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Pirfenidone ameliorates lipopolysaccharide-induced pulmonary inflammation and fibrosis by blocking NLRP3 inflammasome activation

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ABSTRACT

Acute respiratory distress syndrome(ARDS)is a severe clinical disorder characterized by its acute onset, diffuse alveolar damage, intractable hypoxemia, and non-cardiogenic pulmonary edema.

Acute lung injury(ALI) can trigger persistent lung inflammation and fibrosis through activation of the NLRP3 inflammasome and subsequent secretion of mature IL-1β, suggesting that the NLRP3 inflammasome is a potential therapeutic target for ALI, for which new therapeutic approaches are needed. Our present study aims to assess whether pirfenidone,with anti-fibrotic and anti-inflammatory properties, can improve LPS-induced inflammation and fibrosis by inhibiting NLRP3 inflammasome activation. Male C57BL/6 J mice were intratracheally injected with LPS to induce ALI. Mice were administered pirfenidone by oral gavage throughout the entire experimental course. The mouse macrophage cell line (J774 A.1) was incubated with LPS and ATP, with or without PFD pre-treatment. We demonstrated that PFD remarkably ameliorated LPS-induced pulmonary inflammation and fibrosis and reduced IL-1β and TGF-β1 levels in bronchoalveolar lavage fluid(BALF). Pirfenidone substantially reduced NLRP3 and ASC expression and inhibited caspase-1 activation and IL-1β maturation in lung tissues. In vitro, the experiments revealed that PFD significantly suppressed LPS/ATP-induced production of reactive oxygen species (ROS) and decreased caspase-1 activation and the level of IL-1β in J774 A.1 cells. Taken together, the administration of PFD reduced LPS-induced lung inflammation and fibrosis by blocking NLRP3 inflammasome activation and subsequent IL-1β secretion. These findings indicated that PFD can down-regulate NLRP3 inflammasome activation and that it may offer a promising therapeutic approach for ARDS patients.

1. Introduction

Acute respiratory distress syndrome (ARDS), the most serious manifestation of ALI, is typically described as a destructive clinical syndrome characterized by acute hypoxemia, severe respiratory distress and non-cardiogenic pulmonary edema (Ranieri et al., 2012). Despite significant advances in therapy, particularly conservative fluid management and lung protective strategies during mechanical ventilation, the hospital mortality of ARDS remains as high as 40% based on an international, multicentre, prospective cohort study (Bellani et al., 2016). Most ARDS patients survive the acute inflammation phase but inevitably succumb to death, often with evidence of remarkable pulmonary fibrosis (Meduri et al., 1995). Pulmonary fibrosis was observed in 53% of ventilated patients who had ARDS for five days (Papazian et al., 2007), and their mortality rate was 57% compared with 0% in patients without pulmonary fibrosis (Martin et al., 1995). Progressive pulmonary fibrosis also correlates with the restrictive pulmonary physiology and diminished quality of life in ARDS survivors (Burnham et al., 2014a). Currently, there is no effective therapy to prevent or attenuate lung inflammation and pathological fibroproliferation in ARDS; therefore, new advances in elucidating the pathophysiological mechanism and discovery of new therapeutic targets for this

Abbreviations: LPS, lipopolysaccharide; ALI, acute lung injury; ARDS, acute respiratory distress syndrome; NLRP3, nucleotide-binding oligomerization domain-like receptor with pyrin domain 3; ASC, apoptosis-associated speck-like protein containing a caspase-recruiting domain; PFD, pirfenidone; NAC, N-acetylcysteine; ROS, reactive oxygen species

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devastating disease are in urgent need.

