NLRP3 gene silencing ameliorates phosgene-induced acute lung injury in rats by inhibiting NLRP3 inflammasome and proinflammatory factors, but not anti-inflammatory factors

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ABSTRACT — NOD-like receptor protein 3 (NLRP3) is involved in acute lung injury (ALI), but its exact role in phosgene-induced ALI is not clearly understood. The aim of the study is to explore the potential therapeutic effect of NLRP3 inflammasome modulation in the management of phosgene-induced ALI. ALI was induced in rats by phosgene exposure at 8.33 g/m³ for 5 min, 30 hr before intravenous injection of adenovirus-NLRP3 shRNA (Ad/NLRP3-shRNA). The histological changes in the lung were evaluated. Bronchoalveolar lavage fluid (BALF) neutrophils were counted (smear), and protein content was measured using the BCA assay. The wet/dry ratio of lung tissue (W/D) was measured. TUNEL staining for DNA damage was used to indirectly assess pyroptosis. NLRP3 inflammasome was assessed by immunohistochemistry, RT-PCR, western blotting. Cytokines were measured by ELISA. Histological analyses revealed reduced severity in phosgene-induced ALI with Ad/NLRP3-shRNA pretreatment. TUNEL staining indicated decreased pyroptosis in Psg-Ad/NLRP3-shRNA rats. Decreased mRNA and protein levels of NLRP3 and caspase-1 (all P < 0.05), but not ASC (P > 0.05), were found in Psg-Ad/NLRP3-shRNA rats. Immunohistochemistry revealed that Ad/NLRP3-shRNA pretreatment inhibited NLRP3 inflammasome activation. Reduced level of pro-inflammatory interleukin (IL)-1β, IL-18, IL-33, and tumor necrosis factor (TNF)-α (all P < 0.05), but not of anti-inflammatory IL-4 and IL-10 (all P > 0.05), were found in serum and BALF from Ad/NLRP3-shRNA rats. NLRP3 gene silencing exerts beneficial effects on phosgene-induced lung injury by inhibiting NLRP3 inflammasome activation and pro-inflammatory factors, but not anti-inflammatory factors. Disruption of NLRP3 inflammasome activation might be used as a therapeutic modality for the treatment of phosgene-induced ALI.

Key words: Phosgene, Acute lung injury, NLRP3, Inflammasome, Gene silencing, Cytokines

INTRODUCTION

Phosgene (carbonyl chloride, COCl₂) was once extensively used as a chemical weapon during World War I. Currently, phosgene is extensively used in numerous processes of chemical and pharmacological synthesis. Industrial accidents during production and transportation may cause phosgene leaks, threatening the lives of the workers and surrounding inhabitants. Phosgene, like chlorine, hydrogen sulfide, ammonia, and other gases, has medium-to-high water solubility and can immediately react with the mucous membranes of the face, throat, and lung, and exposure symptoms quickly appear (Saed et al., 2016). Even at high doses, phosgene-induced acute lung injury (ALI) may take many hours or even a day to appear (Aggarwal et al., 2019) and may lead to severe acute respiratory distress syndrome (ARDS) and potentially life-threatening pulmonary edema (Rendell et al., 2018). Of note, phosgene-induced ALI is often refractory to conventional treatments (Aggarwal et al., 2019; Rendell et al., 2018). Glucocorticoids are widely used in the management of ALI, but their effect on phosgene-induced ALI is poor, and some potential side effects limit their use (de Lange and Meulenbelt, 2011). No specific therapy is available to cure phosgene-induced ALI (Grainge and Rice, 2010; Rendell et al., 2018). To mit-
igate the serious threats to human and public security posed by potential terrorist attacks and industrial disasters, we need to find out the clinical evaluation index and effective treatment for chemical poisoning.

A feature of phosgene exposure is an unrestrained inflammatory reaction in the lung, as revealed by interleukin (IL)-1β that can be 15 times higher than normal after phosgene inhalation and prior to any symptoms of ALI (Sciuto et al., 2003). Hence, a better understanding of the inflammatory events that occur after phosgene exposure might provide clues for the management of phosgene-induced ALI.

