

# Effects of Z-VaD-Ala-Asp-Fluoromethyl Ketone (Z-VAD-FMK) and Acetyl-Asp-Glu-Val-Asp-Aldehyde(Ac-DEVD-CHO) on Inflammation and Mucus Secretion in Mice Exposed to Cigarette Smoke

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**Background and Objectives:** Smoking can lead to airway inflammation and mucus secretion through the nucleotide-binding domain-like receptor protein 3/caspase-1 pathway. In this study, z-VaD-Ala-Asp-fluoromethyl ketone(Z-VAD), a pan-caspase inhibitor, and acetyl-Asp-Glu-Val-Asp-aldehyde(Ac-DEVD), a caspase-3 inhibitor, were used to investigate the effect of caspase inhibitors on the expression of interleukin(IL)-1 $\beta$  and IL-8, airway inflammation, and mucus secretion in mice exposed to cigarette smoke(CS).

**Methods:** Thirty-two C57BL/6J male mice were divided into a control group, Smoke group, Z-VAD group, and Ac-DEVD group. Except for the control group, the animals were all exposed to CS for three months. After the experiment, lung function was measured and hematoxylin and eosin staining and periodic acid-Schiff staining were performed. The levels of IL-1 $\beta$ , IL-8, and mucin 5ac (Muc5ac) in serum and bronchoalveolar lavage fluid(BALF) were determined by enzyme-linked immunosorbent assay.

**Results:** Compared with the control group, the lung function of mice exposed to smoke was poorer, with a large number of inflammatory cells infiltrating around the airway, collapse of alveoli, expansion and fusion of distal alveoli, and formation of emphysema. The Z-VAD group was relieved compared with the smoke group. Airway inflammation was also reduced in the Ac-DEVD group compared with the Smoke group, but the degree of emphysema was not significantly improved. Although Z-VAD relieved airway inflammation and emphysema, Ac-DEVD only relieved inflammation. Z-VAD and Ac-DEVD decreased serum IL-1 $\beta$  and IL-8 levels. In BALF, IL-1 $\beta$  was decreased in Z-VAD group and IL-8 was highest in Smoke +Ac-DEVD group compared with control group and Ac-DEVD group. There was no significant difference in the expression of Muc5ac in serum. However, in BALF, levels of Muc5ac were higher in the smoking group and the lowest in the Ac-DEVD group.

**Conclusion:** Mice exposed to smoke had decreased lung function and significant cilia lodging, epithelial cell shedding, and inflammatory cell infiltration, with significant emphysema formation. The pan-caspase inhibitor, Z-VAD, improved airway inflammation and emphysema lesions in the mice exposed to smoke and reduced IL-1 $\beta$  and IL-8 levels in serum. The caspase-3 inhibitor, Ac-DEVD, reduced airway inflammation, serum IL-1 $\beta$  and IL-8 levels, and Muc5ac levels in BALF, but it did not improve emphysema.

**Keywords:** cigarette smoke, Z-VAD-FMK, Ac-DEVD-CHO, inflammation, mucin 5ac

## Introduction

Chronic obstructive pulmonary disease (COPD) is a common, preventable, and treatable disease characterized by persistent airflow limitation and respiratory symptoms. Airway and/or alveolar abnormalities caused by significant exposure to harmful particles or gases are the main causes of COPD.<sup>1,2</sup> Moreover, emphysema, remodeling, airway inflammation and high mucus secretion are the main pathological basis of COPD.<sup>3-5</sup> Previous studies detected higher oxidative and inflammatory stress in COPD patients.<sup>5</sup> In addition, several mechanisms, including excess production due

to inflammation, decreased elimination due to impaired ciliary clearance, and reduced coughing efficiency, have been associated with mucus secretion.<sup>6,7</sup> In patients with COPD who smoke, this condition can be further aggravated; cigarette smoke (CS) impairs mucociliary transport by inducing airway dehydration and increasing mucus viscosity.<sup>4,8</sup> However, the exact molecular mechanism associated with this process (ie, inflammation and mucus production, particularly when associated with CS in patients with COPD) is still not well understood.

The nucleotide-binding domain-like receptor protein 3 (NLRP3) inflammasome is an important pattern recognition receptor within the cytoplasm. Studies have found that cigarettes can activate NLRP3 and caspase-1 and promote the expression and release of interleukin (IL)-1 $\beta$  and IL-18, resulting in inflammatory outbursts, which can lead to emphysema and mucus cell proliferation, thus promoting the formation of COPD.<sup>9–11</sup> However, other studies<sup>12</sup> have shown that NLRP3 inflammatory bodies have no inhibitory effect on the pathogenesis of COPD, so further research is needed in this area, and these studies<sup>9–12</sup> further support the studies rationale to improve our understanding of COPD pathogenesis and targeting downstream signaling mediators rather than NLRP3 itself may be important. In addition, the activation of NLRP3/caspase-1 activates caspase-3 expression and promotes the expression of IL-8,<sup>13</sup> and IL-8 promotes mucin 5ac (Muc5ac) expression in airway epithelial cells in a concentration-dependent manner.<sup>14</sup> However, the correlation between the NLRP3 pathway and IL-8 has not been clearly demonstrated.

