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MTOR Suppresses Cigarette Smoke–Induced Epithelial Cell Death and Airway Inflammation in Chronic Obstructive Pulmonary Disease

Yong Wang,*,[1] Juan Liu,*,[1] Jie-Sen Zhou,* Hua-Qiong Huang,* Zhou-Yang Li,* Xu-Chen Xu,* Tian-Wen Lai,* Yue Hu,* Hong-Bin Zhou,* Hai-Pin Chen,* Song-Min Ying,* Wen Li,* Hua-Hao Shen,*,† and Zhi-Hua Chen*

Airway epithelial cell death and inflammation are pathological features of chronic obstructive pulmonary disease (COPD). Mechanistic target of rapamycin (MTOR) is involved in inflammation and multiple cellular processes, e.g., autophagy and apoptosis, but little is known about its function in COPD pathogenesis. In this article, we illustrate how MTOR regulates cigarette smoke (CS)–induced cell death, airway inflammation, and emphysema. Expression of MTOR was significantly decreased and its suppressive signaling protein, tuberous sclerosis 2 (TSC2), was increased in the airway epithelium of human COPD and in mouse lungs with chronic CS exposure. In human bronchial epithelial cells, CS extract (CSE) activated TSC2, inhibited MTOR, and induced autophagy. The TSC2–MTOR axis orchestrated CSE-induced autophagy, apoptosis, and necroptosis in human bronchial epithelial cells; all of which cooperatively regulated CSE-induced inflammatory cytokines IL-6 and IL-8 through the NF-κB pathway. Mice with a specific knockdown of *Mtor* in bronchial or alveolar epithelial cells exhibited significantly augmented airway inflammation and airspace enlargement in response to CS exposure, accompanied with enhanced levels of autophagy, apoptosis, and necroptosis in the lungs. Taken together, these data demonstrate that MTOR suppresses CS-induced inflammation and emphysema—likely through modulation of autophagy, apoptosis, and necroptosis—and thus suggest that activation of MTOR may represent a novel therapeutic strategy for COPD. ***The Journal of Immunology*****, 2018, 200: 2571–2580.**

Chronic obstructive pulmonary disease (COPD), contributing significantly to chronic morbidity and mortality, was responsible for ∼6% of all deaths worldwide in 2012 and will be the fifth-largest public health burden and third leading cause of death by 2020 (1, 2). The disease is characterized by partly reversible airflow obstruction, chronic airway inflammation, and systemic effects or comorbidities (3). Although several factors, including genetic or environmental factors, may contribute to the development of COPD, cigarette smoking is still the highest risk factor (4). Chronic bronchitis and emphysema are pathological features of COPD (5). However, the cellular and molecular mechanisms mediating the pathogenesis of cigarette smoke (CS)–induced COPD remain largely unknown.

The mechanistic (previously known as mammalian) target of rapamycin (MTOR) is a core regulator of cellular metabolism and survival as well as protein synthesis, which has been implicated in cancer, neurodegeneration, obesity, and rheumatic disease (6, 7). Several studies have demonstrated that MTOR participates in the process of inflammation and that it mediates apoptotic cell death (8). By binding with raptor and rictor, MTOR exists in two distinct complexes: MTOR complex (MTORC) 1 (MTORC1) and complex 2 (MTORC2) (9). MTORC1, sensitive to rapamycin, regulates protein synthesis through the activation of ribosomal protein S6 (RPS6) kinase and inhibition of the eukaryotic transcriptional initiation factor 4E binding protein 1. MTORC2 is known to be insensitive to rapamycin and regulates the actin cytoskeleton of cell growth (10).

One of the important functions of MTOR is to suppress autophagy, a cellular digestion process which engulfs cytosolic proteins and damaged organelles to form double membrane–bound vesicles

*Key Laboratory of Respiratory Disease of Zhejiang Province, Department of Respiratory and Critical Care Medicine, Second Affiliated Hospital of Zhejiang University School of Medicine, Hangzhou 310009, China; and †State Key Laboratory of Respiratory Disease, Guangzhou Medical University, Guangzhou 510120, China

[1]Y.W. and J.L. contributed equally to this work.

