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The mTOR Inhibitor Rapamycin Attenuates Ozone-Induced Airway Inflammation and Emphysema In Mice

Xue Tian*, Lei Han*, Yuning Huang*, Yan Zhou, Xue Zhang, Min Zhang

Department of Respiratory and Critical Care Medicine, Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, 200080, People's Republic of China

*These authors contributed equally to this work

Correspondence: Min Zhang; Xue Zhang, Department of Respiratory and Critical Care Medicine, Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, 200080, People's Republic of China, Email zhangmin@sjtu.edu.cn; zx15800562053@163.com

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Background: Ozone exposure is a major risk factor for chronic obstructive pulmonary disease (COPD). In this study, we investigated the potential role of targeting mTOR signaling in the treatment of COPD induced by ozone exposure.

Methods: The public database was chosen to explore the expression of *mTOR* mRNA, *S6K1* mRNA, and *LC3B* mRNA in COPD patients, and potential correlations with FEV_1(%pred). In an ozone-exposed mouse model, large airway and small airway function were evaluated by spirometry. After intraperitoneal injection of a mTOR inhibitor known as rapamycin, the emphysema index, and inflammation scores in lung tissue were measured. Inflammatory cell infiltration in bronchoalveolar lavage fluid (BALF) and levels of cytokines in the lung tissue were also observed. Airway remodeling in the lung tissue was detected using Masson's trichrome stains and immunohistochemical staining. Mucus hypersecretion was evaluated by PAS staining. The protein expression of the mTOR pathway and autophagy marker LC3B in the lung tissue was determined through Western blot.

Results: *mTOR* mRNA and *S6K1* mRNA were upregulated in patients with COPD compared to the control subjects, whereas *LC3B* mRNA showed a downward shift in patients with COPD. Mice that received mTOR inhibitor treatment displayed higher FEV_{50}/FVC, FEF_{25}, FEF_{50}, FEF_{75}, and MMEF. The mTOR inhibitor rapamycin improved the emphysema index and inflammation scores in mice lung tissue. Moreover, it significantly inhibited inflammatory cell infiltration in BALF, IL-1β, TNF-α, and NF-κb in the lung tissue of ozone-exposed mice. The mTOR inhibitor significantly suppressed mucus hypersecretion in large and small airways and decreased the protein expression of collagen I and α-SMA in the lung tissue of ozone-exposed mice. Notably, mTOR repression also decreased the protein expression of S6K1 and increased LC3B expression in the lung tissue.

Conclusion: The mTOR inhibitor rapamycin ameliorates ozone-induced airway inflammation and emphysema in a LC3B-dependent manner. mTOR inhibition may offer a promising therapeutic approach for preventing ozone-induced COPD by mitigating airway inflammation, reducing airway remodeling, and alleviating mucus hypersecretion.

Keywords: chronic obstructive pulmonary disease, mTOR pathway, rapamycin, airway inflammation, emphysema

Introduction

Chronic obstructive pulmonary disease (COPD) is an inflammatory lung disease associated with a high mortality rate. In China, COPD affects approximately 100 million adult individuals and is the third-leading cause of death.[1–4] It is characterized by lung inflammation and airflow obstruction due to the production of pro-inflammatory cytokines or inflammatory interleukins.[5,6] Ozone, cigarette smoke, and other air pollutants are major exogenous sources of oxidative stress in the lungs. Ozone, an air pollutant produced by internal combustion, leads to increased inflammation and lung permeability.[7] It is clear that ozone reacts with cellular membranes and with the epithelial lung lining fluid to generate bioactive mediators that induce airway hyperreactivity, chronic bronchial inflammation, and emphysema-like lung destruction. However, the detailed molecular mechanisms underlying ozone's adverse effects need to be further investigated.

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The mTOR (mechanistic target of rapamycin kinase) signaling pathway is known to be involved in cell metabolism, survival, and growth. Several lines of evidence also indicate that mTOR is a major cell autophagy pathway in inflammatory diseases.[8] mTOR activation induced by the deletion of phosphatase and tensin homolog PTEN or the expression of constitutively active serine/threonine kinase Akt induces normal mouse or human cell senescence.[9] The mechanistic target of mTOR and its key downstream process autophagy play crucial roles in major cellular processes such as metabolism and proliferation.[10,11] According to previous studies, mTOR suppresses the autophagy-mediated production of IL-25 in allergic airway inflammation,[12] and its activation promotes autophagy-mediated epithelial injury in particulate matter-induced airway inflammation.[13] Conventional inhaled therapies provide only limited relief of symptoms and have minimal impact on the progression of COPD.[14] Notably, there is a lack of robust evidence supporting the efficacy of these traditional treatments in reducing pro-inflammatory cytokine levels or alleviating emphysema. In contrast, as a mTOR inhibitor, rapamycin is mainly through inhibition of the mTOR signaling pathway and is one of the few drugs capable of prolonging life in many species.[15] A clinical study demonstrated that rapamycin restores corticosteroid sensitivity in mononuclear cells isolated from patients with COPD, suggesting its potential therapeutic application in COPD.[16] However, no mTOR-targeting drugs are currently approved for clinical use in COPD. Therefore, the mechanisms underlying mTOR inhibition in ozone exposure-induced COPD remain to be clarified.

Here, using a public gene database GSE37147, we analyzed the mRNA levels of the mTOR pathway in patients with COPD. We employed an ozone-induced murine model of COPD, administering rapamycin intraperitoneally to inhibit mTOR signaling. This approach allowed us to assess dynamic changes in several critical aspects of COPD, including emphysema, airway inflammation, lung function, airway remodeling, and mucus hypersecretion. Our primary objective was to explore the potential therapeutic effects of mTOR inhibition in COPD and to elucidate the mechanisms through which the mTOR pathway contributes to airway inflammation and emphysema. We believe that the results of this study will provide a therapeutic target to improve COPD management.

Materials and Methods

Reagents

The primary antibodies used in this study include: anti-mTOR (Proteintech Group, IL, USA), anti-S6K1(Proteintech Group, IL, USA), anti-LC3B (Santa Cruz Biotechnology, TX, USA), anti-collagen I (Cell Signaling Technology, MA, USA), anti-α-SMA antibody (Proteintech Group, IL, USA), and anti-β-actin (BioTNT, Shanghai, China). The mTOR inhibitor rapamycin used for animal studies was obtained from MedChemExpress LLC (NJ, USA). The TRIZol® reagent used for total RNA isolation was purchased from Invitrogen (CA, USA). The PrimeScript qPCR Kit and SYBR Premix Ex Taq kit were from Takara Biology Company (Shiga, Japan).

Analysis of Database GSE37147

To investigate the mTOR pathway profile in COPD, we traced a gene expression dataset (GSE37147) from the National Center for Biotechnology Information (GEO Accession viewer https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE37147). This study was exempt from additional review by the Institutional Review Board at Shanghai General Hospital in accordance with institutional and international guidelines. In this study, there were 139 subjects; 57 of them had COPD and 82 were smokers without COPD. Microarray data from total RNA extracted from bronchial brushings were selected. These subjects were diagnosed as being controls or having COPD as determined by each one's clinical history, lung function and CT scan. In this study, there was no intervention and the study data are cross-sectional.

