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ORIGINAL ARTICLE

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## Overexpression of USP8 inhibits inflammation and ferroptosis in chronic obstructive pulmonary disease by regulating the OTUB1/SLC7A11 signaling pathway

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### KEYWORDS

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USP8

### Abstract

**Background:** Chronic obstructive pulmonary disease (COPD) is a familiar disease, and owns high morbidity and mortality, which critically damages the health of patients. Ubiquitin-specific peptidase 8 (USP8) is a pivotal protein to join in the regulation of some diseases. In a previous report, it was determined that USP8 expression is down-regulated in LPS-treated BEAS-2B cells, and USP8 restrains inflammatory response and accelerates cell viability. However, the regulatory roles of USP8 on ferroptosis in COPD are rarely reported, and the associated molecular mechanisms keep vague.

**Objective:** To investigate the regulatory functions of USP8 in COPD progression.

**Material and Methods:** The lung functions were measured through the Buxco Fine Pointe Series Whole Body Plethysmography (WBP). The Fe level was tested through the Fe assay kit. The protein expressions were assessed through western blot. The levels of tumor necrosis factor- $\alpha$ , interleukin 6, and interleukin 8 were evaluated through enzyme-linked immunosorbent serologic assay. Cell viability was tested through CCK-8 assay.

**Results:** In this work, it was discovered that overexpression of USP8 improved lung function in COPD mice. In addition, overexpression of USP8 repressed ferroptosis by regulating glutathione peroxidase 4 and acyl-CoA synthetase long-chain family 4 expressions in COPD mice. Overexpression of USP8 suppressed inflammation in COPD mice. Furthermore, overexpression of USP8 suppressed ferroptosis in COPD cell model. At last, it was verified that overexpression of USP8 accelerated ubiquitin aldehyde-binding protein 1 (OTUB1)/solute carrier family 7 member 11 (SLC7A11) pathway.

**Conclusion:** This study manifested that overexpression of USP8 restrained inflammation and ferroptosis in COPD by regulating the OTUB1/SLC7A11 signaling pathway. This discovery hinted that USP8 could be a potential target for COPD treatment.

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## Introduction

Chronic obstructive pulmonary disease (COPD) is a common disease with high incidence, and has become a prominent public health problem.<sup>1</sup> COPD is characterized by airway obstruction, abnormal inflammation, cell apoptosis, and alveolar destruction.<sup>2</sup> COPD patients accompanied by increased cell apoptosis and inflammatory response in pulmonary vascular endothelial cells can exacerbate the morphological changes of lung tissue, resulting into damaged lung function.<sup>3,4</sup> Numerous studies have revealed that exposure to cigarette smoke is a major risk factor for COPD.<sup>5</sup> Therefore, further investigating the pathogenesis of COPD and seeking useful therapeutic targets to alleviate the influence of cigarette smoke are of great significance.

Ubiquitination is a reversible process regulated by ubiquitinases and deubiquitinases (DUBs).<sup>6</sup> Ubiquitin-specific peptidase 8 (USP8) acts as a member of DUBs, and is involved in various diseases. For example, USP8 deubiquitinates PD-L1 to aggravate the progression of pancreatic cancer in an immunity-dependent manner.<sup>7</sup> Moreover, USP8 modulates deubiquitination to aggrandize  $\alpha$ -synuclein, thereby facilitating Parkinson's disease.<sup>8</sup> Suppression of USP8 can against drug resistance in hepatocellular carcinoma through inhibiting multiple receptor tyrosine kinases.<sup>9</sup> USP8 regulates the levels of receptor tyrosine kinases to decrease melanoma.<sup>10</sup> Besides, USP8 affects the deubiquitination of TAK1 to restrain hypoxia/reoxygenation-triggered inflammation in renal tubular epithelial cells.<sup>11</sup> Importantly, in a previous report, it was determined that USP8 exhibits lower expression in LPS-treated BEAS-2B cells (human non-tumorigenic lung epithelial cell line derived from human lung tissue), and USP8 restrains inflammatory response and accelerates cell viability.<sup>12</sup> However, the regulatory impact of USP8 on ferroptosis in COPD is not investigated, and the associated molecular mechanisms remain indistinct.