ORCIDs: 0000-0002-0086-0882 (Y.W.); 0000-0003-4013-2473 (J.L.); 0000-0002-8888-6322 (H.-Q.H.).

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Address correspondence and reprint requests to Prof. Zhi-Hua Chen and Prof. Hua-Hao Shen, Key Laboratory of Respiratory Disease of Zhejiang Province, Department of Respiratory and Critical Care Medicine, Second Affiliated Hospital of Zhejiang University School of Medicine, 88 Jiefang Road, Hangzhou, Zhejiang 310009, China. E-mail addresses: zhihuachen@zju.edu.cn (Z.-H.C.) and huahaoshen@zju.edu.cn (H.-H.S.)

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Abbreviations used in this article: ATG5, autophagy related 5; *ATII-mtor*$^{-/-}$, *Mtor* knockout alveolar type II cell; BALF, bronchoalveloar lavage fluid; COPD, chronic obstructive pulmonary disease; CS, cigarette smoke; CSE, CS extract; HBE, human bronchial epithelial; LC3B, microtubule-associated protein 1 L chain 3B; MLI, mean linear intercept; MTOR, mechanistic target of rapamycin; MTORC, MTOR complex; NEC-1, necrostatin-1; p-AMPKα, p-AMP–activated protein kinase α; p-MLKL, phosphorylated mixed lineage kinase domain–like protein; RELA, v-rel reticuloendotheliosis viral oncogene homolog A; RIP, receptor-interacting protein kinase; RPS6, ribosomal protein S6; siRNA, small interfering RNA; SQSTM1, sequestosome-1; TSC2, tuberous sclerosis 2.