NOD-like receptor protein 3 (NLRP3) inflammasome is a multi-protein complex that transforms pro-cytokines IL-1β, IL-18, and IL-33 into their bioactive forms. The NLRP3 inflammasome is involved in many diseases, including diabetes mellitus, atherosclerosis, and neurodegenerative diseases (Guo et al., 2015). In vivo and in vitro experiments confirmed that the NLRP3 inflammasome also plays important roles in ALI from various causes such as lipopolysaccharides (Zhang et al., 2016), hyperoxia (Mizushima et al., 2015), mechanical ventilation (Zhang et al., 2014), cardiopulmonary bypass (CPB) (Hou et al., 2018), bleomycin (BLM) (Huang et al., 2019), and paraquat poisoning (Liu et al., 2015). The inflammatory response in ALI can be inhibited by some interventions that inhibit the activation of the NLRP3 inflammasome, reducing the severity of ALI and achieving lung protection (He et al., 2018; Hou et al., 2018; Huang et al., 2019; Zhang et al., 2016, 2014). On the other hand, in the hyperoxic ALI (HALI) mouse model, NLRP3 deficiency can reduce lung inflammation but increase HAIL mortality (Mizushima et al., 2015). In fact, inflammasome activity is essential for host adaptive immune responses, and the balance between beneficial and harmful inflammasome activation needs to be better understood (Guo et al., 2015).

IL-4 and IL-10 are anti-inflammatory cytokines that play key roles in controlling the immune response. In allergic airway inflammation, Caspase-1 activation and NLRP3/ASC inflammasome complex play key roles in regulating IL-33 and IL-4 (Liu et al., 2018; Madouri et al., 2015). The absence of IL-10 activates the expression of the NLRP3 inflammasome and the production of IL-1β (Ip et al., 2017). The P2X7 antagonist A438079 inhibits NLRP3 ASC/caspase-1 activation, IL-1β production, and neutrophil infiltration, but does not reduce IL-10 production, leading to improved lung injury (Wang et al., 2015). Inhibiting the expression of IL-10 in alveolar macrophages can significantly enhance the activation of pulmonary inflammasome and the secretion of IL-1β (Xu et al., 2013).

Inflammatory caspases (caspases-1, -4, -5, and -11) are activated during microbial infection and cause gasdermin D (GSDMD) Asp276 and Asp275 to generate an amino-terminal cleavage product (GSDMD-NT) to trigger pyroptosis and release inflammatory cytokines such as IL-1β (Liu et al., 2016). As a classical pathway, Caspase-1-dependent NLRP3 inflammasome is activated, inducing the release of IL-1β and pyroptosis (Valderrama et al., 2017).

A previous study by our group (He et al., 2018) showed that elevated NLRP3 levels were associated with downstream inflammatory cytokines such as IL-1β, IL-18, and IL-33 in phosgene-induced ALI. A recombinant adenovirus expression vector angiogenin 1 (Ad Ang 1) was introduced into phosgene-exposed rats, and it was found to have a protective effect on ALI by inhibiting NLRP3 and pyroptosis (He et al., 2018). Nevertheless, it is unclear whether NLRP3 gene silencing inhibits inflammation and protects against phosgene-induced ALI.

Therefore, the aim of the present study was to explore the potential therapeutic effect of NLRP3 inflammasome modulation in the management of phosgene-induced ALI. Our study used NLRP3 gene silencing to study its effects on proinflammatory and anti-inflammatory factors. We also attempted to examine the effect of NLRP3 silencing on IL-4 and IL-10 in ALI induced by phosgene.

**MATERIALS AND METHODS**

**Animals and ethical approval**

Adult male Sprague-Dawley (SD) rats (200 ± 20 g, 4-6 weeks of age) were bought from the Experimental Animal Center of the Second Military University of China and were maintained in cages at 24-26°C under a 12-hr light/dark cycle with free access to food and water. The experiments were approved by the Institutional Animal Care Unit and Use Committee of Jinshan Hospital, Fudan University, and complied with the international guidelines for animal experiments.