Animal Experiment

Male C57BL/6 mice (age 6-8 weeks) were purchased from SLAC Laboratory Animal Co. Ltd. (Shanghai, China) and randomly divided into three groups. The mice were housed at room temperature and given free access to food and water. All animal experiments were conducted in accordance with the guidelines of the Chinese Council on Animal Care and approved by the Ethics Committee of Shanghai General Hospital. The mice in the control group were exposed to room air without any procedures. The ozone-exposed group and the treatment group were exposed to ozone twice per week for

six weeks. Mice were exposed to ozone produced by an ozonizer (Sander 500 ozonizer, Germany) mixed with air for 3 h at a concentration of 2.5 parts per million (ppm) in a sealed Perspex container. The ozone concentration was continuously monitored with an ozone probe (ATi Technologies, Oldham, UK). In addition, the mice in the treatment groups were treated with intraperitoneal injections of rapamycin (0.6 mg/kg) simultaneously. At the end of the study, the mice were weighed, anesthetized with ether, and sacrificed.

Lung Function Measurement

Tracheostomy was performed on anesthetized mice before connecting them to a rodent-specific forced maneuver system (eSpira™ Forced Manoeuvers System, EMMS, Hants, UK) for lung function measurement. A series of preprogrammed forced ventilation maneuvers were used to measure the FVC (forced vital capacity), FEV_{25} (volume expired in first 25 ms of fast expiration), FEV_{50} (volume expired in first 50 ms of fast expiration), FEV_{75} (volume expired in first 75 ms of fast expiration), FEF_{25} (forced expiratory flow at 25% forced vital capacity), FEF_{50} (forced expiratory flow at 50% forced vital capacity), FEF_{75} (forced expiratory flow at 75% forced vital capacity), and MMEF (forced expiratory flow between 25% and 75% FVC). The average values from three repeated measurements were adopted as the final values of lung function measurement.

Lung Histology, Mean Linear Intercept (MLI), Destruction Index, and Mean Alveolar Area (MAA)

The left lung, which was fixed in 10% formalin for 24 h, was dissected in a step-by-step manner, cleansed, and embedded in paraffin. Subsequently, it was cut into several sections (thickness: 5 µm) and stained with the hematoxylin-eosin (H&E), periodic acid-Schiff (PAS), and Masson's trichrome stains. PAS-positive cells were described as blue/purple in the PAS-stained lung sections. The blue areas surrounding the airways were regarded as subepithelial collagen deposition in Masson's trichrome-stained section. Finally, the intensity of Masson or PAS staining was calculated as follows: Masson-positive or PAS-positive area/total epithelial area (Image J Software, MD, USA).

The air space enlargement was evaluated through MLI measurement.[17] At first, 200× magnification of the electronic scanning images was chosen throughout this assessment. To ensure a more accurate assessment of the pathological scale, each lung section scanning image was further divided into 20–30 small image fields in almost full coverage of the whole scanning section. Areas with large airways and large vessels were avoided for counting. A transparent sheet with 10 horizontal lines was laid over the images, and the MLI was derived by counting the number of intercepts of alveolar walls with these lines.

The destructive index was calculated using a light microscope with 42 points.[18] The structures underlying these points were classified as normal, destroyed, and emphysematous. Only points falling on alveolar and/or alveolar duct spaces were counted. Points falling on other structures, such as duct walls and alveolar walls, were excluded. Both alveolar spaces and alveolar duct spaces were considered normal if they were surrounded by intact walls disrupted in only one place. The alveolar wall was considered "destroyed" when it was disrupted in at least two places. A structure was considered "emphysematous" when it was lined by cuboidal epithelium but was clearly not an airway, with or without breaks in the walls, or when a classic emphysematous lesion was present.

Alveolar dimensions were measured in lung areas that remained fully ventilated.[17] According to the extension of lung consolidation, 5–15 noncoincident ventilated fields observed at a magnification of 35 were analyzed on each histological section. The MAA was determined as the average area of the alveoli in all fields.

Inflammation Scores

A scoring system was used to assess lung inflammation,[19] including peribronchial inflammation and perivascular inflammation. The scores of peribronchial and perivascular inflammation were judged by scales from 0 to 3, with 0 indicating no inflammation. If mild inflammation was observed in the bronchi or vascular walls, it was scored 1. When 1–5 layers of inflammatory cells clustered in the walls of bronchi or blood vessels, it was scored 2. If more than 5 layers of inflammatory cells accumulated in walls of bronchi or vascular walls, it was scored 3. Inflammation scores were expressed as mean values and compared between groups.

Immunohistochemistry

The lung sections were dewaxed with xylene and rehydrated in methanol. After blocking the sections for endogenous peroxidase activity using 5% H_2O_2 and boiling the samples in a microwave oven for 2–3 min in citrate buffer for better antigen retrieval of collagen I and α-SMA, samples were washed with PBS and then incubated with the primary antibody for 1 h at room temperature, followed by incubation with an appropriate secondary antibody (Epitomics, Burlingame, CA, USA). Immunoreactivity was visualized using diaminobenzidine (Sigma-Aldrich), and the slides were counterstained with Mayer's hematoxylin. Immunostained sections were analyzed using a Zeiss confocal laser scanning microscope (Carl Zeiss, Oberkochen, Germany) equipped with a bandpass filter for protein localization.

Quantitative PCR (qPCR)

The concentrations of IL-1β, TNF-α, and NF-κb in mouse lung tissues were detected via qPCR. Briefly, the total RNA of fresh lung tissue was isolated, extracted, and purified using chloroform and isopropanol. Next, cDNA was collected using the enzyme kit through Thermocycler. Then, the qPCR amplification was detected using a 7500 Fast Real-Time PCR System (Applied Biosystems, USA) followed by cDNA synthesis. Expression levels of the target genes were normalized to perform analyses. The primers for mouse-specific PCR are presented in Table 1.

Western Blotting

Total protein was extracted from lung tissues using T-PER reagent (Pierce, IL, USA). Protein concentration was determined by the BCA assay. For electrophoresis, 15 μg protein was loaded onto 11% SDS-PAGE, transferred to nitrocellulose membranes, and blocked with 5% BSA for 2 h. Membranes were incubated overnight with primary antibodies at 4°C, followed by secondary antibody incubation for 2 h at room temperature.

Statistical Analysis

GraphPad Prism Statistical Software 9.0 (GraphPad Software Inc., San Diego, CA, USA) was used for the analysis. The data are presented as the mean ± SEM. The differences between two groups were determined with unpaired *t* test, and more than two groups' comparisons were performed using the one-way analysis of variance with Tukey's multiple comparison test. The correlation between different variables was determined via the Spearman analysis, and *p*-values <0.05 were considered statistically significant. Spearman's $r > 0.4$ or < -0.4 was considered indicative of strong correlations, and r values between −0.4 and 0.4 were considered indicative of weak correlations.

Table 1 Primers Information for qPCR

	Primer	
IL-1β	Forward	5'-TACATCAGCACCTCACAAGC-3'
	Reverse	5'-AGAAACAGTCCAGCCCATACT-3'
TNF-α	Forward	5'-TGCCTCCTCTTTTGCTTATGTT-3'
	Reverse	5'-AGGTTCAGTGATGTAGCGACAG-3'
NF-κb	Forward	5'-TGACAAGGTTCAGAAAGATG-3'
	Reverse	5'-GAAGACAATGGCAAACTG-3'
β-actin	Forward	5'-CCTCTATGCCAACACAGT-3'
	Reverse	5'-AGCCACCAATCCACACAG-3'

Results

The *mTOR* mRNA Was Upregulated in COPD patients

To validate whether mTOR increased in patients with COPD, we analyzed *mTOR* mRNA, *S6K1* mRNA, *LC3B* mRNA, and relationships between *mTOR* mRNA with FEV_1 (Forced expiratory volume in one second)(%pred) in database GSE37147. In this study, there were 57 subjects with COPD (average age 62.52 yr) and 82 subjects were smokers without COPD (average age 65.96 yr). The results suggested that *mTOR* mRNA and *S6K1* mRNA were significantly more upregulated in the COPD group than in the control group (p<0.001 and p <0.01 respectively; Figure 1A and B). Furthermore, *mTOR* mRNA was negatively correlated with FEV_1 (%pred) (r=−0.4; Figure 1D and E). *LC3B* mRNA was significantly more downregulated in the COPD group than in the control group (p <0.05) (Figure 1C), while *LC3B* mRNA was positively correlated with FEV_1(%pred) (r=0.28; Figure 1D and F). These results suggest that mTOR/S6K1 may play a significant role in COPD pathogenesis, with a negative correlation observed between its activation and large airway function.

