Research Article

Tussilagone protects acute lung injury from PM2.5 via alleviating Hif-1α/NF-κB-mediated inflammatory response

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Abstract
Environmental pollution, especially particulate matter in the air, is a serious threat to human health. Long-term inhalation of particulate matter with a diameter < 2.5 μm (PM2.5) induced irreversible respiratory and lung injury. However, it is not clear whether temporary exposure to massive PM2.5 would result in epithelial damage and lung injury. More importantly, it is urgent to clarify the mechanisms of PM2.5 cytotoxicity and develop a defensive and therapeutic approach. In this study, we demonstrated that temporary exposure with PM2.5 induced lung epithelial cell apoptosis via promoting cytokines expression and inflammatory factors secretion. The cytotoxicity of PM2.5 could be alleviated by tussilagone (TSL), which is a natural compound isolated from the flower buds of Tussilago farfara. The mechanism study indicated that PM2.5 promoted the protein level of Hif-1α by reducing its degradation mediated by PHD2 binding, which furtherly activated NF-κB signaling and inflammatory response. Meanwhile, TSL administration facilitated the interaction of the Hif-1α/PHD2 complex and restored the Hif-1α protein level increased by PM2.5. When PHD2 was inhibited in epithelial cells, the protective function of TSL on PM2.5 cytotoxicity was attenuated and the expression of cytokines was retrieved. Expectedly, the in vivo study also suggested that temporary PM2.5 exposure led to acute lung injury. TSL treatment could effectively relieve the damage and decrease the expression of inflammatory cytokines by repressing Hif-1α level and NF-κB activation. Our findings provide a new therapeutic strategy for air pollution-related respiratory diseases, and TSL would be a potential preventive medicine for PM2.5 cytotoxicity.

Keywords
Hif-1α, lung injury, NF-κB, PM2.5, tussilagone

1 | INTRODUCTION

With the industrialization progress and population growth, environmental pollution has seriously threatened human health. According to the World Health Organization (WHO), 7 million people die from air pollution every year globally.1 Air pollution is composed of particulates of different sizes and toxic chemicals,2,3 which activate the immune system,4 induce oxidative stress and result in inflammation...

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and tissue damage. Among all kinds of air contaminants, particulate matter with a diameter < 2.5 μm (PM2.5) presents significant adverse effects on human health, especially the respiratory system. The evidence showed that more than 70% of PM is deposited under the trachea of the respiratory tract, while nearly 22% of that reached the alveoli, which may increase oxidative stress of epithelial cells in the respiratory tract and other lung tissues. Long-term exposure to inhaled PM2.5 leads to chronic inflammation in lung tissue and local cytokines increasing. Although the major poisonous chemicals of PM2.5 remain controversial, it is confirmed that multiple components of PM2.5 present different effects on lung epithelial cells and alveolar immune cells. Previous studies have shown that PM2.5 activates NF-κB pathway and then upregulates the expression and secretion of proinflammatory cytokines and eventually led to extensive pulmonary inflammatory lesions. In addition, PM2.5 also triggered oxidative stress to aggravate tissue damage by regulating ROS generation, catalase (CAT) activation, superoxide dismutase (SOD) and glutathione peroxidase (GPX) expression. Therefore, it is significant to reveal the molecular mechanism of the inflammatory response induced by PM2.5 and find the effective medicine for PM2.5 poisonou

Tussilagone (TSL), which is a compound isolated from the flower buds of Tussilago farfara, is used to be considered as a natural product of traditional oriental medicine to act as an immune repressor and alleviate inflammatory reaction. With the deepening of the research on TSL, it has been demonstrated that TSL plays therapeutic roles in many kinds of disease, such as tumor progression, osteoclastogenesis, angiogenesis, allergic responses, inflammatory bowel disease (IBD) and ischemic stroke. What’s more, TSL is involved in the protection of airway damage. However, little is known about the detailed functions and mechanisms of TSL modulating inflammatory response. Several recent studies revealed that TSL attenuated the NF-κB signaling pathway in epithelial cells and colon cancer cells. Therefore, it is reasonable that TSL might alleviate the PM2.5 induced NF-κB activation in lung tissue.

