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PIM1 inhibitor SMI-4a attenuated lipopolysaccharide-induced acute lung injury through suppressing macrophage inflammatory responses via modulating p65 phosphorylation

Jinxuan Wang[a,b,1], Yumeng Cao[b,1], Yuqi Liu[b], Xinyi Zhang[b], Fanceng Ji[c], Jinbao Li[a,b,*], Yun Zou[b,*]

[a] Department of Anesthesiology, Weifang Medical University, Weifang, China
[b] Department of Anesthesiology, Shanghai General Hospital, Shanghai Jiao Tong University, Shanghai, China
[c] Department of Anesthesiology, Weifang People's Hospital, Weifang, China

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ABSTRACT

PIM kinase is involved in the cellular processes of growth, differentiation and apoptosis. However, the role of PIM1 in lipopolysaccharide (LPS)-induced acute lung injury (ALI) remains largely unknown. A trend of PIM1 in the lung tissue of LPS-induced ALI at different time points was detected. Histology, wet/dry (W/D) ratio, inflammatory cells in the bronchoalveolar lavage fluid (BALF) and survival rate analyses were performed when mice received the PIM1 inhibitor SMI-4a intratracheally 3 h before LPS administration. Cytokine production *in vivo* and *in vitro* was measured after SMI-4a pretreatment. NF-κB subunit p65 expression in nuclei and phosphorylation at Ser276 in lung tissues or cells were detected by Western blot analysis. The results showed that PIM1 mRNA and protein were upregulated in the lung tissue of LPS-induced ALI. The PIM1 inhibitor SMI-4a markedly improved the survival rate after lethal LPS administration, reduced the severity of lung edema, attenuated the histologic injuries of the lung tissue and reduced the counts of infiltrated inflammatory cells in the BALF. The PIM1 inhibitor SMI-4a suppressed the production of cytokines in LPS-treated RAW264.7 cell supernatants and BALF. Furthermore, LPS administration upregulated the levels of nuclear p65 and phosphorylated p65 (p-p65) at Ser276, whereas pretreatment with the PIM1 inhibitor SMI-4a reduced p65 upregulation in the nucleus and p-p65 at Ser276. Taken together, these data indicate that the PIM1 inhibitor SMI-4a may serve as a promising therapeutic strategy for LPS-induced ALI by suppressing macrophage production of cytokines via a reduction of p65 activities.

1. Introduction

Acute lung injury/acute respiratory distress syndrome (ALI/ARDS) is a complex and devastating illness with a high mortality rate, which has been described pathologically, as diffuse alveolar damage and infiltration of inflammatory cells into lung tissues [1,2]. The response to intense inflammation is characterized by an accumulation of inflammatory cytokines released from macrophages and neutrophils due to various risk factors, such as sepsis, microbial infection, trauma or ischemia/reperfusion injury [3,4].

Alveolar macrophages (AMs), located at the air-tissue interface, are highly plastic immune response cells. They play a pivotal role in the pathogenesis of inflammation, and subsequently promote neutrophil infiltration [5]. AMs are sentinel cells with relatively high levels of pathogen-related and risk-related molecular model receptors [6], which initiate lung inflammation upon activation by live bacteria, endotoxins or ischemia/reperfusion stimulation [7–9]. It is worth noting that a complex network of cytokines is involved in the inflammatory cascade, which contributes to severe lung damage. For example, TNF-α, IL-1β and IL-6 are the most biologically potent cytokines secreted from activated macrophages during the early stages of ARDS [10]. More importantly, the levels of TNF-α, IL-1β and IL-6 are elevated in bronchoalveolar lavage fluid (BALF) during ARDS and have been associated with disease-associated mortality during a specified period of time [11].

PIM1 is a well-characterized serine/threonine kinase that participates in many cellular processes, including proliferation,

* Corresponding authors at: Department of Anesthesiology, Shanghai General Hospital, Shanghai Jiao Tong University, Shanghai 20080, China.
E-mail addresses: lijinbaoshanghai@163.com (J. Li), zouyun20101211@163.com (Y. Zou).
[1] Jinxuan Wang and Yumeng Cao contributed equally to this work.

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differentiation, apoptosis, *etc.* [12]. Although PIM1 was first reported in hematologic neoplasms as a proto-oncogene, it has also been found that PIM1 kinase is involved in inflammation-related signal transduction pathways [13]. For example, PIM1 inhibition exerted protective roles during the development of airway hyperresponsiveness and airway inflammation in both allergen-sensitized and allergen-challenged mice [14]. Furthermore, a previous study reported that the PIM1 kinase played a critical role in regulating pro-inflammatory mediators in fetal membranes [15]. Therefore, interactions between PIM1 and different proteins and signaling pathways make it a latent anti-inflammatory target.

Based on these interesting findings, we hypothesized that PIM1 may be involved in the pathophysiological development of lipopolysaccharide (LPS)-induced ALI. The purpose of this study was to investigate the effects of PIM1 kinase on LPS-induced ALI and to explore the potential mechanisms both *in vitro* and *in vivo*.