Rapamycin Improves Large and Small Airway Function in Ozone-Induced COPD Mice Model

As shown in Figure 2, the large airway ingredients (FEV_{25}/FVC, FEV_{50}/FVC and FEV_{75}/FVC, and FEF_{25}) and the small airway parameters (FEF_{50}, FEF_{75}, and MMEF) decreased in ozone-exposed mice compared with the mice in the control group ($p < 0.05$ for FEV_{25}/FVC, FEV_{50}/FVC, FEV_{75}/FVC, and FEF_{75}; $p < 0.001$ for FEF_{25}; $p < 0.01$ for FEF_{50} and MMEF), suggesting that the ozone-exposed mice model was successful. To further confirm whether the mTOR pathway takes roles in vivo, we tested the protective effects of rapamycin in an ozone-exposed mouse model. As expected, rapamycin treatment improved the declined FEV_{50}/FVC ($p < 0.05$) and FEF_{25} ($p < 0.05$), as well as the small airway

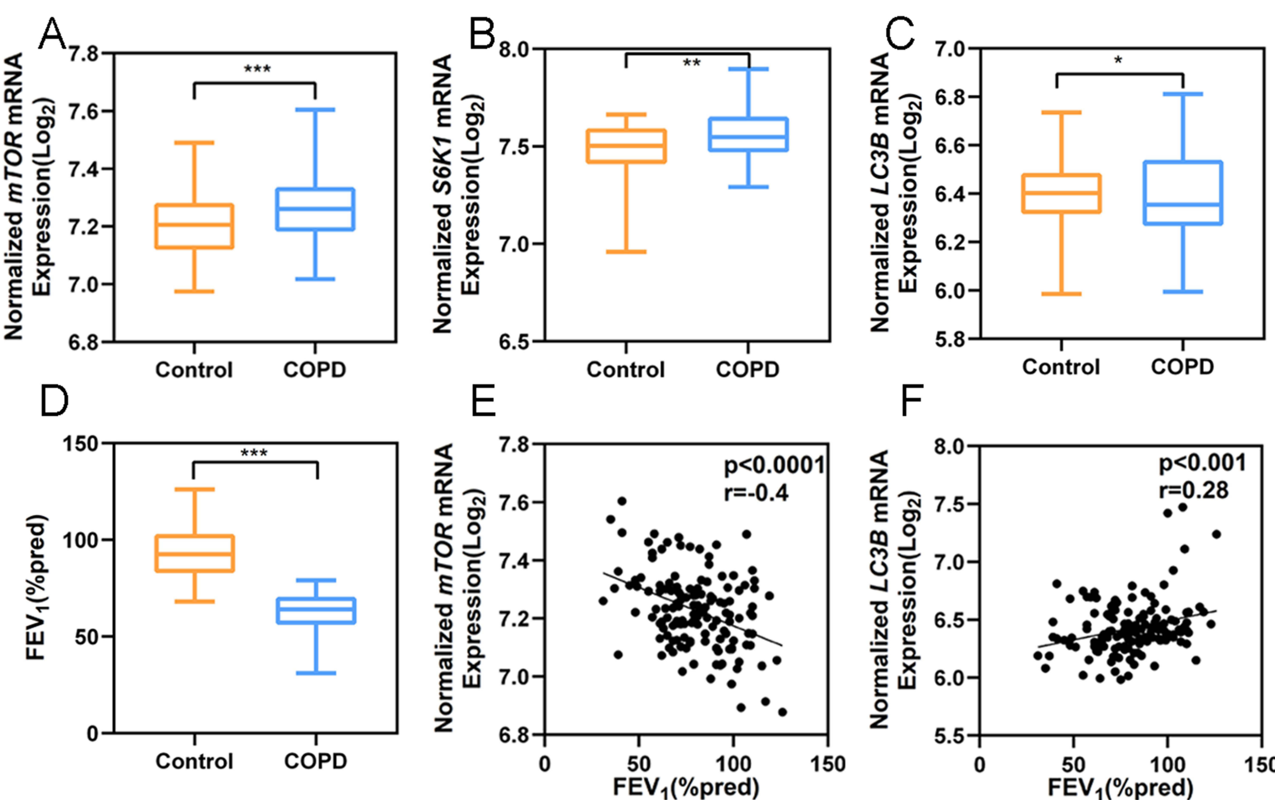

Figure 1 *mTOR* mRNA expression is significantly upregulated in patients with COPD. (**A**) *mTOR* mRNA, (**B**) *S6K1* mRNA, (**C**) *LC3B* mRNA, (**D**) FEV_1(%pred), and correlations of FEV_1(%pred) with *mTOR* mRNA (**E**) and *LC3B* mRNA (**F**) of the control and COPD groups in GSE37147 were analyzed. n=82 in the control group; n=57 in the COPD group. Values are presented as the mean ± SEM. *p<0.05, **p<0.01, ***p<0.001.

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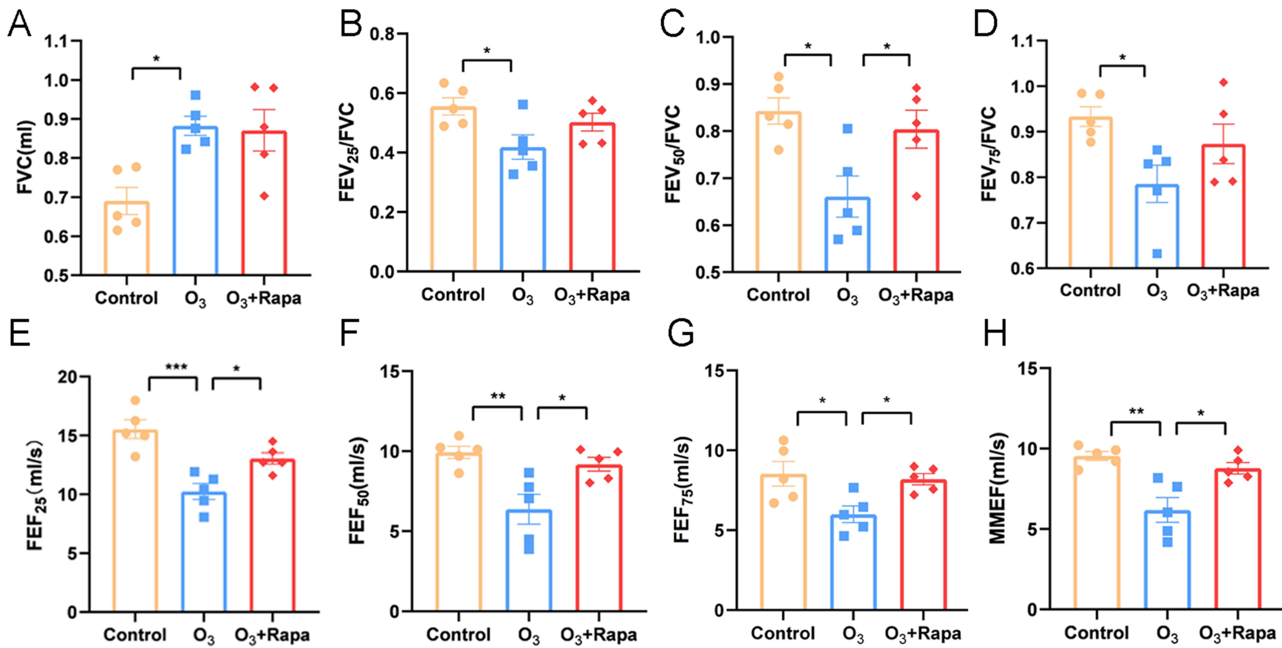