In this study, we constructed the mouse acute lung injury model through short-term exposure of massive dosage PM2.5. Then we demonstrated the protective function of TSL on PM2.5 mediated acute lung damage both in vitro and in vivo, and clarified the mechanisms of TSL repressing NF-κB pathway via Hif-1α signaling. Our findings provide a new therapeutic strategy for air pollution-related respiratory diseases, and TSL would be a potential preventive medicine for PM2.5 poisoning.

2 MATERIALS AND METHODS

2.1 Materials

China fine dust particulate matter (CPM) (No. 28 certified reference material) was purchased from National Institute for Environmental Studies, Ibaraki, Japan. The origin of this material is atmospheric particulate matter collected on filters in a central ventilating system in a building in Beijing city center. The collection period was 10 years, from 1996 to 2005. The particulate matter was recovered from the filters by mechanical vibration and sieved using a 32 μm sieve. The sieved material was homogenized and sterilized by 60Co irradiation. The components of PM2.5 in this study have been identified by elemental analyzes. The element mass fractions were determined by at least two analytical methods. The detailed information of components was shown in Figure 1A. Tussilagone (TSL) was purchased from MedChemExpress Ltd. (MCE, Monmouth Junction, New Jersey, USA). MG132 was purchased from Sigma Aldridge (St. Louis, MO). Dulbecco’s Modified Eagle’s Medium (DMEM), fetal bovine serum (FBS), l-glutamine penicillin and streptomycin were purchased from Thermo Fisher Scientific (Waltham, MA). Cell Counting Kit-8 (CCK-8) and LDH activity detection kit were purchased from Solarbio (Beijing, China). The antibodies for Western blot (WB) were purchased from Thermo Fisher Scientific. Human lung epithelial (A549) cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA).

2.2 Cell culture and transfection

Human lung epithelial cells (A549) were cultured in DMEM containing 10% FBS and 2 mM l-glutamine. The prepared cells were transfected with plasmids by using Lipofectamine™ LTX according to the manufacturer’s instructions (Invitrogen) or treated with PM or/and TSL. After different treatments, A549 cells were cultured in complete DMEM and then collected for further functional detection. All cells were incubated in an atmosphere of 5% CO2 at 37°C. In some experiments, 25 μg/ml of TSL was added for treatment.

2.3 Mice breeding and mouse lung injury model

Mice (C57BL/6 background; male; age, 8 weeks) were purchased from the Shanghai Experimental Animal Center (Chinese Academy of Sciences) and maintained under a specific pathogen-free (SPF) facility. The mice were housed in groups of four and given 5 days to acclimate to the housing facility. The animals were reared under conditions of 22 ± 2 °C, the humidity of 55% ± 10%, light/dark cycle = 12 h, and ad libitum access to water and food. At the start of the experiments, animals weighed (mean ± SD) 22 ± 2 grams. A total of 15 mice were randomly divided into three groups (PBS: 5 mice; PM: 5 mice; PM + TSL: 5 mice). All animal experiments were approved by the Animal Experiment Administration Committee of the Fourth Military Medical University to ensure ethical and humane treatment of animals. For the in vivo experiments, fine dust particulate was used to establish the acute lung injury model. Briefly, C57BL/6 mice were anesthetized by intraperitoneal (i.p) injection of 0.6% pentobarbital sodium (10 μl/g, Sigma-Aldrich, St. Louis, MO). And then, PM was inhaled by the
mouse through the respiratory tract at a concentration of 20 mg/kg for 4 h/day (14:00 to 18:00) and the exposure lasted for 6 days. The total poisoning time reached 24 h. During the interval of exposure, the mice maintained a normal diet. In certain groups, at the same time of PM2.5 inhaling, the TSL was injected intravenously to PM2.5 treated mice with 20 mg/kg every 3 days. After that, the acute lung injury model was identified by detecting the wet and dry weight ratio of the lung (W/D) and assessing the arterial partial pressure of oxygen (PaO₂). Then the lung tissues were isolated for pathology analysis and gene expression determination. All the animal experiments were approved by the Animal Experiment Administration Committee of the Fourth Military Medical University. All methods were carried out in accordance with the recommendations of Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by the National Institutes of Health.