**Pretreatment of experimental animals with adenovirus RNA interference**

Three pairs of short hairpin RNA (shRNA) sequences (shRNA1, shRNA2, and shRNA3) were designed for the NLRP3 gene in rats (supported by Shanghai Honlig Biotechnology Co., Ltd.) (Table 1). The three NLRP3 shRNA sequences were annealed with forward and reverse primers, respectively, to the plasmid u6-cmv-egfp-wpre, and digested with AgeI and EcoRI enzymes. The Snapgene software was used to verify the correct

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sequencing results. The shRNA sequence was constructed in the p-BHG (Delta) E1,3 CRE recombinant shuttle plasmid, followed by the co-transfection of HEK293 cells with the skeleton plasmid, packaging, and amplification. The adenovirus carrier Ad.NLRP3-shRNA was eventually constructed. The best infection time for virus interference was 36-48 hr, and the intervention time point was 30 hr before the phosgene exposure. The rats were injected with 1 × 10⁸ copies of Ad. NLRP3-shRNA into the tail vein 30 hr before exposure (Shen et al., 2013).

**Grouping and treatment**

Sixty SD rats were randomly divided into four groups: air control group (Air, n = 6); phosgene group (Psg, n = 6); phosgene+adenovirus group (Psg+Ad, n = 6); phosgene+adenovirus-NLRP3-shRNA (Psg+Ad/NLRP3-shRNA, n = 6) (Fig. 1). After grouping, the rats in the Psg+Ad and Psg+Ad/NLRP3-shRNA group were

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**Table 1. Primers for NLRP3 shRNA.**

| Primer sequences | Primer sequences |
|------------------|------------------|
| MLK-shRNA-1      | 5'-CCG GGG ATC TTT GCA GCG ATC AAC ATT CAA GAG ATG TTG ATC |
| Primer-F         | GCT GCA AAG ATC CTT TTT Tg-3' |
| MLK-shRNA-1      | 5'-AAT TCA AAA AAG GAT CTT TGC AGC GAT CAA CAT CTC TTG AAT GTT |
| Primer-R         | GAT CGC TGC AAA GAT CC-3' |
| MLK-shRNA-2      | 5'-CCG GGC TTC AGC CAC ATG ACT TTC CTT CAA GAG AGG AAA GTC |
| Primer-F         | ATG TGG CTG AAG CTT TTT Tg-3' |
| MLK-shRNA-2      | 5'-AAT TCA AAA AAG CTT CAG CCA CAT GAC TTT CCT CTC TTG AAG |
| Primer-R         | GAA AGT CAT GTG GCT GAA GC-3' |
| MLK-shRNA-3      | 5'-CCG GGC TGG GAT CTC TCC ACA ATT CTT CAA GAG AGA ATT GTG |
| Primer-F         | GAG AGA TCC CAG CTT TTT Tg-3' |
| MLK-shRNA-3      | 5'-AAT TCA AAA AAG CTG GGA TCT CTC CAC AAT TCT CTC TTG AAG |
| Primer-R         | AAT TGT GGA GAG ATC CCA GC-3' |

Fig. 1. Animal flowchart and group allocation.
intratracheally given Ad or Ad/NLRP3-shRNA (Hanbio, Shanghai, China) at 30 hr, before exposure (Shen et al., 2013). Each rat was placed in a cylinder and exposed to either normal room air (air control group) or 8.33 g/m³ of phosgene (phosgene-exposed group) for 5 min, followed by clean air washout. The dose cited in the previous experiments was consistent with the dose used for the induction of acute lung injury in the animal models (Shen et al., 2013). The rats were sacrificed by cervical dislocation. Serum, bronchoalveolar lavage fluid (BALF), and lung tissues were collected at 6, 24, and 48 hr. Those time points were selected because the inflammatory response to phosgene peaks at 4-8 hr, ALI appears by 24 hr (He et al., 2018), and 48 hr was selected to observe the longer-time effect of the intervention.

H&E staining
Lung tissues were fixed in 10% formalin, embedded in paraffin, and sectioned to 5 μm. Hematoxylin and eosin (H&E) staining was performed using a routine protocol (https://www.leicabiosystems.com/knowledge-pathway/he-staining-overview-a-guide-to-best-practices/). Images were taken using a light microscope (Carl Zeiss GmbH, Oberkochen, Germany).

The average optical density (AOD) value of immunofluorescence was used for staining using a routine protocol (https://www.leicabiosystems.com/knowledge-pathway/he-staining-overview-a-guide-to-best-practices/). Images were taken using a light microscope (Carl Zeiss GmbH, Oberkochen, Germany).