2. Materials and methods

2.1. Animals and reagents

All animal experiments were approved by the Animal Use Committee of Shanghai General Hospital. Briefly, adult C57BL/6 male mice (6–8 weeks old) were obtained from the Animal Experiment Center of the Second Military Medical University. All mice were housed in individually ventilated cages with specific pathogen-free conditions and a standard laboratory diet. PIM1 specific inhibitor SMI-4a (CAS:438190-29-5, purity 100%, molecular formula, $C_{11}H_6F_3NO_2S$) was purchased from Selleck Corporation. SMI-4a was dissolved in vegetable oil for the mouse treatment and dimethyl sulfoxide (DMSO) for cell culture.

2.2. Experimental design

The mouse model of acute lung injury was set up by intratracheal administration of LPS (20 μg in 50 μl PBS). Lung tissue was harvested at different time points (6 h, 12 h, and 24 h) for PIM1 mRNA and protein (34 kDa) detection. Then, the mice were randomly divided into three groups: the sham group, the LPS group and the LPS + SMI-4a group. SMI-4a (60 mg/kg) that was dissolved in vegetable oil was administered by oral gavage 3 h before LPS (Sigma–Aldrich Corporation, St. Louis, MO, USA) treatment. As described previously [16], LPS (20 μg in 50 μl PBS) was intratracheally administered to the mice following anesthetization with sevoflurane. Vegetable oil or phosphate-buffered saline (PBS) was administered in the other two groups as controls. After LPS administration, the mice were euthanized by CO_2 inhalation at scheduled time intervals. BALF and lung tissue were harvested and preserved at −80 °C until use. For the survival experiments, a lethal dose of LPS (1 mg in 50 μl PBS) was given to the mice, and their survival rate was recorded every 12 h for 72 h.

2.3. Histology evaluation

Six hours after LPS administration, the thoracic cavities of the mice were opened under deep anesthesia, and the mice were perfused through the ascending aorta using 50 ml 0.01 M PBS (pH = 7.4), followed by 4% paraformaldehyde in 0.1 M PBS (pH = 7.4). Subsequently, the right upper lobes were fixed in 4% paraformaldehyde, dehydrated with ethanol, and then embedded in paraffin. The lung tissue was cut into 4–5 μm thick sections and subjected to hematoxylin and eosin (H&E) staining. The pathological changes among the three groups were examined under an optical microscope and blindly scored by two experienced pathologists as described previously [17].

2.4. Wet-to-dry weight ratio of lung tissues

The mice were euthanized by CO_2 inhalation 6 h after LPS challenge. After euthanization, right lung tissue was collected, and the "wet" weight was recorded. Subsequently, the lung tissue was covered by silver papers and placed in an incubator at 80 °C until a stable weight, which we recorded as the "dry" weight. Finally, the wet-to-dry ratio (W/D ratio) of lung tissue was calculated to assess the degree of pulmonary edema.

2.5. Inflammatory cell counts of bronchoalveolar lavage fluid

As described previously [18], the mice for bronchoalveolar lavage fluid (BALF) collection were euthanized by CO_2 inhalation. After that, a middle incision was made to open the chest cavity and expose the trachea. The BALF was harvested by washing the pulmonary alveoli twice with 0.5 ml PBS via a tracheal cannula. Inflammatory cells (including total alive cells, $Ly6G^+$ neutrophils, and $F4/80^+$ macrophages) were calculated by a flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) and analyzed using Flowjo software.

2.6. Cell culture and treatment

In the *in vitro* study, the effects of SMI-4a on cytokine production in RAW264.7 cells were examined. First, RAW264.7 cells were cultured in DMEM medium supplemented with 10% FBS, 0.5% penicillin and 0.5% streptomycin. Subsequently, the cells were pretreated with SMI-4a (20, 40, and 80 μM) or an equivalent volume of DMSO (Sigma-Aldrich Corporation, St. Louis, MO, USA) 1 h before LPS (100 ng/ml) stimulation. Twelve hours after LPS stimulation, the supernatants were collected and preserved until use. Then, the RAW264.7 cells were pretreated with SMI-4a (80 μM) or equivalent DMSO for 1 h and stimulated with LPS (100 ng/ml) at different time intervals (6, 12 and 24 h). The supernatants were collected at scheduled time points and preserved until use.

2.7. Measurement of cytokine levels in both in vivo and in vitro supernatants

In this study, BALF was obtained as described previously [18]. The supernatants from BALF were obtained after the BALF was centrifuged at 2000 rpm for 5 min at 4 °C. Eventually, the BALF supernatants and the RAW264.7 cells that were mentioned above were used to measure cytokines, including TNF-α, IL-1β and IL-6, using murine enzyme-linked immunosorbent assay (ELISA) kits (R&D Corporation) according to the manufacturer's instructions.