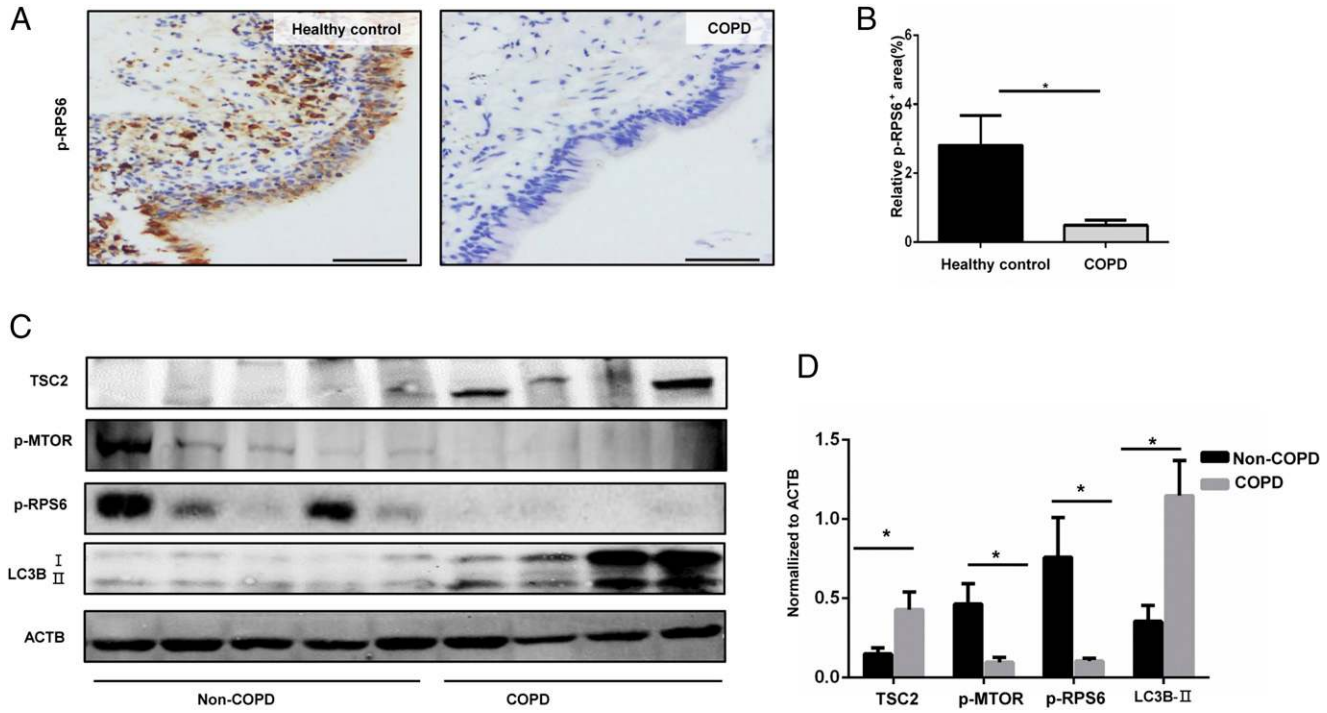

FIGURE 1. CS inactivates MTOR in human COPD lungs. (**A**) Representative micrographs of immunohistochemical staining for p-RPS6 and (**B**) quantification of bronchial p-RPS6 expression from COPD patients ($n = 8$) or healthy controls ($n = 6$). Scale bar, 100 μm. (**C**) Protein levels of TSC2, p-MTOR, p-RPS6, and LC3B in human lung homogenate samples from smokers with COPD ($n = 5$) or nonsmokers without COPD ($n = 6$). (**D**) Densitometry of the immunoblots. Band intensities were normalized to ACTB and the results were represented as fold change. Data are presented as mean ± SEM. $*p < 0.05$.

(autophagosome) (9). The autophagosome then fuses with the lysosome to degrade the engulfed components and to regenerate metabolic precursor molecules. Autophagy performs vital functions in cellular homeostasis and in response to different metabolic stresses (11).

Emerging evidence indicates that CS-induced airway epithelial cell death plays a crucial role in COPD development (12). Recently, we and others have demonstrated that autophagy is essential for CS-induced apoptosis, necroptosis, and mucus hyperproduction in airway epithelium (13–17). Selective autophagy pathways, e.g., mitophagy and ciliophagy, have also been shown to play a significant role in regulating CS-induced mitochondrial depolarization and cilia shortening in lung epithelium (16, 17). As the active suppressor of autophagy, MTOR has been linked closely with COPD as well. Yoshida et al. (18) showed that RTP801, a DNA-damage response gene, critically mediated CS-induced airway inflammation and emphysema through negatively regulating MTOR activity. Also, elastase has been reported to induce autophagy in lung epithelial cells by attenuating MTOR phosphorylation (19). However, the eventual function of MTOR in COPD pathogenesis and the detailed molecular mechanisms of how the MTOR–autophagy axis regulates COPD pathogenesis remain unclear. Moreover, despite the functions of autophagy in regulation of CS-induced apoptosis, cilia loss, and mucus production, the role and mechanisms of autophagy in CS-induced chronic airway inflammation are not well investigated. Interestingly, patients with breast cancer, renal cell carcinoma or other diseases, treated with MOTR inhibitors tend to develop dyspnea, cough or interstitial lung disease (20, 21), but the underlying mechanisms remain unclear.

This study aims to explore the role and mechanisms of MTOR in regulation of CS-induced autophagy, apoptosis, necroptosis, inflammation, and the consequent development of emphysema by using human bronchial and lung specimens from COPD patients, epithelial *Mtor*-specific knockout mice, and cultured human bronchial epithelial (HBE) cell line.

Materials and Methods

Patients

COPD was assessed according to the Global Initiative for Chronic Obstructive Lung Disease. Bronchial biopsy tissues were obtained from six healthy controls with normal lung function and eight smokers with COPD. Lung tissues of six nonsmokers without COPD and five smokers with COPD were acquired from the resected subpleural parenchyma at lung cancer surgery, avoiding areas involving tumors. All COPD patients were in a stable phase and were free from acute exacerbation for at least 4 wk. Participants were excluded if there was any chronic cardiopulmonary disease other than COPD (including asthma) and/or if they received oral or i.v. corticosteroids or any other anti-inflammatory drugs in the preceding 4 wk. All human subjects were of the Chinese Han population, and there were no statistical differences in age and sex between the groups. They were enrolled in the Affiliated Hospital of Guangdong Medical University and the Second Affiliated Hospital of Zhejiang University. The study protocols were approved by the ethics committees of both hospitals, and all participants provided written informed consent.

