) were loaded onto a 10% SDS-PAGE, separated by electrophoresis and then transferred onto a PVDF membrane. After blocking

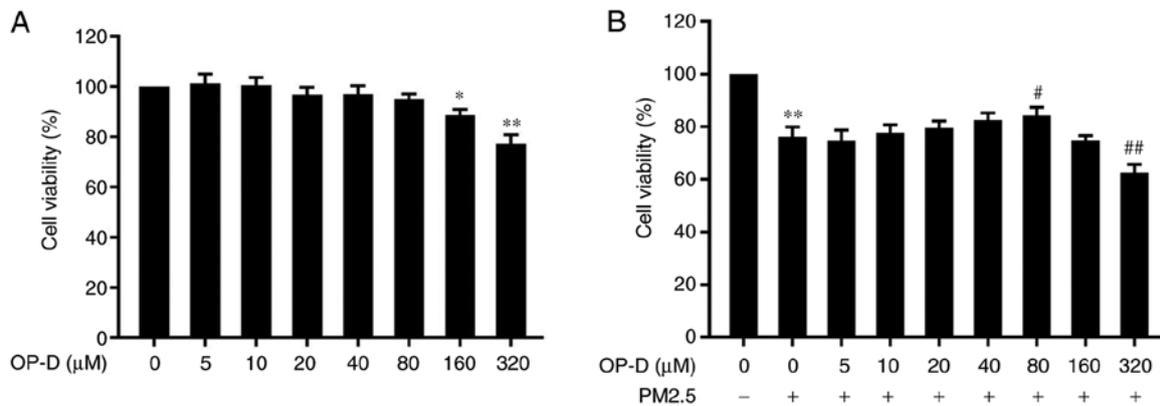


Figure 1. Effect of OP-D on the cellular viability of PM2.5-treated MLE-12 cells. (A) The cellular cytotoxicity of OP-D was evaluated in MLE-12 cells at the indicated concentrations. (B) Cells were pretreated with OP-D (0–320  $\mu\text{M}$ ) for 1 h and then stimulated with PM2.5 (15  $\mu\text{g}/\text{cm}^2$ ) for 24 h. The cellular viability was detected. Data are presented as the mean  $\pm$  SD for three independent assays. \* $P < 0.05$  and \*\* $P < 0.01$  vs. OP-D control group (0  $\mu\text{M}$  OP-D); # $P < 0.05$  and ## $P < 0.01$  vs. PM2.5-treated group (0  $\mu\text{M}$  OP-D with 15  $\mu\text{g}/\text{cm}^2$  PM2.5). OP-D, Ophiopogonin D; PM2.5, particulate matter of  $\leq 2.5 \mu\text{m}$  in diameter.

with 5% skimmed milk in Tris-buffered saline-Tween-20 (TBS-T, 0.1% Tween-20) for 1 h at RT, the membrane was treated with the corresponding primary antibody (AMPK, p-AMPK, NF- $\kappa\text{B}$ p65, p-NF- $\kappa\text{B}$ p65, GAPDH or lamin B) at 4°C overnight, washed with TBST buffer three times and then treated with horseradish peroxidase-conjugated secondary antibody for 1 h at RT. After washing with TBST three times, the membranes were visualized with enhanced chemiluminescence reagents. GAPDH and lamin B were employed as cytosol and nuclear protein loading controls, respectively. Semi-quantitative analysis was performed using ImageJ 1.51t software.

**Statistical analysis.** Data are presented as the mean  $\pm$  SD. Statistical analysis was performed using SPSS 20.0 software (IBM Corp.). Comparison of cell survival rate between the groups were performed using two-way ANOVA, while other experimental groups were compared using one-way ANOVA followed by Bonferroni's post hoc test. Experiments were repeated three times.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**OP-D prevents the PM2.5-induced decrease of MLE-12 cell viability.** Cellular viability was assayed in the OP-D (0, 5, 10, 20, 40, 80, 160 and 320  $\mu\text{M}$ )-treated MLE-12 cells. No cellular cytotoxicity was observed at OP-D concentrations of 0–80  $\mu\text{M}$  for 24 h in the presence or absence of PM2.5 (Fig. 1). However, higher concentrations (160 and 320  $\mu\text{M}$ ) of OP-D caused significant cytotoxicity, thus reducing the cellular viability (Fig. 1A). PM2.5 significantly decreased the MLE-12 cellular viability, compared with the untreated cells (Fig. 1B). Moreover, pretreatment with 80  $\mu\text{M}$  OP-D for 1 h significantly attenuated the PM2.5-induced decrease of cellular viability (Fig. 1B). Lower concentrations (5–80  $\mu\text{M}$ ) of OP-D did not affect cellular viability, however, 320  $\mu\text{M}$  OP-D significantly further inhibited cellular viability after PM2.5 treatment (Fig. 1B). Therefore, concentrations of 10, 20, 40 and 80  $\mu\text{M}$  OP-D were selected for the subsequent experiments.