Figure 2 Rapamycin improves large airway and small airway function in an ozone-exposed mouse model. The evaluation of the large airway index: (**A**) FVC, (**B**) FEV_{25}/FVC, (**C**) FEV_{50}/FVC, (**D**) FEV_{75}/FVC, (**E**) FEF_{25}. The assessment of the small airway parameters: (**F**) FEF_{50}, (**G**) FEF_{75}, (**H**) MMEF. n=5 per group. Values are presented as the mean ± SEM. *p<0.05, **p<0.01, ***p<0.001.
Abbreviations: O_3, ozone. Rapa, rapamycin.

parameters ($p < 0.05$ for FEF_{50}, FEF_{75}, and MMEF) impaired by the ozone, compared with the ozone-exposed group, suggesting that rapamycin treatment effectively alleviates airway obstruction in this murine model of ozone exposure.

Rapamycin Alleviates Ozone-Induced Emphysema

Histological analysis revealed that the lung tissues in the ozone-exposed mouse group showed obvious pathological changes, including intra-alveolar edema, alveolar cavity enlargement, fusion between alveolar walls, and a large number of infiltrating inflammatory cells (Figure 3A). Hence, the above changes also certified that this COPD mice model was successful. On the contrary, the above pathological changes in the rapamycin-treated group were different from the ozone-exposed group: the inflammatory infiltration and emphysema were partially reversed (Figure 3A).

In addition, the MLI, a reflection of the emphysema index, was higher in the ozone-exposed group than in the control group (p <0.01), but rapamycin treatment resulted in a lower MLI than the ozone-exposed group (p <0.05) (Figure 3B). A similar tendency was observed for MAA, as it was significantly elevated in the ozone-exposed group (p <0.001), while decreased in the rapamycin-treated group (p <0.05) (Figure 3D). In contrast, the destructive index decreased in the ozone-exposed group ($p < 0.01$), whereas rapamycin treatment can reverse alveolar destruction caused by ozone exposure ($p < 0.05$) (Figure 3C). These results suggest that rapamycin treatment attenuates some of the phenotypic effects of ozone-induced emphysema in this murine model.

Rapamycin Treatment Mitigates Inflammatory Cell Infiltration in Bronchoalveolar Lavage Fluid (BALF) and Cytokine Expression in the Lung Tissue

To further investigate the efficacy of mTOR inhibitor-rapamycin in ozone-induced inflammation, differential inflammatory cell infiltration of lung tissues in ozone-induced COPD mice was calculated. As shown in Figure 4A, the inflammation scores were higher in the ozone-exposed group than the control group (p <0.001) and were lower in the rapamycin-treated group than the ozone-exposed group (p <0.05).

In addition, differential cell counts of the BALF were counted. As expected, the total cell number in the BALF of ozone-exposed mice was enhanced significantly (p<0.001), with increased neutrophils (p<0.001). Correspondingly,

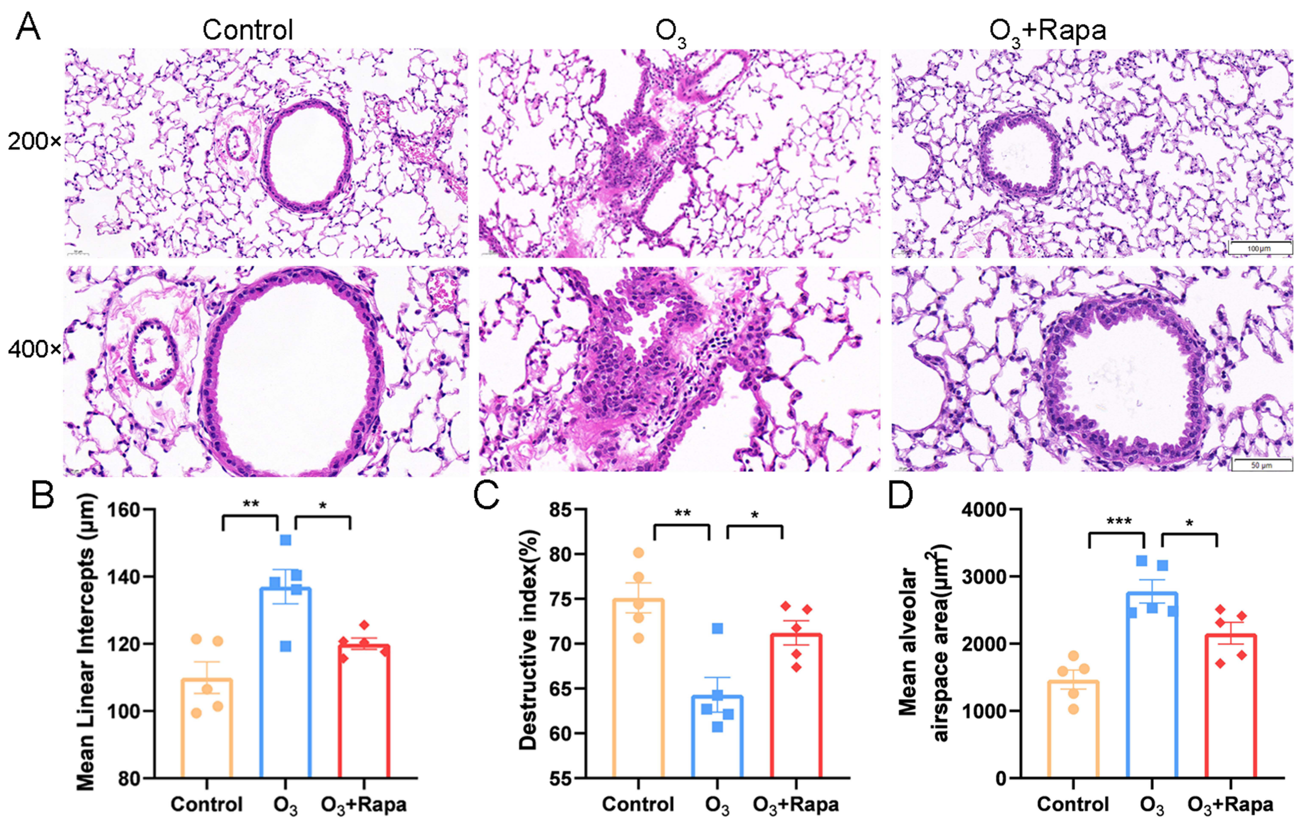

Figure 3 Rapamycin alleviates ozone-exposed emphysema in an ozone-exposed mouse model. (**A**) Lung histopathology of mice using H&E staining at 200x magnification (scale bar: 100 μm) and 400x magnification (scale bar: 50 μm). (**B**) Mean linear intercept. (**C**) Destructive index. (**D**) Mean alveolar airspace area. n=5 per group. Values are presented as the mean ± SEM. *p<0.05, **p<0.01, ***p<0.001.
Abbreviation: H&E, hematoxylin-eosin.

rapamycin treatment significantly reduced the numbers of total cells (p <0.01) and neutrophils (p <0.01) respectively (Figure 4B and C).

