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Original Article

NLRP3 inflammasome is essential for the development of chronic obstructive pulmonary disease

Wenlin Yang, Hongyan Ni, Haifeng Wang, Huiling Gu

Department of Respiration, The Shanghai First People's Hospital of Baoshan Branch, 101 North Tongtai Road, Shanghai 200940, China

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Abstract: Background: Chronic obstructive pulmonary disease (COPD) is now recognized as an inflammatory disease and the nucleotide-binding oligomerization domain-like receptor 3 (NLRP3) inflammasome was speculated to participate into its pathophysiological process, however, a direct role of NLRP3 has yet to be clearly shown. Method: COPD model was established by tobacco inhalation, COPD modeling and NLRP3 knockout mice were treated with similar dose and duration of tobacco inhalation for 12 months, the lung function, lung damage and immune responses were evaluated between control, wild type COPD and NLRP3 knock out C57B1/6 mice. Results: 10 months after tobacco inhalation, the respiratory system resistance indexes of COPD mice was significantly higher than that of control and NLRP3 knockout mice (2.8 ± 0.5 vs. 1.2 ± 0.3 and 1.3 ± 0.1 cm H_2O ml^{-1} s^{-1}, $P < 0.05$); the respiratory system compliance indexes of COPD was significantly lower than that of control and NLRP3 knockout mice (0.31 ± 0.02 vs. 0.43 ± 0.04, and 0.39 ± 0.01 ml/cm H_2O); the NLRP3 knockout mice displayed no distinguishable pathological damage in the lung. Of the broncho-alveolar lavage fluid (BALF), the concentration of IL-1 and IL-18 of the COPD were significantly higher than that of control and NLRP3 knockout mice (IL-1: 286.8 ± 1.7 vs. 23.8 ± 2.1 and 24.2 ± 1.3 pg/mL, $P < 0.05$; IL-18: 104.5 ± 4.2 vs. 12.6 ± 2.1 and 15.7 ± 2.8 pg/mL, $P < 0.05$); the total numbers of macrophages, eosinophils, lymphocyte and neutrophil of control, COPD and NLRP3 knockout mice were 2.3 ± 0.4, 0.5 ± 0.2, 10.3 ± 3.4 and 2.8 ± 2.7; 8.7 ± 1.1, 12.5 ± 1.1, 45.3 ± 3.3 and 29.2 ± 4.2; and 3.2 ± 0.7, 1.8 ± 0.4, 18.1 ± 1.1 and 12.8 ± 3.4 × 10^4 mL, respectively; the rates of NLRP3 positive macrophages in the BALF of control, COPD and NLRP3 knockout mice were 5.0 ± 1.0%, 78.1 ± 9.2% and 2.0 ± 0.9%, respectively. Conclusion: NLRP3 inflammasome is essential for the development of COPD and blockade of NLRP3 might be a possible therapeutic strategy for COPD.

Keywords: Chronic obstructive pulmonary disease, nucleotide-binding oligomerization domain-like receptor 3, inflammasome

Introduction

Chronic obstructive pulmonary disease (COPD) is a multicomponent disease characterized by a progressive and irreversible decline in lung function caused by airflow obstruction, destruction of parenchyma and emphysema, patients with COPD always manifested by the symptoms of emphysema and chronic bronchitis (shortness of breath, increasing cough and sputum production) [1]. COPD was responsible for 5% of deaths globally in 2005 and it is projected to be the fourth leading cause of death by 2030 [2, 3].