Ferroptosis is a type of cell death and is identified as a critical process in the progression of COPD.<sup>13,14</sup> Nevertheless, the regulatory effects of USP8 on ferroptosis in progression of COPD is vague.

In this study, we aimed to probe the regulatory functions of USP8 in the progression of COPD. Results showed that overexpression of USP8 restrained inflammation and ferroptosis in COPD by regulating the ubiquitin aldehyde-binding protein 1 (OTUB1)/solute carrier family 7 member 11 (SLC7A11) signaling pathway. These findings may contribute to understanding the ameliorative functions of SUP8 in COPD.

## Materials and methods

### Animal model

All animals' experiments were executed in line with the Guide for the Care and Use of Laboratory Animals of Wuxi Ninth People's Hospital Affiliated to Soochow University. C57BL six mice (aged 8-10 weeks,  $n = 24$ ) were bought from Beijing Vital River Laboratory Animal Technology Co. Ltd., China. After model establishment, mice were injected with

adenovirus (ad)-NC and ad-USP8. After 14 days, mice were placed into a tobacco smoke inhalation exposure device with cigarettes (Yellow Crane Tower purchased from China Tobacco Hubei Industrial Limited Liability Company; nicotine content in smoke: 1.2 mg/cigarette; tar content: 15 mg/cigarette; and carbon monoxide content in smoke: 14 mg/cigarette). Every time, six cigarettes were kept burning for 1 h to achieve a smoke concentration of 100-120 mg/m<sup>3</sup>. The procedures were performed for 4 weeks, 6 days per week. Mice in the sham group were exposed to normal air and housed normally. The COPD mouse model was established by cigarette smoke (four times a day), and the following four groups ( $n = 6$ ) were created: sham group; cigarette group; cigarette+ad-NC group, and cigarette+ad-USP8 group.

### Cell model

The cigarette smoke was drawn into Dulbecco's modified Eagle's medium (DMEM, 10 mL; Gibco, FL, USA). After being filtered, DMEM was titrated to pH 7.35-7.45 by NaOH, and 100% cigarette smoke extract (CSE) solution was prepared.

Human bronchial epithelial cells BEAS-2B were obtained from Cell Bank, Shanghai Institute for Biological Sciences (Shanghai, China). BEAS-2B cells were cultured in DMEM with 10% fetal bovine serum (FBS; Gibco) under the environment of 5% CO<sub>2</sub> at 37°C. BEAS-2B cells were stimulated with CSE for 24 h to establish COPD cell model.

### Cell transfection

The ad-NC and ad-USP8 vectors with a multiplicity of infection (MOI) of 20 were transfected into BEAS-2B cells through Lipofectamine™ 2000 (Thermo Fisher Scientific, Waltham, MA, USA).

### Detection of lung function

Lung functions, such as tidal volume (TV), peak expiratory flow (PEF), 50% expiratory flow (EP50), forced expiratory volume in 0.3 seconds (FEV0.3), forced vital capacity (FVC), and FEV0.3/FVC, in mice were measured through Buxco® FinePointe Series whole body plethysmography (WBP) (Buxco Research Systems, NC, USA).

### Hematoxylin and eosin (H&E) staining

After fixing in 10% formalin (Sigma, St. Louis, MO, USA), lung tissues were cut into 4- $\mu$ m sections, and mixed with H&E staining solution. H&E images were captured through microscope (Olympus Corporation, Tokyo, Japan).

### Detection of Fe level

The Fe level was measured using the Fe assay kit (ab83366; Abcam, Shanghai, China) according to manufacturer's protocols.

### Western blot assay

The extraction of proteins from lung tissues or BEAS-2B cells was performed through radioimmunoprecipitation assay (RIPA) lysis buffer. Proteins were separated with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). After blocking with 5% skimmed milk, the membranes were incubated with primary and secondary antibodies (1/5000; ab6721). Finally, the blots were inspected under the enhanced chemiluminescence kit (Thermo Fisher Scientific).

Primary antibodies included the following: anti-glutathione peroxidase 4 (GPX4, 1/1000; ab125066; Abcam), USP8 (1/1000; ab228572), acyl-CoA synthetase long-chain family 4 (ACSL4, (1/10,000; ab155282), OTUB1 (1/1000; ab175200), SLC7A11 (1/1000; ab307601), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1/500; ab8245; loading reference).