Consistent with cell infiltration, the expressions of *IL-1β* mRNA, *TNF-α* mRNA, and *NF-κb* mRNA in the lung tissue were significantly elevated in the ozone-exposed group (p <0.05 respectively) but decreased in the rapamycin-treated group (p < 0.05 respectively; Figure 4D–F). These data confirmed and validated the effect of rapamycin in mitigating inflammation.

Rapamycin Ameliorates Large and Small Airway Remodeling in Mice with COPD

Masson staining revealed (Figure 5A) that collagen accumulated in the lung parenchyma in ozone-exposed mice, and these changes were attenuated by rapamycin treatment. To further investigate the role of rapamycin in airway remodeling, collagen I and α-SMA were detected by immunohistochemistry (Figure 5B and C). As an essential component of the extracellular matrix (ECM), collagen I increased in the small and large airways of ozone-exposed mice (p<0.05 for all), and decreased in these airways with the rapamycin treatment (p<0.05 for all). Consistent with collagen I, α-SMA showed an upward tendency in both the small and large airways of ozone-exposed mice (p<0.01 for small airways, p<0.05 for large airways), whereas rapamycin treatment reversed this tendency (p<0.05 for all). Taken together, rapamycin improves airway remodeling induced by ozone exposure.

Rapamycin Mitigates Mucus Hypersecretion in the Airway Epithelium of COPD Mice

To illustrate the function of rapamycin in airway hypersecretion, mucus secretion was determined using PAS staining (Figure 6). Mucus secretion was elevated in both large and small airways compared with the control group (p<0.05 for

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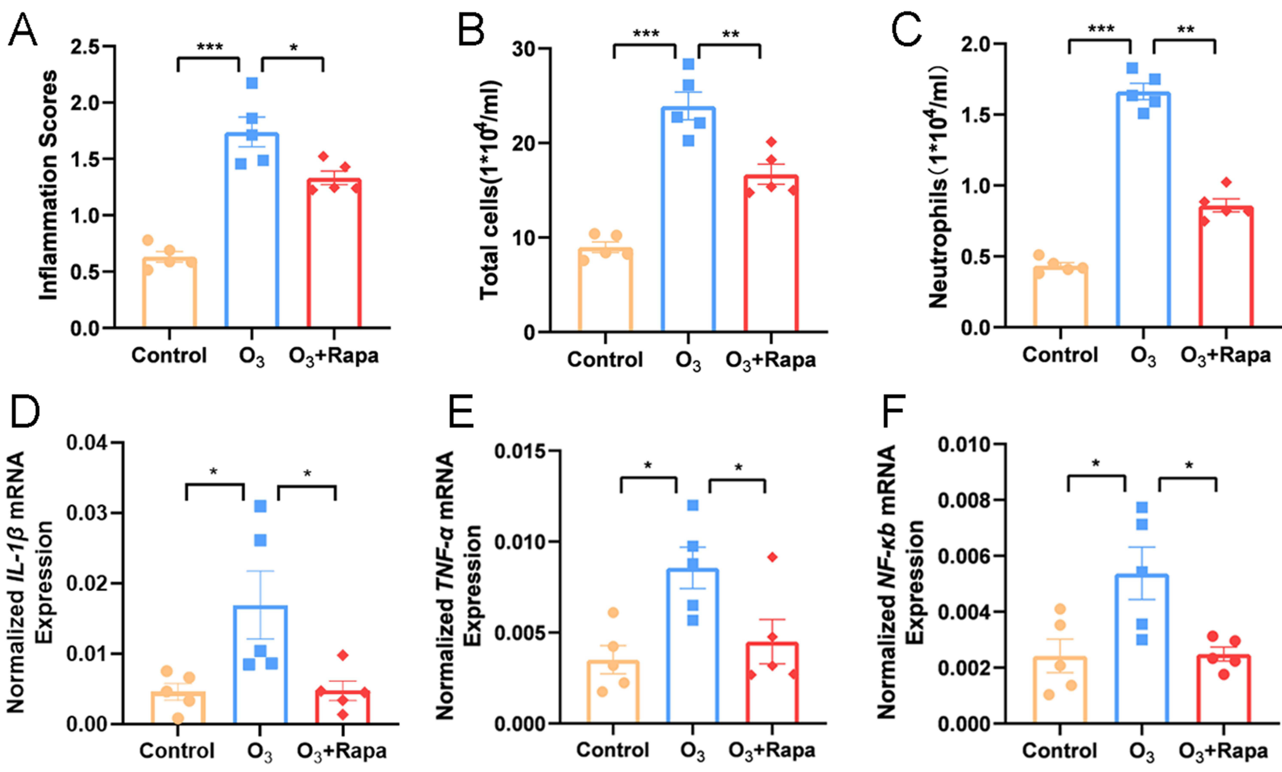

Figure 4 Rapamycin mitigates inflammatory cell infiltration in BALF and cytokine expression in the lung tissue of ozone-exposed mice. (**A**) Inflammation scores. (**B** and **C**) total cells and neutrophils in BALF. (**D**–**F**) *IL-1β* mRNA, *TNF-α* mRNA, and *NF-κb* mRNA in the lung tissue. n=5 per group. Values are presented as the mean ± SEM. *p<0.05, **p<0.01, ***p<0.001.
Abbreviation: BALF, bronchoalveolar lavage fluid.

all). Surprisingly, rapamycin treatment significantly alleviated ozone-induced mucus secretion (p<0.05 for both large and small airways), which was also the basis of rapamycin in improving lung function.

Rapamycin Promotes LC3B Expression Through the mTOR/S6K1 Pathway

To verify whether the mTOR/S6K1 pathway is involved in the autophagy of COPD, mTOR, S6K1, and the autophagy marker LC3B were detected by Western blot analyses of the lung tissue. Consistent with the analysis from the database, the expression of mTOR and S6K1 both increased in the ozone-exposed group ($p < 0.05$ respectively) but decreased in the rapamycin-treated group ($p < 0.05$ respectively; Figure 7A–C). In contrast, LC3B decreased in the ozone-exposed group ($p < 0.05$), whereas rapamycin treatment can reverse the downward trend caused by ozone exposure ($p < 0.05$; Figure 7A, D). These results suggest that rapamycin attenuating ozone-induced airway inflammation and emphysema may be due to a LC3B-dependent mechanism.

mTOR Expression is Correlated with Lung Function, TNF-α, and LC3B in the Lung Tissue

To further evaluate whether mTOR expression was correlated with the severity of airway inflammation and emphysema, Spearman's rank analysis was used to analyze the relationship of small airway parameters, large airway elements, cytokines, inflammatory cells, MLI, mTOR, and LC3B expression (Figure 8).

As expected, both small-airway and large-airway variables showed negative correlations with MLI, indicating that small airway and large airway dysfunction prompted emphysema. In another way, lung function ingredients were negatively correlated with neutrophils in BALF and cytokines in the lung tissue, especially TNF-α. The Spearman r values between neutrophils and FEF_{50}, FEF_{75}, and MMEF were −0.74 (p <0.001), −0.67 (p <0.01), and −0.75