Cell culture, CS extract treatment, and cell viability

HBE cells were obtained from American Type Culture Collection (CRL-2741; ATCC) and were cultured in RPMI 1640 containing 10% FBS and antibiotics at 37°C in the presence of 5% CO_2. CS extract (CSE) was prepared and treated as described previously (13, 15). Cell viability was determined by Cell Counting Kit-8 assay (Liankebio, Hangzhou, China) following the manufacturer's instructions.

Chemicals and reagents

Necrostatin-1 (NEC-1) and Z-VAD were from Sigma-Aldrich (St. Louis, MO). The following Abs were used: microtubule-associated protein 1 L chain 3B (LC3B), purchased from Sigma-Aldrich; ACTB, v-rel reticuloendotheliosis viral oncogene homolog A (RELA), p-RELA, p-AMP–activated protein kinase α (p-AMPKα), tuberous sclerosis 2 (TSC2), p-TSC2, MTOR, p-MTOR, p-RPS6, sequestosome-1 (SQSTM1), receptor-interacting protein kinase (RIP) 1, and cleaved caspase-3, -9, obtained from Cell Signaling Technology (Danvers, MA); phosphorylated mixed lineage kinase domain–like protein (p-MLKL) from Millipore (Billerica, MA); and RIP3 from Abcam (Cambridge, MA).