2.4 RNA extraction and quantification assay

According to the manufacturer’s instructions, total RNAs were extracted from A549 cell line or lung tissues with TRIzol reagent (Invitrogen). The cDNA was reverse-transcribed by using the HiScriptII Q RT SuperMix Reagent Kit (Vazyme, Nanjing, China) for different experiments. Quantitative real-time PCR was performed with the SYBR Premix EX Taq Kit (Takara, Dalian, China) using an ABI PRISM 7500 Real-time PCR system (Life Technologies, Waltham, MA), and β-actin was used as the internal control. The sequences of PCR primers used in this study were shown as follows: IL-6 forward, 5′-ACTACACCTTCTCAGGAGAATGG-3′ and reverse, 5′-CCATCTTTGGAAGGTTAGGTG-3′; IL-1β forward, 5′-ATGAG TGGCTTATTACAGTGCA-3′ and reverse, 5′-GTCGGAATTCGTAGC TGGA-3′; IL-12 forward, 5′-ACCCGTGATCCATCTCAGAAA-3′ and reverse, 5′-TTGCGCTCCAGATCTGAGGAC-3′; TNF-α forward, 5′-CCT GCTCTTAACTACGCCCCCTCGTG-3′ and reverse, 5′-GAGGACCTGGAGAT TAGTG-3′; TGF-β forward, 5′-GGCCAGATCCGCTGCAAGC-3′ and reverse, 5′-GTGGGTTCCTCAGCATCCACGC-3′; GAPDH forward, 5′-CTT CAACGACCACTTTGTG-3′ and reverse, 5′-TGGTCCAGGCTCTTACT-3′.

2.5 Western blotting

For Western blotting analysis, the cells were harvested and lysed on ice for 30 min in RIPA buffer supplemented with protease inhibitors (100 mM Tris–HCl at pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1% deoxycholate acid, 0.1% SDS, 2 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 2 mM DTT, 2 mM leupeptin, 2 mM pepstatin). Cells lysates were centrifuged at 12,000 rpm for 15 min, and the supernatants were collected as total proteins. After the concentrations of protein samples were determined by the BCA method (Beyotime, Haimen, China), an equal amount of each sample was separated by SDS-PAGE and transferred onto PVDF membranes. Membranes were blocked with 5% nonfat dried milk solution for 2 h and incubated with primary antibodies, respectively. The antibodies used were against cl-caspase 3, Bcl2, Kra, Hif1α, PHD2 (CST), phospo-P65, total P65 (Abcam, Cambridge, MA) and β-actin (Boster Bio Tec, Wuhan, China). After washing three times with PBST, the membranes were incubated with HRP conjugated secondary antibody and visualized with an ECL detection system. Protein expression was measured by ImageJ software.

2.6 Immunofluorescence staining

The fixed tumor tissues of the mice and clinical specimens were blocked with 5% BSA after antigen retrieval. The sections were stained with Ki67 or TUNEL detection kit and incubated with 4′,6-diamidino-2-phenylindole (DAPI). The sections were observed with a laser scanning confocal microscope (FV-1000, Olympus, Tokyo, Japan).

2.7 Luciferase reporter assay

A549 cells were cotransfected with Hif1α- or NF-κB-recognized reporter systems and the Renilla luciferase plasmid (phRL-TK, Promega, Madison, WI) as an internal control. Simultaneously, PM or/and TSL were added to the cultured cells as a stimulation. The cells were harvested and lysed after transfection for 24 h, and the relative luciferase activity was read out using the Dual-Luciferase Reporter Assay with a Glomax 20/20 Luminometer (Promega).

2.8 Proliferation assays

The proliferation of A549 cells was analyzed with CCK8 assay. Briefly, A549 cells were seeded into 96-well plates with PM or/and TSL treatment, and evaluated cell proliferation at 24, 48, 72, and 96 h using the CCK8 reagent Kit. In some experiments, the agonist of NF-κB was used for rescue. After the incubation for 4 h at 37°C, the supernatant was removed and the precipitation was dissolved in DMSO (Sigma). Spectrophotometric absorbance was measured at the wavelength of 490 nm by a microplate reader (BioTek Instruments Inc., Winooski, VT).