### Enzyme-Linked Immunosorbent Serologic Assay (ELISA)

The commercial ELISA kits of TNF- $\alpha$  (ab208348, Abcam), IL-8 (ab214030), and IL-6 (ab222503) were followed according to manufacturer's protocols.

### Detection of malondialdehyde (MDA), superoxide dismutase (SOD), and glutathione (GSH)

The factors of oxidative stress were inspected through MDA (ab118970, Abcam), SOD (ab65354), and GSH (ab65322) commercial kits.

### Cell Counting Kit-8 (CCK-8) assay

BEAS-2B cells (1000 cells/well) were seeded into a 96-well plate. After 24 h, CCK-8 solution, 10  $\mu$ L (Dojindo Laboratories, Kumamoto, Japan) was placed into each well for 2 h. Finally, cell viability was ascertained through a spectrophotometer (Thermo Fisher Scientific).

### Immunohistochemistry (IHC) assay

Dewaxing and rehydration were performed for 4- $\mu$ m lung sections embedded in paraffin. After blocking with serum, GPX4 antibody (1/100, ab125066; Abcam), ACSL4 antibody (1/200, ab155282), OTUB1 antibody (1/250, ab270959), or SLC7A11 antibody (1/500, ab307601) was mixed for 12 h, next adding the horseradish peroxidase-conjugated secondary antibody (1:1000, ab7090, Abcam, Shanghai, China). Dyeing was executed with diaminobenzidine and re-dyeing with hematoxylin. Protein expressions in lung tissues were examined through microscope (Olympus Corporation).

### Statistical analysis

The data were exhibited as mean  $\pm$  standard deviation (SD), and GraphPad Prism 9 (GraphPad Inc, La Jolla, CA, USA) was used for statistical analysis. Comparisons were performed using the one-way analysis of variance (ANOVA) with Tukey's *post hoc* test;  $P < 0.05$  was considered statistically significant.

## Results

### Overexpression of USP8 improved lung functions in COPD mice

The results showed that the levels of TV, PEF, EP50, FEV0.3, FVC, and FEV0.3/FVC decreased after cigarette treatment, but these impacts were reversed after USP8 overexpression (Figure 1A). Moreover, USP8 expression was reduced after cigarette treatment, but this change was offset by USP8 overexpression (Figure 1B). Inflammatory cells were infiltrated by H&E staining, and terminal ends of the bronchi were enlarged after cigarette treatment, but these phenomena improved after USP8 overexpression (Figure 1C). These results indicated that overexpression of USP8 improved lung functions in COPD mice.