In this study, the pan-caspase inhibitor z-VaD-Ala-Asp-fluoromethyl ketone (Z-VAD) and the caspase-3 inhibitor acetyl-Asp-Glu-Val-Asp-aldehyde (Ac-DEVD) were used to investigate the effect of caspase inhibition on IL-1 $\beta$  and IL-8 expression, airway inflammation, and mucus secretion in mice exposed to CS.

## Materials and Methods

### Animals

Thirty-two 6–7-week-old male C57BL/6J mice were obtained from the GemPharmatech Co., Ltd, China (Certificate no: 202104256). All the animals had been bred in the animal facility at the JRDUN Biotechnology (a branch of Shanghai Rat & Mouse Biotech Co., Ltd) and were housed in an environment with a temperature of 22°C  $\pm$  1°C, a relative humidity of 50%  $\pm$  1%, and a light/dark cycle of 12/12 h, where they were fed and watered ad libitum. All the studies (including the mice euthanasia procedure) were conducted in compliance with the regulations and guidelines of the Biomedical Research Ethics Committee of Zhongshan Hospital Wusong Branch, Fudan University and carried out according to the guidelines of National Institutes of Health Guide for the Care and Use of Laboratory Animals.<sup>15</sup>

### Cigarette Smoke Exposure

The mice were randomly divided into four groups (eight mice/group): a control group, Smoke group (only exposed to CS), Z-VAD group (exposed to CS + Z-VAD), and Ac-DEVD group (exposed to CS + Ac-DEVD). Except for the control group, the animals were exposed to CS from five cigarettes (tar: 13 mg/cig; nicotine: 1.0 mg/cig; carbonic oxide: 14 mg/cig; Daqianmen, Shanghai, China) 15 minutes every time, four times a day, and the two smoke periods had a 30-minute interval. Cigarette smoke exposure was performed every two days for three months.

### Administration of Caspase-1 and Caspase-3 Inhibitors

The pan-caspase inhibitor Z-VAD (Beyotime, Shanghai, China) and caspase-3 inhibitor Ac-DEVD (Beyotime, Shanghai, China) were reconstituted in dimethyl sulfoxide and diluted according to the supplier's instructions. Both compounds were administered at a dose of 3 mg/kg intraperitoneally 1 h before CS exposure (every other day).

### Pulmonary Function Tests

After three months of smoke exposure, lung function was evaluated with an invasive lung function instrument to measure total airway resistance (lung resistance), lung dynamic compliance, and maximum minute ventilation (MVV). The mice were anesthetized using an intraperitoneal injection of 1% pentobarbital sodium (50 mg/kg). After the neck skin was disinfected, it was cut, and the subcutaneous tissue was separated with blunt dissection. The trachea was exposed, a transverse incision was made, and a venous cannula needle (about 5–6 mm deep) was implanted and fixed with

a suture. The mice were then placed in a body plethysmograph box (AniRes2005, Beilanbo, Beijing, China) and connected to a small animal ventilation machine for mechanical ventilation. The following parameters were used: tidal volume (7 mL/kg), respiratory frequency (90 times/min), and inspiratory-to-expiratory ratio (10:20). Once the ventilation was set up, the lung ventilation function was measured.

## Lavage Procedures

After the lung function test was complete, the mice were euthanized, their chests were opened up, and the left main bronchus was ligated. A volume of 0.7 mL of saline was injected into the right lung for alveolar lavage three times. The recovery was about 60%–70%. The BALF was directly placed in a fully automated blood sphere analyzer for cell counting and classification, after which it was centrifuged at  $1000 \text{ r} \cdot \text{min}^{-1}$  for 10 min. The supernatant was then collected and stored at  $-80^{\circ}\text{C}$  for an enzyme-linked immunosorbent assay (ELISA).

## Morphometric Measurements of Airway and Lung Tissue

The left lung upper lobe tissue was fixed in 4% formaldehyde for 24 h, paraffin embedded, cut into 5- $\mu\text{m}$ -thick sections, and stained with hematoxylin and eosin (Biovisualab, Shanghai, China). Inflammatory cell infiltration and bronchial tube pulmonary alveolus structural changes were verified by histopathology. The alveolar intercept was measured randomly at five high-magnification visual fields for each pathological piece, avoiding the airway and blood vessels, and the number of alveoli on the cross wire was counted. The average alveolar intercept (AAI) was calculated as the wire length divided by the number of alveoli. Periodic acid–Schiff (PAS) staining was performed to detect mucus secretion in the airways.

## Serum and Bronchoalveolar Lavage Fluid, Interleukin-1 $\beta$ , Interleukin-8, and Mucin 5ac Levels

Eye blood samples were collected, and a total of 0.8–1 mL of blood was centrifuged at  $4000 \text{ r} \cdot \text{min}^{-1}$  for 10 min. The supernatant was removed and stored at  $-80^{\circ}\text{C}$  for testing. R&D Systems (USA) supplied ELISA kits for detecting the IL-1 $\beta$ , IL-8, and Muc5ac levels, and the serum and BALF supernatant ELISA detection was performed according to the manufacturer's instructions.