COPD is now recognized as an inflammatory disease of the airways [4]. Development of the chronic inflammatory pathological process in COPD is caused by inhalation of noxious particles or gas, most commonly cigarette smoke. Acute symptomatic exacerbations in COPD patients are commonly brought about by secondary viral or bacterial infections of the lung [1-4]. The inhaled substances can activate the inflammatory cascade in the airways resulting in the production of a number of potent cytokines, chemokines and tissue-degrading enzymes which play a critical role in the induction of chronic inflammation and subsequent tissue destruction [5, 6]. Epithelial cells, alveolar macrophages, and dendritic cells as well as immune cells recruited from the periphery, respond to the noxious inhaled substances that cause COPD in the lung [5, 6]. These early

inflammatory responses may lead to infiltration of inflammatory cells into the mucosa, submucosa, and glandula [7].

Inflammasomes, a complex of proteins that oligomerize and activate the caspase-1 cascade, has a substantial impact on tissue inflammation. The inflammasomes produce the active proinflammatory cytokines interleukin (IL)-1β and IL-18 when triggered by various environmental, pathogenic or endogenous danger signals [8]. The inflammasome contains the adaptor protein apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), pro-caspase 1 and the receptor which activated its formation [8, 9]. The nucleotide-binding oligomerization domain-like receptor 3 (NLRP3) is the most studied inflammasome [10]. Studies showed that two-step mechanism is required for the full activation of the NLRP3 inflammasome; the first is a priming step that is initiated by various damage-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs), which results in upregulation of pro-IL-1β, pro-IL-18 and the components of the inflammasome. The second step is the activation step, which is the assembly of the components into the inflammasome structure and production of proinflammatory interleukins [8-10]. Increasing reports showed that inflammasome activation occurs in the lungs of COPD. Cigarette smoking leads to IL-1 release in the human lung [11]; elevated levels of IL-1 and IL-1 are found in the lungs of COPD patients, and their secretion is amplified in lungs during disease exacerbations [12, 13]; mice overexpressing IL-1 in the lung present a phenotype similar to COPD, including lung inflammation, emphysema, and pulmonary fibrosis [14]; in patients with COPD, IL-18 levels are elevated in blood and lungs [15]; IL-18 levels in sputum and even serum inversely correlate with lung function in COPD patients [16, 17].

Although above indirect lines of evidence link inflammasome-dependent cytokines to disease pathology of COPD, a direct role for the NLRP3 inflammasome has yet to be clearly shown. In this report, COPD model was established by tobacco inhalation, COPD modeling and NLRP3 knock out mice were treated with similar dose and duration of tobacco inhalation for 12 months, the lung function, lung damage and immune responses were evaluated between control, COPD and NLRP3 knock out C57B1/6 mice.

Materials and methods

Mice

C57Bl/6-based mice with a targeted deletion of the NLRP3 ($NLRP3^{-/-}$) were obtained from JAX Labs (Bar Harbor, ME) [18]. C57Bl/6 mice (Charles River, Boston, MA) were used as controls. For all the following experiments, 8 week old female mice, at least 6 in each group, were used. All animal experiments were conducted in accordance with internationally recognized guidelines for animal experiments ("Animal Research: Reporting in Vivo Experiments" (ARRIVE) guidelines) and were approved by the Animal Ethics Committee of the Shanghai First People's Hospital of Baoshan Branch.

Cigarette smoke exposure

We followed the protocol reported previously [19, 20]. In brief, the system consists of a vented nose only chamber, into which serial aliquots of 20 mL of fresh cigarette smoke were injected, with each cigarette consumed over a period of approximately 10 min. The animals received the smoke of 10 cigarettes a day for 5 days a week during a 12-month total time period. For COPD modeling, C57B1/6 and $NLRP3^{-/-}$ mice were treated as under the same protocol with same duration. Mice, followed all procedures of COPD modeling except cigarette smoke exposure, were used as controls. All following pathological, pathophysiological and immune assessments were performed at 10 and 12 month after cigarette smoke exposure.

Pulmonary function tests

As reported previously [18-21], the animals were anaesthetized and intubated with a 14 gauge intravenous cannula. After intubation, the mice were placed in a pressure sensitive 7.5 L small animal plethysmograph, and ventilated at 80 breaths/min with a tidal volume of approximately 1.5 mL. A water-filled esophageal tube with a multiholed tip was used to measure pleural pressure; transpulmonary pressure was calculated as the difference between mouth and pleural pressure.