2.8. Quantitative PCR assay

Total RNA was extracted from lung tissue at different time intervals after LPS administration using a commercial kit (miRNeasy Mini Kit, Qiagen). cDNA was synthesized from total RNA by reverse transcriptase (Takara) after evaluating the RNA quality via the A260/A280 ratio. Each sample was prepared in triplicate for a total reaction volume of 20 μl, with 250 nM forward and reverse primers, 10 μl SYBR Green (Takara, Japan), ROX Reference Dye II and 20 ng cDNA. All reactions were carried out in a QuantStudio 3 Real-Time PCR system. GAPDH was used as an internal control to normalize the variability in expression levels. The quantitative PCR primers were as follows: PIM1 forward, GCTCGGTCTACTCTGGCATC and reverse, CCGAGCTCACCTTCT TCAAC; GAPDH forward, AACTTTGGCATTGTGGAAGG and reverse, GGATGCAGGGATGATGTTCT.

2.9. Western blot analysis

The extraction of nuclear and total protein from lung tissue or RAW264.7 cells was performed using nuclear and total protein

extraction reagents, respectively [19,20]. Protein concentrations of the lysed lung tissue or the cells were determined using a BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA). Equal amounts of protein were loaded in each well and separated with 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The separated proteins were transferred onto a polyvinylidene difluoride membrane (PVDF). After blocking in 5% nonfat milk, the membranes were probed with specific primary antibodies, including anti-NF-κB-p65 (1:1000, CST), anti-phospho-NF-κB-p65 at Ser276 (1:1000, Affinity), anti-PIM1 (1:1000, CST), anti-α-tubulin (1:2000, CST), anti-GAPDH (1:2000, Affinity) and anti-H3 (1:2000, Bioss) at 4 °C overnight under gentle agitation. Subsequently, the membranes were incubated with the corresponding secondary antibodies for 1 h at room temperature. Protein bands were detected with an enhanced chemiluminescence (ECL) Western blot kit and visualized by a ChemiDoc XRS System with Image Lab software (Bio-Rad). The intensity of the blots was quantified via densitometry using Image Lab software (Bio-Rad). Target protein expression was normalized to the corresponding internal reference signal.

2.10. Statistical analysis

All data are expressed as the mean ± standard deviation (SD). Statistical analysis was performed using SPSS Statistics version 16.0 Program (SPSS Inc., USA). Graphs were plotted by Prism 5.0 (GraphPad Software, San Diego, CA). Kaplan-Meier plots and log-rank tests were used to analyze the survival data. The differences among the groups were compared by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. PIM1 was upregulated in the lung tissue of LPS-induced ALI mice

Lung tissue was harvested after LPS challenge at different time points (6, 12 and 24 h) for PIM1 mRNA and protein level measurement. As shown in Fig. 1A, the mRNA expression of PIM1 increased dramatically and reached its peak at 6 h after LPS administration. Then, the mRNA of PIM1 decreased gradually. In accordance with PIM1 mRNA expression, the protein level of PIM1 was significantly upregulated when compared with the Sham group (Fig. 1B and C). These findings suggest that PIM1 may be involved in the development of LPS-induced ALI.

3.2. PIM1 inhibitor SMI-4a ameliorated lung damage induced by LPS challenge

In this study, we used the PIM1-specific inhibitor SMI-4a to investigate whether PIM1 inhibition could exert a protective effect on LPS-induced ALI. As shown in Fig. 2A, the structure of alveoli was diminished at 6 h after LPS administration. Intragastric pretreatment with SMI-4a significantly suppressed the thickness of alveolar walls. Compared with the Sham group, LPS administration upregulated the W/D ratio, which reflects pulmonary edema, whereas the PIM1 inhibitor SMI-4a reduced the W/D ratio. This phenomenon represents a protective role of SMI-4a (Fig. 2C). Furthermore, the survival rate of mice that received the lethal dose of LPS was 20%; however, in the LPS + SMI-4a group, the survival rate reached 60% (Fig. 2D). These findings suggest that SMI-4a plays a positive protective role in lethal LPS-induced lung injury.

3.3. SMI-4a reduced the infiltration of activated inflammatory cells in the BALF

To investigate the protective roles of SMI-4a during LPS-induced acute lung injury, we calculated the inflammatory cells in the BALF, which contribute to the damage of lung tissue. As Fig. 3A–C shows, the counts of total live cells, $Ly6G^+$ neutrophils and $F4/80^+$ macrophages were significantly increased after LPS administration when compared with the Sham group. However, pretreatment with oral SMI-4a reduced the counts of the inflammatory cells that infiltrated in the BALF compared with the LPS group.

3.4. SMI-4a inhibited inflammatory cytokine production in the BALF of ALI mice

The levels of inflammatory cytokines, including TNF-α, IL-6 and IL-1β, were used as parameters to assess the severity of LPS-induced ALI. Notably, the levels of the cytokines (TNF-α, IL-6 and IL-1β) were significantly increased in the LPS group compared to the Sham group. In addition, SMI-4a pretreatment attenuated the production of these cytokines. This anti-inflammatory phenomenon indicates a protective role for the PIM1 inhibitor SMI-4a (Fig. 4).