## Statistical Analysis

Statistical processing was performed using SPSS software (version 22.0). The graphics were performed using GraphPad Prism (version 5.0). Measurement data obeying the normal distribution were analyzed using analysis of variance, and the data were presented as mean  $\pm$  standard deviation for each experimental group. Data with a non-normal distribution were analyzed using the Kruskal–Wallis *H*-test and presented as quartiles.  $P < 0.05$  was considered statistically significant.

## Results

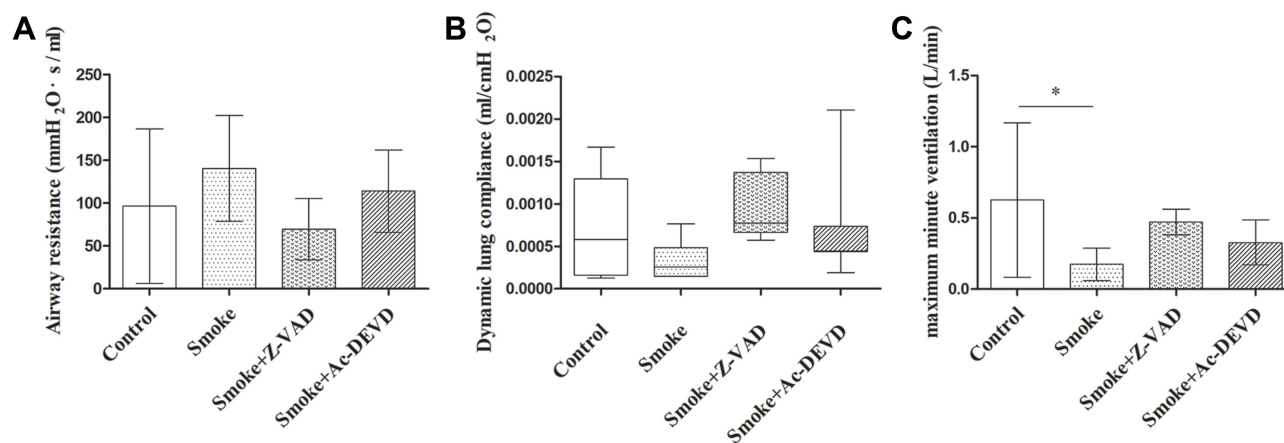
### General Conditions

Compared to control mice, the smoke-exposed animals clustered together more, reacted more slowly, were lethargic and had dull fur. Two of the mice in the Z-VAD group died.

### Pulmonary Functional Assessments

The mice in the Smoke group showed increased lung airway resistance and decreased lung compliance, but the difference was not statistically significant compared with the other three groups ( $P > 0.05$ , Figures 1A and B). The Z-VAD group showed lower airway resistance and greater compliance than the control and Smoke groups. The Ac-DEVD group exhibited higher airway resistance than the control group, but there was no significant difference ( $P > 0.05$ , Figures 1A and B).

In addition, the MVV was lower in the Smoke group than in the control group ( $P = 0.015$ , Figure 1C), and the MVV in the Z-VAD and Ac-DEVD groups was higher than in the Smoke group, but there was no significant difference.



**Figure 1** Lung function assessment after smoke exposure and treatment with caspase-1 inhibitor Z-VAD-fluoromethylketone or the caspase-3 inhibitor Ac-DEVD-CHO. (A) Airway resistance was higher in the Smoke group and lower in the Z-VAD group but with  $P$  value  $>0.05$ . (B) Dynamic lung compliance decreased in Smoke group and increased in the Z-VAD group compare with the control. (C) MVV were lower in the Smoke group. Values are mean  $\pm$  SD for airway resistance and MVV. Values are quartiles for dynamic lung compliance, and  $n = 6, 6, 5,$  and  $7$  for each group.  $*P < 0.05$ .

## Pathological Assessments: Inflammation and Emphysema

The control mice had normal bronchial mucosa, neat cilia, few lumen secretions, no significant inflammatory cell infiltration around the airway, alveoli of uniform size, no significant alveolar collapse, and no thickening of the alveolar interval (Figures 2A–C). The airways of the Smoke group were deformed, with obvious epithelial cell shedding, cilia lodging, more secretions in the lumen, a large amount of inflammatory cell infiltration (lymphocytes, neutrophils) surrounding the airway, alveolar collapse around the trachea, distal alveolar expansion and fusion, and the formation of emphysema (Figures 2D–F). In the Z-VAD group, there was still a certain degree of inflammatory cell infiltration and emphysema formation, visible secretions in the airway, and some ciliary lodging, but it was less than that in the Smoke group (Figures 2G–I). The mice in the Ac-DEVD group also showed reduced airway inflammation compared with the Smoke group, but no significant improvement in the degree of emphysema (Figures 2J–L).

The AAI was shorter in the control group than in the other three groups ( $P < 0.001$ ), and it was shorter in the Z-VAD group than in the Smoke group ( $P = 0.026$ ), but it was not significantly shorter in the Ac-DEVD group than in the Smoke group ( $P = 0.056$ , Figure 3).

