

# Naringin alleviates pneumonia caused by *Klebsiella pneumoniae* infection by suppressing NLRP3 inflammasome

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## ABSTRACT

*Klebsiella pneumoniae* (Kpn) is an important pathogen of hospital-acquired pneumonia, which can lead to sepsis and death in severe cases. In this study, we simulated pneumonia induced by Kpn infection in mice to investigate the therapeutic effect of naringin (NAR) on bacterial-induced lung inflammation. Mice infected with Kpn exhibited increases in white blood cells (WBC) and neutrophils in the peripheral blood and pathological severe injury of the lungs. This injury was manifested by increased expression of the inflammatory cytokines interleukin (IL)-18, IL-1 $\beta$ , tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and IL-6, and elevated the expression of NLRP3 protein. NAR treatment could decrease the protein expression of NLRP3, alleviate lung inflammation, and reduce lung injury in mice caused by Kpn. Meanwhile, molecular docking results suggest NAR could bind to NLRP3 and Surface Plasmon Resonance (SPR) analyses also confirm this result. In vitro trials, we found that pretreated with NAR not only inhibited nuclear translocation of nuclear factor (NF)- $\kappa$ B protein P65 but also attenuated the protein interaction of NLRP3, caspase-1 and ASC and inhibited the assembly of NLRP3 inflammasome in mice AMs. Additionally, NAR could reduce intracellular potassium (K<sup>+</sup>) efflux, inhibiting NLRP3 inflammasome activation. These results indicated that NAR could protect against Kpn-induced pneumonia by inhibiting the overactivation of the NLRP3 inflammasome signaling pathway. The results of this study confirm the efficacy of NAR in treating bacterial pneumonia, refine the mechanism of action of NAR, and provide a theoretical basis for the research and development of NAR as an anti-inflammatory adjuvant.

## 1. Introduction

*Klebsiella pneumoniae* (Kpn) is a Gram-negative pathogenic bacterium belonging to the Enterobacteriaceae family, which has classically been considered an opportunistic pathogen because it typically causes infections in hospitalized or otherwise immunocompromised individuals [1]. When the immunity of severe patients is decreased, Kpn is easily transported to the lungs, where it releases various virulence factors,

including lipopolysaccharide (LPS), and immune cells such as alveolar macrophages and dendritic cells are stimulated to secrete inflammatory factors to resist bacterial invasion. However, the continuous increase in the release of inflammatory factors leads to immune imbalance, and the resulting inflammatory cascade can lead to the release of large numbers of cytokines. This so-called "cytokine storm" induces pneumonia, acute lung injury and other respiratory system diseases [2,3] and is the leading cause of organ damage and even sepsis [4,5].

**Abbreviations:** AMs, Alveolar macrophages; Co-IP, Co-Immunoprecipitation; ELISA, enzyme-linked immunosorbent assay; H&E, hematoxylin and eosin; IL-18, Interleukin-18; IL-1 $\beta$ , Interleukin-1 $\beta$ ; IL-6, Interleukin-6; Kpn, *Klebsiella pneumoniae*; LPS, lipopolysaccharide; NAR, naringin; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; WBC, white blood cell.

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Alveolar macrophages (AMs) are widely distributed in alveoli and on bronchial surfaces and represent a vital defense barrier for the lungs in innate immunity [6]. AMs can express pattern recognition receptors to detect pathogen-related molecular patterns (PAMPs) and damage-related molecular patterns [7,8]. NLRP3 (NLR family pyrin domain-containing protein 3) inflammasome is an essential member of the NOD-like receptors family, one of the crucial undertakers of innate immunity. When pathogens invade the AMs, the nuclear transfer of p-P65 acts as the first signal (Priming signal) to promote the expression of NLRP3, pro-IL-18, and pro-IL-1 $\beta$  genes. At the same time, the second signal (Activation signal) mediated by stimuli (e.g., K<sup>+</sup> efflux) induces the assembly of NLRP3, ASC, and Caspase-1 complex proteins, as well as the production of active Caspase-1. IL-18 and IL-1 $\beta$  are matured and secreted under caspase-1 and are further involved in the immune response. This over-activation of NLRP3 aggravates the imbalance of the macrophage activation state, which leads to the dysregulation of the inflammation cascade, which is the primary pathophysiological mechanism of lung diseases and is considered to be an essential target for its treatment [9]. A recent study found that Kpn-induced pneumonia could be alleviated by inhibiting the expression of NLRP3 [10].

Naringin (NAR), a type of dihydro flavone, is mainly derived from *Exocarpium Citri Grandis*, the dried unripe fruit peel of *Citrus grandis* 'Tomentosa' or *Citrus grandis* (L.), which has been an established botanical treatment for cough and sputum for thousands of years in China [11]. Studies showed that NAR is potentially anti-inflammatory in expressing inflammatory cytokines in rats with chronic obstructive pulmonary disease (COPD) induced by cigarette exposure [12]. NAR has also been shown to suppress eosinophilic airway inflammation in ovalbumin-induced asthma and attenuate acute neutrophil infiltration induced by airway hyperreactivity [13,14]. With the effect of NAR on relieving pulmonary inflammation initially confirmed, its advantages of high drug safety and wide range of sources have been gradually discovered. NAR is also being developed as a therapeutic adjunct to clear the throat and moisten the lungs [15,16]. Although the anti-inflammatory effect of NAR has been established, the role of NAR on NLRP3, a critical inflammatory target of pneumonia, has not been thoroughly studied so far, which has affected the disclosure of the anti-inflammatory mechanism of NAR. Given previous reports, we used molecular docking to confirm the association between NAR and NLRP3 theoretically. We demonstrated the inhibitory effect of NAR on NLRP3 in pneumonia mice and the AMs inflammation model caused by Kpn infection.

## 2. Materials and methods

### 2.1. Chemicals and reagents

NAR was purchased from Sigma Aldrich Chemical (St. Louis, MO, USA). HPLC $\geq$ 98%, chemical formula C<sub>27</sub>H<sub>32</sub>O<sub>14</sub>, molecular weight 580.54, CAS No. 10236-47-2), Dulbecco's modified Eagle's medium (DMEM) and trypsin were purchased from JS Biosciences Co., Ltd. (Lanzhou, Gansu Province, China). Fetal bovine serum (FBS) and penicillin-streptomycin antibiotics were purchased from Thermo Fisher Scientific (MA, USA). LDH assay kit, ELISA kits of IL-6, TNF- $\alpha$ , and nucleoprotein extraction kit were purchased from Solarbio Science & Technology Co., Ltd. (Beijing, China). ELISA kits of IL-1 $\beta$  and IL-18 were purchased from Jianglai Industrial Limited by Share Ltd (Jiangsu, China). RNA extraction kit, TRIzol® Reagent RT-PCR kit, and SYBR® green PCR master mix were bought from TaKaRa (Tokyo, Japan). The primary antibodies against NF- $\kappa$ B P65, p-P65, NLRP3, Caspase-1, ASC, Pro-Caspase-1, IL-1 $\beta$  p31, IL-1 $\beta$  p17, GAPDH, Lamin B, HRP-conjugated goat anti-rabbit IgG, HRP-conjugated goat anti-mouse IgG and Alexa Fluor 488 labeled anti-mouse IgG were obtained from Cell Signaling Technology, Inc. (Beverly, MA, USA). Bovine serum albumin (BSA) and the ECL detection kit were supplied by Thermo Fisher Scientific (MA, USA). McConkey's medium was purchased from Sigma-Aldrich (Merk,

Germany). All the other reagents employed in the study were of analytic grade and are commercially available unless otherwise stated.

### 2.2. Molecular docking studies of NAR

The crystal structure of mice NLRP3-NACHT in complex with ADP and allosteric inhibitor NP3-146 (PDB ID: 7ALV) was downloaded from RCSB PDB Bank (<http://www.pdb.org>) [17]. The structure file in pdb format was imported into AutoDock Tools 1.5.7 (ADT) (Ref2). Only one protein chain and ligands remained, while others were removed. Protein structures were automated, manually checked, and finally protonated. The 3D structures of NAR were downloaded from PubChem (<http://www.pubchem.org>) and imported into ADT. The docking grid files were generated by autogrid4 [18]. The molecular docking was performed with AutoDock-GPU on a Linux server. NAR was docked into the ATP- or allosteric binding sites [19], respectively. Receptors were kept rigid during the docking process, and ligands were flexible. Finally, 20 poses were kept and scored with an inherent scoring function. Ligand poses with the best score were used for protein-ligand interaction analysis by LigPlot+ (version 2.2.4) [20]. The 3D visualization and plot were generated by VMD 1.9.1 [21].