2.9 Apoptosis assay

The cell apoptosis was examined by Dead Cell Apoptosis Kit with FITC-Annexin V and PI (Thermo Fisher Scientific). Briefly, FITC-Annexin V was added into the single cell suspension for incubating 15 min at room temperature, and followed by and propidium iodide staining. Afterwards, the cells were washed with 1 × Annexin-binding buffer and gently mixed for further analysis by flow cytometry.

For the LDH activity determination, the cell suspension of prepared A549 cells was added to each well of a flat-bottom 96-well plate. And then, an equal volume of reaction substance buffer was added to the cell suspension for incubation 10 min at 37°C in a CO₂ incubator. After that, add 10 μl of the Lysis Buffer to each well for
incubation 30 min at 37°C, followed by the addition of Working Solution. Protect the plate from light and incubate it at room temperature for 30 min and add Stop Solution to each well. Then the LDH activity was measured with the absorbance at 490 nm by a microplate reader.

2.10 | ROS generation detection

The collected A549 cells were stained by 2′,7′-dichlorofluorescein diacetate (DCFDA) (Abcam, Cambridge, MA) and incubated for 30 min at 37°C. Then the flow cytometry was used to measure the fluorescence intensity. For some experiments, the tissue specimens were stained with DCFDA using immunofluorescence.

2.11 | Co-immunoprecipitation (Co-IP)

A549 cells were co-transfected with Flag-Hif1α and Myc-PHD2 plasmid. The cells were treated with PM or/and TSL for 24 h, and MG-132 (Selleck, Houston, Texas) was added 6 h before harvesting the cells. The cell lysates were incubated with an anti-Flag antibody conjugated to protein-G beads (Dynabeads®, Pharmacia, Uppsala, Sweden) at 4°C overnight with rotation. The protein complexes were precipitated, and the levels of Flag-Hif1α and Myc-PHD2 were detected.

2.12 | Statistical analysis

The data were analyzed by SPSS 12.0 software. Unpaired Student’s t-test or one-way ANOVA test was performed to compare the differences between groups by using Graph Pad Prism 5 software, version 5.0. The results were presented as mean ± SD. p value less than .05 was considered statistically significant.

3 | RESULTS

3.1 | PM2.5 exposure repressed proliferation and facilitated apoptosis of A549 cells

It is known that fine particulate matter (PM2.5) induces cell damage via different mechanisms and the inflammation degree is positively correlated with the exposure duration even under low dosage.7,8,28 However, whether short-term and massive dosage treatment of PM2.5 could accelerate acute lung injury remains unclear. Therefore, we added PM2.5 with different concentrations (small dosage: 30 μg/ml; mid dosage: 100 μg/ml and massive dosage: 300 μg/ml) into the culture media of A549 lung epithelial cells. The further detection suggested that PM2.5 decreased A549 viability, and the massive dosage of PM2.5 presented the most significant effect (Figure 1B). The addition of PM2.5 inhibited A549 cells proliferation (Figure 1C). In addition, apoptosis evaluation by FACS displayed that, with the increase of exogenous PM2.5 content, percentages of both early and late apoptosis were promoted in A549 cells (Figure 1D). The conclusion was also confirmed that massive PM2.5 increased LDH activity in A549 cells (Figure 1E). The further expression detection of apoptosis and cell damage-associated molecules suggested that massive PM2.5 exposure increased caspase 3 cleavage and Kras expression (Figure 1F). It has been demonstrated that PM2.5 stimulation induced the cleavage of caspase 3 directly induced mitochondria damage and cell apoptosis. Therefore, we inferred that the apoptosis promotion function of PM2.5 is dependent on the cleavage and activation of caspase 3. The above data indicated that short time exposure of PM2.5 repressed proliferation and facilitated apoptosis of A549 cells in a dosage-dependent manner.