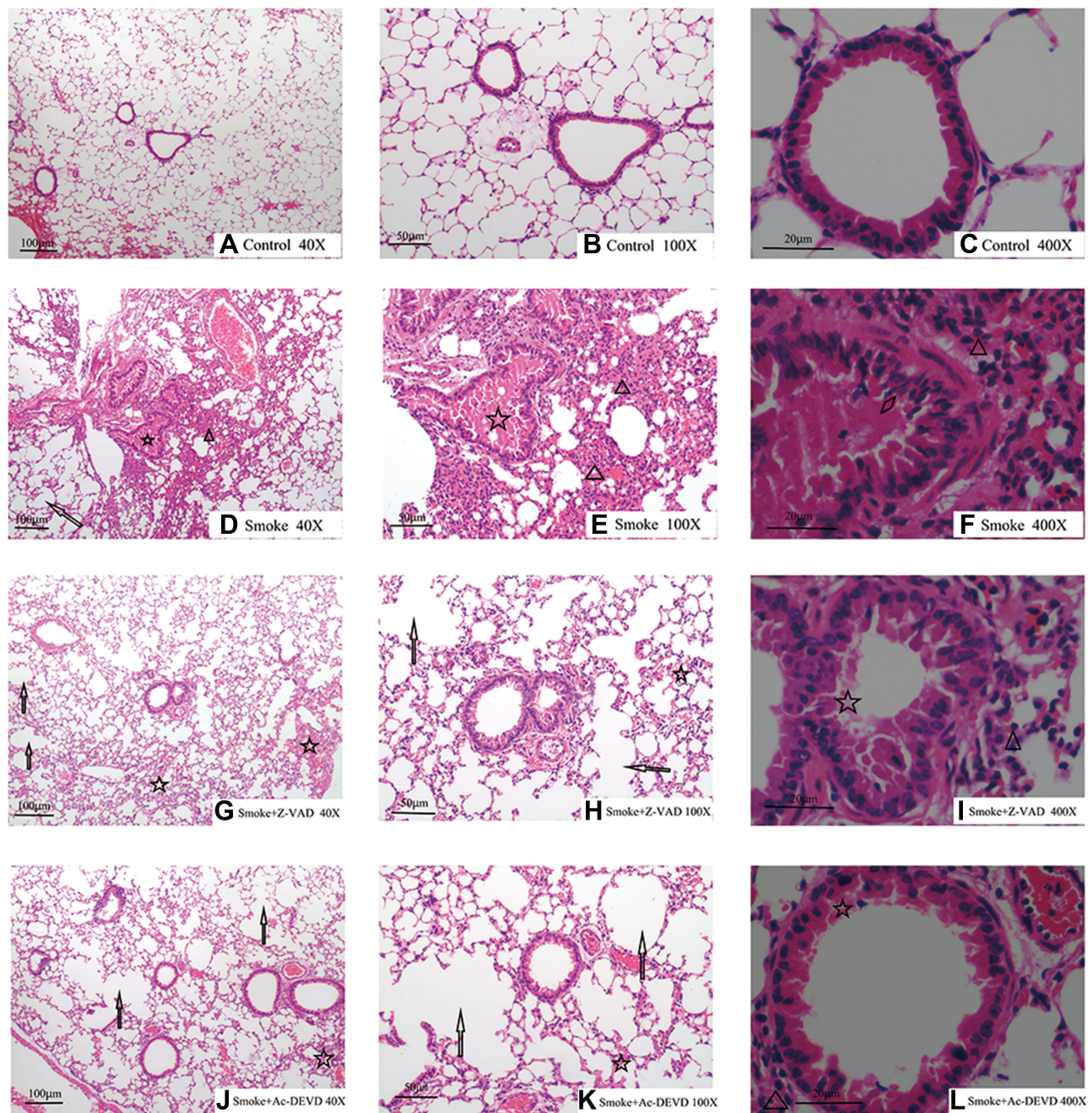
## Inflammatory Factors in Serum and Bronchoalveolar Lavage Fluid

The IL-1 $\beta$  and IL-8 levels in serum were higher in the Smoke group than in the control group ( $P < 0.05$ ). However, they were lower in both the Z-VAD and Ac-DEVD groups than in the Smoke group ( $P < 0.05$ ). Moreover, the IL-1 $\beta$  concentration was lower in the BALF in the Z-VAD group than in the other three groups, and there was a statistical difference between the Z-VAD group and control and Ac-DEVD groups, but showed a decreasing trend compared with the Smoke group, and the difference was not statistically significant. The lowest level of IL-8 was detected in the Smoke group and the highest in the Ac-DEVD group ( $P < 0.05$ , Figure 4).