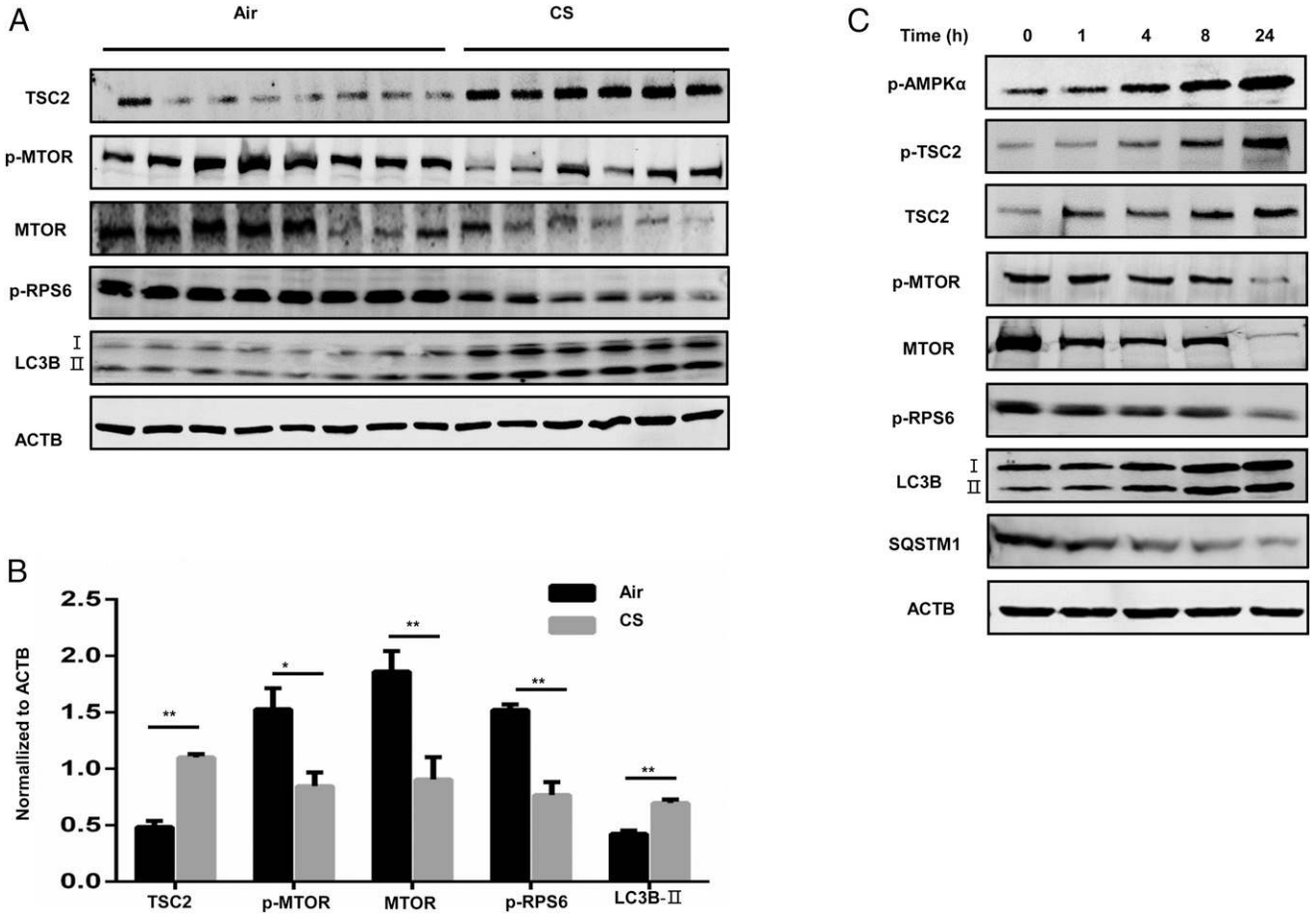

FIGURE 2. CS inactivates MTOR in mouse lungs and in HBE cells. (**A** and **B**) Protein levels of TSC2, p-MTOR, MTOR, p-RPS6, and LC3B in wild-type mouse lung exposed to air or CS were measured by immunoblotting and further assessed by the relative densitometry of bands. Band intensities were normalized to ACTB and the results were represented as fold change. (**C**) Expression of p-AMPKα, p-TSC2, TSC2, p-MTOR, MTOR, p-RPS6, LC3B, and SQSTM1 in HBE cells stimulated with 1% CSE for indicated times. Blots represent three independent experiments. Data are presented as mean ± SEM. $*p < 0.05$, $**p < 0.01$.

Small interfering RNAs (siRNAs) for control, *LC3B*, *autophagy related 5* (*ATG5*), and *RELA* were purchased from Santa Cruz (Dallas, TX); and an additional set of siRNAs for *TSC2* and *MTOR* were obtained from OriGene (Rockville, MD). The siRNA transfection reagent (GeneMute) were purchased from SignaGen Laboratories (Rockville, MD).

Transfection

All transfection was achieved according to the manufacturer's protocol as described previously (22). The *ATG5* plasmids (Addgene plasmid 22952, deposited by N. Mizushima) were transfected into HBE cells by MegaTran reagent (OriGene). HBE cells were treated with CSE 24 h after transfection.

RNA isolation and quantitative RT-PCR

Total RNA was isolated from cells with TRIzol (Takara Bio, Beijing, China). RNA was reverse transcribed using the Reverse Transcription Reagents (Takara Bio). Quantitative RT-PCR was carried out with SYBR Green system (Takara Bio) to measure the levels of human *IL-6* and *IL-8* mRNA. The levels of mRNA were normalized to GAPDH.

ELISA

Supernatants were collected from treated cells. IL-6 and IL-8 were measured in supernatants by the ELISA Kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

Immunoblotting

Cells lysates and lung tissue homogenates containing protease and phosphatase inhibitors were boiled for 10–15 min. Protein samples were separated on 6–15% SDS-PAGE and immunoblotted using the above Abs following standard methods. Band densities were quantified using densitometry (Odyssey; LI-COR, Lincoln, NE) and results were normalized by ACTB and represented as fold change.

Flow cytometry

After exposure to 4% CSE, HBE cells were stained with Annexin V/PI (MultiSciences, Hangzhou, China) and flow cytometry was performed following the manufacturer's protocol.

Immunohistochemistry staining

Paraffin-embedded tissues were deparaffinized and rehydrated. After treatment with 3% H_2O_2, tissue sections were incubated with Abs against p-RPS6 following standard methods. The Olympus BX53 inverted microscope was used to scan and analyze stained areas. The average percentages of positively stained areas were quantified by using ImageJ.