### 2.3. Affinity test

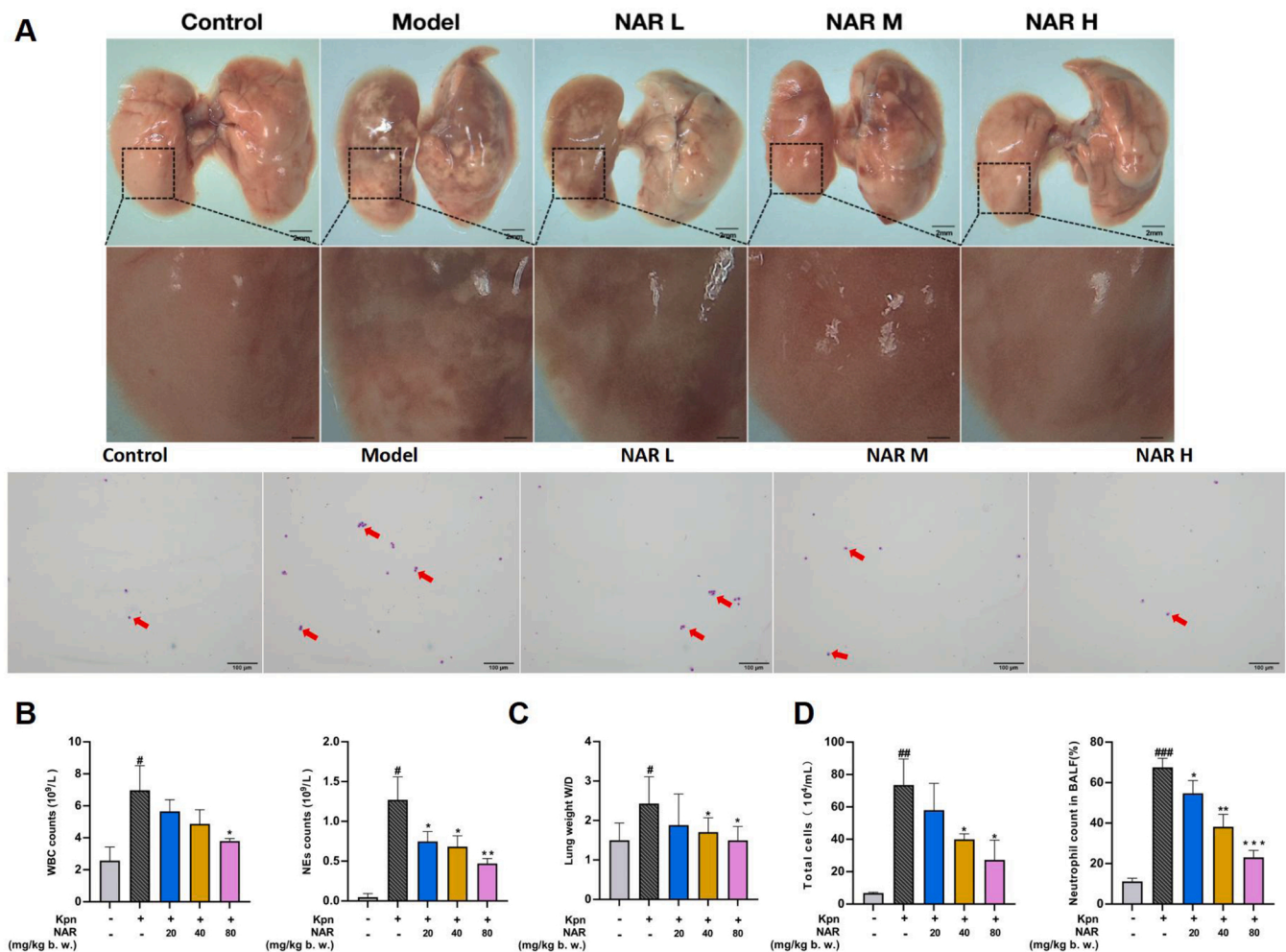
Surface Plasmon Resonance (SPR) experiments were performed on Biacore 8 K instruments (Cytiva). SA (streptavidin, Cytiva) chip was used to immobilize NLRP3 to around 5000RU using immobilization buffer (10 mM HEPES pH 7.4, 200 mM NaCl, two mM MgCl<sub>2</sub>, 0.05% Tween-20, one mM DTT, 0.5 mM ADP). NAR (0.19  $\mu$ M - 200.0  $\mu$ M) was titrated using a Multi-Cycle Kinetic model (contact time: 60 s, dissociation time: 300 s) with running buffer of 10 mM HEPES pH 7.4, 200 mM NaCl, two mM MgCl<sub>2</sub>, 0.05% Tween-20, one mM DTT, 0.5 mM ADP, 2% DMSO. The measurement was conducted at 10 °C with a flow rate of 30  $\mu$ L/min.

### 2.4. Bacteria, cellular inflammation models and NAR treatment

*Klebsiella pneumoniae* (Kpn), which was kept in our laboratory, was isolated from pig farms and verified by blast sequencing; LB medium (1% tryptone, 0.5% yeast exact, 1%NaCl) was used for culture at 37 °C before use. Mice AMs (MH-S) were acquired from the Procell Life Science & Technology Co., Ltd and cultured in DMEM, including 1% penicillin-streptomycin and 10% FBS in the humidified atmosphere with 5% CO<sub>2</sub> at 37 °C. After cell confluency reached about 70–80%, cells were trypsinized and subcultured. To observe the protective effect of NAR on MH-S, the cells were pretreated with NAR (10  $\mu$ g/mL, 20  $\mu$ g/mL, and 30  $\mu$ g/mL) for 12 h and then stimulated with Kpn bacterial solution (1  $\times$  10<sup>8</sup> CFU/ mL) for one h. After removing the bacterial solution, the culture was continued as usual for 12 h. The cells were collected for Western blotting and RT-PCR, and the supernatant was collected for ELISA.

### 2.5. Establishment of animal model and treatment of NAR

60 Kunming mice (18–22 g) were provided by the Lanzhou Veterinary Institute of CAAS (License No. SCXK Gansu 2022-0009). Animal welfare statement: institutional ethical and animal care guidelines were observed, and all procedures were conducted according to the China Guide for the Care and Use of Laboratory Animals (protocol number: IACUC-157031). A total of 5 groups were divided into a control group, model group, NAR low-dose group (20 mg/kg), NAR medium-dose group (40 mg/kg), and NAR high-dose group (80 mg/kg) randomly (n = 12). The specific drug administration and challenge schemes are shown in Fig. 1 A. Before infection of Kpn, mice from drug treatment groups received NAR of the corresponding concentrations by gavage once a day for eight consecutive days; the control group and model group were given normal saline. On day 7, the mice were narcotized by



**Fig. 1.** The therapeutic effect of NAR on Kpn-induced pneumonia in mice. (A) Mice necropsy results indicated that NAR alleviates inflammatory damage in Kpn-induced pneumonia and observation of cell smears in BALF by Swiss-Giemsa staining after Kpn infection. (B) Effect of NAR on white blood cells (WBC) and neutrophils in peripheral blood of mice after Kpn infection. (C) The wet/dry (W/D) lung weight of mice. (D) The total number of cells and neutrophil ratio amount in BALF. Data are presented as means  $\pm$  SEMs;  $n \geq 3$  independent experiments. #  $p < 0.05$ , ##  $p < 0.01$ , ###  $p < 0.001$  vs. control. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$  vs. model group.

the intraperitoneal injection of sodium pentobarbital (50 mg/kg b.w.). The Kpn was injected into the lungs of the mice through endotracheal tubes (100  $\mu$ L for each body; the concentration of a bacterial solution is  $1 \times 10^8$  CFU/mL), and the control group was injected with an equal volume of sterile normal. Three days after infection, blood was collected from the orbital venous plexus for physiological analysis, and then the mice were anesthetized and sacrificed; the different samples were taken for the following steps.

## 2.6. Blood analysis

For blood physiological analysis, the whole blood was analyzed using Abaxis Blood Chemistry Analyzer according to the manufacturer's instructions.

## 2.7. Bronchoalveolar lavage fluid (BALF) preparation and analysis

After the mice were sacrificed, three mice in each group were randomly selected to open the chest cavity and undergo endotracheal intubation; the bronchoalveolar lavage was performed on the lungs with pre-cooled PBS (chilled in the refrigerator at 4  $^{\circ}$ C in advance) for three times, 0.5 mL for each time, and about a total of 1.2 mL lavage solution (80% recovery rate). Then, the BALF was stained with Swiss-Giemsa

solution to account for the total cell and neutrophil using a hemocytometer. Finally, the BALF was centrifuged at 5000 g for 10 min at four  $^{\circ}$ C, and the supernatant was aspirated and set aside.

## 2.8. Lung wet/dry weight ratio (W/D)

The W/D of the lung was assessed for the severity of pulmonary edema induced by Kpn. Precisely, the lung of each group of mice was excised and weighed (weight of wet lung) and then dried in an oven at 70  $^{\circ}$ C until a constant weight was obtained (weight of dry lung), and then the W/D ratio was calculated.

W/D = weight of wet lung (g) / weight of dry lung (g).