## Mucin Expression in Mice

Periodic acid–Schiff-positive cells were occasionally detected in the airways of the control group (Figure 5A–C), but the highest number of PAS-positive cells was detected in the Smoke group (Figure 5D–F) and the lower in the Z-VAD group (Figure 5G–I). Ac-DEVD group showed an increasing trend compared with control group, but decreased compared with the smoke group (Figure 5J–L). Moreover, there were no significant differences in serum Muc5ac expression between the groups (Figure 6A). However, Muc5ac levels in the BALF were significantly higher in the Smoke group than in the control group ( $P < 0.05$ ). In Ac-DEVD group, we cannot detect the Muc5ac, because its concentration was below the test line and significantly lower than the smoke group ( $P < 0.05$ ) (Figure 6B).

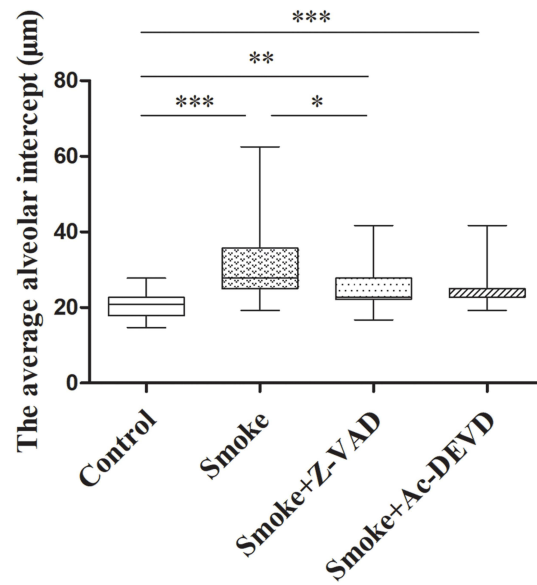




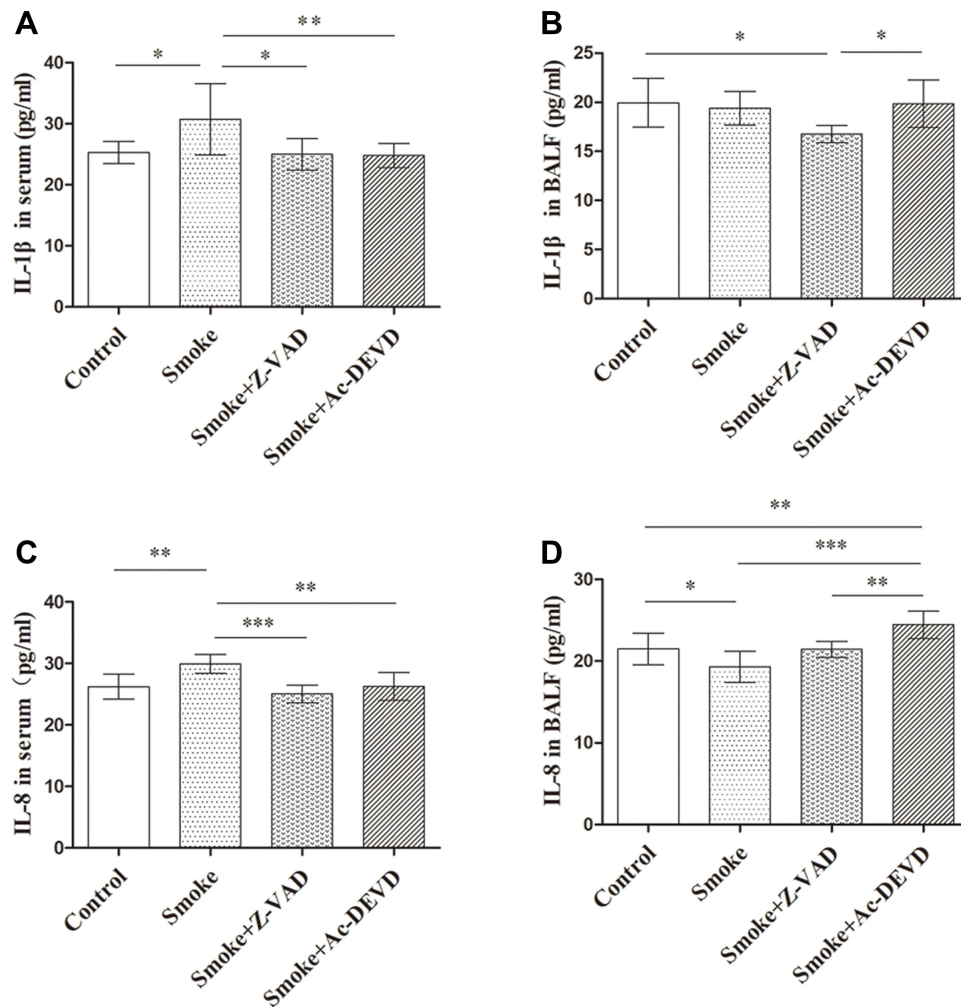
**Figure 2** Representative photomicrographs of hematoxylin and eosin staining of lung sections (original magnification  $\times 40$ ,  $100$ , and  $400$ ). ( $\uparrow$ ) Arrowheads represent pulmonary emphysema. ( $\star$ ) represent exudates. ( $\Delta$ ) represent inflammatory cell infiltration. ( $\diamond$ ) represent epithelial proliferation. Control group (**A-C**) normal lung tissues. Smoke group (**D-F**) inflammation and pulmonary emphysema. Z-VAD group (**G-I**) reduced inflammation and emphysema than the smoke group. Ac-DEVD group (**J-L**) reduced inflammation than the smoke group.