3.2 | Exogenous PM2.5 promoted inflammatory cytokines expression via activating NF-κB signaling

To clarify the mechanism of PM2.5 induced cell apoptosis, we examined the expression of cytokines and inflammatory factors in A549 cells with different concentrations of PM2.5. The data showed that both mid and massive dosages of PM2.5 obviously elevated the cytokines expression (Figure 2A) and inflammatory factors secretion (Figure 2B). What’s more, FACS assay was performed to detect ROS production in PM2.5 stimulated A549 cells, which suggested that PM2.5 increased intracellular ROS generation (Figure 2C). As it is known that, NF-κB signaling plays significant role in the inflammatory response. Phosphorylation of transcription factor P65 was required for NF-κB signaling activation.31 Therefore, we detected the phosphorylation of P65 in PM2.5 stimulated A549 cells. The results displayed that P65 activation was activated gradually as PM2.5 concentration increased (Figure 2D,E). To further evaluate the activation of NF-κB signaling, we constructed the reporter system with NF-κB recognized motif inserting into upstream of luciferase open read frame (ORF) region. And then A549 cells were transfected by this NF-κB activation reporter system with PM2.5 treatment. The monitoring results indicated that PM2.5 indeed promoted NF-κB signaling activation (Figure 2F). JSH-23, which is an inhibitor of NF-κB signaling through repressing P65 phosphorylation and nuclear translocation, was added together with PM2.5. The detection of cytokines displayed that JSH-23 retrieved the function of PM2.5 completely (Figure 2G, H). Taken together, these data suggested PM2.5 promoted inflammatory cytokines expression via activating NF-κB signaling.

3.3 | TSL rescued the effect of PM2.5 on A549 cell survival and inflammatory response

Several studies and our previous research have demonstrated that TSL possessed anti-inflammatory function in multiple diseases.18–23 Therefore, TSL with different concentrations was added into the
culture media of A549 cells, as well as massive dosage of PM2.5 (300 μg/ml). We found that TSL rescued the repression of viability and proliferation mediated by PM2.5 (Figure 3A). On the other hand, both FACS analysis and LDH activity determination indicated that PM2.5 induced apoptosis was completely restored by the high concentration of TSL therapy (Figure 3C). Meanwhile, the WB was performed to detect the apoptosis of A549 cells, which indicated that TSL reduced both caspase 3 cleavage and Kras expression mediated by PM2.5 (Figure 3E). These results demonstrated that TSL retrieved the influence of PM2.5 on A549 cells proliferation and survival.

Next, we detected the inflammatory condition of A549 incubated with TSL by qRT-PCR and ELISA assay. The determination data displayed that TSL alleviated the inflammatory cytokines expression and factors production obviously (Figure 4A-C). Notably, Hif-1α, which is responsible for hypoxia signaling transduction, was upregulated in PM2.5 treated A549 cells, while sharply reduced by TSL (Figure 5A). It has been reported that Hif-1α stimulated P65 nuclear translocation and NF-κB signaling activation. What’s more, Hif-1α alone could evoke an inflammatory response and trigger cytokines and inflammatory factors generation. It should be noticed that Hif-1α was quickly degraded under an oxygen-rich environment after translation. To validate the regulation of Hif-1α by TSL, we examined the mRNA and protein expression of Hif-1α in different groups. The results demonstrated that PM2.5 promoted the protein level but not mRNA expression of Hif-1α, and TSL rescued the Hif-1α protein elevation mediated by PM2.5 (Figure 5B,C). Although HIF-1β, as another subunit of HIF-1, has been demonstrated to regulate NF-κB