**OP-D attenuates the PM2.5-induced levels of TNF- $\alpha$ , IL-6, IL-8 and IL-1 $\beta$  in MLE-12 cells.** The anti-inflammatory activity of OP-D was investigated in PM2.5-treated MLE-12 cells via ELISA to examine levels of IL-1 $\beta$ , IL-6, IL-8 and TNF- $\alpha$  in the culture supernatant. PM2.5 treatment significantly induced the expression of IL-1 $\beta$  (Fig. 2A), IL-6 (Fig. 2B), IL-8 (Fig. 2C) and TNF- $\alpha$  (Fig. 2D) in the culture medium of MLE-12 cells, compared with the negative controls. Cells treated with OP-D alone did not demonstrate any effect on the levels of proinflammatory cytokines. However, pretreatment with OP-D at 20–80  $\mu\text{M}$  significantly reduced the levels of the PM2.5-triggered cytokines in a dose-dependent manner, compared with those of the control PM2.5-treated cells (Fig. 2).

**OP-D inhibits the NF- $\kappa\text{B}$  inflammatory signaling induced by PM2.5 in MLE-12 cells.** OP-D has been reported to reduce inflammation via activation of the NF- $\kappa\text{B}$  signaling pathway (19), which can be suppressed by AMPK. To further examine the anti-inflammatory activity of OP-D, the effects of OP-D were detected on both NF- $\kappa\text{B}$  subcellular translocation and the protein phosphorylation of AMPK and NF- $\kappa\text{B}$ p65 in MLE-12 cells using immunofluorescence and western blot analysis.

PM2.5 treatment significantly induced NF- $\kappa\text{B}$  translocation from the cytoplasm to the nucleus, compared with the negative control in which most NF- $\kappa\text{B}$  expression still remained in the cytoplasm, according to immunofluorescence staining (Fig. 3B). In contrast, the PM2.5-induced nuclear translocation of NF- $\kappa\text{B}$ p65 was partly suppressed by pretreatment with OP-D (Fig. 3). Taken together, these findings suggest the potential anti-inflammatory effect of OP-D in PM2.5-induced cellular inflammation.

**OP-D phosphorylates AMPK in PM2.5-treated MLE-12 cells.** Next, the activation of AMPK in MLE-12 cells was investigated via western blot analysis. Exposure to PM2.5 did not significantly alter the phosphorylation of AMPK; however, 10–80  $\mu\text{M}$  OP-D significantly increased the phosphorylation of AMPK in a dose-dependent manner (Fig. 4A). Moreover, there were no significant changes in the OP-D alone group.