Inflammasomes are innate immune system sensors that recognize a variety of stimuli that include pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs); inflammasomes promote the maturation of caspase-1 and the release of important pro-inflammatory cytokines, including interleukin-1β(IL-1β) and IL-18 (Guo et al., 2015). One of the most important inflammasomes is the NLRP3 inflammasome, which has been implicated in many inflammatory diseases; the NLRP3 inflammasome is composed of nucleotide-binding oligomerization domain-like receptor with pyrin domain 3(NLRP3), apoptosis-associated speck-like protein containing a caspase-recruiting domain(ASC) and caspase-1 domains (Netea et al., 2015). Recently, emerging evidence indicates that the NLRP3 inflammasome plays crucial roles in various lung diseases, including chronic obstructive pulmonary disease(COPD),asthma,fibrotic lung disease,cystic fibrosis,ALI and ARDS (De Nardo et al., 2014; Lee et al., 2016). Various studies suggested that reactive oxygen species (ROS) are key factors contributing to the activation of the NLRP3 inflammasome, which in turn activates caspase-1 and leads to the cleavage of the IL-1β and IL-18 precursors, producing biologically active cytokines (Heid et al., 2013; Tang et al., 2017; Xie et al., 2016). Lipopolysaccharide, a potent inducer of acute lung injury, can significantly increase intracellular ROS generation, which can injure pulmonary parenchyma cells and activate the NLRP3 inflammasome, leading to secretion of active IL-1β (Liu et al., 2016a; b; Netea et al., 2015; Yin et al., 2016; Yu et al., 2017). Using the LPS-induced model of ALI, NLRP3$^{-/-}$or caspase-1$^{-/-}$mice demonstrated reduced pulmonary inflammation and IL-1β levels in BALF, compared with wild-type mice (Grailer et al., 2014). Additionally, there is growing evidence that IL-1β plays key roles in acute inflammation injury, tissue remodelling, and fibrosis in lung diseases. IL-1β, a pleiotropic cytokine product of the inflammasome, promotes the production of transforming growth factor β1 (TGF-β1),which is a prototypical pro-fibrotic mediator of the development of pulmonary fibrosis via stimulating fibroblast proliferation and differentiation, inducing the epithelial mesenchymal transition and inducing collagen production (Wynn, 2011; Xu et al., 2012). Treatment of mice with recombinant IL-1β induced significant pulmonary inflammatory injury and collagen deposition (Gasse et al., 2007). In lung fibrosis models induced by bleomycin, silica and asbestos,NLRP3$^{-/-}$,ASC$^{-/-}$ and caspase-1$^{-/-}$ mice had decreased secretion of IL-1β and abrogated pulmonary inflammation and fibrosis (Dostert et al., 2008; Gasse et al., 2009a). All the evidence mentioned above suggest that the NLRP3 inflammasome is an important regulator of lung inflammation and fibrosis.

The activation of the NLRP3 inflammasome in various immune cells, including alveolar macrophages(AMs), plays an important role in ALI and pulmonary fibrosis (Dos et al., 2012). Under balanced-state conditions, AMs predominate the leukocyte population in the alveolar spaces, accounting for more than 90% of the total cell population. In the animal model of ALI, depletion of AMs down-regulatedthe expression of IL-1β in BALF by 70% (Grailer et al., 2014) and ameliorated alveolar barrier dysfunction and inflammatory injury (Frank et al., 2006). Furthermore, conditional depletion of macrophages at early stages of fibrosis inhibited fibrosis progression in a fibrosis model (Murray et al., 2011; Wynn and Barron, 2010). Research suggests that ARDS patients who had a large number of fibroblasts in bronchoalveolar lavage fluid (BALF) had an increased collagen-1 concentration and a high proportion of AMs(Quesnel et al., 2010). AMs produce profibrotic mediators such as TGF-β1,platelet-derived growth factor, extracellular matrix (ECM) proteins and metalloproteases, which directly cause abnormal lung repair and lung fibrosis (Kolahian et al., 2016).