## 2.9. Histopathologic examination

The lung tissues of mice treated with different NAR doses were harvested, fixed in 4% formaldehyde for 24 h, embedded in paraffin, and then sliced into four  $\mu$ m-thick sections with a slicer. The sections were stained with hematoxylin-eosin (H&E) or Masson. The microscope (DM 4000B, Leica, Germany) was used for histological observation. The degree of lung injury and inflammatory cell infiltration was recorded using Image J software.

## 2.10. ELISA test

Collecting samples in vivo and in vitro (cell supernatant, lung tissue, and BALF), a commercially available LDH assay kit for detecting the degree of pyroptosis, while C-reactive protein (CRP), IL-1 $\beta$ , IL-6, IL-18 and TNF- $\alpha$  reflected the level of inflammation. All procedures were performed according to the manufacturer's instructions.

## 2.11. Quantitative real-time PCR analysis

Total RNA was isolated from cells or lung tissues of the different groups using the TRIzol reagent. The cDNA was prepared using a PrimeScript RT Master Mix according to the manufacturer's instructions. The expression was quantified using an applied biosystems real-time PCR system with SYBR Premix Ex Taq II. The quantification results were calculated using the  $2^{-\Delta\Delta CT}$  method compared to  $\beta$ -actin as the reference mRNA. The gene-specific oligonucleotide primers used for qPCR:  $\beta$ -actin F: GGTCACCAGGGCTGCTTT,  $\beta$ -actin R: ACTGTGCCGTTGACCTTGC; IL-1 $\beta$  F: GCAGGCAGTATCACTCATTGT, IL-1 $\beta$  R: GGCTTTTGTGTTTCATCTC; NLRP3 F: TCTGCACCCGAGCTGTAAAC, NLRP3 R: CACCCAACTGTAGGCTCTGC; Caspase-1 F: TGCCCAGAGCA-CAAGACTTC, Caspase-1 R: TCCTTGTTCTCTCCACGGC.

## 2.12. Western blotting analysis

The collected lung tissue homogenate or cells were lysed with buffer A PMSF and then centrifuged at 15000 g for 10 min at 4 °C; the supernatants were harvested as the protein extracts, and the concentration of the protein specimens was measured according to bicinchoninic acid assay (BCA). After the preparation of the protein extracts, identical quantities of the protein specimens were detached in 8–12% SDS-PAGE and then electrotransferred into the polyvinylidene difluoride membrane (Millipore, MA, USA). The membranes were sealed with 5% skimmed milk at room temperature for 1.5 h and incubated at 4 °C overnight by the following specific primary antibodies against NF- $\kappa$ B P65 (1:1000), NF- $\kappa$ B p-P65 (1:1000), NLRP3(1:1000), Caspase-1 (1:1000), Pro-Caspase-1(1:1000), IL-1 $\beta$ p31(1:1000), IL-1 $\beta$ p17(1:1000) ASC(1:1000) respectively. Then, HRP-conjugated goat anti-mouse IgG secondary antibody (1:5000) or HRP-conjugated goat anti-rabbit IgG secondary antibody (1:5000) was appended and incubated at 37 °C for one h. Ultimately, the membranes were visualized by the chemiluminescence detection kit and examined with a Biosciences Imager. GAPDH or Lamin-B served as the internal control normalized against the overall proteins. Further, the phosphorylated proteins were normalized against their respective total proteins. The densities of the bands were quantified by Image J software and calculated by the normalization against the densitometric value of the internal loading control.

## 2.13. Immunofluorescence staining

MH-S cells were placed on coverslips in 24-well plates and incubated in 5% CO<sub>2</sub> and humidified air at 37 °C. After treatment, the MH-S cells were fixed with 4% paraformaldehyde for one h and permeabilized with 0.1% Triton X-100 for 30 min at 25 °C. Then, the cells were incubated overnight at 4 °C with P65 primary antibody (1: 100). After three washes with PBS, cells were incubated with the working solution, including Alexa Fluor 488-conjugated goat anti-mouse secondary antibodies (1:1000) at room temperature for one h. 1  $\mu$ g/mL blue-fluorescent DAPI dye was added to the plates for 10 min to stain cell nucleic DNA before the end of incubation. The stained slides were observed and captured under the IF laser scanning confocal microscope with the imaging system (Zeiss Germany, Oberkochen, Germany). Fluorescence images of the stained cells were acquired using the excitation wavelengths of 488 nm (green) and 405 nm (blue).

## 2.14. Co-Immunoprecipitation analysis

MH-S cells in the logarithmic growth phase were taken and inoculated in 6-well plates. After treating the cells according to the experimental grouping, the cell precipitate was collected and added to 800  $\mu$ L of pre-cooled protein lysate. Centrifuge at 12000 r/min for 10 min at 4 °C. The supernatant was collected and transferred to EP tubes for nonspecific binding of Protein G (PG) beads. After mixing for 30 min at 4 °C on a shaker, all the supernatant from each EP tube was transferred to a new EP tube. The ASC antibody was added and incubated overnight at 4 °C on a shaker. The next day, the samples were removed from the four °C shaker, and the appropriate amount of 5  $\times$  SDS-PAGE loading buffer was added. After sufficient mixing, metal bath at 100 °C for 10 min and cooling, the samples were directly analyzed by immunoblotting.

## 2.15. Statistical analysis

GraphPad Prism 8.0 (GraphPad Software Inc., CA, USA) was used for graphing, and SPSS 24.0 was used for statistical analysis (SPSS Inc., Chicago, USA). Values were expressed as mean  $\pm$  SEM. Student's t test was used for comparison of two groups. Differences between three or more groups were analyzed by one-way ANOVA followed by least significant difference (LSD) or Tamhane's T2 post-hoc test.  $p < 0.05$  was considered significant.

## 3. Results

### 3.1. NAR alleviates Kpn-induced pneumonia in mice

A Mice model of Kpn pneumonia was established by tracheal intubation, and the therapeutic effects of NAR at different doses (20, 40, and 80 mg/kg) on Kpn pneumonia were assessed by several parameters. On day three after Kpn infection, the levels of WBCs and neutrophils in the peripheral blood of mice in the model group were significantly higher than those in the control group ( $6.97 \pm 1.46 \times 10^9$  cells/L and  $1.27 \pm 0.52 \times 10^9$  cells/L vs.  $2.57 \pm 0.86 \times 10^9$  cells/L and  $0.04 \pm 0.01 \times 10^9$  cells/L, respectively). After NAR intervention, the levels of WBCs and neutrophils were significantly lowered in a dose-dependent manner; in the NAR high-dose group specifically, these levels were reduced to  $3.79 \pm 0.16 \times 10^9$  cells/L and  $0.47 \pm 0.06 \times 10^9$  cells/L, respectively (Fig. 1B). To visualize the efficacy of NAR, we took out the intact lungs of mice and analyzed them for observation. Postmortem examinations revealed that the control mice had healthy pink lungs. In contrast, the model mice exhibited blunt edema with the formation of red masses and spots that gradually developed into scattered inflammatory lesions in both lungs. By contrast, the NAR treatment groups showed only localized areas of mild solid change, a few sites of red spots, and lesions that appeared to be in remission upon visual observation, with the most pronounced relief in the high-dose group (Fig. 1A). The degree of lung swelling was usually evaluated from the wet/dry lung weight (W/D) in a quarter of the mice in each group. The W/D of lung tissue of mice in the model group was significantly higher than that in the control group; the W/D in the NAR high and medium groups was significantly lower than that in the model group; and the W/D of the NAR high-dose group was close to that in the control group (Fig. 1C). In addition, we examined the number of total cells and percentage of neutrophils (NEs), in the BALF to further assess the anti-infective efficacy of NAR in vivo. BALF smears were used for cell counting by the Swiss-Giemsa staining method after centrifugation. Compared with the control group, dramatic increases in total cells and NEs were found in the BALF of Kpn-infected model mice, reaching  $7.35 \times 10^5$  cells/mL and 72.0%, respectively. After NAR intervention, the total cell count and NEs percentage were significantly reduced, and the degree of reduction was positively correlated with the dose. Specifically, compared with the model group, the total cell count was reduced by almost half in the medium-dose group and was reduced

to  $2.73 \times 10^5$  cells/mL in the high-dose group, while the NEs percentage was reduced to 39.5% in the medium-dose group and 25.5% in the high-dose group (Fig. 1A, D).