The integrated optical density (IOD) value and pixel area (AREA) of each positive image were obtained by analyzing each image. The AOD value was calculated using the formula (IOD/AREA). The higher the AOD value, the higher the positive expression level will be.

Neutrophil count and protein content of BALF
The right upper lobe was intubated through the right bronchus, and 5 mL of ice normal saline (4°C) was slowly injected at low pressure. The lavage fluid was withdrawn repeatedly until the total amount of lavage fluid reached 15 mL. The cells were counted by a smear on a blood cell counting plate. The obtained lavage solution was centrifuged (1000 rpm, 10 min). The supernatant was collected in a 15-mL centrifuge tube and stored at -80°C. The protein content of the supernatant was determined.

Wet-to-dry ratio of lung tissue
After thoracotomy, the lower left lung was removed, weighed (wet mass), dried in an oven at 80°C for 48 hr or until constant weight, and weighed (dry mass). The wet-to-dry (W/D) ratio of lung tissue was calculated.

TUNEL assay for DNA damage
Pyroptosis is defined as a programmed cell death pattern that relies on the classical caspase-1 pathway and is characterized by the release of a large number of pro-inflammatory cytokines (Valderrama et al., 2017). It is pyroptosis, based on the results of TUNEL and the comprehensive judgment from caspase-1 activation and increased cytokine production. Therefore, the TUNEL assay for DNA damage was used to indirectly evaluate pyroptosis in lung tissue. Lung tissues were assessed with the In situ Cell Death Detection Kit (Roche, Basel, Switzerland) according to the manufacturer’s instructions. The green fluorescence was observed under a fluorescence microscope (Carl Zeiss GmbH). Paraffin sections were dewaxed to water, and antigens were retrieved. Reagents 1 and 2 were added at 2:29. The sections were slightly shaken dry, and each section was added with an appropriate amount of reactant 3 (converter-POD), placed flat in a wet box, and incubated at 37°C for 30 min. A freshly prepared DAB solution was added. The nuclei were stained with hematoxylin.

Real-time reverse transcription-polymerase chain reaction (RT-PCR)
Total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA, USA). RT-PCR was performed using a One-Step SYBRVR PrimeScript RT-PCR kit (Takara, Dalian, China) and an iQ5 Real-time PCR Detection System (Bio-Rad, Hercules, CA, USA). Expression of the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene was assessed at the same time as an internal control. Relative gene expression was determined using the 2^(-ΔΔCT) method. The primers specific for NLRP3, ASC, caspase-1, and GAPDH are listed in Table 2. The lung tissue blocks of rats in each group were ground into powder with liquid nitrogen in a mortar, passed into a glass homogenizer and then 1 mL Trizol was added to make a homogenate. Next, the supernatant was centrifuged at 12,000g/min for 5 min, followed by addition of 200 μL chloroform, mixing back and forth for 15 sec, and then maintaining at room temperature for 15 min. After centrifugation at 12000g/min for 15 min, the upper water phase was carefully transferred to 1.5 mL sterile EP tube for removing RNA. After that, 0.5 mL isopropyl alcohol was added and gently mixed. After maintaining at room temperature for 10 min, the supernatant was centrifuged at 12000g/min for 10 min. Then RNA
washing, RNA redissolution, determination of total RNA purity and concentration were performed. All primers were designed and synthesized by Primer Premier 5 combined with Dnastar analysis software and BLAST analysis online.

### Western blotting

The lung tissues were lysed in RIPA buffer (Beyotime Institute of Biotechnology, Haimen, China), followed by high-speed (10,000-14,000 rpm) centrifugation. Protein concentrations were tested using a BCA kit (Beyotime Institute of Biotechnology). Proteins were separated by SDS-PAGE (Beyotime Institute of Biotechnology) and transferred to PVDF membranes (Millipore Corp., Billerica, MA, USA). The membranes were blocked with skimmed milk containing Tween-20 and incubated with the primary antibody overnight. The membranes were incubated with the anti-rabbit or anti-mouse HRP-conjugated IgG secondary antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) for 1 hr. The protein bands were developed with SuperSignal Ultra Chemiluminescent Substrate (Pierce Chemical, Dallas, TX, USA) on X-ray films (Kodak, Tokyo, Japan). Anti-NLRP3, anti-ASC, anti-Caspase-1, and anti-GAPDH were purchased from Santa Cruz Biotechnology, Inc.