After measurement of functional residual capacity, the mice were given supplementary do-

ses of Innovar Vet, and were rendered apnoeic by hyperventilation. Pressure-volume curves were constructed by deflation to -30 cm H_2O (expiratory reserve volume), inflation to +30 cm H_2O, and deflation to -30 cm H_2O (vital capacity). Two inflation-deflation procedures were performed prior to measurement of the curve, and calculation of lung volumes. Static lung compliance was calculated between functional residual capacity and 10 cm H_2O transpulmonary pressure. A flow-volume curve was constructed by inflation to +30 cm H_2O, and rapid deflation to -50 cm H_2O pressure. From this curve, forced vital capacity, forced mid-expiratory flow, forced expiratory volume in one second, and peak expiratory flow were calculated. The flow volume curve itself was plotted, using the volumes at 95-30% total lung capacity as the independent variable.

Cytokine measurement and cell subset analysis

Blood was collected by retro-orbital bleeding and the serum was isolated and stored at -80°C until measurements. The BALF was collected by lavaging the lungs three times with 0.5 mL of phosphate-buffered saline (PBS), and the cell suspensions were then centrifuged at 1500 rpm for 5 min at 4°C. After centrifugation, supernatants were collected and stored at -80°C for cytokine analysis. The BALF cells were resuspended in PBS, and the total leucocyte counts were obtained using a hemocytometer. Differential counts were determined by cytocentrifugation of 30 μL aliquots of BALF cells at 1500 rpm for 3 min onto slides. Next, the slides were stained with Wright-Giemsa (Biovisualab, Shanghai, China) and counted in a blinded fashion. A minimum of 200 cells was counted per sample under light microscopy. The cytokine levels in the BALF and serum were determined using a commercially available ELISA kits according to the manufacturer's instructions. ELISA kits for IL-1 and IL-18 were purchased from Biovisualab, Shanghai, China.

Fluorescence-activated cell sorting analysis

To learn the NLRP3 inflammasome expression in macrophage, macrophage of BALF was analyzed by FACS (BD Biosciences, San Jose, CA, USA). The cells were stained using mouse NLRP3 antibody (R&D Systems, Inc., Minneapolis, MN). A minimum of 10^4 events within the gated live population were collected per sample. The data were analyzed with the Cell Quest Pro analysis software (BD Biosciences, San Jose, CA, USA) by gating on the live cell populations.

Histopathology

Bulging and bloodshot inflammatory cell infiltration and bronchial tube pulmonary alveolus structural changes were verified by histopathology. Hematoxylin and eosin (H&E, Biovisualab, Shanghai, China) staining were performed. The mouse lungs were removed and inflated with 4% paraformaldehyde. The issues were then embedded in paraffin and cut into 5-μm-thick sections.

Statistical analysis

The results are expressed as the mean ± standard deviation (SD). An analysis of variance (ANOVA) was used to determine the difference between all groups. Pairs of groups were compared using Student's t-test. The results were considered statistically significant for *P*-values < 0.05.

Results

Pulmonary functional assessments

To confirm the establishment of COPD mice model and to observe the role of NLRP3 in the pathological process of COPE, pulmonary functional assessments were performed firstly. As we know, the elevation of respiratory system resistance and the decline of respiratory system compliance are the topic pathophysiological characteristics of COPD [18-21]. The respiratory system resistance indexes of control, tobacco inhalation and NLRP3 knock out treated with tobacco inhalation mice at 0, 10, 12 months were 1.3 ± 0.2, 1.2 ± 0.3 and 1.4 ± 0.3; 1.2 ± 0.3, 2.8 ± 0.5 and 1.3 ± 0.1; and 1.3 ± 0.1, 2.9 ± 0.2 and 1.4 ± 0.2 cm H_2O ml^{-1} s^{-1}, respectively (**Figure 1A**). The respiratory system resistance index of tobacco inhalation treated mice elevated significantly than that of control and NLRP3 knock out mice at 10 and 12 months. The respiratory system compliance indexes of control, tobacco inhalation treated and NLRP3 knock out treated with tobacco inhalation mice at 0, 10, 12 months were 0.41 ± 0.02, 0.42 ± 0.03 and 0.40 ± 0.02; 0.43 ±