3.5. SMI-4a suppressed LPS-induced cytokine production in vitro

To explore whether PIM1 inhibition could affect inflammatory responses, RAW264.7 cells were cultured and treated with SMI-4a *in vitro*. First, RAW264.7 cells were pretreated with different doses of SMI-4a 1 h before LPS (100 ng/ml) stimulation. As shown in Fig. 5A–B, SMI-4a pretreatment remarkably suppressed the production of TNF-α and IL-6 in a dose-dependent manner. However, SMI-4a exerted little effect on IL-1β secretion from macrophages (Fig. 5C). In addition, RAW264.7 cells were stimulated with LPS at different time intervals after SMI-4a pretreatment. In accordance with our results demonstrated before, the levels of the inflammatory cytokines TNF-α and IL-6 were significantly

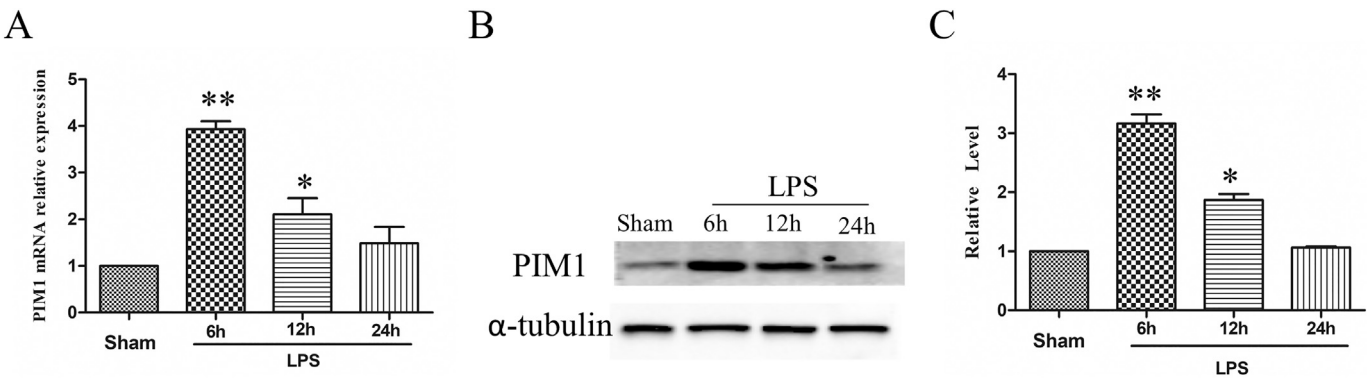

Fig. 1. PIM1 was upregulated in the lung tissue of ALI mice. Lung tissue was harvested at different time points to detect the mRNA and protein level of PIM1. (A). PIM1 mRNA expression in lung tissue at different time points after LPS-induced acute lung injury. (B). PIM1 protein (34 kDa) expression in lung tissue at different time points after LPS-induced acute lung injury. $^*P < 0.05$ *vs.* Sham group, $^{**}P < 0.01$ *vs.* Sham group. All experiments were repeated three times.