Mice

All the mice were of the C57BL/6 strain. Wild-type mice were purchased from the Animal Center of Slaccas (Shanghai, China). For the *Mtor* knockout bronchial epithelial cells (*mBE-mtor*$^{-/-}$) and alveolar type II cells (*ATII-mtor*$^{-/-}$), *Mtor*$^{flox/flox}$ (Jackson Laboratory) mice were crossed with *CC10-rtTA/(tetO)7-cre* or *SPC-rtTA/(tetO)7-cre* transgenic mice. CC10-rtTA/(tetO) 7-cre and SPC-rtTA/(tetO)7-cre transgenic mice were provided by Y. Ke (Zhejiang University School of Medicine). Doxycycline (Sigma-Aldrich) in drinking water (2 mg/ml) was fed to 4-wk-old mice to induce the deletion of Mtor. Transgene-negative littermates were used as the control mice, which were also fed doxycycline. Genomic DNA were extracted from tails or lungs for genotyping. Mice were exposed to filtered room air or total body CS for 6 mo. Total body CS exposure was performed in a stainless-steel chamber using a whole-body smoke exposure device (TE-10; Teague Enterprises, Woodland, CA) as described (15). The mice were exposed to 100 cigarettes/d (around 2 h CS exposure), 5 d/wk. The total particulate matter concentrations