### Overexpression of USP8 repressed ferroptosis in COPD mice

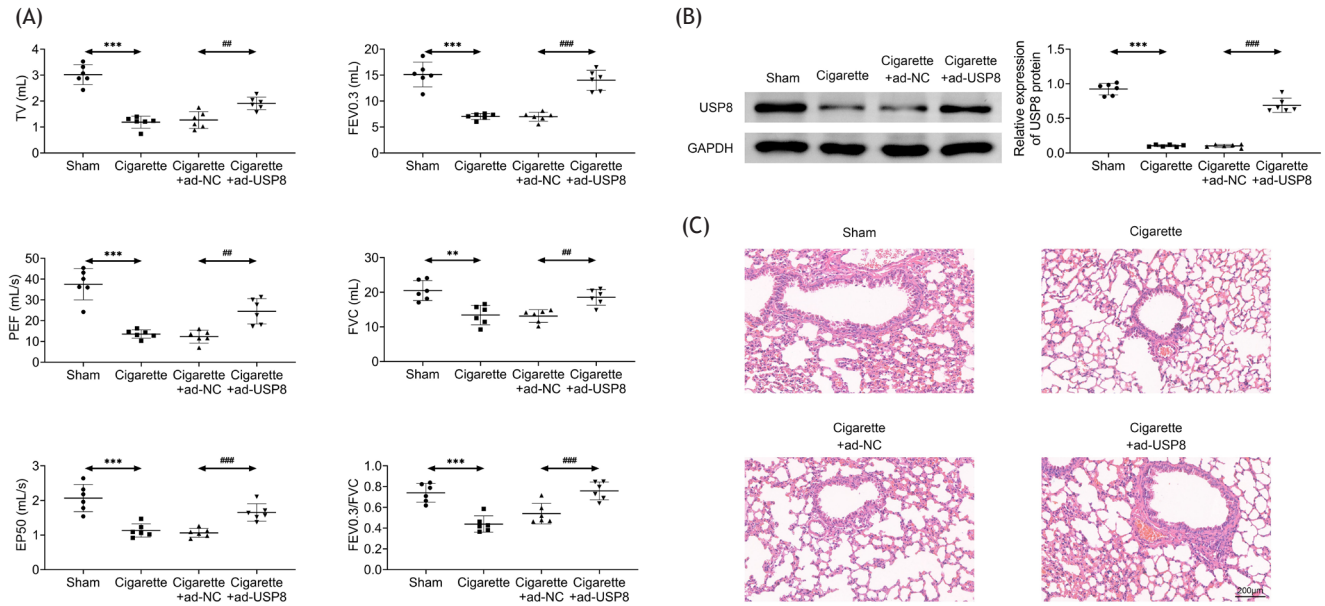
The Fe level was enhanced after cigarette treatment, but this change was attenuated after USP8 amplification (Figure 2A). Furthermore, the protein expression of GPX4 was decreased and ACSL4 was increased after cigarette treatment, but these effects were restored after USP8 augmentation (Figures 2B and 2C). IHC assay demonstrated that the protein expression of GPX4 was decreased and ACSL4 was elevated after cigarette treatment, but these impacts were reversed after USP8 augmentation (Figure 2D). In general, overexpression of USP8 repressed ferroptosis in COPD mice.

### Overexpression of USP8 restrained inflammation in COPD mice

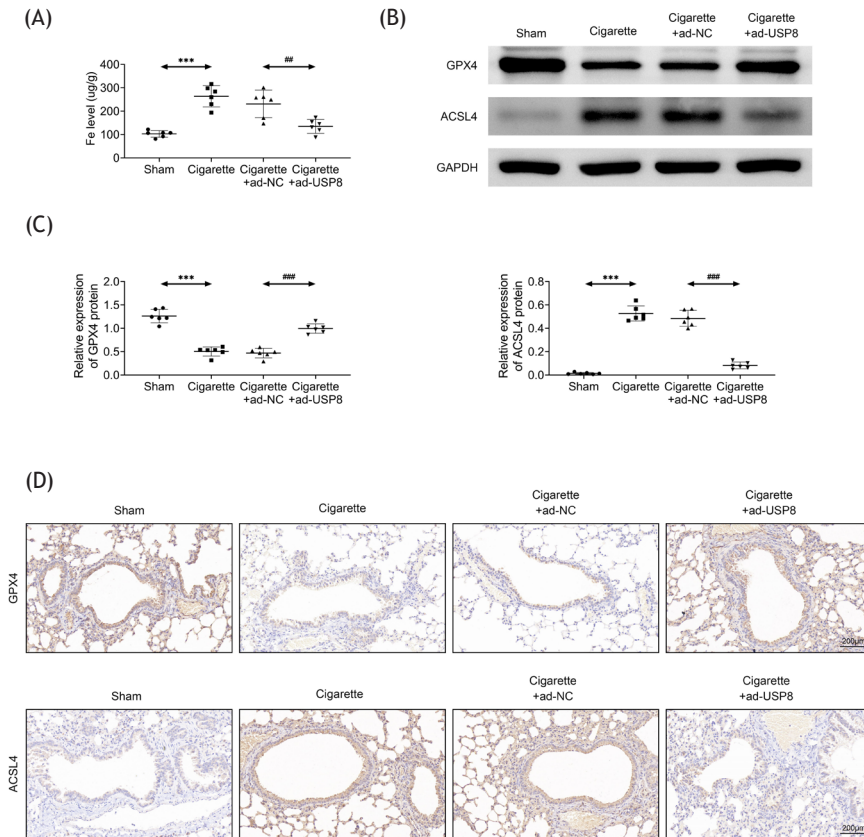
It was demonstrated through ELISA that the levels of TNF- $\alpha$ , IL-6, and IL-8 increased after cigarette treatment, but these influences were relieved after USP8 overexpression (Figure 3A). Furthermore, the MDA level was strengthened and the SOD and GSH levels were weakened after cigarette treatment, but these changes were rescued after USP8 overexpression (Figure 3B). Taken together, overexpression of USP8 restrained inflammation in COPD mice.