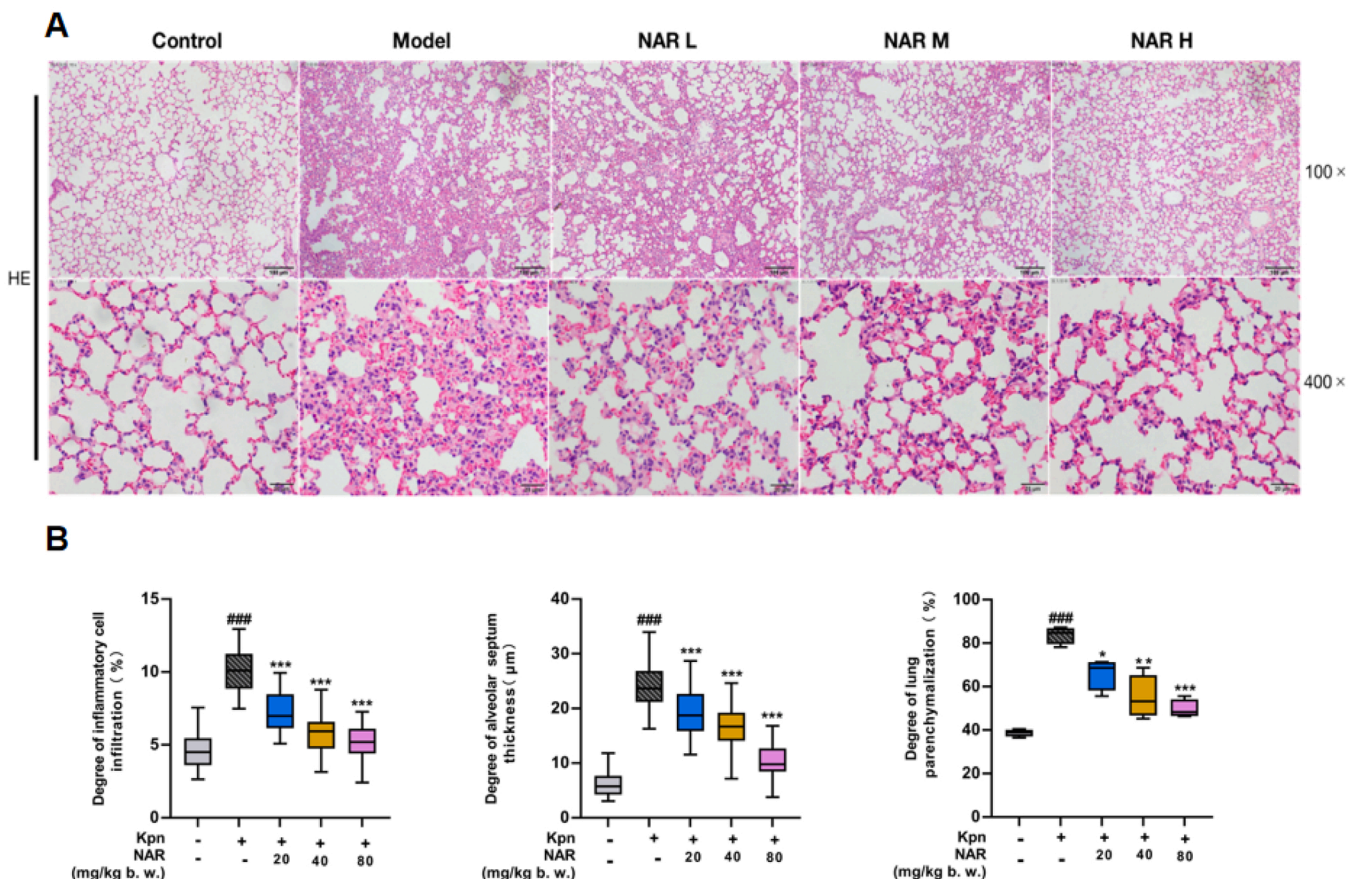
### 3.2. NAR repairs the histopathological injury of lung tissue induced by Kpn in mice

To show the therapeutic effect of NAR Mice lungs in more depth. The lung tissues of each group of mice were stained with hematoxylin and eosin and evaluated for pathological damage. In the control group, the fine bronchial mucosal epithelium and alveolar lumen structures were intact, and the pulmonary lobules were structured. In the model group, neutrophils, red blood cells, and flocculent secretions exuded into the bronchioles, the mucosal epithelium was damaged, and many inflammatory cells infiltrated the surrounding tissues. The alveolar structure was damaged, and hemorrhaging occurred in the alveolar lumen. The alveolar septum was thickened and filled the pulmonary lobules, the blood vessels in the septum were dilated and congested, and the septum was infiltrated with inflammatory cells (Fig. 2A). To show the difference of lung tissue in different groups more objectively, the degrees of lung inflammatory cell infiltration, lung parenchyma, and alveolar thickening in each group of pathological sections were evaluated from calculations of the area ratio of inflammatory cells, the area ratio of lung tissue, and thickness of the alveolar septum (Fig. 2B), respectively, using Image J. This method replaces traditional subjective scoring with specific values, and the calculation method is shown in [Supplementary Material 2](#). The results showed that the area ratios of inflammatory cells

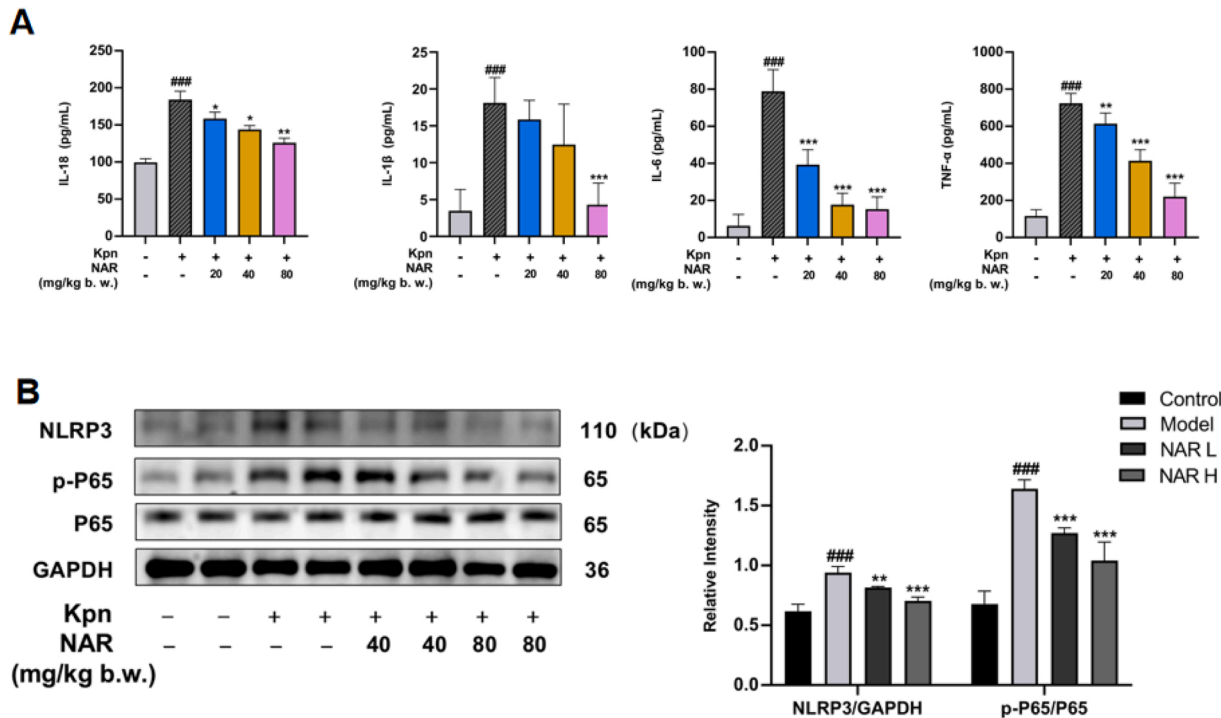
in the control, model, and low-, medium- and high-dose NAR treatment groups were 4.58%, 10.05%, 7.06%, 5.94%, and 5.12%, respectively; those in the lung tissue were 38.70%, 83.27%, 64.55%, 54.10%, and 49.28%, respectively; and the thickness measurements of the alveolar septum were 7.08  $\mu$ m, 22.95  $\mu$ m, 15.98  $\mu$ m, 15.40  $\mu$ m, and 10.27  $\mu$ m, respectively. Statistical analysis showed that compared with the control group, the degrees of inflammatory cell infiltration, lung parenchyma, and alveolar thickening were significantly increased in the lungs of mice in the model group; compared with the model group; these parameters were significantly decreased in a dose-dependent manner in each NAR treatment group.