## Discussion

Various inflammatory cells participate in airway inflammation in COPD, including macrophages, neutrophils, and lymphocytes. Activated inflammatory cells release inflammatory mediators that act on airway epithelial cells, inducing hypersecretion of goblet cell and airway mucus and promoting peri-airway smooth muscle and fibroblast proliferation.<sup>1</sup> In this study, the effects of the NLRP3/caspase pathway on airway inflammation and mucus secretion in mice exposed to CS for three months were mainly explored by inhibiting the caspase-1/IL-1 $\beta$  and caspase-3 pathways using Z-VAD, which is a pan-caspase inhibitor, and Ac-DEVD, which is a strong caspase-3 inhibitor.

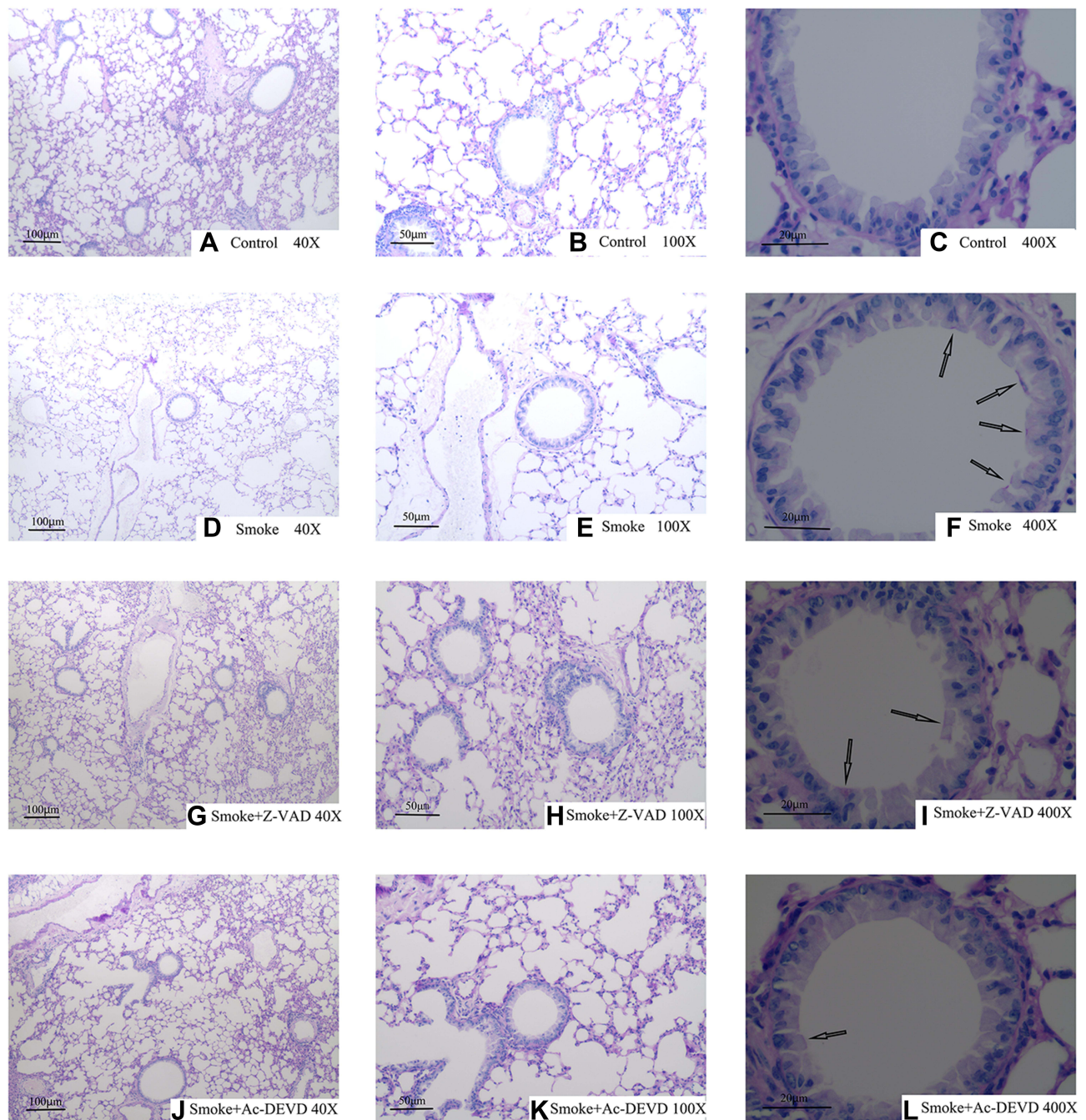


**Figure 3** The average alveolar intercept in the corresponding groups. The AAI was shorter in the control group than in the other three groups, and it was shorter in the Z-VAD group than in the Smoke group, but it was not significantly shorter in the Ac-DEVD group than in the Smoke group. Data are expressed as quartiles, and n = 8, 8, 6, and 8 for each group. \**P* < 0.05, \*\**P* < 0.01, and \*\*\**P* < 0.001.