### Overexpression of USP8 suppressed ferroptosis in COPD cell model

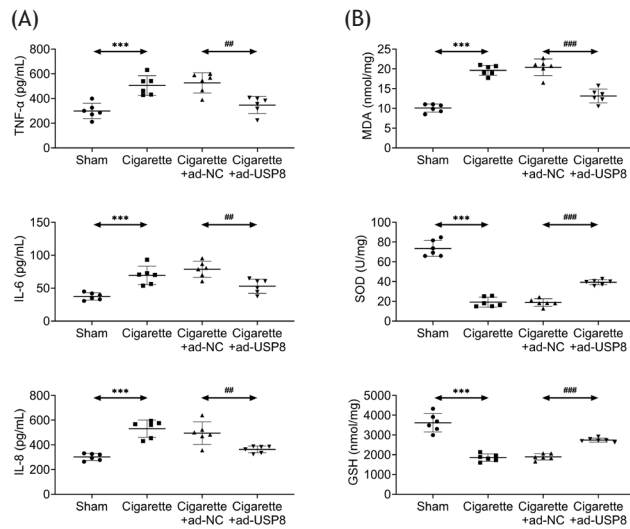
Next, *in vitro* experiments discovered that Fe level was enhanced after cigarette treatment, but this change was



**Figure 1** Overexpression of USP8 improved lung function in COPD mice. Groups were separated into the Sham, Cigarette, Cigarette+ad-NC and Cigarette+ad-USP8 group. (A) The levels of tidal volume (TV), peak expiratory flow (PEF), 50% expiratory flow (EP50), forced expiratory volume in 0.3 seconds (FEV0.3), forced vital capacity (FVC) and FEV0.3/FVC were measured through the Buxco Fine Pointe Series Whole Body Plethysmography. (B) The protein expression of USP8 was tested through western blot. (C) The pathological changes in lung tissues were observed through HE staining. \*\*p < 0.01, \*\*\*p < 0.001 vs the Sham group; ##p < 0.01, ###p < 0.001 vs the Cigarette+ad-NC group.



**Figure 2** Overexpression of USP8 repressed ferroptosis in COPD mice. Groups were separated into the Sham, Cigarette, Cigarette+ad-NC and Cigarette+ad-USP8 group. (A) The Fe level was tested through the Fe kit. (B-C) The protein expressions of GPX4 and ACSL4 were assessed through western blot. (D) The protein expressions of GPX4 and ACSL4 in lung tissues were confirmed through IHC assay. \*\*\*p < 0.001 vs the Sham group; ##p < 0.01, ###p < 0.001 vs the Cigarette+ad-NC group.