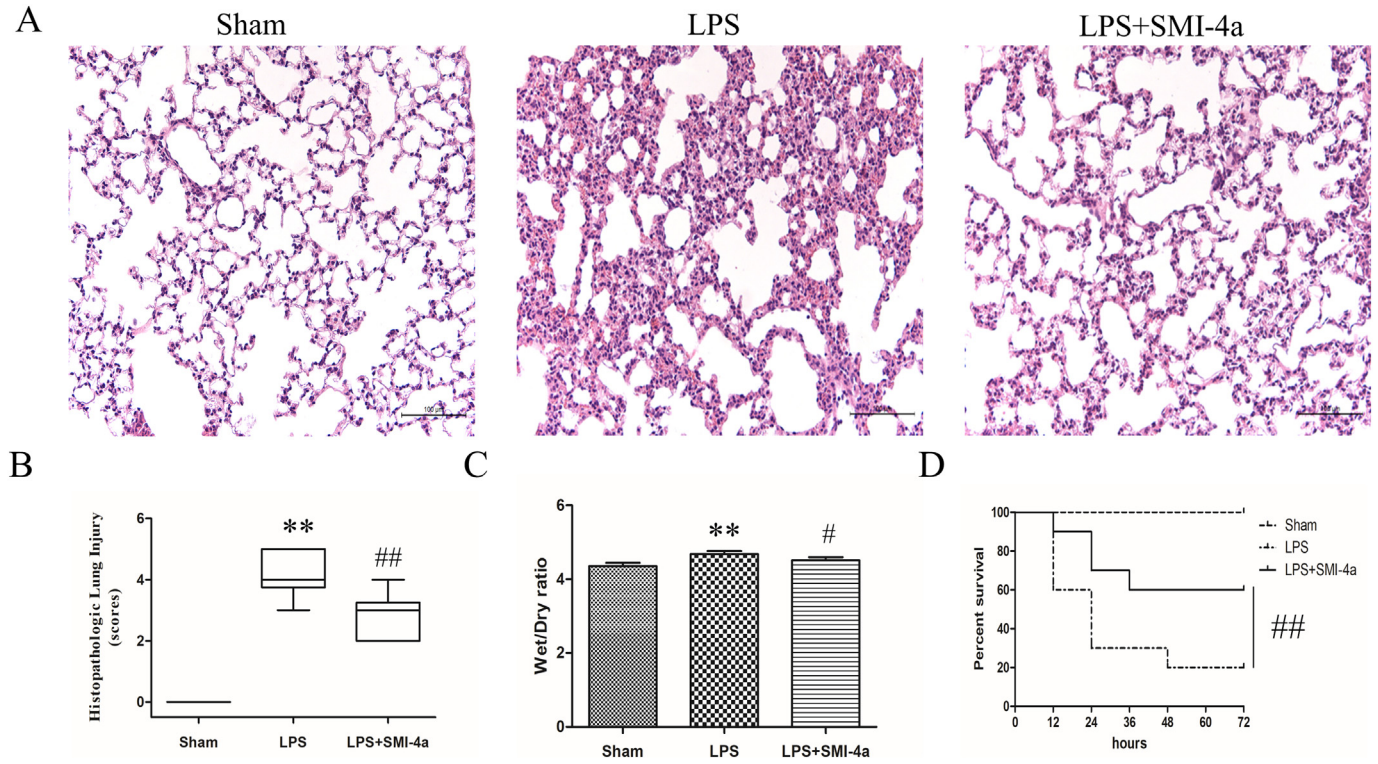

Fig. 2. PIM1 inhibitor SMI-4a ameliorated lung damage induced by LPS challenge. (A). Representative images of pulmonary H&E-stained cross sections from scheduled groups 6 h after LPS administration (×100). (B). Histopathologic scores that reflect effects of SMI-4a on the severity of lung injuries 6 h after LPS challenge. (C). The effects of SMI-4a on W/D ratio. (D). SMI-4a pretreatment improved the survival rate within 72 h after lethal LPS administration. n = 10. $^{**}P < 0.01$ *vs.* Sham group, $^{\#}P < 0.05$ *vs.* LPS group, $^{\#\#}P < 0.01$ *vs.* LPS group.

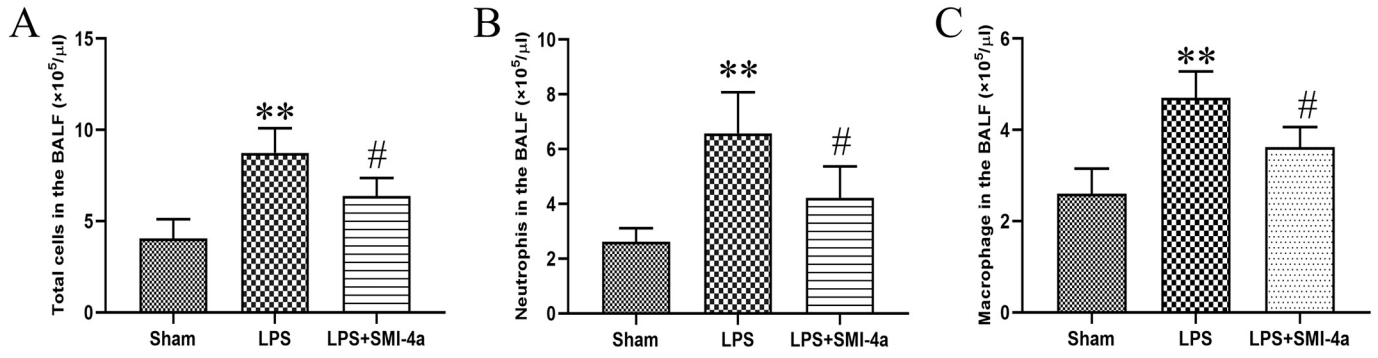

Fig. 3. SMI-4a reduced the infiltration of activated inflammatory cells in the BALF of ALI mice. BALF was harvested 6 h after intratracheal LPS administration, then the counts of inflammatory cells were calculated by flow cytometry. (A). SMI-4a reduced the counts of total activated inflammatory cells in BALF. (B). SMI-4a decreased the number of $Ly6G^+$ neutrophils that infiltrated BALF. (C). SMI-4a attenuated the counts of infiltrated $F4/80^+$ macrophages. $^{**}P < 0.01$ *vs.* Sham group. $^{\#}P < 0.01$ *vs.* LPS group.