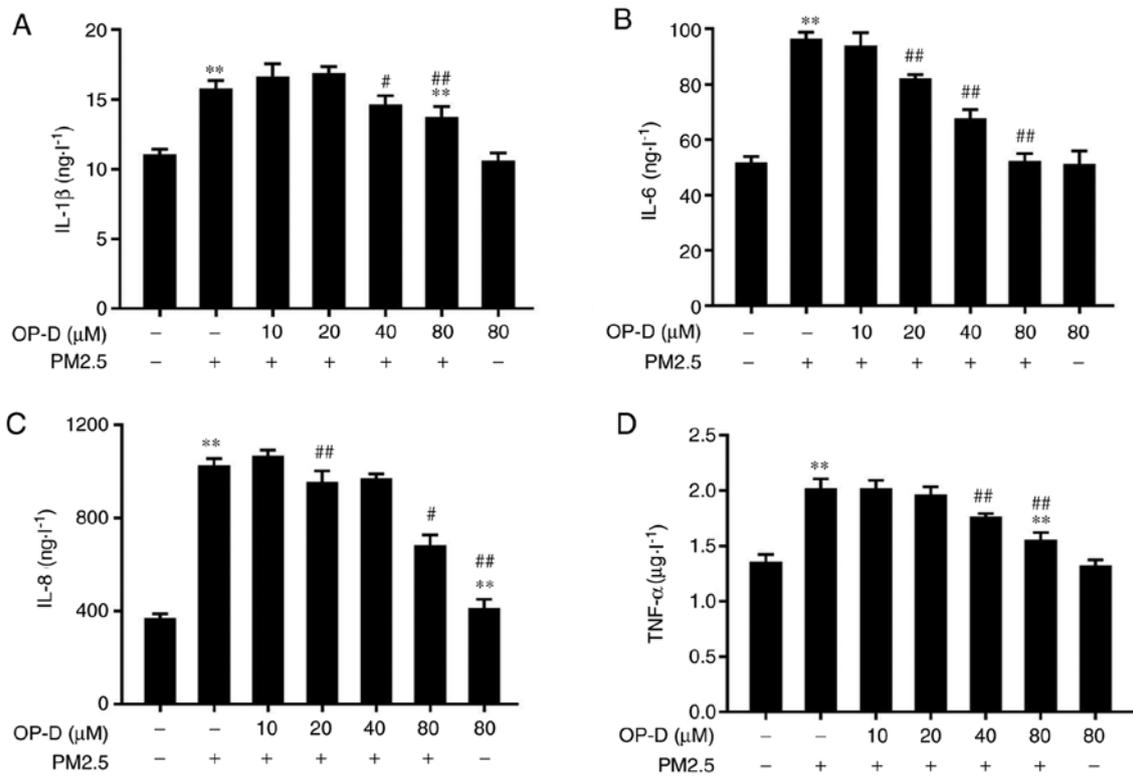


Figure 2. OP-D inhibits PM2.5-induced proinflammatory cytokine expression of IL-1 $\beta$ , IL-6, IL-8 and TNF- $\alpha$  in MLE-12 cells. MLE-12 cells were pretreated with the indicated concentrations of OP-D for 1 h and then stimulated with PM2.5 (15  $\mu\text{g}/\text{cm}^2$ ) for another 24 h. The levels of (A) IL-1 $\beta$ , (B) IL-6, (C) IL-8 and (D) TNF- $\alpha$  in the culture medium were determined using ELISA. Data are presented as the mean  $\pm$  SD (n=5). \*\*P<0.01 vs. controls; #P<0.05 and ##P<0.01 vs. PM2.5-treated cells. OP-D, Ophiopogonin D; PM2.5, particulate matter of  $\leq 2.5$   $\mu\text{m}$  in diameter.

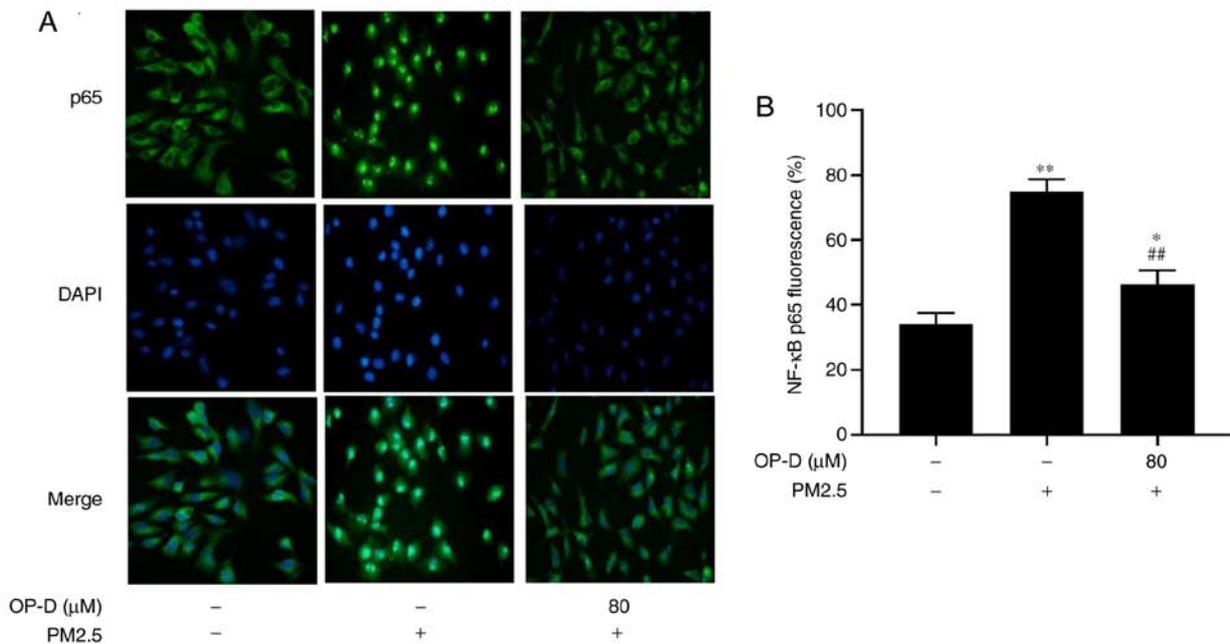


Figure 3. Immunofluorescence staining of NF- $\kappa$ Bp65 in MLE-12 cells. MLE-12 cells were pretreated with 80  $\mu\text{M}$  OP-D for 1 h and then stimulated with 15  $\mu\text{g}/\text{cm}^2$  PM2.5 for 24 h. (A) NF- $\kappa$ Bp65 (green) was visualized via immunofluorescence staining. Nuclei were stained by DAPI (blue). Magnification, x400. (B) The overall fluorescence was semi-quantified. Data are presented as the mean  $\pm$  SD (n=3). \*P<0.05 and \*\*P<0.01 vs. controls; ##P<0.01 vs. PM2.5-treated cells. OP-D, Ophiopogonin D; PM2.5, particulate matter of  $\leq 2.5$   $\mu\text{m}$  in diameter.