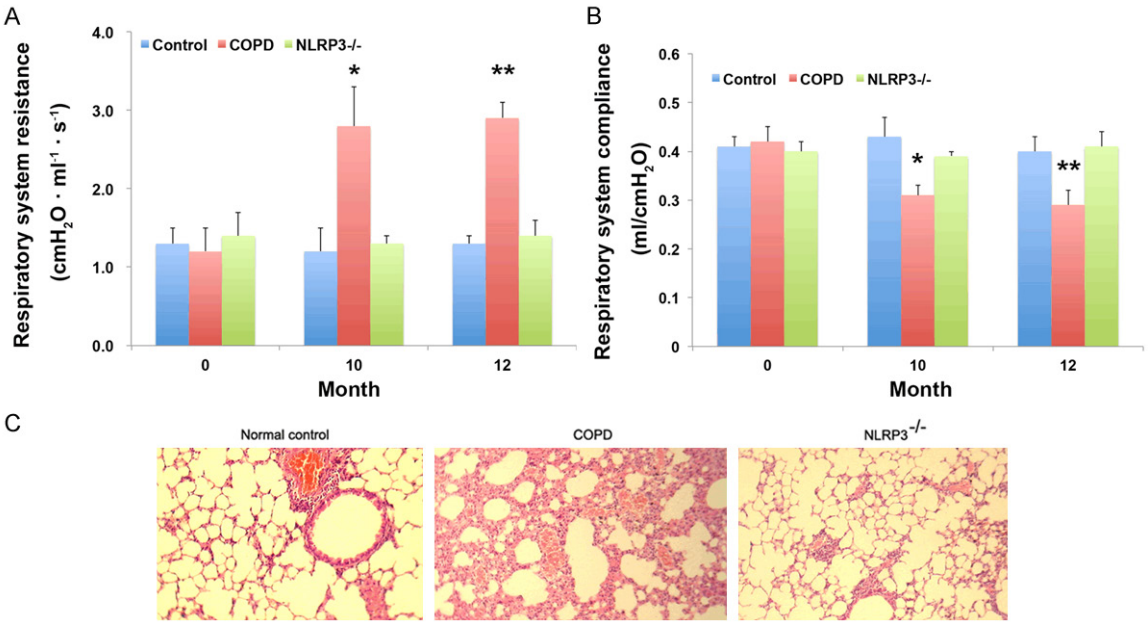

Figure 1. Functional and pathological assessments in lung. A. The respiratory system resistance indexes of control, tobacco inhalation and NLRP3 knock out treated with tobacco inhalation mice at 0, 10, 12 months, * and ** indicate the significances ($p < 0.05$) when compared with control or NLRP3 knockout mice. B. The respiratory system compliance indexes of control, tobacco inhalation treated and NLRP3 knock out treated with tobacco inhalation mice at 0, 10, 12 months, * and ** indicate the significances ($p < 0.05$) when compared with control or NLRP3 knockout mice. C. Hematoxylin and eosin staining showed bulging and bloodshot inflammatory cell infiltration and bronchial tube pulmonary alveolus structural changes.