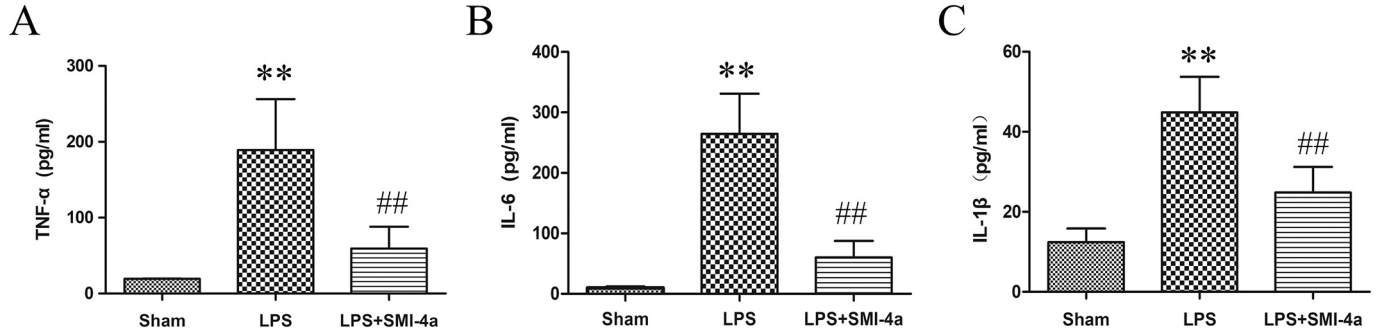

Fig. 4. SMI-4a inhibited inflammatory cytokine production in the BALF of ALI mice. BALF was collected 6 h after intratracheal LPS administration. The levels of cytokines in the supernatants of the BALF were detected by ELISA. (A–C). SMI-4a suppressed proinflammatory cytokine production, including TNF-α, IL-6 and IL-1β. $^{**}P < 0.01$ *vs.* Sham group. $^{\#\#}P < 0.01$ *vs.* LPS group.

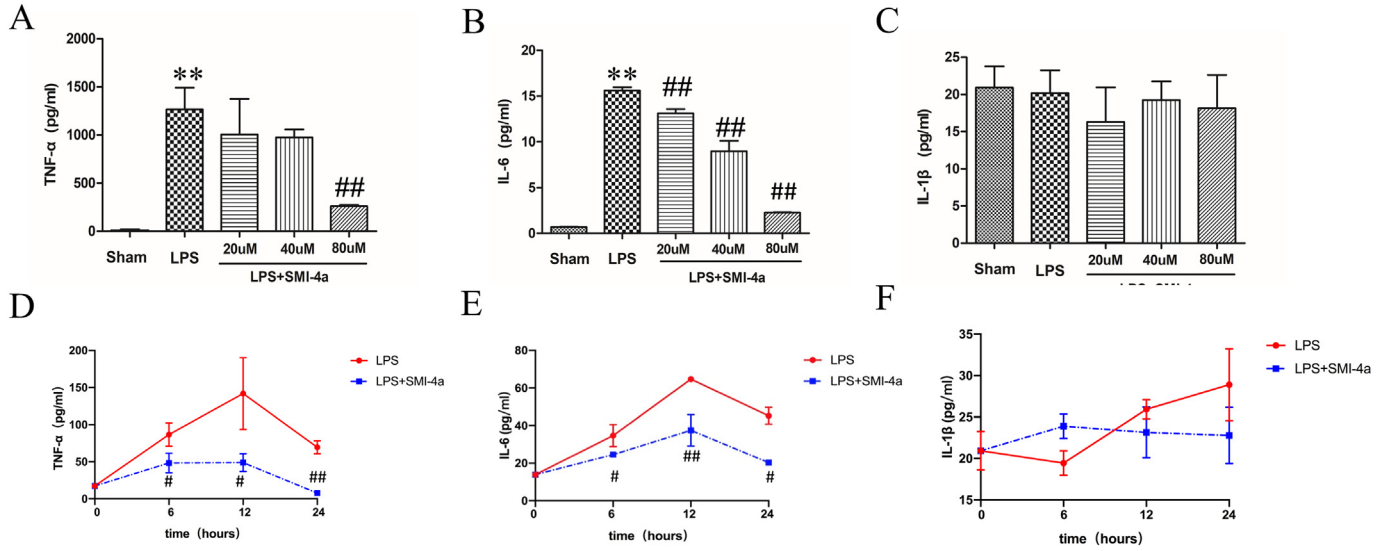

Fig. 5. SMI-4a suppressed LPS-induced cytokine production *in vitro*. RAW264.7 cells were cocultured with different doses of SMI-4a (20, 40, and 80 μM) for 1 h before LPS (100 ng/ml) stimulation. The supernatants were collected for ELISA. (A–B). Different doses of SMI-4a suppressed pro-inflammatory production of cytokines, including TNF-α and IL-6. (C). SMI-4a had little effect on IL-1β production. RAW264.7 cells were cocultured with SMI-4a (80 μM) for 1 h before LPS (100 ng/ml) stimulation. (D–E). The inhibitory effects of SMI-4a on cytokine production at different time points, including TNF-α and IL-6. (F). SMI-4a had no effect on IL-1β production. $^{**}P < 0.01$ *vs.* Sham group, $^{\#}P < 0.05$ *vs.* LPS group, $^{\#\#}P < 0.01$ *vs.* LPS group.

increased and reached their peak 12 h after LPS stimulation (Fig. 5D–E). Furthermore, it was noted that SMI-4a pretreatment reduced the levels of TNF-α and IL-6 at different time points. IL-1β production exhibited little relationship with LPS stimulation and SMI-4a treatment (Fig. 5F).