### 3.3. NAR reduces the pulmonary inflammatory reaction induced by Kpn

To examine the severity of inflammatory response and tissue injury induced by Kpn, the expression of inflammatory factors in lung tissue samples was detected by enzyme-linked immunosorbent assay kits. The results showed that the contents of inflammatory factors IL-18, IL-1 $\beta$ , IL-6, and TNF- $\alpha$  in the lung tissues of the model group were significantly increased (184 pg/mL, 18.12 pg/mL, 78.9 pg/mL, and 723.8 pg/mL, respectively) compared with those in the control group (99.5 pg/mL, 3.47 pg/mL, 6.31 pg/mL and 115.86 pg/mL, respectively). After NAR intervention, the contents of all four inflammatory factors in the lung tissues were significantly decreased, with similar values detected in the high-dose group and the control group (Fig. 3A). Meanwhile, we examined the protein expression levels of NLRP3, P65, and p-P65 in lung tissues by Western blotting to validate the effects of NAR on NLRP3



**Fig. 2.** Effect of NAR on Kpn-induced histopathological damage in mice lung. (A) H&E staining showed that the alveolar wall thickened and inflammatory infiltration in the alveolar interstitium after Kpn infection, and the inflammatory damage was alleviated after NAR intervention (20, 40, or 80 mg/kg.bw). (B) Degree of inflammatory cell infiltration, Degree of lung parenchymalization and the thickness of the alveolar wall. The bar in  $10 \times$  is 100  $\mu$ m and the bar in  $40 \times$  is 20  $\mu$ m. Data are presented as means  $\pm$  SEMs;  $n \geq 3$  independent experiments. #  $p < 0.05$ , ##  $p < 0.01$ , ###  $p < 0.001$  vs. control. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$  vs. model group.

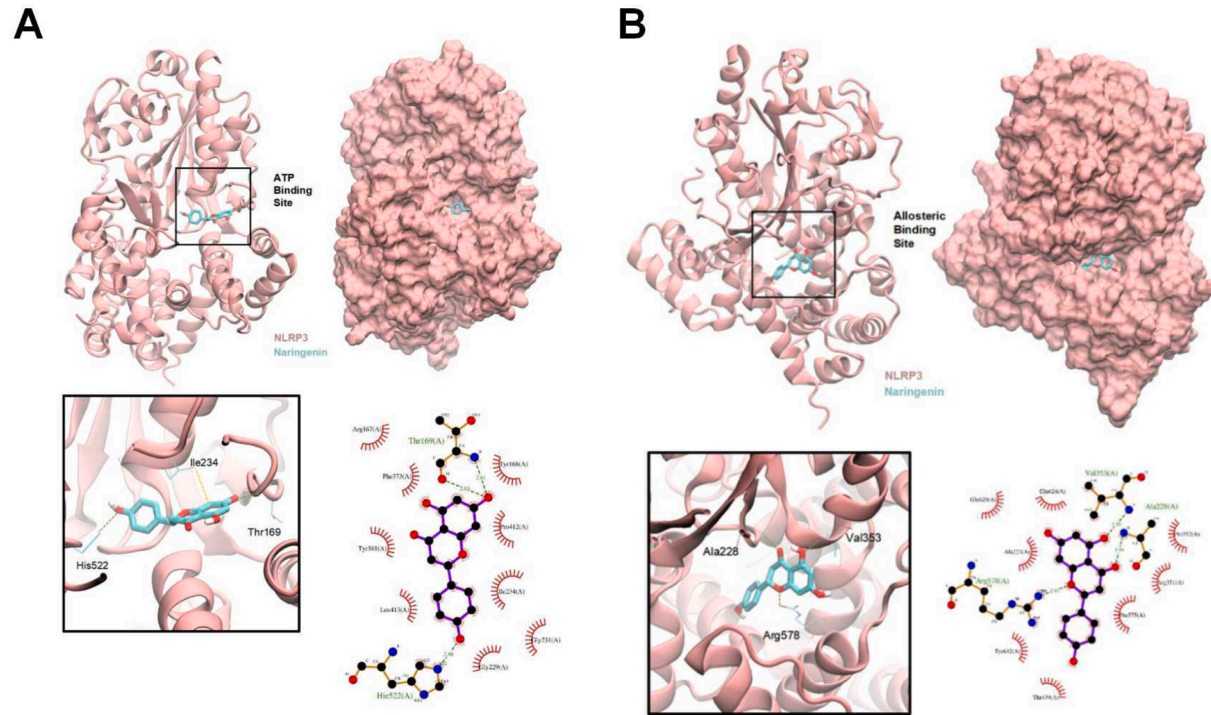


**Fig. 3.** Relief effect of NAR on Kpn-induced lung inflammation in mice. (A) NAR (20, 40, or 80 mg/kg-bw) reduces the elevation of IL-18, IL-1β, IL-6 and TNF-α in lung tissue caused by Kpn pneumonia. (B) Western Blotting analysis of the protein of NLRP3, P65, p-P65 and Relative quantitative protein expression levels indicated that NAR intervention reduces the protein expression of NLRP3/GAPDH and p-P65/P65 in lung tissue. Data are presented as means ± SEMs;  $n \geq 3$  independent experiments. #  $p < 0.05$ , ##  $p < 0.01$ , ###  $p < 0.001$  vs. control. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$  vs. model group.

preliminarily. It was shown that NAR significantly reduced Kpn-induced abnormal elevation of NLRP3 protein and p65 phosphorylation and exerted anti-inflammatory effects (Fig. 3B).

### 3.4. Molecular docking and affinity results of NAR and mice NLRP3 protein

To explore the treatment mechanism of NAR for pneumonia by Kpn-



induced in mice, we hope to discover potential targets of NAR through molecular docking. NLRP3 comprises an N-terminal pyrin effector domain (PYD), a central NACHT domain, and a C-terminal leucine-rich repeat (LRR) domain. The known structure of NLRP3-NACHT shows that there are four subdomains of NACHT: nucleotide-binding domain (NBD), helical domain1 (HD1), winged-helix domain (WHD) and helical domain2 (HD2). The ADP was bound in the nucleotide-binding site (ATP binding site), while a novel allosteric inhibitor, NP3-146, was found to bind to a pocket (allosteric binding site) formed by all four NACHT subdomains. We hypothesized that NAR could bind to the ATP or allosteric binding sites. To test such a hypothesis, a molecular docking study was performed. Molecular docking results revealed that in the docking model of NAR-ATP binding site, hydrogen bonding interactions were formed between NAR and Thr169 and His522 in the ATP binding site, while a solid hydrophobic packing interaction was found between the aromatic ring of NAR core fragment and the side chain of Ile234; In the docking model of NAR-allosteric binding site, hydrogen bonding interactions were observed between NAR and Ala228, Val353, and Arg578, while a weak hydrophobic packing interaction was found between the aromatic ring of NAR core fragment and the side chain of Pro352. NAR was generally well suited for the NLRP3-NACHT ATP or allosteric binding sites (Fig. 4A, B). The calculated binding free energy for NAR bound to the ATP binding site was  $-7.88$  kcal/mol, while that for NAR bound to the allosteric site was  $-6.90$  kcal/mol. Therefore, the docking models support NAR binding to NLRP3-NACHT at either ATP- or allosteric binding sites. In addition, To validate the direct binding between NLRP3 and NAR, the SPR assays were performed. The results showed that NAR could bind to NLRP3 directly. In particular, in the concentration range of  $0.19\text{ }\mu\text{M}$  to  $200\text{ }\mu\text{M}$ , the NAR demonstrated a concentration-dependently increase of signal, and the maximum relative response (RU) was  $16.42$  (Table 1).

3.5. NAR inhibits the nuclear translocation of the transcription factor NF- $\kappa$ B

Based on the in vivo model, we pretreated MH-S cells with different concentrations of NAR. We induced a cellular pneumonia model with Kpn and used this as an in-depth investigation of the action mechanism of NAR. In the priming signal step, the entry of the p-P65 protein into the nucleus is a primary signal for activation of the NLRP3 inflammasome. P65 enters the nucleus and binds to specific sequences on DNA to promote the transcription of NLRP3-related genes. Therefore, it is vital to determine the amount of P65 protein that enters the nucleus. We isolated cytoplasmic and nuclear proteins from cell samples and examined the expression of P65 in both compartments by Western blotting. We found that, following Kpn infection, cytoplasmic P65 gradually decreased and nuclear P65 gradually increased; NAR intervention significantly reversed the amount of P65 protein entering the nucleus (Fig. 5B). The subcellular localization of P65 in the Kpn-induced MH-S cell inflammation model was detected by confocal microscopy, confirming that NAR was able to reduce the transport of P65 from the cytoplasm to the nucleus. As shown in Fig. 5A, NAR ( $30\text{ }\mu\text{g/mL}$ ) significantly reduced the nuclear translocation of P65 from the cytoplasm to the nucleus in Kpn-induced inflammatory MH-S cells compared with that in the model group. The present findings confirm that NAR could reduce the expression of NLRP3 by inhibiting the nuclear translocation of NF- $\kappa$ B.