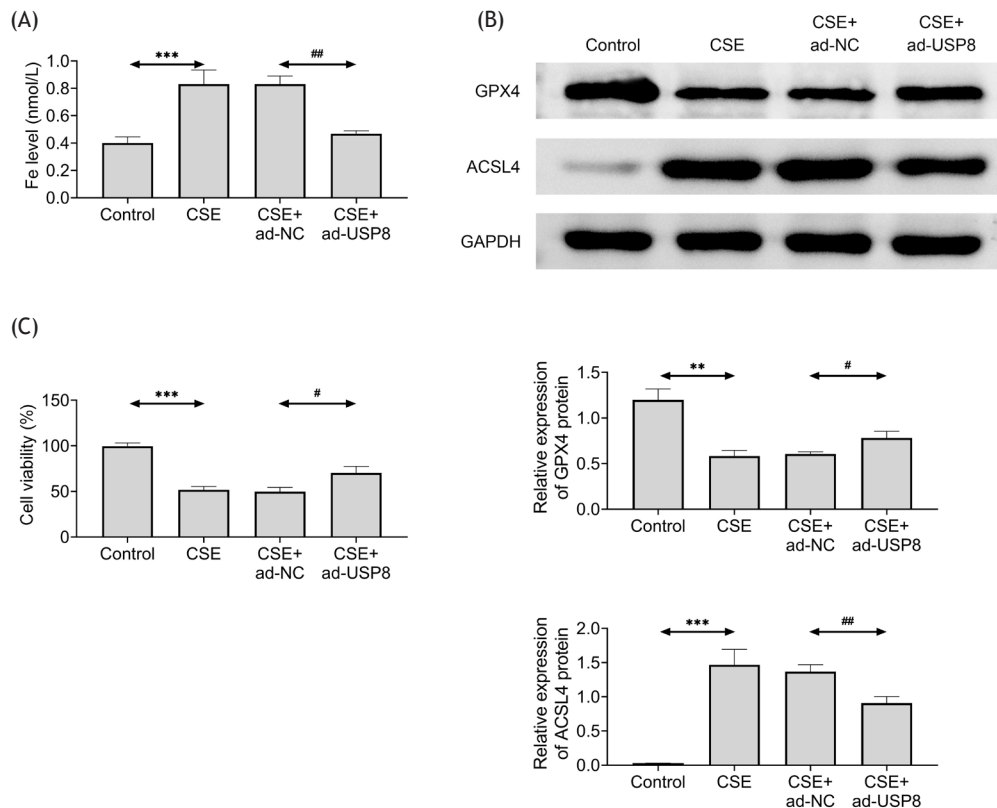


**Figure 3** Overexpression of USP8 restrained inflammation in COPD mice. Groups were separated into the Sham, Cigarette, Cigarette+ad-NC and Cigarette+ad-USP8 group. (A) The levels of TNF- $\alpha$ , IL-6 and IL-8 were evaluated through ELISA. (B) The levels of MDA, SOD and GSH were examined through the commercial kit. \*\*\* $p < 0.001$  vs the Sham group; ## $p < 0.01$ , ### $p < 0.001$  vs the Cigarette+ad-NC group.

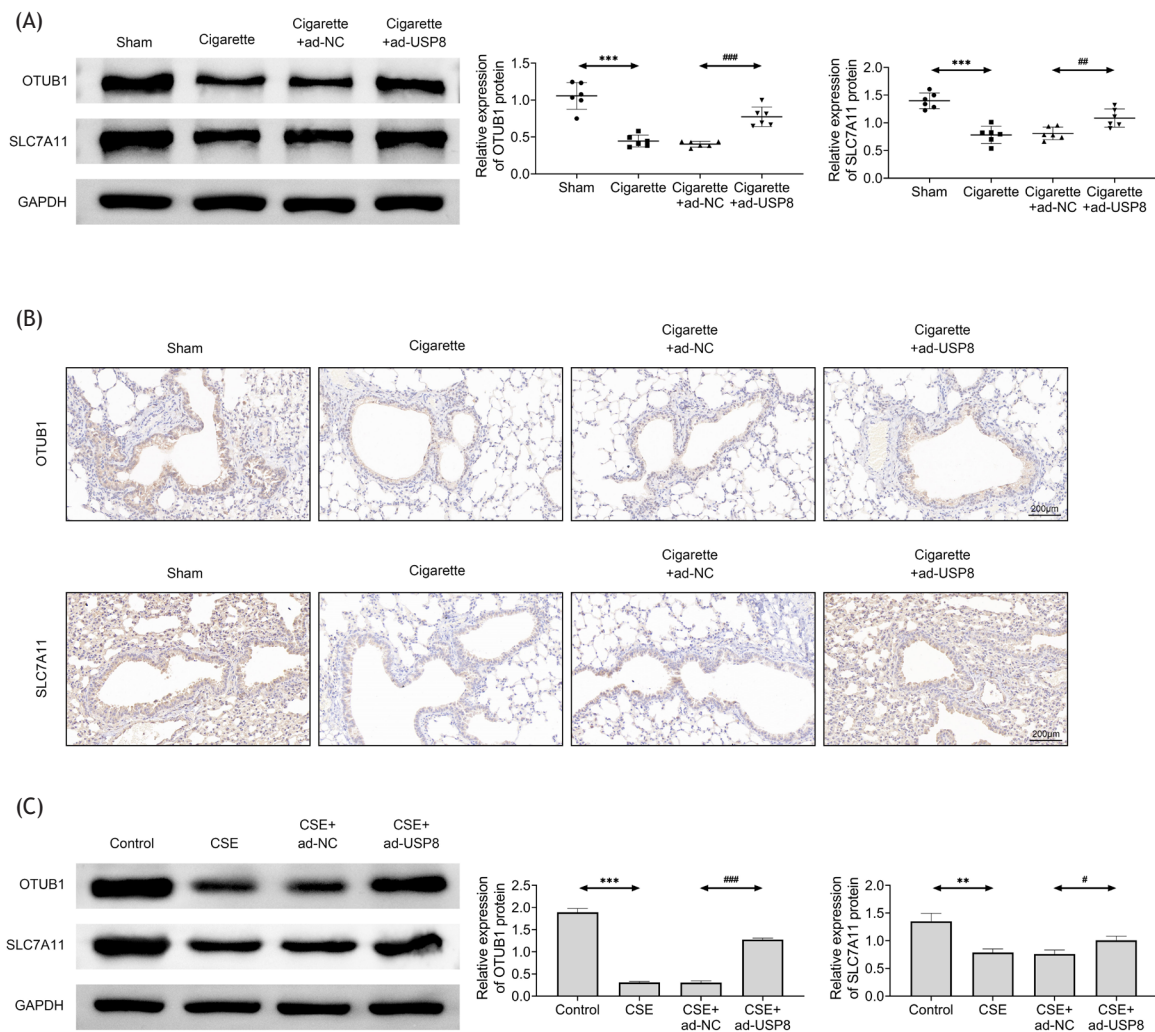
weakened by USP8 amplification (Figure 4A). Additionally, the GPX4 protein expression was down-regulated and that of ACSL4 was up-regulated after cigarette treatment, but these impacts were neutralized by USP8 overexpression (Figure 4B). Moreover, cell viability was reduced after cigarette treatment, but this phenomenon was neutralized after USP8 augmentation (Figure 4C). In a word, overexpression of USP8 suppressed BEAS-2B ferroptosis.