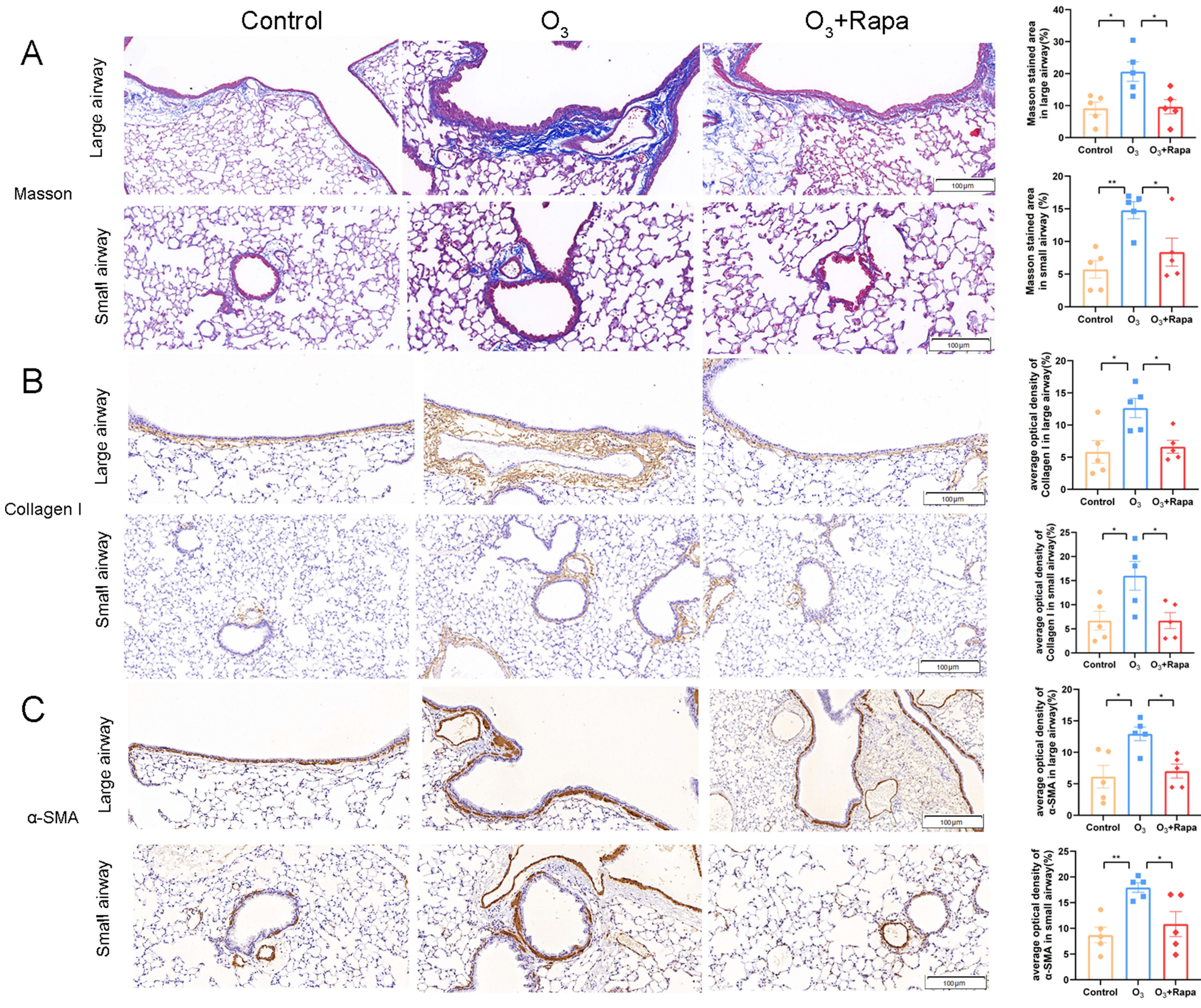

Figure 5 Rapamycin improves large and small airway remodeling in COPD mice. Representative images and quantification of Masson staining (**A**), immunohistochemistry of collagen I (**B**) and α-SMA (**C**). Scale bar: 100 μm. n=5 per group. Values are expressed as the mean ± SEM. *p<0.05, **p<0.01.

(p <0.001), respectively. A similar relevance was observed for TNF-α, as the Spearman r values for the FEF_{50}, FEF_{75}, and MMEF were −0.67 (p <0.01), −0.55 (p <0.05), and −0.64 (p <0.01), respectively. mTOR expression in the lung tissue was negatively correlated with both small airway and large airway function components. The Spearman r values between mTOR with FEF_{25}, FEV_{25}/FVC, FEV_{50}/FVC, and MMEF were −0.52 (p <0.05), −0.63 (p <0.01), −0.50 (p <0.05), and −0.52 (p <0.05), respectively. Interestingly, mTOR was also strongly correlated with TNF-α (r=0.50, p <0.05) and LC3B (r=−0.47, p <0.05), suggesting that mTOR was associated with airway inflammation. As an indicator of autophagy, LC3B had negative correlations with TNF-α in the lung tissue (r=−0.47, p <0.05), neutrophils in BALF (r= −0.70, p <0.01), and MLI (r=−0.64, p <0.01).

Discussion

In this study, we demonstrate that ozone exposure activates the mTOR pathway and induces airway inflammation and emphysema. We further reveal that the mTOR inhibitor improves both large and small airway function, inhibits inflammatory cytokine release, reduces ozone exposure-induced collagen-I and α-SMA synthesis, and mitigates mucus secretions in both large and small airways in vivo. mTOR pathway activation contributes to downstream dysregulated

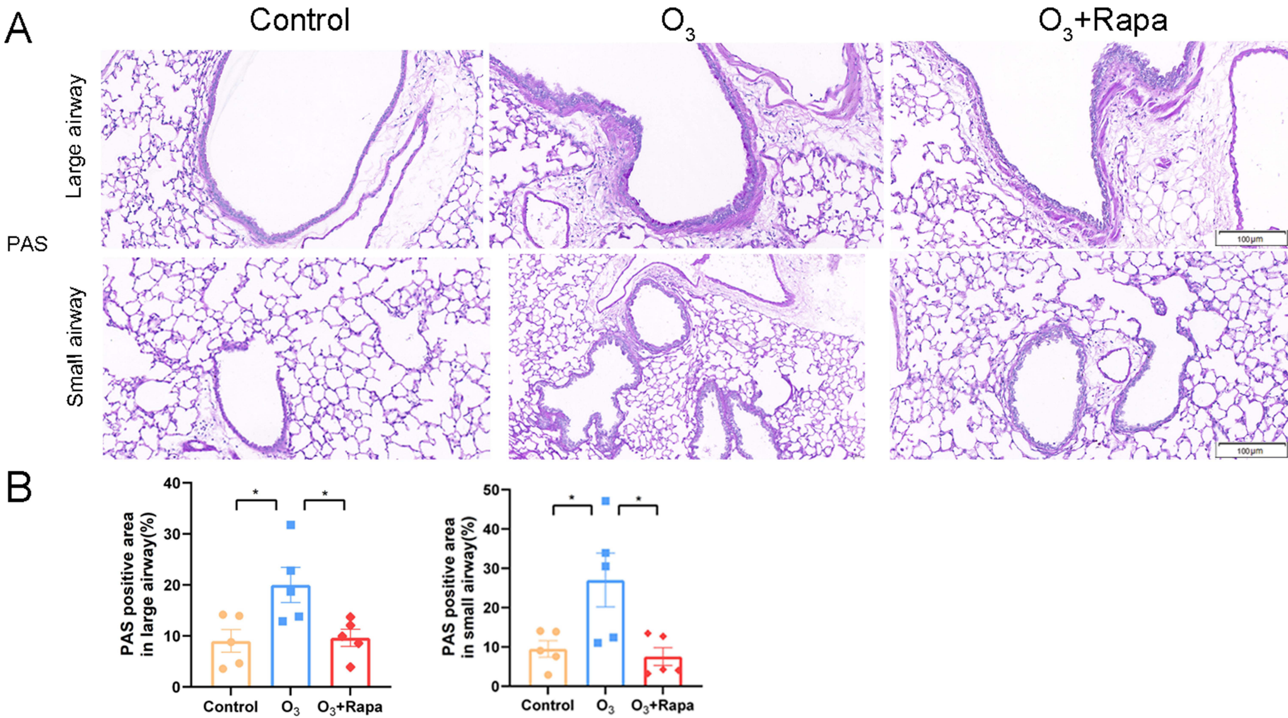

Figure 6 Rapamycin mitigates mucus hypersecretion in the airway epithelium of COPD mice. (**A**) Representative images of PAS staining in large and small airways. Scale bar: 100 μm. (**B**) Quantification of PAS staining in large and small airways. n=5 per group. Values are presented as the mean ± SEM. *$p<0.05$.
Abbreviation: PAS, periodic acid-Schiff.