**Figure 4** Inflammatory factor in serum and bronchoalveolar lavage fluid. IL-1β and IL-8 were detected using an enzyme-linked immunosorbent assay: (A) IL-1β in serum; (B) IL-1β in and bronchoalveolar lavage fluid (BALF); (C) IL-8 in serum; and (D) IL-8 in BALF. The results are expressed as mean ± SD, n = 5, 6, 5, and 7 for serum and n = 6, 5, 5, and 8 for BALF. \**P* < 0.05, \*\**P* < 0.01, and \*\*\**P* < 0.001.

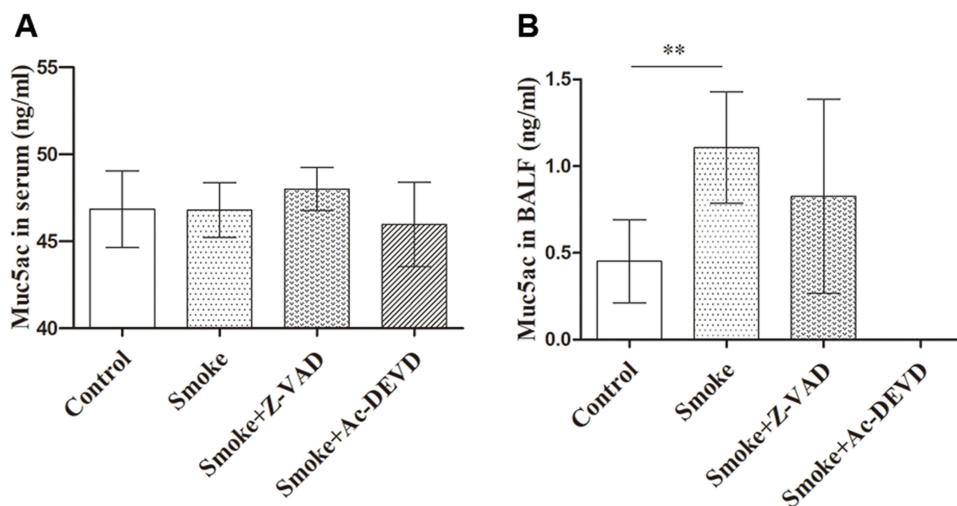




**Figure 5** Sections were stained with periodic acid-Schiff. (A-C) the control group; (D-F) the Smoke group; (G-I) the Z-VAD group; (J-L) the Ac-DEVD group. (†) Arrowheads represent pulmonary emphysema.

CS can affect the activation of the NLRP3 inflammasome in various ways. Cigarettes contain abundant free radicals, including reactive oxygen species (ROS) and reactive nitrogen species, and many toxic, carcinogenic, and mutagenic chemicals, which cause tissue damage to the lung. ROS are known to be upstream activators of the NLRP3 inflammasome.<sup>16,17</sup>

Educed smoke-induced increases in central airways resistance, but not reduced inflammation, emphysema or changes in lung function.<sup>12</sup> In this study, mice exposed to smoke had decreased lung function and significant cilia lodging, epithelial cell shedding, and inflammatory cell infiltration (including neutrophils and lymphocytes), with significant emphysema formation and increased mean alveolar intercept, which is consistent with previous studies.<sup>18,19</sup>



**Figure 6** Mucin 5ac measurements in serum and bronchoalveolar lavage fluid. Mucin 5ac were detected using an enzyme-linked immunosorbent assay. **(A)** Muc5ac in serum. There were no significant differences in serum Muc5ac expression between the groups. **(B)** Muc5ac in BALF. Muc5ac in Smoke group was higher than other groups. The data are expressed as mean  $\pm$  SD, n = 5, 6, 5, and 7 for serum and n = 6, 5, 5, and 8 for bronchoalveolar lavage fluid. \*\* $P < 0.01$ .

After three months of CS exposure, neutrophil infiltration and lymphocyte infiltration in the lung tissue were evident, whereas the degree of airway inflammatory infiltration was somewhat reduced in the Z-VAD and Ac-DEVD groups compared with the Smoke group. It appeared that the Z-VAD inhibited airway inflammation and reduced the occurrence of emphysema, which is consistent with previous research.<sup>20</sup> Inhibition of the caspase-3 pathway only alleviated the airway inflammation but did not significantly reduce the degree of emphysema in the Ac-DEVD mice, suggesting that the caspase-3 pathway has little effect on the occurrence of emphysema.