Table 1. NLRP3 gene knock-out mice displayed no COPD symptoms and pathological damage of lung

Symptoms and Pathology	Control (n = 6)	COPD (n = 6)	NLRP3$^{-/-}$ (n = 6)
COPD manifestations			
Dysphoria or asthenia	No	Yes	No
Shortness of breath	No	Yes	No
Increasing sputum production	No	Yes	No
Respiratory rate (beats per minute)	109 ± 3	179 ± 17*	111 ± 6
Pathological damage of lung			
Bulging and bloodshot	No	Yes	No
Inflammatory cell infiltration	No	Yes	No
Bronchial tube pulmonary alveolus structure	Normal	Pathological changes	Normal

NLRP3, nucleotide-binding oligomerization domain-like receptor 3; COPD, chronic obstructive pulmonary disease; NLRP3$^{-/-}$, NLRP3 gene knock-out mice. *$P < 0.05$, compared with control or NLRP3$^{-/-}$.

0.04, 0.31 ± 0.02 and 0.39 ± 0.01; and 0.40 ± 0.03, 0.29 ± 0.03 and 0.41 ± 0.03 ml/cm H_2O, respectively (**Figure 1B**). The respiratory system compliance index of tobacco inhalation treated mice decreased significantly than that of control and NLRP3 knock out mice at 10 and 12 month. Taken together, mice displayed pathophysiological characteristics of COPD after 10 months of tobacco inhalation, while, NLRP3 knock out mice tolerated similar dose and duration tobacco inhalation and displayed no significant lung function change.

Pathological assessments in lung

After tobacco inhalation for 12 months, all mice as well as controls were subjected for lung pathological assessments. The lungs of COPD mice appeared bulging and bloodshot with bronchial tube pulmonary alveolus structural

Table 2. NLRP3 gene knock-out mice eased inflammatory responses to tobacco challenge

Cell Classification and Cytokine Level	Control (n = 6)	COPD (n = 6)	NLRP3$^{-/-}$ (n = 6)
BALF			
Macrophages (×10^4/mL)	2.3 ± 0.4	8.7 ± 1.1$^{\#}$	3.2 ± 0.7*
Eosinophils (×10^4 mL)	0.5 ± 0.2	12.5 ± 1.1$^{\#}$	1.8 ± 0.4*
Lymphocyte (×10^4 mL)	10.3 ± 3.4	45.3 ± 3.3$^{\#}$	18.1 ± 1.1$^{*,\&}$
Neutrophil (×10^4 mL)	2.8 ± 2.7	29.2 ± 4.2$^{\#}$	12.8 ± 3.4$^{*,\&}$
IL-1 beta (pg/mL)	23.8 ± 2.1	286.8 ± 1.7$^{\#}$	24.2 ± 1.3*
IL-18 (pg/mL)	12.6 ± 2.1	104.5 ± 4.2$^{\#}$	15.7 ± 2.8*
Sera			
IL-1 beta (pg/mL)	20.7 ± 1.6	44.8 ± 1.4$^{\#}$	21.9 ± 1.7
IL-18 (pg/mL)	15.7 ± 3.3	27.5 ± 5.6	12.7 ± 3.9

BALF, bronchoalveolar lavage fluid; IL, interleukin; COPD, chronic obstructive pulmonary disease. $^{*}P < 0.05$, compared with COPD group; $^{\#}P < 0.05$, compared with control group; $^{\&}P < 0.05$, compared with control group.

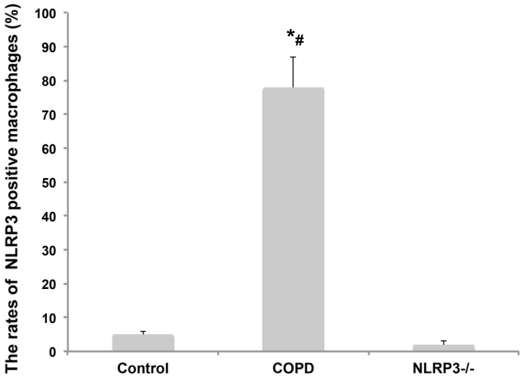

Figure 2. NLRP3 inflammasome in BALF. The rates of NLRP3 positive macrophages in the BALF of control, COPD and NLRP3 knock out mice were measured respectively. * indicates the significances ($p < 0.05$) when compared with control, # indicates the significances ($p < 0.05$) when compared with NLRP3 knockout mice.