Pirfenidone is an FDA-approved oral anti-fibrotic drug for the treatment of idiopathic pulmonary fibrosis(IPF) and for its improvement in lung function, progression-free survival, exercise tolerance and its effect in shortening the course for pulmonary diseases (King et al., 2014). Pirfenidone attenuates fibroblast proliferation and fibroblast differentiation into myofibroblasts and inhibits the expression of fibrogenic mediators, such as TGF-β, platelet-derived growth factor (PDGF)and IL-1β (Conte et al., 2014; Schaefer et al., 2011).Furthermore, pirfenidone can inhibit the production of ROS and pro-inflammatory cytokines (Liu et al., 2017a; Liu et al., 2017b; Pourgholamhossein et al., 2018). An earlier study showed that pirfenidone could improve hypertension-induced myocardial fibrosis via inhibiting NLRP3 activation-induced inflammation and fibrosis (Wang et al., 2013). Recent research (Li et al., 2018) has proved that pirfenidone can directly inhibit silica-induced NLRP3 inflammasome activation in a human bronchial epithelial cell line (16HBE). Additionally, pirfenidone could ameliorate oleic acid-induced acute lung injury and hypoxemia (Mei et al., 2005). However, the therapeutic effects of pirfenidone and its underlying mechanisms in LPS-induced acute lung injury and subsequent fibrosis remain unclear.

In the current study, we explored the protective effects of pirfenidone and its possible mechanism in LPS-induced pulmonary inflammation and fibrosis. We demonstrated that pirfenidone could attenuate LPS-induced acute lung injury and subsequent fibrosis by suppressing NLRP3 inflammasome activation.

2. Materials and methods

2.1. Animals

Male C57BL/6 mice(6–8 weeks old, 20–25 g)were obtained from the Experimental Animal Center of Central South University (Changsha, China). All mice were housed in a specific pathogen-free(SPF)animal facility with water and food ad libitum and were maintained in a 12-hour light,12-hour dark cycle. All mouse experiments were approved by the Animal Care and Use Committee of the Central South University and were consistent with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

2.2. Induction of LPS-induced pulmonary fibrosis and treatment

Male C57BL/6 mice were randomly assigned to five groups: 1) control group(control,n = 20); 2)pirfenidone group(PFD, n = 20); 3) LPS group(LPS,n = 20); 4)LPS + pirfenidone group (LPS + PFD,n = 20), and 5)LPS + caspase-1 inhibitor (LPS + Cp1 inhibitor, n = 20). The mice were anaesthetized with ketamine (50 mg/kg) and xylazine (10 mg/kg) by intraperitoneal injections. After the administration of anaesthesia, Orotracheal intubation was performed by using a 20 G intravenous cannula. The mice were instilled with a single intratracheal dose of LPS (*Escherichia coli* 0111:B4; Sigma-Aldrich, St. Louis, MO, USA) at 5 mg/kg (Zhang et al., 2015a) in 30 μl of phosphate-buff ;ered saline (PBS). The same volumes of PBS served as vehicle controls. After LPS or PBS administration, in order to distribute the instilled fluid throughout each lung, the mice were injected with 200 μl of air. In our study, the fine powder of pirfenidone (Sigma-Aldrich,St.Louis,MO,USA) was suspended in 0.4% sodium carboxymethylcellulose(CMC) (Sangon Biotech, Shanghai, China). Three days before LPS exposure, the mice were treated with pirfenidone(400 mg/kg,once per day) (Du et al., 2017; Kakugawa et al., 2004; Tanaka et al., 2012) by oral gavage. After they were injected with LPS, the mice were given once daily doses of 400 mg/kg pirfenidone delivered by oral gavage for 22 consecutive days. The mice were injected intraperitoneally with caspase1inhibitor AcYVADcmk(10 mg/kg) at eight hours and four hours before LPS administration (Gasse et al., 2009b; Song et al., 2016). Then, caspase-1inhibitor Ac-YVAD-cmk (10 mg/kg)was administered once every two days by intraperitoneal injection after LPS challenge. The vehicle control group was gavaged with an equal volume of 0.4% sodium carboxymethylcellulose. Randomly, ten mice in each group were euthanized separately on day 1 and day 22 after LPS challenges. Part of the left lung was collected for Masson's trichrome staining and haematoxylin and eosin stain; the right lung tissues were snap frozen in