The lung tissues of rats in each group were cut into small fragments, RIPA buffer (100 uL of lysis buffer was added for every 20 mg of tissue) was added, and then homogenized using a glass homogenizer until fully decomposed. After lysis, the samples were centrifuged at 10000-14000r/min for 5 min and the supernatant was extracted. The protein concentration was calculated using BCA kit according to the standard curve. Sample solution and protein MaAer were sampled. The protein was isolated using SDS-PAGE. The sealant was freshly prepared and the PVDF membrane was immersed in the sealant, and then was oscillated for 1 hr at room temperature. The primary antibody was diluted and the membrane after oscillation was incubated at room temperature. The dilution ratio of primary antibodies were: NLRP3 antibody 1:1000, ASC antibody 1:100, Caspase-1 antibody 1:1000, respectively. Tris buffered saline Tween-20 was used to wash the membranes three times for 5 min each time. Diluted secondary antibodies were added and incubated at room temperature for 1 hr after oscillation. HRP goat anti-rabbit 1:5,000 was used as a secondary antibody. The film was washed three times for 5 min each. Finally, chemiluminescence detection was carried out.

### Immunohistochemistry for NLRP3 in lung tissues

Paraffin sections of left lung tissues were dewaxed to water. After antigen retrieval and cooling, the slides were placed in PBS (pH 7.4) and washed three times for 5 min each time. BSA (3%) was added to cover the sections evenly at room temperature for 30 min. A primary antibody prepared in PBS (pH 7.4) was added to the sections, followed by three washes in PBS (pH 7.4). The secondary antibody was added and incubated at room temperature for 50 min. The sections were washed three times in PBS (pH 7.4), 5 min each time. Freshly prepared DAB was added. The sections were observed under the microscope (Carl Zeiss GmbH). The positive color was brown and yellow. Harris hematoxylin restaining of the nuclei was performed. The staining was observed at × 200 and determined as being weak or strong.

The AOD value of immunohistochemistry was used for quantitative analysis. Image-pro Plus 6.0 software was used to select the same brown and yellow color as the unified standard for determining all positive photos. The IOD value and AREA of each positive image were obtained by analyzing each image. The IOD value and AREA of each positive image were obtained by analyzing each image. The AOD value was calculated using the formula (IOD/AREA). The higher the AOD value, the higher the positive expression level will be.

### Collection of BALF and serum, and cytokine measurement

BALF was obtained by using 0.9% saline irrigation of the right main bronchial through tracheal intubation. BALF was collected by washing the lungs with 1 mL of PBS three times. The serum was collected from the abdominal artery. BALF and serum were centrifuged at 6000 × g for 5 min and stored at -80°C. The levels of IL-1β, IL-18, IL-33, tumor necrosis factor (TNF)-α, FIL-4, and IL-10 were measured using the appropriate ELISA kits (R&D Systems, Minneapolis, MN, USA), according
to the manufacturer’s protocols.

Statistical analysis
All statistical analyses were carried out using GraphPad Prism 8 (GraphPad Software Inc., San Diego, CA, USA). Comparisons among groups were performed using the Student t-test (two groups) or ANOVA followed by the post hoc Student-Newman-Keuls (SNK) (multiple groups). Comparisons within groups over time were performed using repeated measure ANOVA and the SNK post hoc test. Data are presented as means ± standard deviations. Two-sided P-values < 0.05 were considered statistically significant.