S6K1 expression in response to ozone exposure. Moreover, it is likely that the downstream LC3B-mediated autophagy exerts a protective role in the ozone exposure-induced mouse model.

mTOR is a serine-threonine protein kinase of the phosphatidylinositol 3-kinase (PtdIns3K)-related family.[20] Extensive studies have established a dominant role of mTOR in regulating cellular growth and metabolism in response to growth factors and nutrients, and these studies reveal that the mTOR signaling pathway is implicated in the progression of cancer, obesity, autoimmune diseases, and senescence.[21–23] Evidence has revealed that mTOR activation promotes lung inflammation, oxidative stress, and alveolar wall destruction,[24] which was accompanied by driving lung cell senescence and lung alterations in COPD.[25] Moreover, the mTOR pathway also regulates autophagy to induce apoptosis of alveolar epithelial cells in COPD. Blocking the mTOR pathway can inhibit the NF-κB signaling pathway to reduce inflammatory cell infiltration and inflammatory mediator release,[26] further alleviating inflammation. It is noteworthy that the protective role of mTOR inhibitor is observed not only in PM-induced airway epithelial injury but also in COPD.[27] COPD is strongly correlated with inflammation and cell ageing, both of which are linked to increased mTOR activity.[25] Accordingly, inhibition of the mTOR pathway has been proposed as a promising therapeutic approach and is under investigation in several chronic airway diseases. Previous studies have shown that rapamycin, a well-established mTOR inhibitor, can mitigate smoke-induced inflammatory and oxidative stress responses, as well as reduce cell apoptosis-key drivers of COPD progression.[28] Moreover, rapamycin treatment was reported to protect mice against cigarette smoke-induced emphysema.[29] However, despite these findings, the precise mechanisms by which mTOR activation contributes to COPD pathogenesis remain to be fully elucidated. Here, in samples obtained from patients with COPD, we observed a significant upregulation of both mTOR and its downstream effector S6K1. Importantly, the expression levels of these molecules were negatively correlated with large airway function, indicating that enhanced activation of the mTOR/S6K1 signaling may contribute to airway obstruction. This association suggests a potential pathogenic role of the mTOR/S6K1 pathway in COPD, whereby its dysregulation could exacerbate structural and functional impairment of the large airways.

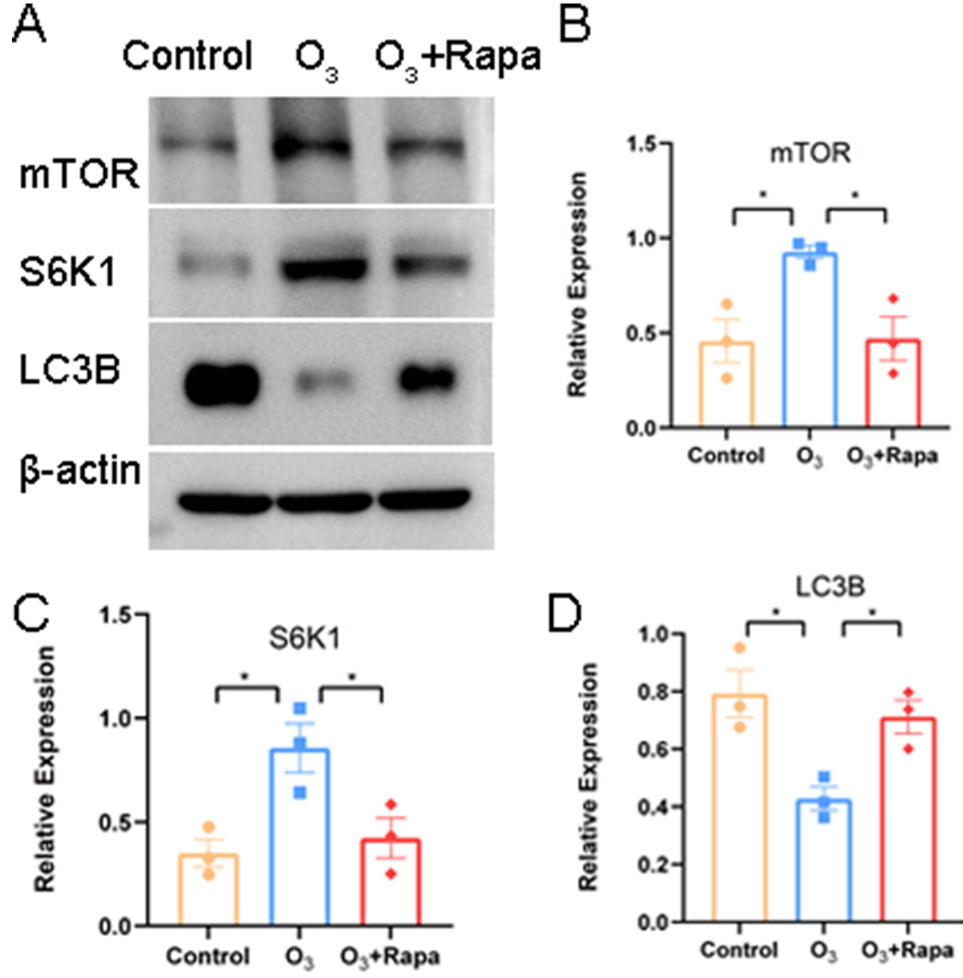

Figure 7 Rapamycin promotes LC3B expression through the mTOR/S6K1 pathway. (**A**)The expression of mTOR, S6K1, and the LC3B protein in the lung tissue by Western blot. (**B**–**D**) Relative protein expression. Values are presented as the mean ± SEM. **p*<0.05.

To further elucidate the potential mechanisms underlying the involvement of the mTOR pathway in COPD, we conducted in vivo experiments using an ozone-exposed mouse model. Consistent with our previous studies,[30,31] we successfully established a COPD mouse model induced by ozone exposure, with remarkable emphysema, dysfunction of both large and small airways, inflammatory cell infiltration, airway remodeling, and mucus hypersecretion. Notably, for the first time, the mTOR pathway was activated in ozone-exposed COPD mice, as evidenced by elevated expression of its downstream effector S6K1. These findings align closely with our clinical data observed in patients with COPD, highlighting the pathological relevance of the mTOR/S6K1 signaling in disease progression. Building upon this observation, we next investigated whether blocking the mTOR pathway could attenuate emphysema and airway inflammation in vivo. By using rapamycin to inhibit the mTOR pathway, we demonstrated that rapamycin treatment improved lung function and emphysema index, ameliorated collagen-I and α-SMA synthesis, and eliminated airway mucus hypersecretion, suggesting the therapeutic potential of mTOR inhibitors in COPD. Moreover, rapamycin treatment significantly decreased inflammatory cell infiltration, with a downward trend in IL-1β, TNF-α, and NF-κB expression. Collectively, these results provide compelling preclinical evidence that mTOR inhibitor exerts protective effects against ozone-induced COPD pathogenesis.