*OP-D inhibits PM2.5-triggered NF- $\kappa$ B activation.* PM2.5 exposure significantly phosphorylated NF- $\kappa$ B, and pretreatment with OP-D (20-80  $\mu\text{M}$ ) significantly attenuated this activation

in a dose-dependent manner (Fig. 4A) in MLE-12 cells. In addition, similar to the immunofluorescence staining results (Fig. 3), PM2.5 treatment significantly decreased NF- $\kappa$ B expression in

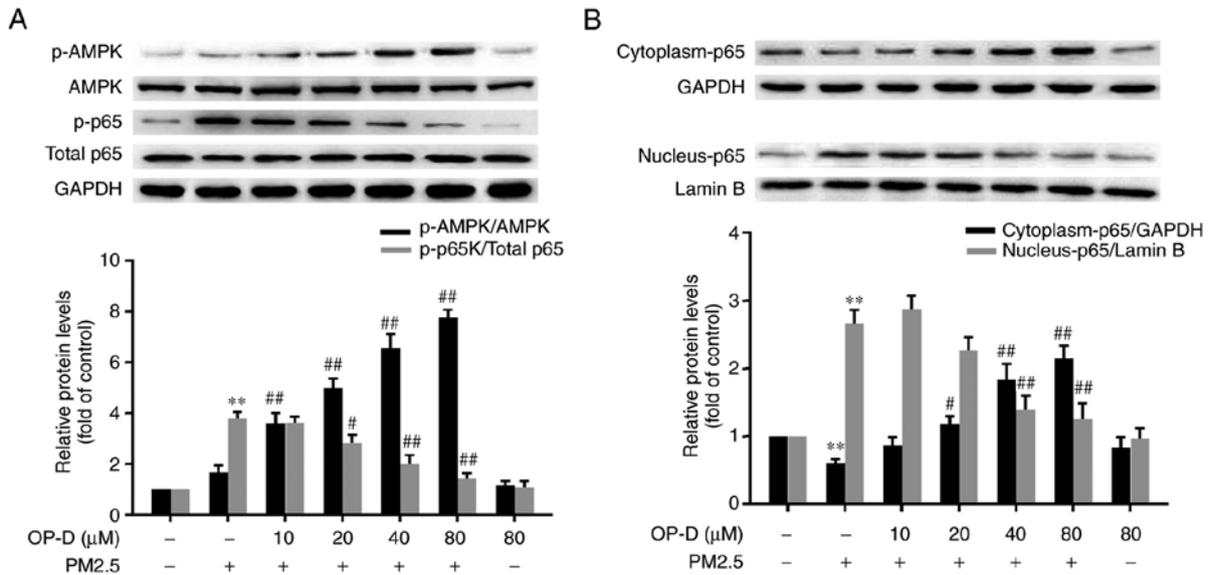


Figure 4. OP-D phosphorylated AMPK and inhibited PM2.5-induced NF-κB activation in MLE-12 cells. Representative western blotting images demonstrating (A) the activation state of p-AMPK and p-p65 in 15 μg/cm<sup>2</sup> PM2.5-treated (24 h) MLE-12 cells with or without pretreatment of OP-D (1 h) at different concentrations. (B) Expression of p-p65 was detected in the isolated cytoplasm and nuclei. Total AMPK, NF-κB, GAPDH and lamin B were used as the corresponding protein loading references. The relative phosphorylation levels of AMPK (to total AMPK), NF-κB (to total NF-κB), cytoplasmic NF-κB (to GAPDH) and nuclear NF-κB (to lamin B) were analyzed. The relative phosphorylation data are presented as the mean ± SD (n=3 in each group). \*\*P<0.01 vs. controls; #P<0.05 and ##P<0.01 vs. PM2.5-treated cells. AMPK, AMP-activated protein kinase; OP-D, Ophiopogonin D; PM2.5, particulate matter of ≤2.5 μm in diameter; p-, phosphorylated.