RESULTS
NLRP3 gene silencing attenuates lung injury
Previous animal experiments suggested that ALI from phosgene poisoning may manifest as increased lung dry-wet weight ratio and exudation of plasma proteins and neutrophils into BALF (Aggarwal et al., 2019). According to our previous work, we successfully built a phosgene-induced ALI model in rats (Shen et al., 2013). To verify the protective effects of NLRP3 gene silencing in phosgene-induced ALI, we established the phosgene-induced ALI rat model with NLRP3 gene silencing or control adenovirus. Lung tissue in the phosgene group showed impaired alveolar integrity, hemorrhage, leukocyte infiltration, and edema (Fig. 2A). At 6, 24, and 48 hr, the NLRP3 gene silencing group showed a relative-
ly integral alveolar structure. Alleviated hemorrhage and edema were found in the NLRP3 gene silencing group compared with the other groups. The number of cells and protein content in BALF were low in the Air group, but after phosgene exposure, a large number of granulocytes could be seen in BALF accompanied by increased protein content, which is closely related to local inflammatory response in the lungs. Compared with the Air group, the number of neutrophils and protein content in the BALF in the Psg group were significantly increased (P < 0.05). Compared with the Psg group, the number of neutrophils and protein content in BALF in the Psg+Ad/NLRP3-shRNA group were decreased (P < 0.05) (Fig. 2B&C). The lung W/D in the Psg group was significantly higher than in the Air group (P < 0.05). Compared with the Psg and Psg+Ad groups, lung W/D was significantly decreased in the Psg+Ad/NLRP3-shRNA group (P < 0.05) (Fig. 2D).

NLRP3 gene silencing decreases pyroptosis during phosgene-induced ALI

Since the activation of the NLRP3 inflammasome could result in pyroptosis, we performed TUNEL staining for DNA damage to indirectly test the pyroptosis in lung tissues. TUNEL staining was observed in the NLRP3 gene silencing group, indicating slight pyroptosis after NLRP3 gene silencing pretreatment. In the Air control group, the green fluorescence was weak, and there was no obvious pyroptosis (Fig. 3). After phosgene poisoning, green fluorescence was observed, and the cells were pyroptosis-positive. Compared with the phosgene group, the fluorescence green of lung tissue in the Psg+Ad/NLRP3-shRNA group was decreased, and pyroptosis was decreased. Light green fluorescence was observed in the NLRP3 gene silencing group, indicating slight pyroptosis under NLRP3 gene silencing. Compared with the other two groups, the NLRP3 gene silencing group demonstrated obviously decreased pyroptosis.

NLRP3 gene silencing decreases NLRP3 in phosgene-induced ALI

We examined the mRNA and protein levels of NLRP3 in lung tissues. At 6, 24, and 48 hr, decreased mRNA and protein levels of NLRP3 were observed in the Psg+Ad/NLRP3-shRNA group compared with the Psg group and Psg+Ad groups (all P < 0.05) (Fig. 4A-B). Immunohistochemistry was performed to measure NLRP3. The lung structure of the air control group rats was clear, the alveolar wall was thin, and there were almost no NLRP3-positive cells. The alveolar septum of Psg rats was thickened, and NLRP3 positive cells were found. In the NLRP3 gene silencing intervention group, the alveolar structure of rats was relatively clear, the alveolar wall was slightly thickened, and a small number of NLRP3 positive cells were seen in the lung tissue. NLRP3-positive cells in lung tis-
issues of rats with phosgene inhalation lung injury were significantly reduced after NLRP3 gene silencing (Fig. 4C). Those data suggest that NLRP3 gene silencing alleviated phosgene-induced ALI, partly by suppressing NLRP3.

**NLRP3 gene silencing reduces the levels of inflammatory cytokines during phosgene-induced ALI**

After the observation of the alleviation of lung injury severity under H&E staining, we performed an evaluation of the inflammatory factors in BALF at 6, 24, and 48 hr. Compared with the Air group, the inflammatory factors such as IL-1β, IL-18, IL-33 and TNF-α levels, and the anti-inflammatory IL-4 and IL-10 levels showed significant increase in Psg and Psg+Ad groups (all $P < 0.05$) (Fig. 5A-L). At 6, 24, and 48 hr, significant decreases in the inflammatory factors IL-1β, IL-18, IL-33, and TNF-α levels (all $P < 0.05$) (Fig. 5A-H), but not in the anti-inflammatory IL-4 and IL-10 levels ($P > 0.05$) (Fig. 5I-L), were found in the NLRP3 gene silencing group compared with the Psg and Psg+Ad groups. The levels of the above-mentioned factors were also tested in serum, and similar results were observed (all $P < 0.05$) (Fig. 5). These data suggested that NLRP3 gene silencing modulated inflammation in phosgene-induced ALI.
NLRP3 gene silencing decreases Caspase-1 in phosgene-induced ALI, but not ASC

We examined the mRNA and protein levels of ASC and caspase-1 in lung tissues. Compared with Air group, the mRNA and protein levels of ASC and caspase-1 were significantly increased in Psg and Psg+Ad groups (all P < 0.05) (Fig. 6). At 6, 24, and 48 hr, decreased mRNA and protein levels of caspase-1 were observed in the Psg+Ad-shNLRP3 group compared with the Psg group and Psg+Ad group, but not in ASC levels (P > 0.05) (Fig. 6). Those data suggest that NLRP3 gene silencing alleviated phosgene-induced ALI, partly by suppressing Caspase-1.