3.4 TSL inhibited Hif-1α signaling via promoted degradation mediated by PHD2

To clarify the molecular mechanisms of TSL regulating NF-κB signaling, the antibody array of inflammation-associated molecules was performed in A549 cells with PBS, PM2.5 (300 μg/ml) and PM2.5/TSL. The results showed many transcription factors involved in inflammatory response were decreased by TSL treatment (Figure 5A). Notably, Hif-1α, which is responsible for hypoxia signaling transduction, was upregulated in PM2.5 treated A549 cells, while sharply reduced by TSL. PM2.5 promoted the protein level but not mRNA expression of Hif-1α, and TSL rescued the Hif-1α protein elevation mediated by PM2.5 (Figure 5B,C). Although HIF-1β, as another subunit of HIF-1, has been demonstrated to regulate NF-κB
activation via TRAF6, the expression of HIF-1α were not affected by PM2.5 stimulation (Figure 5B,C). We further constructed the reporter system with Hif-1α binding site to determine the activation of Hif-1α. The reporter assay indicated that exogenous TSL effectively promoted the trans-activation of Hif-1α (Figure 5D). Hif-1α is involved in multiple signaling pathways and biology progress, including metabolism and inflammatory regulation. The activation of Hif-1α would stimulate NO generation produced by iNOS. The relative NO concentration was measured by Griess Kit, which suggested that additional TSL inhibited NO production dramatically compared with PM2.5 alone (Figure 5E). The data demonstrated that TSL repressed the protein expression and activation of Hif-1α.

It has been demonstrated that ROS generation was induced by PM2.5 and rescued by TSL administration (Figure 4). Lots of studies indicated that oxidative stress and ROS increasing promote the expression of Hif-1α. To evaluate whether PM2.5 regulated Hif-1α signaling dependent on ROS, the ROS inhibitor (ALK/ROS1-IN-1) was added together with PM2.5 into A549 culture media. The data
suggested that ROS inhibition did not alleviate the Hif-1α expression and activation mediated by PM2.5, which indicated that PM2.5 regulated Hif-1α independent on ROS generation (Figure 5F,G). Generally, Hif-1α protein was hydroxylated and then bound with PHD2 at normoxia. The interaction of the Hif-1α/PHD2 complex induced ubiquitination and further degradation of Hif-1α.36 To validate the regulatory mechanism of TSL on Hif-1α, A549 cells were transfected with shRNA of Hif-1α for functional detection. Expectedly, the pro-inflammation of PM2.5 was attenuated when Hif-1α was inhibited (Figure 5J,K). The above results demonstrated that PM2.5 prevented the interaction of Hif-1α and PHD2 to maintain the stability of Hif-1α and activate NF-κB signaling, which was retrieved by TSL.

FIGURE 3 Additional TSL rescued the effect of PM on A549 cell survival. (A,B) A549 cells were incubated with PM2.5, as well as the different concentrations of TSL. Then the proliferation was determined by using the CCK-8 assay (A) (n = 5) and Ki67 staining (B) (n = 5). (C,D) A549 cells were treated as same as (A), and the FACS assay (C) (n = 5) and LDH activity detection (D) (n = 5) were performed to assess the apoptosis. (E) The expression of apoptosis-associated molecules, including cl-Cas3 and Kras, was detected in A549 cells with different treatments as (A) (n = 5). Bars, means ± SEM; *p < .05; **p < .01; ***p < .001
3.5 | TSL administration retarded the acute lung injury affected by PM2.5 in vivo

Although the therapeutic function of TSL on PM2.5-induced inflammation has been demonstrated in vitro, the in vivo effect of TSL remains unclear. Next, we constructed the acute lung injury mice model with short-term exposure of PM2.5, and then TSL was administered through tail vein injection. The body weight of mice was measured every 2 days, suggesting that PM2.5 triggered mice present a slower speed of weight obtain and TSL therapy reversed the phenotype (Figure 6A). After 4 days, the mice were sacrificed and the lung tissues were weighed. Although the body weight decreased, both lung weight and wet/dry weight (W/D) ratio of lung were elevated by PM2.5 and rescued by TSL therapy (Figure 6B,C). We also assessed the arterial partial pressure of oxygen (PaO₂) of different mice, indicating that TSL retrieved the regulation function of PM2.5 exposure (Figure 6D). Then the lung tissue was fixed by paraformaldehyde for histomorphological analysis. It is obvious that PM2.5 poisoning destroyed the normal alveolar structure and promoted apoptosis of lung epithelial cells, which were both rescued by TSL therapy (Figure 6E,F). The further molecular detection was consistent with in vitro experiments that PM2.5 induced stability of Hif-1α and NF-κB signaling (Figure 6G). As expected, TSL administration also eliminated the inflammatory response resulting from PM2.5 poisoning, including reducing cytokines expression (Figure 6H,I), inhibiting inflammatory factors secretion (Figure 6J) and alleviating ROS generation (Figure 6K). Collectedly, short-term exposure of massive dosage...
PM2.5 would induce heavy acute lung injury by activating the Hif-1α-NF-κB regulatory axis, and TSL administration completely retrieved the influence of PM2.5 poisoning both in vitro and in vivo. Our findings provided a new therapeutic strategy for air pollution-related respiratory diseases, and TSL would be a potential preventive medicine for PM2.5 poisoning.