**Table 1**  
Affinity test between NAR and NLRP3 using SPR assay.

|                                         | 1    | 2    | 3    | 4     | 5     | 6      |
|-----------------------------------------|------|------|------|-------|-------|--------|
| Analyte Concentration ( $\mu\text{M}$ ) | 0.19 | 0.78 | 3.13 | 12.50 | 50.00 | 200.00 |
| Relative response (RU)                  | 0.65 | 1.64 | 1.90 | 2.15  | 4.41  | 16.42  |

3.6. NAR inhibits Kpn-induced over-activation of NLRP3 inflammasome signaling pathway in MH-S cells

To explore the effects of Kpn and NAR on the NLRP3 signaling pathway, we examined the level of NLRP3 signaling pathway factors in MH-S cells. The levels of IL-18 and IL-1 $\beta$  in cell supernatants were measured by Elisa assay. It was detected that the IL-18 and IL-1 $\beta$  contents in the supernatants of cells in the model group were significantly increased after Kpn intervention. At the same time, the NAR pretreatment was able to significantly inhibit the secretion of IL-18 and IL-1 $\beta$  (Fig. 6A). Furthermore, we examined NLRP3 inflammasome signaling pathway-related factors in MH-S cells at gene and protein levels. Compared with the control group, Kpn was able to significantly elevate the mRNA levels of NLRP3 and Caspase-1, as well as increase the protein expression of NLRP3, Pro-Caspase-1, Caspase-1, IL-1 $\beta$  p31, and IL-1 $\beta$  p17 in MH-S cells, and different doses of NAR were able to dose-dependently alleviate the elevated expression of NLRP3, Caspase-1 and other factors induced by Kpn (Fig. 6B, C). It inhibited the activation of the NLRP3 inflammasome pathway.

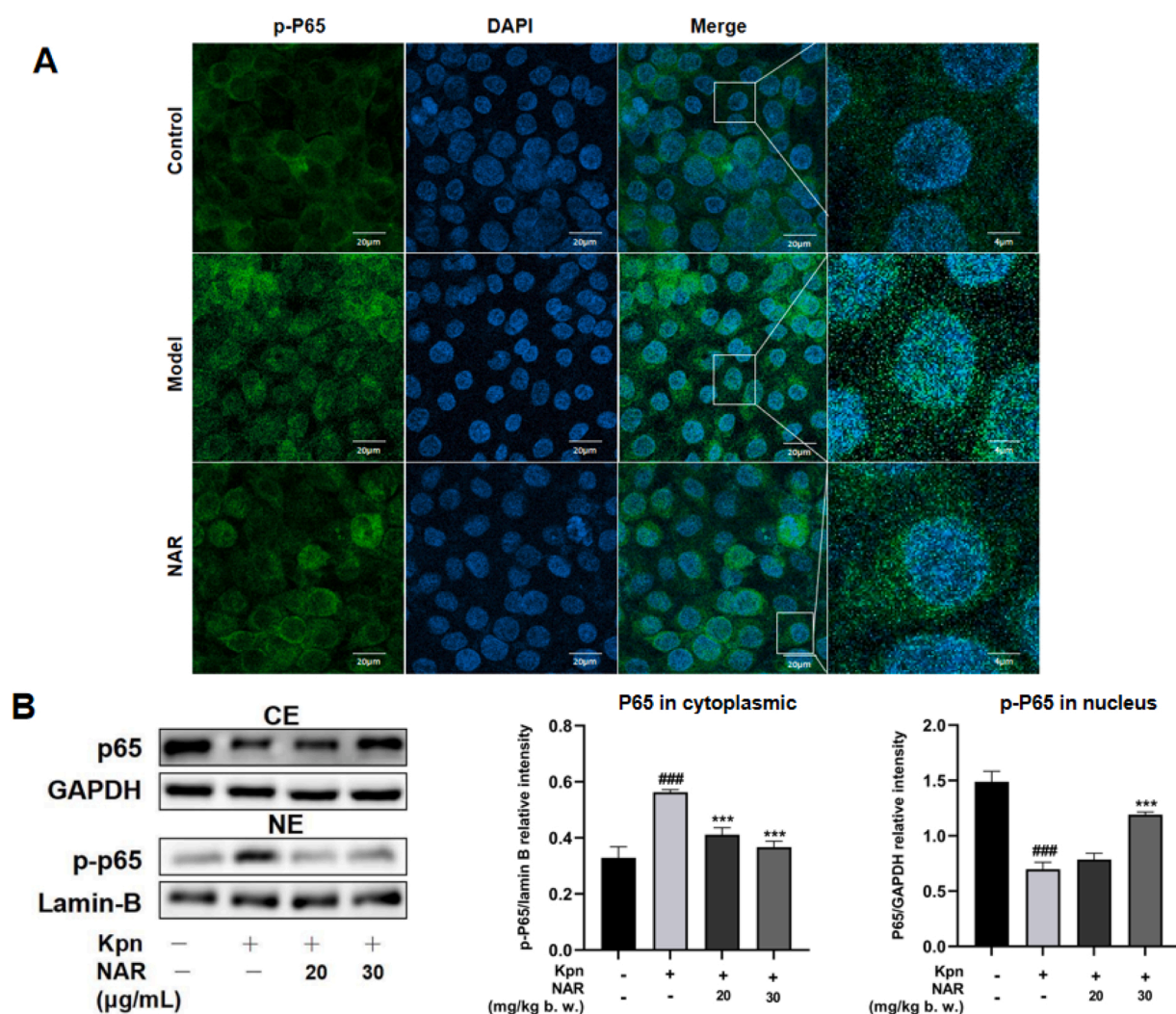
3.7. NAR inhibits the activation and assembly of the NLRP3 inflammasome

In further research, we explored the role of NAR during the activation of NLRP3 in the MH-S cell model and thus revealed the mechanism of NAR's action on NLRP3.  $\text{K}^+$  efflux is one of the standard second signals causing NLRP3 activation, and we found that  $\text{K}^+$  levels were significantly reduced in the cells of the model group. NAR was able to relieve the Kpn-induced decrease in  $\text{K}^+$  content of intracellular, and it was demonstrated that NAR could prevent excess  $\text{K}^+$  efflux with Kpn-induced and thus inhibit the activation of NLRP3 (Fig. 7A). The oligomerization of ASC was critical for the assembly of NLRP3 inflammasome. In the present research, it was detected that the level of ASC oligomerization was significantly increased in the MH-S inflammatory cell model induced by Kpn. At the same time, pretreatment with NAR was able to significantly reduce ASC oligomerization (Fig. 7B). Since the function of the inflammasome is dependent on the assembly of NLRP3, ASC and Caspase1 complex proteins, we further probe the assembly of NLRP3, Caspase-1, and ASC protein by Co-IP technique, the results showed that the levels of NLRP3 and Caspase-1, which interacts with ASC were increased by Kpn induction, whereas after intervention of NAR the NLRP3 and Caspase-1 were restricted interacted with ASC, thus inhibit NLRP3 inflammasome assembly (Fig. 7C).

4. Discussion

Considered an important zoonotic pathogen, Kpn has been identified as one of the leading causes of hospital-acquired infections in the USA. Meanwhile, Kpn has been identified as the third most common cause of hospital-acquired pneumonia [22]. Additionally, Kpn is the leading cause of ventilator-associated pneumonia among intensive care unit patients [23], for which the mortality rate can reach 50% [1]. As a result, lung diseases induced by Kpn pose a heavy burden on public healthcare. Furthermore, over the past few decades, the inappropriate use of antibiotics in medicine and agriculture has made Kpn one of several bacteria that have shown a dramatic increase in antibiotic resistance [36], posing a severe threat to human and animal health. Therefore, searching for new plant sources of complementary treatment strategies has gradually become a research focus. NAR is enriched in Exocarpium Citri Grandis but is also widely found in grapefruits, tomatoes, beans, cherries, and many other citrus fruits and is relatively easy to extract [24]. The exploration of the biological activity of NAR has attracted much interest from scholars because of its advantages, the low cost of acquisition and safety of use.

Regarding the treatment of lung diseases, there have been several reports demonstrating the remarkable pharmacological activity of NAR