3.6. SMI-4a attenuated NF-κB activation by inhibiting p65 phosphorylation at Ser276 and impending p65 nuclear translocation

In this study, we firstly checked whether SMI-4a could inhibit the protein of PIM1. As shown in Fig. 6A, D, G and J, SMI-4a could effectively suppress the upregulation of PIM1 protein induced by LPS *in vivo* and *in vitro*. The NF-κB signaling pathway controls the expression of multiple proteins involved in the regulation of cell survival and immune response [21]. Phosphorylation of p65 at Ser276 could be indispensable due to its ability to function as an activator of gene expression [22]. As revealed in Fig. 6B and E, after LPS administration, the level of p65 in the nucleus of lung tissue significantly increased, while oral SMI-4a pretreatment reduced the trend of p65 in the nucleus. In accordance with the trend of p65 in the nucleus, the level of phosphorylated p65 at Ser276 was significantly increased in the lung tissue of the LPS-treated group, and SMI-4a administration downregulated the level of p-p65 at Ser276 (Fig. 6C&F). In the *in vitro* study of RAW264.7 cells (Fig. 6H, I, K, and L), we found a similar phenomenon, where LPS stimulation upregulated p65 in the nucleus and p-p65 at Ser276, while SMI-4a reduced this LPS challenge-induced increase.

4. Discussion

Acute lung injury/acute respiratory distress syndrome (ALI/ARDS) represents a main manifestation of pulmonary dysfunction and is seen in pulmonary inflammation or systemic inflammation in lung tissue among critically ill patients. Pathogen clearance, pro-inflammatory signaling inactivation, activated cell removal and transition to anti-inflammatory subtypes are the coordinated processes that can ameliorate lung inflammation [23–25]. Macrophages, which display remarkable heterogeneity, play an essential role during the inflammatory response process in ALI/ARDS [26,27]. In the present study, we demonstrated that SMI-4a, a PIM1 kinase-specific inhibitor, improved lung tissue damage induced by LPS challenge through suppression of p65 nuclear translocation and phosphorylation at Ser276 mediated macrophage activation.

As a serine/threonine kinase, PIM1 is focused on the roles of invasions of different types of tumors [28]. Interestingly, the association between PIM1 and inflammatory mediated diseases was reported recently [29–31]. In our study, we found that PIM1 mRNA and protein in the lung tissue were increased significantly after intratracheal LPS administration, which suggests that PIM1 may be involved in the development of LPS-induced ALI. Furthermore, pretreatment with the PIM1 specific inhibitor SMI-4a improved the outcomes of lung injuries induced by LPS challenge.

Based on these findings, we proposed that PIM1 displays a protective role in LPS-induced ALI. In our study, we found that pretreatment with oral SMI-4a suppressed the level of TNF-α, IL-6 and IL-1β in BALF compared with the LPS group. This suggests that PIM1 inhibition can suppress production of the pro-inflammatory cytokines. Our result was in accordance with a previous study, where PIM1 inhibition decreased the pro-inflammatory cytokine (IL-1β, IL-6, etc.) production in the placenta when stimulated with LPS, TNF-α and IL-1β [32]. However, there is still controversy regarding the role of PIM1 in an inflammatory response. Nawijn MC et al. have shown that inhibition of the PIM1 kinase increased house dust mite (HDM)-induced proinflammatory activity as measured by IL-1α secretion [33]. To clarify the inflammatory roles of PIM1, we investigated cytokine production *in vitro*. In parallel with the *in vivo* study, we found that SMI-4a reduced the levels of proinflammatory cytokines TNF-α and IL-6 in LPS-stimulated RAW264.7 cells. Interestingly, in the *in vitro* study, SMI-4a had little effect on IL-1β production from macrophages, whereas we found that oral SMI-4a pretreatment attenuated the IL-1β level in BALF *in vivo*. These findings suggest that there are some intricate pathways involved in inflammatory and compensatory mechanisms that are activated independently of PIM1 under inflammatory stimuli *in vivo*.

NF-κB is an inducible transcription factor that controls the expression levels of multiple proteins involved in the modulation of cell survival and the immune response [34,35]. Originally, Ser536 phosphorylation of p65 was thought to enhance its import into the nucleus and be required for the expression of a subset of NF-κB target genes, which can be elicited by cytokines and growth factors. According to our previous study, the level of p-p65 at Ser536 in lung tissue was significantly increased after intratracheal LPS administration [36], but we found that SMI-4a had little effect on the level of Ser536 phosphorylation of p65 in