DISCUSSION

Exposure to phosgene leads to ALI, but this specific condition is refractory to the classical anti-inflammatory drugs (de Lange and Meulenbelt, 2011; Grainge and Rice, 2010; Rendell et al., 2018). NLRP3 is involved in ALI (Hou et al., 2018; Huang et al., 2019; Liu et al., 2015; Mizushima et al., 2015; Zhang et al., 2016, 2014), but its exact role in phosgene-induced ALI is not clearly understood. Therefore, this study aimed to explore the potential therapeutic effect of NLRP3 inflammasome modulation in the management of phosgene-induced ALI. The results suggest that NLRP3 gene silencing exerts beneficial effects on phosgene-induced lung injury by inhibiting NLRP3 inflammasome activation and pro-inflammatory factors, but not anti-inflammatory factors. Disruption of NLRP3 inflammasome activation might be used as a therapeutic modality for the treatment of phosgene-induced ALI.

A major consequence of inflammasome activation is pyroptosis, which plays some role in ALI (Valderrama et al., 2017). Inflammatory caspases trigger pyroptosis and the release of inflammatory cytokines (Liu et al., 2016). The maturation of caspase-1 can also directly induce a programmed cell death named pyroptosis, which has recently been proposed as being caspase-1-dependent and to involve an important release of pro-inflammatory cytokines (Coll et al., 2011; Cookson and Brennan, 2001). Once pyroptosis is activated, NLRP3 inflammasome regulated by caspase-1 is activated, and DNA fragmentation occurs. Pyroptosis is a type of cell death that shares many characteristics of apoptosis and necrosis (Bergsbaken et
TUNEL staining can be performed to evaluate the degree of pyroptosis. In the present study, the silencing of the NLRP3 inflammasome significantly suppressed pyroptosis. Inhibition of pyroptosis is an important mechanism in the protection against phosgene-induced ALI.

As an explanation for the observation of pyroptosis after phosgene inhalation, a previous study by our group (He et al., 2018) confirmed that the expression of the NLRP3 inflammasome was increased in ALI induced by phosgene inhalation, accompanied by an increase in the levels of the downstream inflammatory factors. In addition, an intervention using a recombinant adenovirus expression vector for Ang-1 showed that Ang-1 could protect the lungs against phosgene-induced ALI by inhibiting the NLRP3 inflammasome. Nevertheless, the effect of silencing the NLRP3 inflammasome itself was not tested.

The activation of the NLRP3 inflammasome leads to the maturation of cytokine precursors into IL-1β, IL-18, and IL-33 (Cassel et al., 2009), and those cytokines...
are involved in ALI (Martinon et al., 2002; Wang et al., 2002). In the present study, NLRP3 inflammasome expression levels were significantly increased in ALI induced by phosgene inhalation and, accordingly, the levels of IL-1β, IL-18, and IL-33 in the BALF and serum were elevated, correlating with the histopathological signs of ALI. It can be considered that phosgene inhalation activates the NLRP3 inflammasome, thereby causing waterfall inflammatory response leading to ALI. After NLRP3 gene silencing, the expression of NLRP3 inflammasome in lung tissue was decreased, and the downstream products of the NLRP3 inflammasome (IL-1β, IL-18, and IL-33) were also significantly decreased in the BALF and serum, also correlating with alleviated histopathological indicators of ALI. This is supported by a previous study that showed that the NLRP3 inflammasome signaling pathway is involved in the occurrence and development of phosgene-induced ALI (He et al., 2018).