Inflammatory factors play a key role in the development of COPD. The activation of IL-1 $\beta$  can activate an immune reaction and cause an aggregation of immune cells, leading to neutrophil inflammation, emphysema formation, fibrosis, and mucous cell hyperplasia.<sup>21,22</sup> The knockout of NLRP3 reduces airway inflammation and goblet cell hyperplasia in mice, and this improvement may be caused by IL-1 $\beta$ .<sup>23</sup> Studies have shown that most NLRP3 and IL-1 $\beta$  mRNA in stable COPD lung tissues are inactive, but they show an up-regulation trend, while activated caspase-1 and IL-1 $\beta$  in sputum are significantly increased in the state of infection.<sup>24</sup> IL-1 $\beta$  is a proinflammatory mediator that drives many types of acute inflammatory responses,<sup>25</sup> and it has been reported that IL-1 $\beta$  levels in the BALF of NLRP3 knock-out mice did not increase after ten months of cigarette exposure, nor did they cause significant lung damage.<sup>26</sup> Despite the significantly high-expression of NLRP3 in patients with COPD, neither anti-IL-1 $\beta$  nor anti-IL-1 $\beta$ R monoclonal antibody therapy has been proven to be effective.<sup>27,28</sup>

IL-8 is a neutrophil chemokine, and inhibition of IL-8 can suppress Muc5ac expression.<sup>29</sup> Noy et al revealed that smoke extract could increase IL-1 $\beta$  and IL-8 in vitro.<sup>30</sup> Similar to these results, the present study found that IL-1 $\beta$  and IL-8 levels in serum were increased in the Smoke group compared with the control group ( $P < 0.05$ ), and Z-VAD and Ac-DEVD reduced IL-1 $\beta$  and IL-8 levels in the serum of mice exposed to smoke. Compared with the other three groups, Z-VAD also reduced the IL-1 $\beta$  concentration in BALF. There was a statistically significant difference between the Z-VAD group and the control and Ac-DEVD groups, but the difference was not statistically significant in the Smoke group. In addition, IL-8 levels did not decrease in BALF in the Ac-DEVD group, and they were higher than in the other groups. These data suggest that Z-VAD suppresses the level of inflammatory factors in mice exposed to smoke. In addition, the inhibition of the caspase-3 pathway does not reduce the level of IL-8 in BALF, and its improvement of airway inflammation may therefore be caused by other factors.

In addition to inflammation, airway mucus hypersecretion promotes the development of COPD,<sup>31</sup> and cigarette exposure can lead to mucus hypersecretion. Muc5ac and Muc5b are the most important components of secretory mucin, secreted by goblet cells and submucosal glands;<sup>32,33</sup> thus increased Muc5ac concentration in the airways might contribute to the initiation, progression, exacerbation risk, and overall pathogenesis of COPD.<sup>34</sup> In this study, the number of PAS-positive airway cells was significantly increased in the Smoke group and slightly higher in the Z-VAD



and Ac-DEVD group, suggesting that CS promotes airway mucus secretion. A previous study showed that the Muc5ac level in BALF increased in mice after CS and lipopolysaccharide exposure, accompanied by an increase in IL-8 and tumor necrosis factor.<sup>35</sup> Notably, no significant difference in serum Muc5ac levels was found among the four groups in the present study, but Muc5ac levels were significantly increased in the Smoke group. Caspase-3 inhibitor Ac-DEVD significantly reduced Muc5ac levels in BALF to below the normal limit, suggesting the importance of the role played by the caspase-3 pathway in airway mucus hypersecretion. Nevertheless, Muc5ac and IL-8 exhibited a negative correlation, suggesting the effect in reducing mucus secretion may not function through the IL-1 $\beta$  and IL-8 pathways.

In conclusion, our data suggest that the pan-caspase inhibitor Z-VAD improves airway inflammation and emphysema, reduces IL-1 $\beta$  levels in serum and BALF, and decreases serum IL-8 levels in mice exposed to smoke, but it does not significantly improve airway mucus hypersecretion. However, the caspase-3 inhibitor Ac-DEVD reduces airway inflammation, serum IL-1 $\beta$  and IL-8 levels, and the Muc5ac levels in BALF, but it does not significantly improve the degree of emphysema in mice exposed to smoke. The decrease in Muc5ac levels in BALF in the Ac-DEVD group was not accompanied by a reduction of IL-1 $\beta$  and IL-8 levels in BALF, suggesting that there may be additional mechanisms for mucus secretion resulting from exposure to CS. Thus, further studies are needed to reveal the specific mechanism of mucus hyper-secretion in COPD.

## Abbreviations

NLRP3, NOD-like receptor family, pyrin domain containing 3; IL, interleukin; HE, hematoxylin and eosin; PAS, Periodic Acid Schiff; BALF, bronchoalveolar lavage fluid; ELISA, Enzyme-linked immunosorbent assay; Muc5ac, mucin 5ac; Muc5b, mucin 5b; COPD, chronic obstructive pulmonary disease; AAALAC, American Association for Accreditation of Laboratory Animal Care; IACUC, Institutional Animal Care and Use committee; CS, cigarette smoke; RL, lung resistance; Cdyn, dynamic compliance; MVV, maximum minute ventilation; AAI, average alveolar intercept; ANOVA, analysis of variance; SD, standard deviation; ROS, reactive oxygen species; RNS, reactive nitrogen species; TNF, tumor necrosis factor.

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## Disclosure

The authors declare no conflicts of interest in this work.

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