damage (**Figure 1C** and **Table 1**). The NLRP3 knock out mice, treated with similar dose and duration tobacco inhalation of COPD, displayed no distinguishable pathological damage in the lung (**Figure 1C** and **Table 1**). In summary, wide type mice with COPD displayed inflammatory changes in lung; while, NLRP3 knock out mice lost inflammatory evidences in lung.

NLRP3 gene knock out mice displayed no COPD symptoms

The COPD symptoms were observed and recorded; COPD mice displayed dysphoria, asthenia, shortness of breath and sputum production increasing. However, the NLRP3 knock out mice treated with similar dose and duration of tobacco inhalation displayed no COPD symptom (**Table 1**). The respiratory rate of control, COPD mice and NLRP3 knock out mice were 109 ± 3, 179 ± 17 and 111 ± 6 beats per minute, respectively. COPD mice showed significant higher respiratory rate than that of control and NLRP3 knock out mice (**Table 1**).

NLRP3 gene knock-out mice eased inflammatory responses to tobacco challenge

Cellular immunity and humoral immunity are both activated in the pathophysiological process of COPD. To figure out the role of NLRP3 in the immune responses in the COPD developing, the NLRP3 representative cytokines, IL-1 and IL-18, as well as immune cell classification were evaluated both in the BALF and sera. Of the sera, only the concentration of IL-1 is significantly higher in COPD mice than that of control and NLRP3 knock out mice, the IL-18 levels are similar among three mice groups (**Table 2**). Of the BALF, the concentration of IL-1 and IL-18 of the control, COPD and NLRP3 knock out mice were 23.8 ± 2.1 and 12.6 ± 2.1; 286.8 ± 1.7 and 104.5 ± 4.2; and 24.2 ± 1.3 and 15.7 ± 2.8 pg/mL, respectively (**Table 2**). Both IL-1 and IL-18, were significantly elevated in COPD mice, while, NLRP3 knock out mice displayed no increasing in IL-1β and IL-18 levels when treated with similar dose and duration of tobacco inhalation. The total numbers of macrophages, eosinophils, lymphocyte and neutrophil of control, COPD and NLRP3 knock out mice were 2.3 ± 0.4, 0.5 ± 0.2, 10.3 ± 3.4 and 2.8 ± 2.7; 8.7 ± 1.1, 12.5 ± 1.1, 45.3 ± 3.3 and 29.2 ± 4.2; and 3.2 ± 0.7, 1.8 ± 0.4, 18.1 ± 1.1 and 12.8 ± 3.4 ×10^4 mL, respectively (**Table 2**). The total numbers of macrophages, eosinophils, lymphocyte and neutrophil increased significantly in COPD mice but deletion of NLRP3 gene eased the inflammatory responses to tobacco challenge. NLRP3 knock out mice displayed no significant elevation in the numbers of macrophages and eosinophils (**Table 2**). Although the total numbers of lymphocyte and neutrophil of NLRP3 knock out mice were significantly higher than that of con-

trol, these numbers were still significantly lower than that of COPD mice (**Table 2**). Taken together, NLRP3 gene deletion eased inflammatory responses in the COPD developing.

NLRP3 inflammasome in BALF

To evaluate if NLRP3 inflammasome was involved in COPD and confirm if NLRP3 knock out mice lost NLRP3 inflammasome formation, the NLRP3 inflammasome was studied in the BALF macrophage. As showed in **Figure 2**, the rates of NLRP3 positive macrophages in the BALF of control, COPD and NLRP3 knock out mice were 5.0 ± 1.0%, 78.1 ± 9.2% and 2.0 ± 0.9%, respectively. The number of NLRP3 positive macrophages was significantly higher in mice with COPD than that in control mice, which suggests that NLRP3 inflammasome participated in the developing of COPD. The NLRP3 knock out mice lost the generating capacity of NLRP3 inflammasome.