4 | DISCUSSION

Air pollution, especially PM2.5 in the air, presents a great contribution to the development of respiratory diseases. On the one hand, we should strengthen the implementation of supervisory measures and environmental protection policies to reduce man-made contributions
to PM2.5 emissions. More importantly, it is necessary to clarify the process and mechanism of PM2.5 induced disease in depth, which might find potential therapeutic targets and provide effective medicine and therapeutic strategies. The previous studies tend to pay more attention to chronic diseases caused by long-term PM2.5 stimulation, such as chronic obstructive pulmonary disease,\textsuperscript{37–39} chronic bronchitis,\textsuperscript{40} chronic kidney disease,\textsuperscript{41,42} and chronic cardiovascular diseases.\textsuperscript{43} In fact, industrial activities with a transient outbreak and seasonal climate change often cause the air pollution index elevation and PM2.5 concentration increasing in a short period. Therefore, it is more valuable and meaningful to explore the potential influence of such a massive dosage and short-term exposure to PM2.5 on human health. In this research, we mainly elucidated the regulatory effect and mechanism of short-term with massive dosage PM2.5 exposure
on acute lung injury. Our study preliminarily confirmed that with the increase of PM2.5 exposure dosage, the proportion of cell damage and apoptosis increased significantly, and cell proliferation and survival were repressed at the same time (Figure 1).

Accumulating evidence has pointed out that multiple components of PM2.5 could regulate the activation of the NF-κB signaling pathway.44 NF-κB signaling plays a critical role in the modulation of inflammatory response. The triggering of inflammation is always rapid, accurate, and controllable, which requires the expression of inflammatory cytokines and factors accumulating to an abundant level in a short period, and quickly recovering in quiescent condition.31 In fact, in a quiescent condition, the promoter region of many inflammation-associated genes has been activated and the transcription initiation is a start-up. However, the transcription elongation is blocked by multiple repressor proteins to inhibit gene expression temporarily.45 Once the inflammation is stimulated, the activated NF-κB signaling removes the repressor proteins and switches on the suppressive state of gene expression in a very short time, which triggered the inflammatory response quickly.46,47 Our data indicate that PM2.5 could induce the production of inflammatory cytokines and ROS in a dose-dependent manner. The further detection showed that massive dosage and short-term PM2.5 exposure strongly resulted in P65 phosphorylation and nuclear translocation to activate NF-κB signaling. When NF-κB signaling was blocked by specific inhibitors, the influence of PM2.5 on inflammatory response and pulmonary epithelial cell damage was completely retrieved (Figure 2). Our findings elucidated the regulatory effect of PM2.5 on NF-κB signaling in vitro, which partially explains the reason of short-term exposure to PM2.5 can cause a strong cellular inflammatory response.