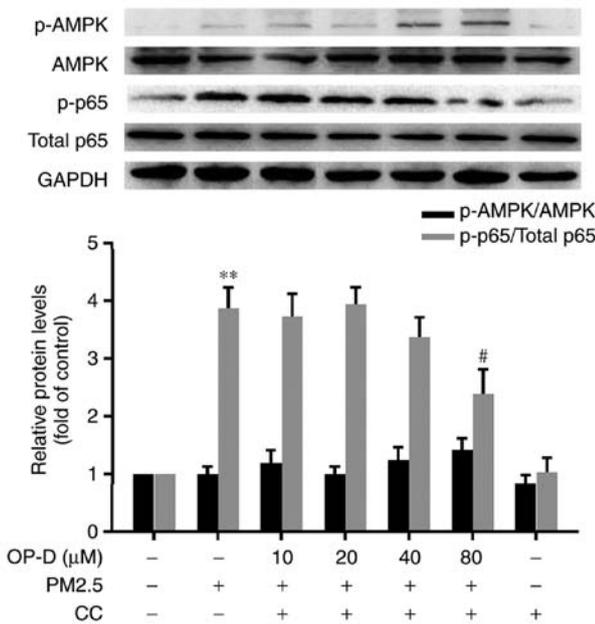


Figure 5. CC blocks the OP-D-mediated inhibition of NF-κB activation in PM2.5-exposed MLE-12 cells. MLE-12 cells were pretreated with different concentrations of OP-D (10, 20, 40 or 80 μM) and 10 μM CC for 1 h, and then they were exposed to 15 μg/cm<sup>2</sup> PM2.5 for 24 h. The expression levels of NF-κBp65, p-NF-κBp65, AMPK and p-AMPK were assessed using western blotting and were semi-quantified. Data are presented as the mean ± SD (n=3 in each group). \*\*P<0.01 vs. controls; #P<0.05 vs. PM2.5-treated cells. AMPK, AMP-activated protein kinase; CC, Compound C; OP-D, Ophiopogonin D; PM2.5, particulate matter of ≤2.5 μm in diameter; p-, phosphorylated.

the cytoplasm, while NF-κB expression in the nuclei was significantly increased. These alterations were significantly reversed by pretreatment with OP-D in a dose-dependent manner (Fig. 4B).

*CC inhibits the OP-D-induced dephosphorylation of NF-κB in MLE-12 cells.* To elucidate whether the anti-inflammatory effect of OP-D is mediated via AMPK, all the OP-D-treated cells were incubated with 10 μM CC simultaneously. The CC-treated cells did not demonstrate any significant activation of AMPK (Fig. 5). In the PM2.5-exposed MLE-12 cells cotreated with OP-D and CC, the 20-40 μM OP-D-mediated decrease of NF-κB was significantly blocked; however, CC did not inhibit this effect in the 80 μM OP-D-treated cells compared with the negative control cells (Fig. 5). These results indicated that AMPK mediated NF-κB activation during PM2.5-induced inflammation.

*CC upregulates the levels of TNF-α, IL-6, IL-8 and IL-1β inhibited by OP-D in MLE-12 cells.* The aforementioned results suggested that the anti-inflammatory activity of OP-D is AMPK dependent. In order to verify this finding, the downregulation of IL-1β (Fig. 6A), IL-6 (Fig. 6B), IL-8 (Fig. 6C) and TNF-α (Fig. 6D) by OP-D was measured in the presence or absence of CC. CC alone did not demonstrate any notable effect on the levels of the proinflammatory cytokines. Pretreatment with 10 μM CC significantly reversed the downregulation of IL-1β, IL-6, IL-8 and TNF-α induced by OP-D (80 μM), compared with the group without CC treatment. Furthermore, the inhibition by OP-D did not completely reverse to the control levels of TNF-α, IL-8 and IL-1β, compared with the PM2.5-treated group. These results indicated that downregulation of the inflammatory factors by OP-D was partly via the AMPK pathway, which is consistent with the western blotting results. Thus, the present results suggested that OP-D attenuated the PM2.5-induced cell inflammation via activation of AMPK and suppression of the NF-κB signaling pathway.