The balance of proinflammatory and anti-inflammatory factors plays an important role in the inflammatory response. In addition to the effect of NLRP3 on IL-1β, IL-18, IL-33, and TNF-α, the present study examined whether silencing the NLRP3 inflammasome had effects on the anti-inflammatory cytokines IL-4 and IL-10. In this study, it was found that IL-4 and IL-10 levels in the BALF and serum were significantly increased during ALI induced by phosgene. Interestingly, the NLRP3 gene silencing did not decrease IL-4 and IL-10 levels. In allergic inflammatory response, neutralization of IL-33 inhibits the inflammatory response, while recombinant IL-33 enhances the allergic inflammatory response in caspase-1-deficient mice (Madouri et al., 2015). Caspase-1-deficient mice exposed to house dust mite (HDM) had increased airway inflammation, accompanied by significant eosinophil recruitment, increased IL-4, IL-5, and IL-13 levels, and increased full-length and bioactive IL-33 expression (Madouri et al., 2015), indicating the key role of caspase-1 activation and NLRP3/ASC inflammasome complex in down-regulating IL-33. In a study of bronchial asthma, NLRP3 overexpression increased IL-4, while NLRP3 silencing down-regulated IL-4 secretion (Liu et al., 2018). The effect of NLRP3 silencing on IL-4 levels is partially the same as our results. The possible reasons for the partial difference include different pathogeneses, and the interventions against the inflammatory reaction were different. The P2X7 antagonist A438079 inhibits NLRP3 ASC/activate caspase-1 and reduces IL-1β, IL-17, and other inflammatory factors and neutrophil infiltration, but does not target IL-10 production, leading to significant improvements in lung injury (Wang et al., 2015). Pyrin is an NLRP that negatively regulates inflammasome activation through nucleotide-binding interactions, and IL-10 is known to be an effective inducer of pyrin expression in macrophages (Xu et al., 2013). Pyrin inhibits the expression of IL-10 in alveolar macrophages and significantly enhances the activation of the inflammasome and the secretion of IL-1β in the lungs. In the present study, the expression of IL-10 was not decreased after NLRP3 silencing compared with the phosgene group. Therefore, inhibiting pro-inflammatory cytokines without reducing anti-inflammatory cytokines should further shift their balance toward anti-inflammation, contributing to the alleviation of phosgene-induced ALI.

Some interventions that inhibit inflammatory response can improve ALI. The administration of an IL-1 receptor antagonist (IL-1RA) has been proved to reduce the severity of ALI in Gram-negative pneumonia models (Herold et al., 2011). Clinical studies have shown that patients with ARDS and elevated IL-18 levels had poor long-term prognosis (Dolnay et al., 2012; Rogers et al., 2019). Jordan et al. (2001) also showed that an IL-18 antibody could alleviate ALI in mice. Caspase-1 activation and the NLRP3/ASC inflammasome complex play key roles in the down-regulation of IL-33 in allergic pulmonary inflammatory responses (Madouri et al., 2015). Mizushima et al. (2015) found that in a HALI mouse model, compared with wild-type and IL-1β (-/-) mice, NLRP3 (-/-) mice had a higher mortality rate, but there was no significant change in IL-1β production in each group. NLRP3 protein deficiency increases the mortality of HALI through Stat3 signaling but does not rely on IL-1β generation (Mizushima et al., 2015). In the present study, NLRP3 silencing had no significant effect on ASC expression in lung tissues, which is consistent with our previous study on Ang-1 (He et al., 2018). Therefore, NLRP gene silencing mainly plays the role of phosgene lung protection by inhibiting NLRP3 and caspase-1 levels, and the role of ASC in acute phosgene lung injury needs to be clarified in future studies.

The present study has limitations. Only a small panel of cytokines and proteins were examined, and further study is necessary to obtain a more comprehensive understanding of the changes occurring in the lungs after phosgene exposure and NLRP3 silencing. In addition, in the present study, NLRP3 silencing was done prior to exposure to phosgene, which, of course, cannot be used in the context of accidental exposure. Future studies should examine the role of NLRP3 inhibitors.

In conclusion, we found that NLRP3 gene silencing exerts beneficial effects on phosgene-induced ALI by inhibiting IL-1β, IL-18, and IL-33, but not IL-4 and IL-10, thereby shifting the cytokine balance toward anti-
inflammation. Inhibition of the NLRP3 inflammasome (i.e., NLRP3 and caspase-1) and pyroptosis are important mechanisms in the protection process, but ASC does not seem to be involved. Nevertheless, how NLRP3 silencing balances pro-inflammatory and anti-inflammatory factors still needs further investigation.

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