### Overexpression of USP8 accelerated OTUB1/SLC7A11 pathway

The protein expressions of both OTUB1 and SLC7A11 were decreased after cigarette treatment, but these impacts were compensated by USP8 overexpression in COPD mouse model (Figure 5A). Through IHC assay, the protein expressions of both OTUB1 and SLC7A11 were decreased after cigarette treatment, but these impacts were neutralized after USP8 overexpression in lung tissues (Figure 5B). Moreover, the same protein changes in OTUB1/SLC7A11 signaling pathway were observed in COPD cell model (Figure 5C). In general, overexpression of USP8 accelerated OTUB1/SLC7A11 signaling pathway.



**Figure 4** Overexpression of USP8 suppressed ferroptosis in COPD cell model. Groups were separated into the Control, CSE, CSE+ad-NC and CSE+ad-USP8 group. (A) The Fe level was examined through the Fe kit. (B) The protein expressions of GPX4 and ACSL4 were determined through western blot. (C) The cell viability was tested through CCK-8 assay. \*\* $p < 0.01$ , \*\*\* $p < 0.001$  vs the Control group; # $p < 0.05$ , ## $p < 0.01$  vs the CSE+ad-NC group.



**Figure 5** Overexpression of USP8 accelerated OTUB1/SLC7A11 pathway. (A) The protein expressions of OTUB1 and SLC7A11 were tested through western blot. Groups were separated into the Sham, Cigarette, Cigarette+ad-NC and Cigarette+ad-USP8 group. \*\*\*p < 0.001 vs the Sham group; ##p < 0.01, ###p < 0.001 vs the Cigarette+ad-NC group. (B) The protein expressions of OTUB1 and SLC7A11 in lung tissues were inspected through IHC assay. (C) The protein expressions of OTUB1 and SLC7A11 were inspected through western blot. Groups were separated into the Control, CSE, CSE+ad-NC and CSE+ad-USP8 group. \*\*p < 0.01, \*\*\*p < 0.001 vs the Control group; #p < 0.05, ##p < 0.01, ###p < 0.001 vs the CSE+ad-NC group.