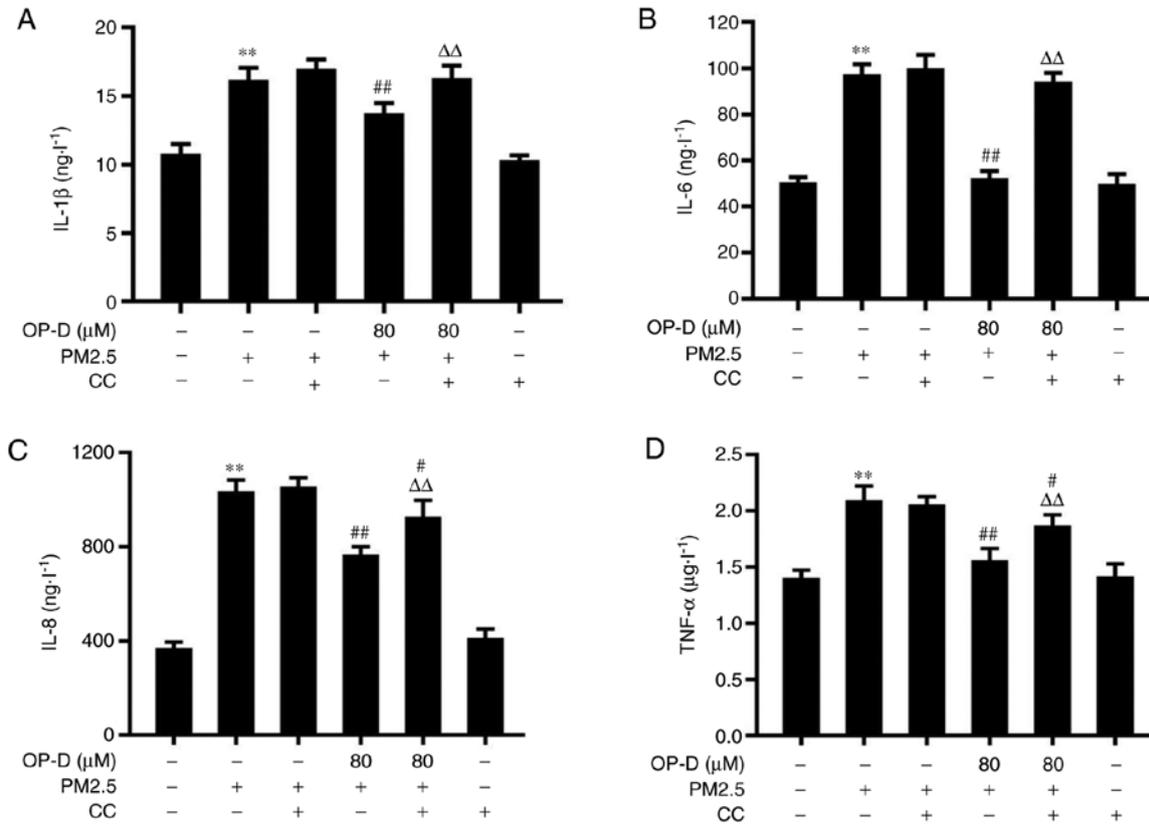


Figure 6. CC reverses the OP-D-mediated inhibition of proinflammatory cytokine expression in MLE-12 cells. MLE-12 cells were pretreated with different concentrations of OP-D (10, 20, 40 or 80  $\mu$ M) and 10  $\mu$ M CC for 1 h, and then they were exposed to 15  $\mu\text{g}/\text{cm}^2$  PM2.5 for 24 h. The levels of (A) IL-1 $\beta$ , (B) IL-6, (C) IL-8 and (D) TNF- $\alpha$  in the culture medium were determined via ELISA. Data are presented as the mean  $\pm$  SD (n=5). \*\*P<0.01 vs. controls; #P<0.05 and ##P<0.01 vs. PM2.5-treated cells;  $\Delta\Delta$ P<0.01 vs. PM2.5 + 80  $\mu$ M OP-D group. CC, Compound C; OP-D, Ophiopogonin D; PM2.5, particulate matter of  $\leq 2.5$   $\mu$ m in diameter.

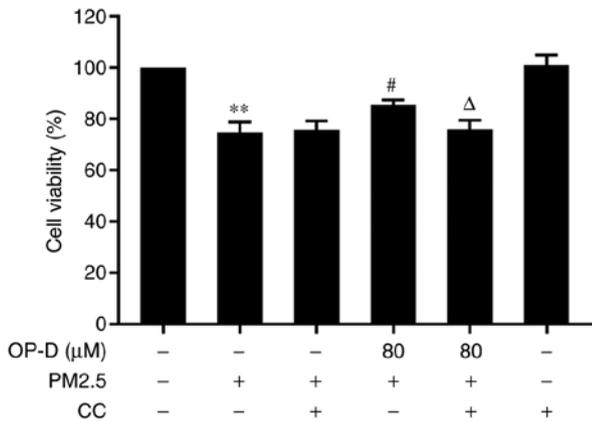


Figure 7. Effect of CC on the cellular viability of PM2.5-treated MLE-12 cells. MLE-12 cells were pretreated with different concentrations of OP-D (10, 20, 40 and 80  $\mu$ M) and 10  $\mu$ M CC for 1 h, and then exposed to 15  $\mu\text{g}/\text{cm}^2$  PM2.5 for 24 h. Data are presented as the mean  $\pm$  SD for three independent assays. \*\*P<0.01 vs. controls; #P<0.05 vs. PM2.5-treated cells;  $\Delta$ P<0.05, vs. PM2.5 + 80  $\mu$ M OP-D treated group. CC, Compound C; OP-D, Ophiopogonin D; PM2.5, particulate matter of  $\leq 2.5$   $\mu$ m in diameter.