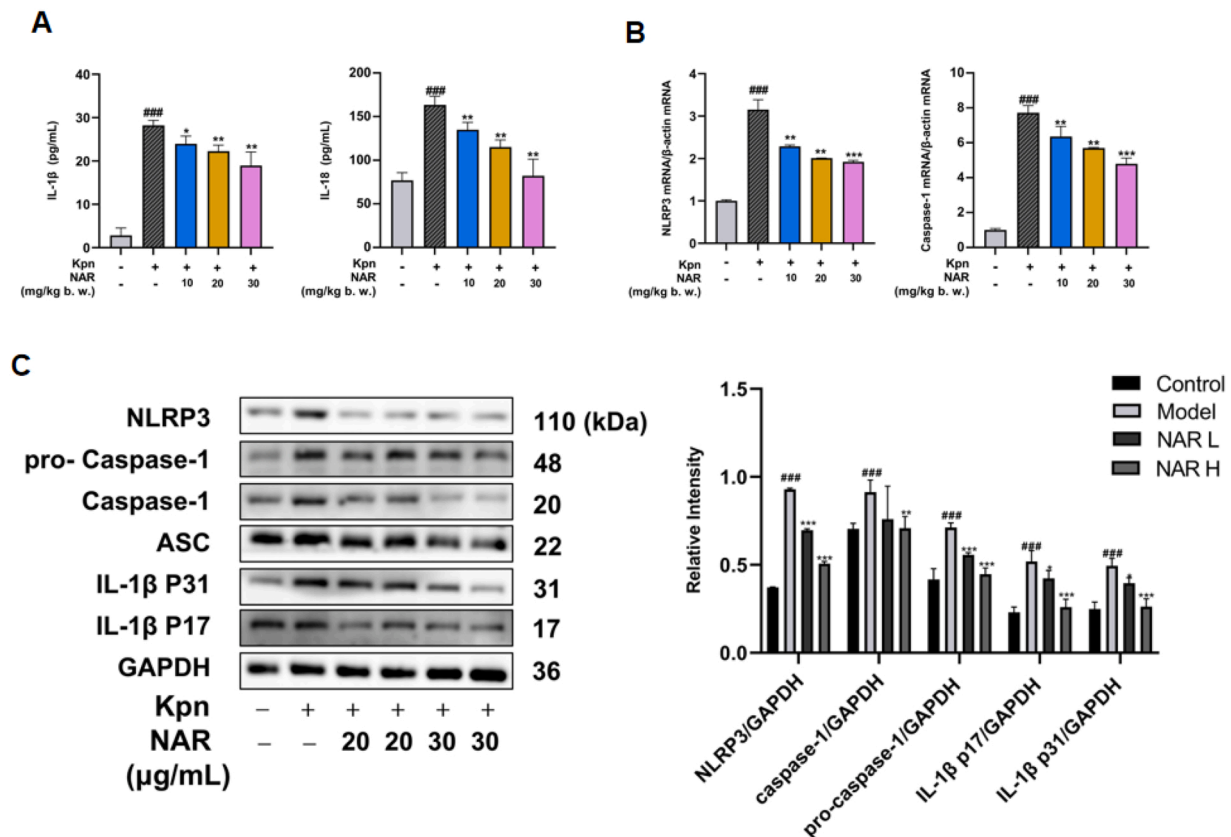
**Fig. 5.** Antagonistic effect of NAR on nuclear translocation of P65. (A) The confocal microscope shows NAR (30 μg/mL) significantly reduced the nuclear translocation of P65 from the cytoplasm to the nucleus in Kpn-induced inflammatory MH-S cells. One-way ANOVA statistical tests and Tukey's post hoc analysis were used. (B) Western Blotting analysis showed a P65 protein decrease in the cytoplasm and an increase in the nucleus after kpn infection, while NAR intervention significantly reversed the amount of P65 protein entering the nucleus, and the relative quantitative protein expression levels. Data are presented as means ± SEMs;  $n \geq 3$  independent experiments. #  $p < 0.05$ , ##  $p < 0.01$ , ###  $p < 0.001$  vs. control. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$  vs. model group.

through different lung disease models [25]. While NAR has been shown to inhibit the growth of Kpn and control the formation of bacterial biofilms in vitro, the regulation of innate immunity, reversal of excessive inflammation, and repair of pathological damage have become hot topics in the study of natural products to treat pneumonia [26–29]. This study provides the research basis for the development of natural drugs for the treatment of lung diseases induced by bacteria.

During the initial phase of Kpn infection, moderate expression of chemokines or pro-inflammatory cytokines to attract leukocytes such as monocytes and neutrophils to the lung helps clear pathogens [30]. However, an excessive inflammatory response can lead not only to severe lung cases [4], in which the main clinical manifestations are increased WBCs in peripheral blood, as well as pulmonary edema and inflammatory damage to lung tissue due to the infiltration of inflammatory factors [3]. Our results suggested that NAR not only reduces the number of WBCs and neutrophils in the peripheral blood of Kpn-infected mice but also significantly reduces the secretion of inflammatory cytokines IL-18, IL-1β, IL-6, and TNF-α in lung tissue, thereby playing a significant anti-inflammatory role. BALF is often used in the clinical diagnosis of various lung diseases because it allows for assessing large alveolar compartments by providing cells and non-cellular constituents

from the lower respiratory tract [31]. Additionally, changes in the number and proportion of leukocytes in BALF reflect pathological changes in the lung parenchyma [32]. The results of this study suggested that NAR administration remarkably reduced the large number of neutrophils induced by Kpn in BALF. Pathologic results showed that NAR can relieve lung tissue damage, reduce the infiltration of inflammatory cells in the alveoli, and restore the structure of the alveolar wall to normal, indicating that NAR has an inhibitory effect on tissue inflammation and injury.

After inducing a Mice pneumonia model by Kpn and demonstrating the efficacy of NAR, we set out to elucidate the anti-inflammatory mechanism of NAR. Activation of NLRP3 inflammasome leads to secretion of IL-1β family cytokines and induces increased inflammation. As a result of the critical role of NLRP3 in inflammatory diseases, it is recognized as a key target for drugs to alleviate inflammation [33]. In the present research, we found by molecular docking technique that NAR was well suited for the NLRP3-NACHT ATP binding site or allosteric binding site. NLRP3 was theoretically demonstrated to be one of the targets of NAR action. Moreover, the results of the SPR assay showed that NAR could bind to NLRP3 directly. In particular, in the concentration range of 0.19 μM to 200 μM, the NAR demonstrated a



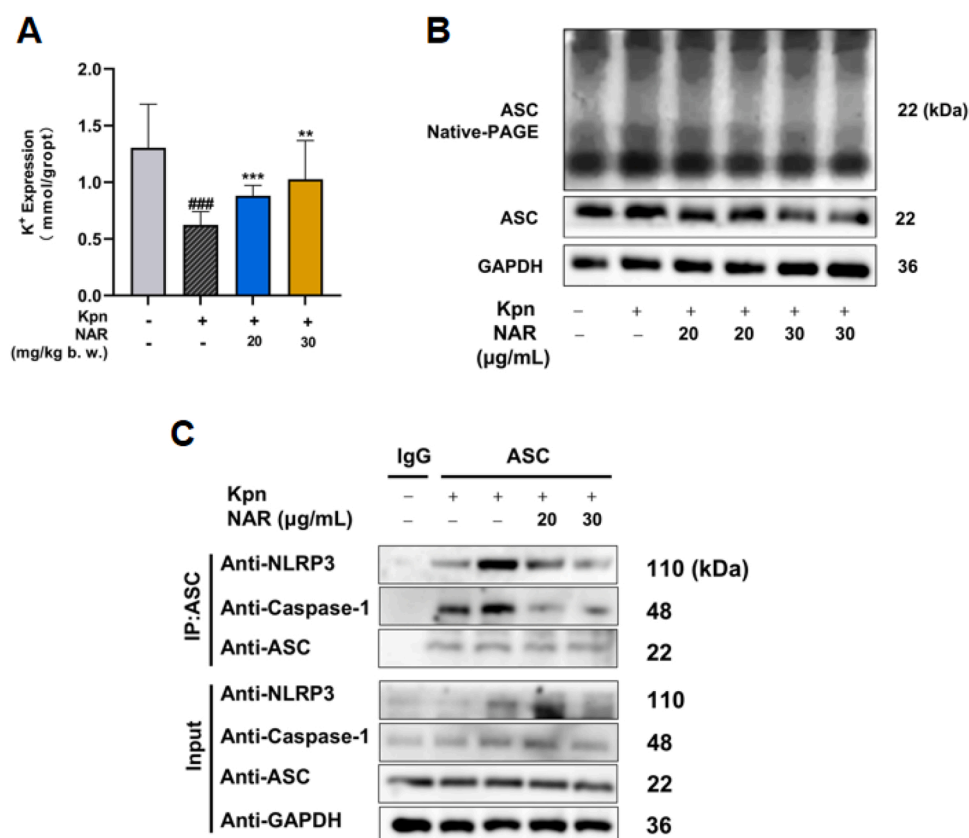
**Fig. 6.** Inhibitory effect of NAR on Kpn-induced NLRP3 overactivation in MH-S cells. (A) NAR pretreatment reduces the levels of IL-18 and IL-1β in the supernatant of Kpn-infested MH-S cells. (B) NAR decreased NLRP3 and Caspase-1 in MH-S cells. (C) Relative quantitative protein expression levels indicated that NAR intervention reduce the protein expression of NLRP3, pro-Caspase-1, Caspase-1, ASC, IL-1β p31, and IL-1β p17. Data are presented as means ± SEMs;  $n \geq 3$  independent experiments.  $\# p < 0.05$ ,  $\#\# p < 0.01$ ,  $\#\#\# p < 0.001$  vs. control.  $* p < 0.05$ ,  $** p < 0.01$ ,  $*** p < 0.001$  vs. model group.

concentration-dependently increase of signal, and the maximum relative response (RU) was 16.42. Meanwhile, we further found in a Kpn-induced mice pneumonia model that NLRP3 protein expression in the lung tissues of mice was significantly reduced under the intervention of NAR. The inhibitory effect of NAR on NLRP3 was demonstrated preliminarily. Based on the results of the in vivo model, we constructed an ex vivo model using Kpn-infested MH-S cells to show the mechanism of NAR action on NLRP3 properly. In this study, we found that the gene expression of NLRP3 and Caspase-1, as well as the protein content of NLRP3, pro-Caspase-1, Caspase-1, IL-1β p31, and IL-1β p17, were significantly elevated in Kpn-infested MH-S, while the levels of IL-18, and IL-1β were also significantly increased in the cell supernatant. These results suggest that Kpn can activate the NLRP3 signaling pathway in MH-S cells.