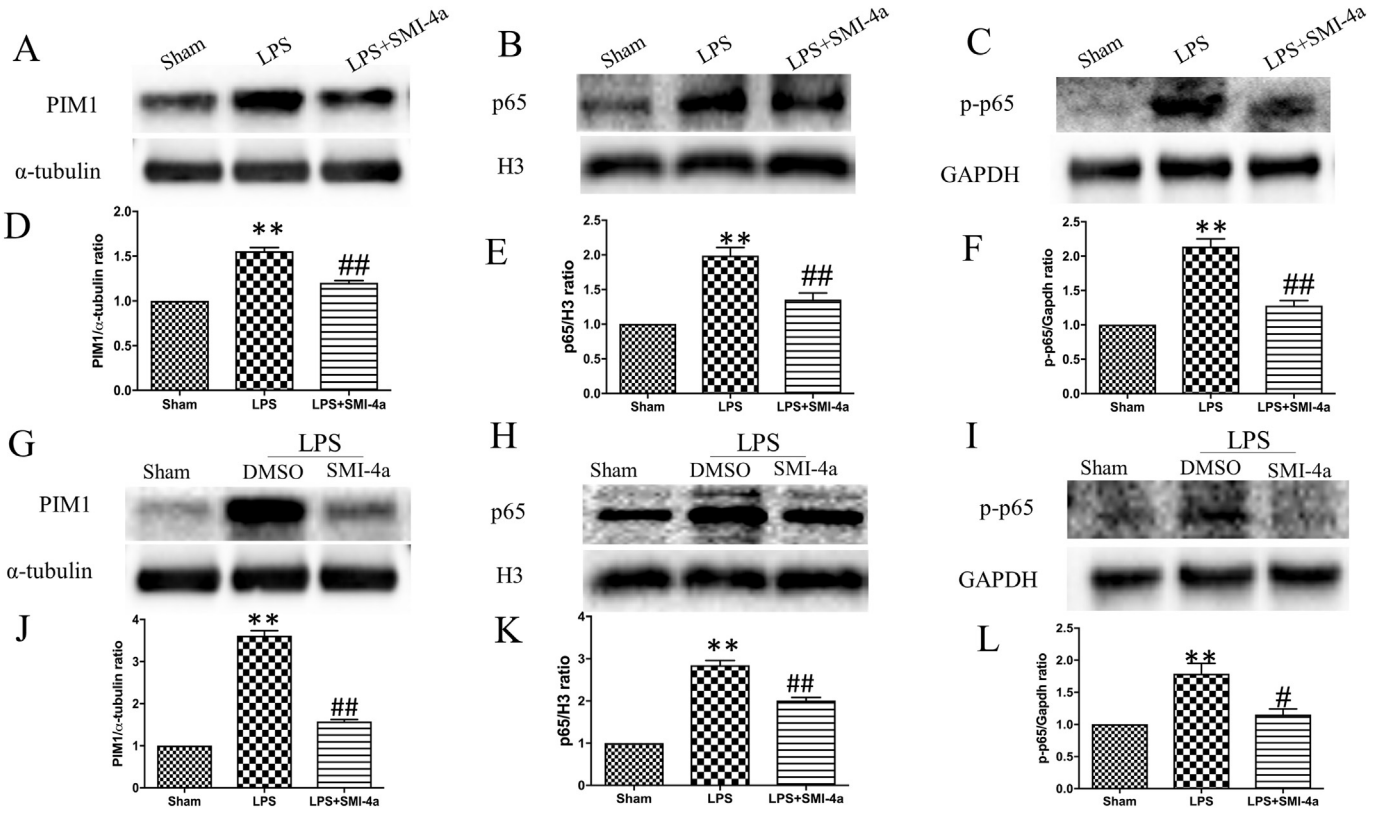

Fig. 6. SMI-4a attenuated NF-κB activation by inhibiting p65 phosphorylation at Ser276 and impending p65 nuclear translocation. A–F: the lung tissue from scheduled groups were collected and detected. (A, D). SMI-4a inhibited the upregulation of PIM1 induced by LPS administration. (B, E). SMI-4a reduced the nucleus p65 expression when compared with the LPS group. (C, F). SMI-4a reduced p65 phosphorylation at Ser276, which was induced by LPS administration. G–L: The proteins of treated cell lines were collected and detected. (G, J). SMI-4a reduced PIM1 protein expression after LPS stimulation in RAW264.7 cells. (H, K). The level of p65 in the nucleus was increased after LPS stimulation, and SMI-4a precultured with RAW264.7 cell lines reduced the nucleus p65 expression when compared with the LPS group. (I, L). SMI-4a reduced p-p65 at Ser276, which was induced by LPS stimulation *in vitro*. $^{**}P < 0.01$ *vs.* Sham group. $^{\#}P < 0.05$ *vs.* LPS group, $^{\#\#}P < 0.01$ *vs.* LPS group.

lung tissue compared with the LPS group (data not shown). Given that Ser276 phosphorylation is critical for the transcriptional activity of p65 and that PIM1 is thought to be responsible for the phosphorylation of p65 at Ser276 [22], we detected roles for SMI-4a in p65 translocation as well as phosphorylation at Ser276 in the lung tissue or cell lines. As expected, LPS administration upregulated expression of p65 in the nucleus while SMI-4a treatment reduced p65 level in the nucleus. Importantly, SMI-4a pretreatment mitigated the level of p-p65 at Ser276, which was induced by LPS administration. These findings may account for decreased production of cytokines.

In conclusion, we focused on the activation of macrophages and the production of cytokines in an LPS-induced acute lung injury model. PIM1 inhibition remarkably suppressed the release of inflammatory factors through the inhibition of p65 phosphorylation at Ser276. The findings from our experiments reveal that the inhibition of PIM1 expression is of great significance in reducing lung injury.

Declaration of Competing Interest

The authors declare that there are no conflicts of interests regarding the publication of this paper.

Acknowledgments

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