Several mechanisms contribute to the pathogenesis of COPD. The disruption of the balance between autophagy and proliferation in the lung tissue contributes to the COPD pathogenesis.[32] Because mTOR is an essential negative regulator of autophagy, its activation may decrease autophagy.[10,33] Therefore, autophagy, which is associated with upstream

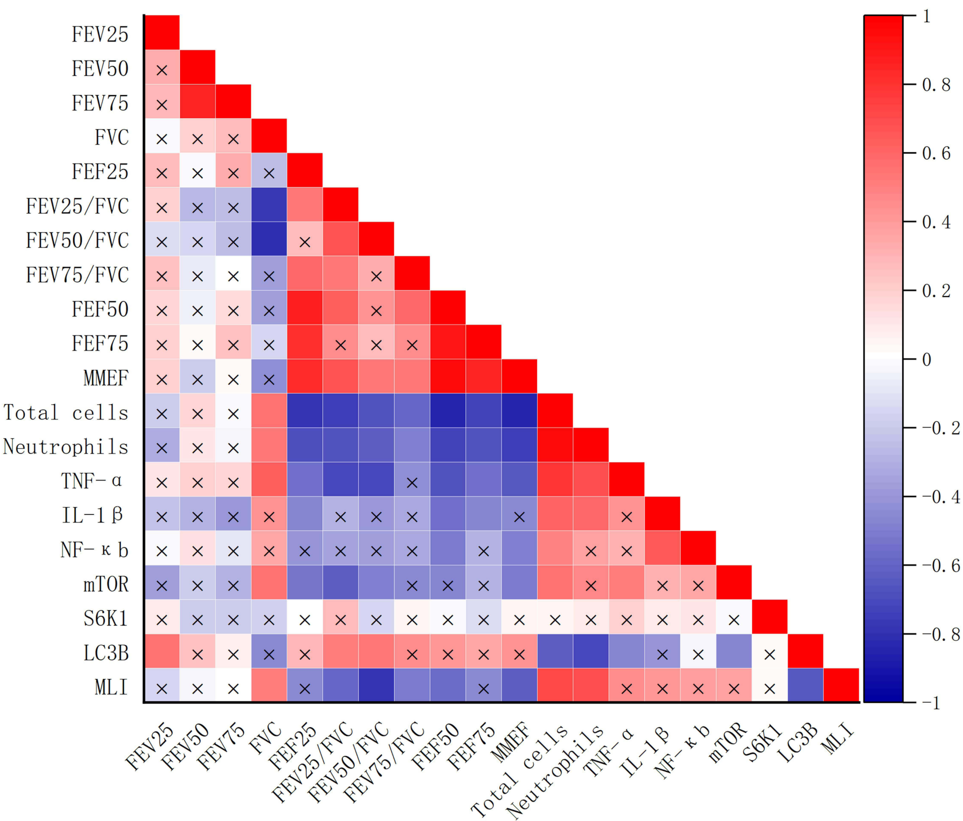

Figure 8 Heatmap of Spearman correlation between outcomes. The mTOR expression was correlated with lung function, TNF-α, and LC3B in the lung tissue. A Spearman correlation coefficient matrix was constructed and Spearman rank correlation tests were performed. A cross "×" indicates no statistical significance.

signaling mTOR, plays an important role in regulating airway epithelial inflammatory responses in COPD[27] and asthma.[12] LC3B, as an autophagosome biogenesis-related protein, participated in it to formulate autophagic vesicles.[34] Interestingly, mTOR deficiency was dependent on the downstream autophagy in which LC3B was involved.[35] In general, mTOR activation inhibited autophagy with the suppression of autophagy-associated proteins such as Atg13, ULK1, and LC3B.[34,36] Consistent with this possibility, the autophagic mechanism of LC3B in the nucleus is considered a regulatory factor of ciliary differentiation and airway epithelial cell function.[37] As expected, using a public database, we found a decrease in *LC3B* mRNA in patients with COPD, showing a positive correlation with the large airway function. To further explore this relationship, we conducted in vivo experiments to examine whether blockade of the mTOR pathway could modulate autophagy-associated proteins in vivo. By using rapamycin to inhibit the mTOR pathway, we demonstrated an enhanced LC3B expression in the lung tissue, demonstrating that LC3B-mediated autophagy may be involved in ozone-mediated inflammation. Importantly, the upregulation of LC3B expression following rapamycin treatment suggests that LC3B-mediated autophagy exerts a protective effect in ozone-exposed mice. This finding highlights a potential mechanistic link between mTOR inhibition and LC3B-mediated autophagy, offering valuable insight for future studies aimed at elucidating the role of LC3B in ozone-induced COPD pathogenesis and for identifying novel therapeutic strategies.

Furthermore, we sought to determine whether activation of the mTOR pathway was associated with the severity of airway inflammation and emphysema. In the current study, lung function ingredients were negatively correlated with neutrophils in BALF and cytokines in the lung tissue, especially TNF-α. mTOR expression in the lung tissue was negatively correlated with both small airway and large airway function components, suggesting that mTOR activation contributes to airway dysfunction. On the other hand, mTOR was positively correlated with TNF-α and inversely correlated with LC3B, further supporting its close association with airway inflammation. As a key marker of autophagy, LC3B expression exhibited negative correlations with TNF-α in the lung tissue, neutrophils in BALF, and MLI,

highlighting that reduced LC3B expression is linked to exacerbated airway inflammation and emphysematous changes. Collectively, these findings highlight that high mTOR activity combined with diminished LC3B-mediated autophagy promotes airway obstruction, inflammation, and emphysema in ozone-exposed mice. Taken together, we further identify that the role of targeting the mTOR pathway in airway inflammation and emphysema may be LC3B-dependent, which is relevant to autophagy.

In this study, we established a murine model of COPD through chronic ozone exposure, which successfully recapitulated the key pathological features of the disease, including impaired lung function, severe emphysema index, infiltration of inflammatory cytokines and inflammatory cells, airway remodeling, and mucus hypersecretion. Treatment with rapamycin effectively improved pulmonary function, attenuated emphysematous changes, reduced airway inflammation and airway remodeling, and alleviated mucus overproduction. These findings provide strong evidence that inhibition of the mTOR pathway plays a pivotal role in maintaining the balance between cell survival and death in the context of COPD. To our knowledge, it was the first study to clearly elaborate the physiological relevance of mTOR/S6K1 in regulating ozone-induced airway inflammation and emphysema in vivo. Furthermore, our data confirmed that the potential role of mTOR pathway was dependent on the downstream of LC3B-mediated autophagy, highlighting a potential mechanistic pathway through which mTOR influences disease progression. Taken together, our results identify mTOR not only as a promising therapeutic target for COPD but also as a potential serum biomarker for disease evaluation. Importantly, this study provides critical preclinical evidence supporting the efficacy of rapamycin, underscoring its potential as a novel therapeutic candidate for COPD. Future clinical investigations will be essential to validate these findings and to further evaluate the safety, efficacy, and optimal clinical application of rapamycin in patients with COPD.

Nevertheless, this study had several limitations. First, there is an insufficiency of in vitro experiments on the mechanisms of targeting the mTOR pathway through LC3B in epithelial cells. In future studies, the underlying mechanism in vitro should be further investigated in our subsequent work. Second, we did not further explore the relationships between autophagy and the mTOR pathway, which would be a vital interpretation of the mechanisms of mTOR inhibitors in inflammation. The potential reaction mechanism between autophagy and the mTOR pathway requires further validation.

Conclusion

In conclusion, mTOR pathway activation, which occurs in airway inflammation and emphysema, may be LC3B-dependent. The mTOR inhibitor rapamycin eliminated airway inflammation and emphysema caused by ozone exposure, suggesting that targeting the mTOR pathway may be a novel therapeutic strategy for COPD. These findings provide strong evidence that rapamycin could serve as a novel therapeutic agent for COPD.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

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.

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