Discussion

COPD is now recognized as a complex and multicomponent lung diseases, the role of inflammasome in the immunopathogenesis is a brand new topic [22, 23]. However, the solid role of inflammasome in the pathophysiological process of COPD remains unknown. In this study, to figure out the above question, NLRP3 knock out mice were adapted, the NLRP3 knock out mice were treated as same dose and duration of COPD modeling condition. We found that: 1) COPD mice displayed pathophysiological characteristics of COPD after 10 months of tobacco inhalation, while, NLRP3 knock out mice tolerated similar dose and duration tobacco inhalation and displayed no significant lung function change; 2) wide type mice with COPD displayed inflammatory changes in lung while, NLRP3 knock out mice lost inflammatory evidences in lung; 3) NLRP3 knock out mice displayed no COPD symptoms; 4) NLRP3 knock out mice showed immune silence both in humoral immunity and cellular immunity levels against tobacco challenge; and 5) NLRP3 knock out mice lost the generating capacity of NLRP3 inflammasome. Our data is the first one to reveal the role of inflammasome NLRP3 in the pathophysiological process of COPD comprehensively. Data of our report will not only advance our understanding on the COPD but also provided a possible therapeutic strategy for COPD in the feature.

There are four major members were found in NLR family relevant to lung diseases [22]. All NLRs have a tripartite domain organization comprising a C-terminal LRR, middle NACHT, and a variable N-terminal domain [22]. The defining feature of NLRP3 is the N-terminal pyrin domain that homotypically binds pyrin domain of ASC (apoptosis-associated speck-like protein containing a C-terminal CARD). The activation of NLRP3 requires two distinct signals [23-25]. The preassembly “priming” signal comes from toll-like receptors activation that induces NLRP3 expression via NF-kB activation; once the amount of NLRP3 in cytoplasm reaches a threshold, NLRP3 assembly is initiated in response to a second signal originating from one or more NLRP3 ligands [23-25]. Once NLRP3 activated, it will induce downstream molecular pathways to generate cytokines and chemokines that play important roles in the pathophysiology of many lung diseases including COPD [23-25]. The IL-1 and IL-18 are two markers for NLRP3 activation, in our report, in NLRP3 knock out mice, the NLRP3 positive macrophage rate was very low and the IL-1 and IL-18 levels in the sera and BALF were also significantly lower than wild type mice when challenged with tobacco inhalation. These evidences not only confirmed the NLRP3 gene deletion but also explained the why NLRP3 knock out mice had tolerated to tobacco challenge.

There are three major experimental approaches to mimic COPD encompassing inhalation of noxious stimuli, tracheal instillation of tissue-degrading enzymes to induce emphysema-like lesions and gene-modifying techniques leading to a COPD-like phenotype [26]. The challenge is in the measurement of lung function in small mammals such as mice, in this report, we adopted the method established by Brown et al [19], following their protocol, we had successfully measured baseline pulmonaryfunction measurements, the respiratory system resistance indexes and the respiratory system compliance indexes, these measurements are essential for COPD diagnosis. Together with following assessments on COPD symptoms and pathological damage of lung, the pulmonary-function measurements were consistent with these COPD symptoms and pathological changes in lung.

In conclusion, NLRP3 inflammasome is immune hub for the COPD developing and blockade of NLRP3 might be modified into a therapeutic strategy for COPD in the feature.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Wenlin Yang, Department of Respiration, The Shanghai First People's Hospital of Baoshan Branch, 101 North Tongtai Road, Shanghai 200940, China. Tel: 86-21-56162417; Fax: 86-21-56162417; E-mail: yangwl15@sina.com

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