CC reduces the protective effect of OP-D in MLE cells. CC was used to block the activation of AMPK in order to observe whether the AMPK pathway is essential to protect cellular viability. The present results demonstrated that blocking the AMPK pathway with CC significantly reduced

the protective effect of OP-D on the PM2.5-induced cells (Fig. 7; P<0.05). These results indicated that OP-D had a protective effect on MLE cells via activation of the AMPK signaling pathway.

## Discussion

To the best of our knowledge, the present study was the first to demonstrate that OP-D has an anti-inflammatory effect on PM2.5-injured alveolar epithelial cells. The present study identified that PM2.5 activated the NF- $\kappa$ B signaling pathway and then induced high expression levels of inflammatory factors, such as IL-6, IL-8, IL-1 $\beta$  and TNF- $\alpha$ , leading to inflammatory stress in the alveolar epithelial cells. In contrast, OP-D had an anti-inflammatory role via activation of AMPK to inhibit the PM2.5-activated NF- $\kappa$ B pathway. These findings suggested that the anti-inflammatory activity of OP-D may be a valuable strategy for protection against PM2.5- or other cause-induced pulmonary injury progression.

Due to the use of heating systems during the long winter season, air pollution in the city of Changchun is a serious issue during this time period, which markedly increases the concentrations of PM2.5. According to the environmental data released by the Ministry of Ecological Environment of the P.R. China, during the winter of 2017, the concentration of PM2.5 was 60-90  $\mu\text{g}/\text{m}^3$  in Changchun, which reached 2-3 times that of other seasons in the same area (21), the concentration,

composition and properties of the particles vary with the region and season. Our previous study found that PM<sub>2.5</sub> contained numerous chemical components, including Al, Ca, Fe, K, Zn, Mg, polycyclic aromatic hydrocarbons, organic carbon and elemental carbon, of which Al and organic carbon were the most abundant in the PM<sub>2.5</sub> samples in Changchun, China, during the winter (22). In the present study, the samples of PM<sub>2.5</sub> were collected over five months in winter. Therefore, the present data may be more reflective of what individuals may be exposed to during the winter. Since PM<sub>2.5</sub> is composed of extremely complex components, the toxic effects of different batches may reveal different results. Further work will be conducted to observe the anti-inflammatory activity of OP-D on additional samples collected from different cities.

As the first defense barrier between air and lung tissue, the airway epithelium is extremely vulnerable to the stimulation of harmful substances, such as PM<sub>2.5</sub> or cigarette smoke. For example, He *et al* (9) have established that PM<sub>2.5</sub> can cause inflammatory effects in MLE-12 cells, which are derived from a female mouse. In addition, multiple studies in male mice have reported that PM<sub>2.5</sub> can activate inflammation and lead to lung injury (23-25). Altogether, these findings suggest that sex differences do not affect the outcome of PM<sub>2.5</sub> exposure; therefore, the MLE-12 cell line was selected to perform this research.

Previous investigations have demonstrated that PM<sub>2.5</sub> can lead to an inflammatory response, which is suggested to be the basic pathogenesis of respiratory disorders of the respiratory system (26) and can damage pulmonary function (27), making the lungs more susceptible to infection. For example, Jeong *et al* (28) have revealed that the epidermal growth factor receptor/mitogen-activated protein kinase/NF- $\kappa$ B/IL-8 pathway may be a possible mechanism for PM<sub>2.5</sub>-induced lung toxicity. In addition, Li *et al* (29) observed that PM<sub>2.5</sub> may induce inflammatory responses via the Toll-like receptor 4/p38/NF- $\kappa$ B pathway. Notably, activated NF- $\kappa$ B serves a crucial role in PM<sub>2.5</sub>-induced inflammatory diseases. To date, NF- $\kappa$ B is known to consist of five family member protein monomers (p65/RelA, RelB, cRel, p50 and p52) that form homodimers or heterodimers to bind DNA differentially (30). Moreover, p65/RelA is activated and translocated into the nucleus via the formation of different heterodimers with p50 or p52 (31,32). The present study only determined the translocation of p65, not of the other forms, to investigate the activation of NF- $\kappa$ B. It has previously been reported that PM<sub>2.5</sub>-induced p65 translocation results in the release of certain inflammatory cytokines (20). After encountering inflammatory irritants, p65, the most important subunit of NF- $\kappa$ B, undergoes phosphorylation (activation) and translocates to the nucleus, acting as a transcriptional activator to promote the expression of various downstream proinflammatory mediators, including TNF- $\alpha$ , IL-6 and IL-1 $\beta$  (8). A previous study revealed that PM<sub>2.5</sub> exposure activates the NF- $\kappa$ B complex via phosphorylation of nuclear p65 and cytoplasmic I $\kappa$ B kinase- $\alpha$ , leading to nuclear p65/p50 DNA binding in human lung epithelial cells in a time- and concentration-dependent manner (33). These findings are consistent with the present results.