## Discussion

Increased proteins participate into the regulation of COPD. For example, inhibition of RTEL1 affects M1/M2 macrophage polarization and inflammation to alleviate COPD progression.<sup>15</sup> In addition, miR-23a-5p targets receptor able to bind advanced glycation end-products-reactive oxygen species (RAGE-ROS) pathway to ameliorate the progression of COPD.<sup>16</sup> Deubiquitinase USP21-mediated TET2 relieves cigarette smoke-stimulated cell apoptosis in COPD.<sup>17</sup> Moreover, mechanistic target of rapamycin (MTOR) controls cigarette smoke-triggered inflammation in COPD.<sup>18</sup> USP8 has been uncovered as a pivotal protein involved in regulating some diseases.<sup>7-11</sup> Significantly, it was determined in previous report that USP8 expression is down-regulated in LPS-treated BEAS-2B cells, and USP8 restrains inflammatory response and accelerates cell viability.<sup>12</sup> However, the regulatory effects of USP8 on ferroptosis in COPD are rarely

reported, and the associated molecular mechanisms are vague. In this study, it was discovered that overexpression of USP8 improved lung function in COPD mice.

Ferroptosis is a type of cell death, and its key participants include GPX4 and ACSL4.<sup>19</sup> Ferroptosis is a critical process of multiple diseases, including COPD. More and more studies have focused on the modulation of ferroptosis in the progression of COPD. For instance, dihydroquercetin stimulates the nuclear factor erythroid 2-related factor 2 (Nrf2)-mediated pathway to reduce cigarette smoke-triggered ferroptosis in COPD.<sup>20</sup> Additionally, deubiquitinase USP14 stabilizes MFG-E8 to inhibit cigarette smoke-mediated ferroptosis in COPD.<sup>21</sup> Furthermore, knockdown of TET2 contributes to ferroptosis in cigarette smoke-stimulated COPD.<sup>22</sup> Interestingly, USP8 has been shown to work against ferroptosis, thereby positively modulating tumorigenesis in hepatocellular carcinoma.<sup>23</sup> Besides, inhibition of USP8 affects SQSTM1/p62-mediated ferritinophagy

to aggravate ferroptosis.<sup>24</sup> Similar to previous reports, in this study, overexpression of USP8 repressed ferroptosis by regulating GPX4 and ACSL4 expressions in COPD mice. Overexpression of USP8 restrained inflammation and oxidative stress in COPD mice. Furthermore, overexpression of USP8 suppressed ferroptosis in COPD cell model.

Ferroptosis is stimulated by lipid peroxidation, and can be controlled by SLC7A11.<sup>25</sup> OTUB1 serves as a major modulator for SLC7A11 stability, and OTUB1/SLC7A11 signaling pathway suppresses ferroptosis by lessening ROS.<sup>26</sup> Interestingly, it was discovered that USP8 modulates SLC7A11 to inhibit ferroptosis, thereby retarding the progression of hepatocellular carcinoma.<sup>27</sup> Similar to the results from previous studies, we verified in this study that overexpression of USP8 also accelerated OTUB1/SLC7A11 signaling pathway in COPD.

## Conclusion

Overexpression of USP8 restrained inflammation and ferroptosis in COPD by regulating the OTUB1/SLC7A11 signaling pathway. This study has certain limitations, such as the absence of human samples, clinical experiments, and other malignant phenotypes *in vivo* and *in vitro*. In the future studies, more investigations must be performed for other regulatory functions of USP8 in COPD progression.

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## Availability of Data and Materials

All data generated or analyzed in this study are included in the published article. Data sharing is not applicable to this article as no new data were created or analyzed in this study. The datasets used and/or analyzed in the present study are available from the corresponding author on reasonable request.

## Competing interests

The authors stated that there were no conflicts of interest to disclose.

## Ethical approval

Ethical approval was obtained from the Ethics Committee of Wuxi Ninth People's Hospital Affiliated to Soochow University.

## Author Contributions

Conceptualization, methodology, and writing—original draft was prepared by Lu Liu. Formal analysis, resources,

and investigations were accomplished by Yu Zhang. Formal analysis, visualization, and data curation were executed by Di Xu. Project administration, supervision, and validation were done by Dan Zhu. Validation, supervision, and writing (review and editing) were done by Ying Zhou, Zhihai Chen, and Xiufeng Huang. All authors read and approved the final manuscript.

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