After understanding the mechanism of PM2.5 induced cell damage, it is important to find out the appropriate therapies or mitigation treatments. Specific inhibitors of NF-κB signaling have certain cytotoxicity, and systemic inflammatory response is often unstable when administered in vivo. In fact, many natural compounds have been proved to present certain functions of regulating inflammatory response and promoting tissue damage repair.48,49 TSL is a ketone compound isolated from Tussilago farfara. According to the view of traditional oriental medicine, TSL administration alleviates immune response in acute and chronic inflammatory injury.22-24 With the development of modern medicine and the application of molecular biology techniques, the detailed efficacy and mechanisms of TSL have been revealed. Some studies have suggested that TSL, as an immune repressor, inhibits NF-κB signaling and MAPK pathway, thereby reducing the inflammatory activation and M1 polarization of RAW264.7.50 In addition, TSL has a good therapeutic effect on many autoimmune diseases.49 In our study, different concentrations of TSL decelerated PM2.5-induced cell damage and inflammation. Particularly, a high dosage of TSL completely improved and rescued the functions of PM2.5 exposure on lung epithelial cells, not only reducing cell apoptosis and inhibiting inflammatory response, but also significantly limiting the activation of NF-κB signaling (Figures 3 and 4). We also used the acute lung injury model to explore the in vivo therapeutic effect of TSL on PM2.5-induced acute lung injury. Consistent with the in vitro experiment, TSL obviously attenuated lung injury degree and lung epithelial cell damage. Simultaneously, the expression of inflammatory factors in lung tissues was also decreased (Figure 6). Our findings demonstrated that TSL depressed the inflammatory response and protected lung epithelial cells from apoptosis mediated by PM2.5 poisoning both in vitro and in vivo.

Previous literature and our findings have revealed the activation of NF-κB signaling by PM2.5.10,11 And our data also supported the repression effect of TSL on NF-κB signaling. However, the modulation mechanisms of TSL affecting NF-κB signaling remain unclear. Many pathways and factors present crosstalk with NF-κB signaling, including MAPK pathway, Notch pathway, HIF-1α signaling.21 Similarly, HIF-1α signaling displays a stimulating effect on immune cell activation and inflammatory response.22,23 The activation of HIF-1α signaling aggravates acute lung injury caused by mobilizing immune cells recruitment and activation. Interestingly, it has been reported that the extract of flower bud from Tussilago farfara could decrease the expression of iNOS, TNF-α and Hif-1α in mice ischemic stroke.24 Therefore, this natural extract repressed the inflammatory reaction mediated by activated microglia, protected neuron apoptosis, and brain damage.24 On the other hand, PM2.5 promoted Hif-1α nuclear localization and further transactivation function.51 Our study also showed that PM2.5 could significantly increase HIF-1α protein expression, while TSL restored the effect of PM2.5 on HIF-1α signaling. Considering that HIF-1α is extremely unstable in normoxia conditions, it would be degraded by VHL mediated ubiquitination after interaction with PHD2. Therefore, the interaction of Hif-1α and PHD2 is crucial for the stability and function of Hif-1α. In our study, we further revealed that PM2.5 stimulation greatly blocked the binding of PHD2 and Hif-1α, thus protecting Hif-1α from degradation. However, after TSL administration, the formation of the Hif-1α/PHD2 complex was restored and the constitutive activation of Hif-1α was abolished. Furtherly, the activation of NF-κB signaling and inflammatory response was depressed by TSL in a Hif-1α dependent manner (Figure 5).

5 | CONCLUSIONS

In summary, our study elucidated the effects of massive dosage and short-term PM2.5 exposure on acute lung injury in vivo and in vitro, as well as the therapeutic function and detailed molecular mechanisms of TSL. Our findings provide new targets and strategies for the prevention and treatment of PM2.5 poisoning, and TSL is expected to become a potential natural medicine for the therapy of PM2.5.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.
AUTHOR CONTRIBUTIONS
Conceptualization: Faguang Jin. Data curation: Faguang Jin and Hongwei Lin. Formal analysis: Hongwei Lin and Yanjun Gao. Funding acquisition: Faguang Jin. Investigation: Faguang Jin. Methodology: Hongwei Lin. Project administration: Faguang Jin. Resources: Faguang Jin. Software: Hongwei Lin and Min Chen. Supervision: Faguang Jin. Validation: Min Chen, Yanjun Gao, and Zaiqiang Wang. Visualization: Faguang Jin. Writing—original draft preparation: Faguang Jin. All authors have read and agreed to the published version of the manuscript.

INSTITUTIONAL REVIEW BOARD
The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Institutional Review Board (or Ethics Committee) of the Fourth Military Medical University (No: FMMU-20200269).

DATA AVAILABILITY STATEMENT
The study data are available by email to the correspondence author.

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