In the current study, immunofluorescence staining demonstrated that there was a greater level of NF- $\kappa$ Bp65 in the nuclei of PM<sub>2.5</sub>-treated MLE-12 cells, compared with

that of the blank control group (Fig. 3). Semi-quantitative analysis via western blotting indicated that the nuclear translocation of NF- $\kappa$ Bp65 and the phosphorylation level of NF- $\kappa$ Bp65 were  $\sim$ 3 (Fig. 4B) and four (Fig. 4A) times greater, compared with the blank control group. In addition, the cytoplasmic NF- $\kappa$ Bp65 was reduced significantly only in the PM<sub>2.5</sub>-treated MLE-12 cells, compared with that in the blank control group (Fig. 4B). It was also found that PM<sub>2.5</sub> stimulation significantly induced the levels of IL-1 $\beta$ , IL-6, IL-8 and TNF- $\alpha$  in MLE-12 cells, compared with control cells (Fig. 2). However, the PM<sub>2.5</sub>-induced inflammatory cytokine response may induce the death of pulmonary epithelial cells, inhibit the junctional gap between these cells to block intercellular communication and impair their function, further leading to alveolar collapse (34,35).

OP-D significantly downregulated the expression of the PM<sub>2.5</sub>-triggered cytokines in a dose-dependent manner (Fig. 2), indicating that OP-D may have protective effects against PM<sub>2.5</sub>-induced inflammation. PM<sub>2.5</sub>-induced nuclear translocation and phosphorylation of NF- $\kappa$ Bp65 were partly suppressed by OP-D (Figs. 3 and 4), which suggested that OP-D had a protective effect against PM<sub>2.5</sub>-induced inflammation via the NF- $\kappa$ B signaling pathway.

AMPK is a crucial sensor that regulates the intracellular ATP to AMP ratio in all eukaryotic cells (36). It has been reported that AMPK activation attenuates cigarette smoke-induced inflammatory responses in human lung epithelial cells to protect against the development of emphysema (37). Moreover, activated AMPK inhibits the inflammatory response via the attenuation of NF- $\kappa$ B phosphorylation to block the production of proinflammatory cytokines (38). Based on these findings, it was hypothesized that the protective mechanism of OP-D may be partly attributed to activation of AMPK signaling in PM<sub>2.5</sub>-stimulated MLE-12 cells. Indeed, the present results supported this hypothesis, as it was demonstrated that the activity of OP-D inhibited the release of IL-6, IL-8, IL-1 $\beta$  and TNF- $\alpha$  via activation of AMPK to block the NF- $\kappa$ B pathway in PM<sub>2.5</sub>-stimulated MLE-12 cells (Figs. 5 and 6). Furthermore, the AMPK signaling pathway was identified to have a significant protective effect on cellular viability (Fig. 7). However, AMPK activation cannot completely inhibit the activation of NF- $\kappa$ B and the production of cytokines, which suggests that other signaling pathways may be involved in the anti-inflammatory effect of OP-D in PM<sub>2.5</sub>-treated alveolar epithelial cells. Therefore, the specific anti-inflammatory mechanism of OP-D should be investigated in future studies. The present study demonstrated the dose effects of OP-D in the cells; however, whether the doses are equivalent in the *in vivo* exposure has not been elucidated. Hence, in order to further evaluate the pharmacological effect and value of OP-D, an additional study on the function and dose correlation of OP-D is underway in a mouse model of PM<sub>2.5</sub>-induced emphysema.

In conclusion, the present study demonstrated that OP-D has anti-inflammatory effects against the PM<sub>2.5</sub>-induced damage in alveolar epithelial cells. The mechanism of OP-D activity may be via inhibiting the expression of inflammatory cytokines, such as IL-6, IL-8, IL-1 $\beta$  and TNF- $\alpha$ , activating phosphorylated AMPK and inhibiting activation of the NF- $\kappa$ B pathway. Therefore, OP-D could potentially be an efficient and

therapeutic drug for assisting in the treatment of respiratory inflammation caused by PM<sub>2.5</sub>. The present research also provides theoretical evidence for the transformation of OP-D into clinical applications.

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### Availability of data and materials

The data used and/or analyzed during the current study are available from the corresponding author on reasonable request.

### Authors' contributions

HD and YW designed most of the investigation, performed data analysis and wrote the manuscript. YW provided experimental technical assistance, and DL and LS contributed to interpretation of the data and analyses. All authors read and approved the final manuscript.

### Ethics approval and consent to participate

Not applicable.

### Patient consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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