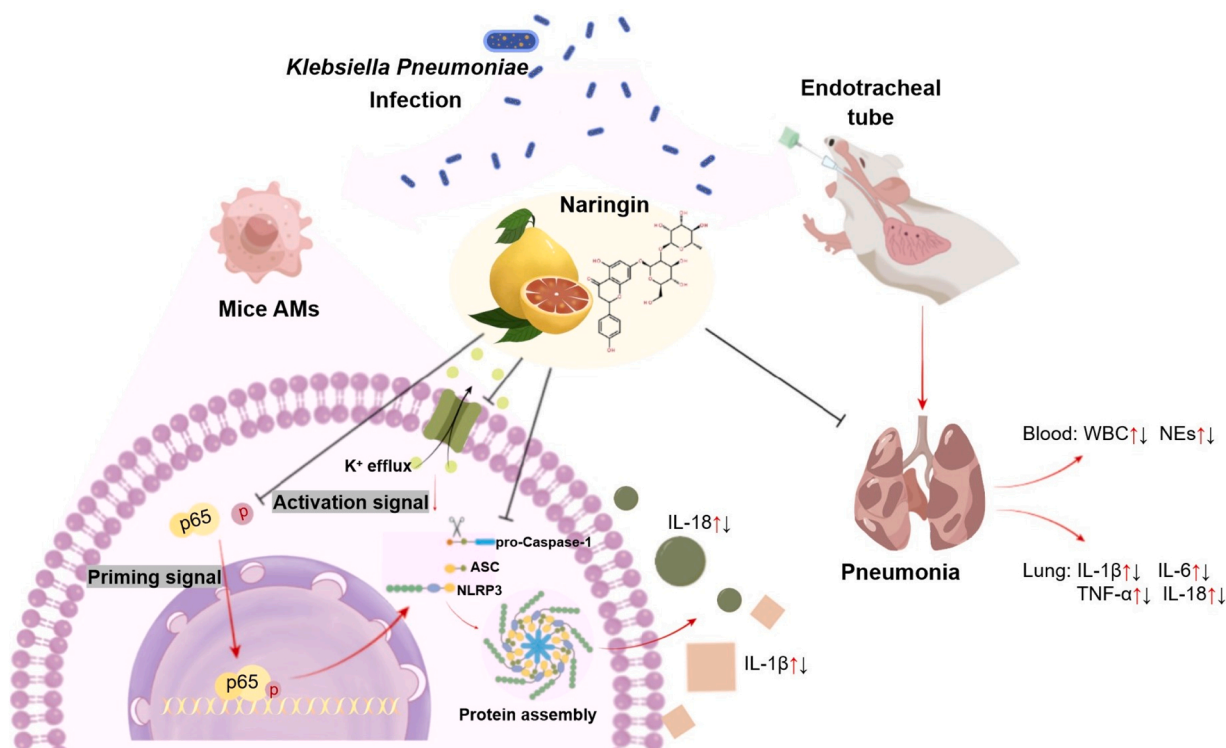
In contrast, NAR pretreatment significantly alleviated the stimulation of NLRP3 in MH-S cells by Kpn and inhibited the elevated expression of factors such as NLRP3, Caspase-1, and IL-1β. The classical activation of NLRP3 is recognized to experience two significant transduction steps: Priming signaling and activation signaling. We investigated the nuclear transcription of p-P65 and  $K^+$  efflux in MH-S cells to probe the inhibitory role of NAR in NLRP activation. The protein expression of P65 and p-P65 in cytoplasm and nucleus was detected by Western blot, respectively, and it was found that the content of P65 in the cytoplasm was decreased after Kpn intervention, the content of p-P65 in the nucleus was significantly increased, and the phosphorylation level of P65 was significantly increased. In contrast, NAR reduced the protein expression of p-P65 in the nucleus and inhibited the phosphorylated nuclear translocation of P65. Meanwhile, indirect immunofluorescence results revealed that P65 in MH-S cells significantly aggregated in the nucleus under the intervention of Kpn.

In contrast, this phenomenon was significantly suppressed in NAR-treated cells, confirming our conclusion. Excess  $K^+$  efflux can cause the conformational change of NLRP3 and thus activate NLRP3. In the present research, it was found that  $K^+$  expression in MH-S cells showed a significant decrease under Kpn stimulation, and NAR was able to resist Kpn-induced  $K^+$  efflux. These results demonstrated that NAR inhibited the activation of NLRP3 at multiple stages to reduce the activation level of the NLRP3 signaling pathway. On the other hand, the assembly of NLRP3 inflammasome is a prerequisite for its efficacy. Dimers formed by ASC oligomerization play a linking role in NLRP3 inflammasome complex proteins [34]. Therefore, we demonstrated the effect of NAR on the assembly process of NLRP3 inflammasome by detecting the oligomerization level of ASC. The research results showed that pretreatment with NAR reduced the elevated levels of oligomerized ASC in MH-S cells after the intervention of Kpn, which demonstrated the inhibitory effect of NAR on the assembly of NLRP3 inflammasome. To further improve the reliability of our conclusions, we obtained similar results by Co-IP, NLRP3 and caspase-1, which interacted with ASC and were significantly increased after Kpn infestation. At the same time, NAR was shown to block the association between NLRP3, ASC, and caspase-1, acting as an inhibitor of the assembly of the NLRP3 inflammasome.

In short, NAR attenuated Kpn-induced pneumonia and was associated with inhibiting inflammatory mediator production and NLRP3 signaling pathway activation. Specifically, NAR decreased the production of WBCs and NEs in peripheral blood and inhibited the number of NEs in BALF, reducing IL-1β, IL-6, and TNF-α expression and inhibiting activation of the NLRP3 signaling pathway and assembly of NLRP3 inflammasome. In this research, NLRP3 was identified as one of the binding targets of NAR through molecular docking and affinity assay. We initially found that NAR could alleviate lung inflammation by



**Fig. 7.** Suppression of NLRP3 inflammasome activation by NAR. (A) The level of K<sup>+</sup> efflux in MH-S cells was significantly decreased after treatment with NAR. (B) Relative quantitative protein expression levels indicated that NAR intervention reduced the protein expression of Native-ASC, and Kpn-induced ASC oligomerization was inhibited. (C) NAR reduces the expression of NLRP3 and Caspase-1, which interact with ASC under the action of Kpn, in MH-S cells. Data are presented as means  $\pm$  SEMs;  $n \geq 3$  independent experiments. #  $p < 0.05$ , ##  $p < 0.01$ , ###  $p < 0.001$  vs. control. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$  vs. model group.



**Fig. 8.** The underlying mechanism of NAR attenuates pneumonia induced by Kpn.

inhibiting NLRP3 expression in a Kpn-induced animal model. Afterward, we analyzed the mechanism of action of NAR in the MH-S cell model. We found that NAR was able to achieve its inhibitory effect on NLRP3 inflammasome through multiple pathways, including inhibiting the nuclear translocation of p-P65, preventing K<sup>+</sup> efflux, and preventing ASC dimerization from blocking the assembly of NLRP3 inflammasome. Taken together, the results of this study suggest that NAR has a significant therapeutic effect on lung diseases under Kpn induction, with NLRP3 being an essential target for NAR's effect. NAR has the potential as a drug for lung inflammation (Fig. 8).

## 5. Conclusions

In recent years, developing plant-derived immunomodulators with anti-inflammatory functions has become a new trend [35]. The results of this study expand our understanding of the therapeutic effects of NAR on bacterial pneumonia and provide a theoretical basis for the research and development of NAR as a potential therapeutic drug. In this study, the protective effect of NAR on Kpn-induced inflammation was clarified by in vivo and in vitro assays and the mechanism of action of NAR in inhibiting NLRP3 inflammasome, and thus kpn-induced pneumonia, through multiple pathways.

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## CRediT authorship contribution statement

**Zou Wenshu:** Formal analysis. **Huang Lina:** Visualization, Methodology. **Wang Guibo:** Resources, Methodology. **Huang Qilin:** Methodology, Formal analysis, Conceptualization. **Jing Xiaohan:** Writing – original draft, Methodology. **Zhao Guanyu:** Writing – original draft, Software, Methodology, Data curation. **Qiu Zhengying:** Writing – review & editing, Visualization, Software, Funding acquisition, Conceptualization. **Xin Ruihua:** Writing – review & editing, Writing – original draft, Validation, Project administration, Funding acquisition, Data curation, Conceptualization. **Li Wei:** Writing – review & editing, Investigation.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.biopha.2023.116028](https://doi.org/10.1016/j.biopha.2023.116028).

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