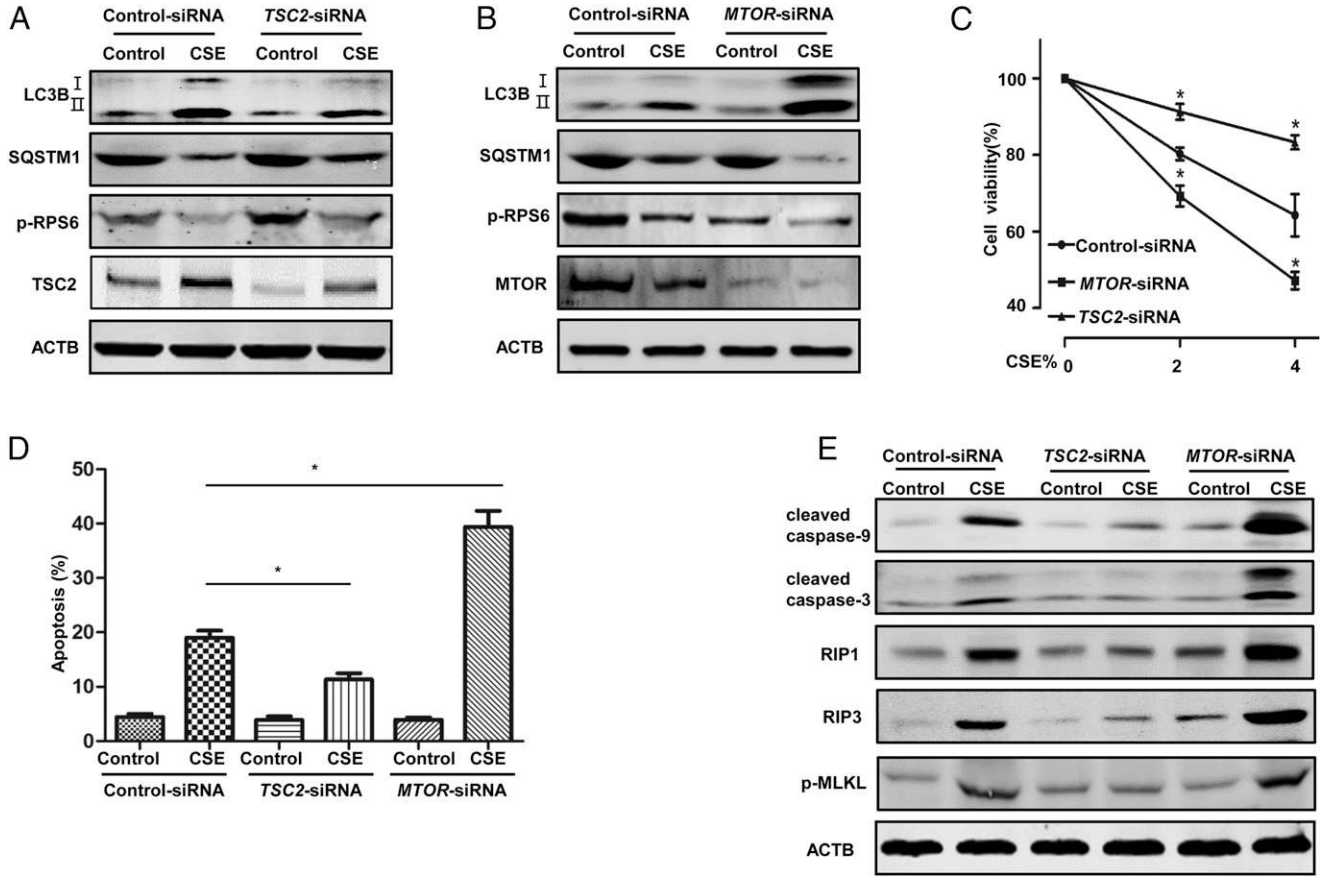

FIGURE 3. TSC2–MTOR axis regulates CSE-induced cell death in HBE cells. HBE cells were pretreated with control, *TSC2*, or *MTOR* siRNA for 24 h. (**A** and **B**) Cells were then incubated with 1% CSE for an additional 8 h and cell lysates were then subjected to immunoblotting for MTOR, TSC2, p-RPS6, LC3B, or SQSTM1. After siRNA transfection, HBE cells were treated with (**C**) indicated concentrations or (**D** and **E**) 4% CSE for 24 h, and (C) cells were subjected to Cell Counting Kit-8 assay, (D) staining of Annexin V, or (E) immunoblotting for cleaved caspase-9, cleaved caspase-3, RIP1, RIP3, and p-MLKL. Data (C and D) are mean ± SEM of three independent experiments. Blots (A, B, and E) also represent three independent experiments. $*p < 0.05$.

measured in the chamber were 150–180 mg/m^3. At 24 h after the last exposure to CS, bronchoalveloar lavage fluid (BALF) was collected and the number of inflammatory cells was counted as described previously (22). All the animal experimental protocols were approved by the Ethical Committee for Animal Studies at Zhejiang University, China.

Lung morphometry

After euthanasia, the left lungs of mice were fixed with 4% paraformaldehyde inflated at 30-cm H_2O pressure for 15 min and then stored in 4% paraformaldehyde for 24 h. The collected lungs were embedded in paraffin and stained with H&E using standard protocols. The right lungs of mice were removed from the thorax for immunoblot analysis. Airspace enlargement was represented by mean linear intercept (MLI) method as described (13, 15).

TUNEL

Paraffin-embedded mice lung tissues were deparaffinized and rehydrated. Apoptosis was measured using the TUNEL kit (Roche, Shanghai, China) according to the manufacturer's instructions. TUNEL-positive cells were counted and cells positive for TUNEL staining were expressed as a percentage of total cells.

Statistical analysis

Results are expressed as mean ± SEM. Statistical tests were analyzed using GraphPad Prism software (version 5.01; San Diego, CA). Statistical analyses were performed using the parametric two-tailed Student *t* test or nonparametric Mann–Whitney *U* test for the comparison of two groups, and ANOVA with post hoc Tukey corrections for the comparison of more than two groups. A p value <0.05 was considered to be statistically significant.

Results

CS inactivates MTOR through induction of TSC2 in human COPD lungs, in mouse lungs, and in HBE cells

To study the possible dysregulation of MTOR in COPD, we first examined the expression of MTOR in airway epithelium from COPD patients. As expected, the MTOR activity, as revealed by the expression of p-RPS6, was markedly decreased in airway epithelial cells from COPD patients when compared with the healthy controls (Fig. 1A, 1B). We found that the expression of p-MTOR and p-RPS6 was also decreased in lung tissue homogenates from COPD patients relative to that in control subjects (Fig. 1C, 1D). In contrast, the levels of TSC2, a master negative regulator of MTORC1, and the downstream autophagy marker LC3B were significantly increased in COPD lung tissues (Fig. 1C, 1D). These findings were consistent with our previous observation that autophagy is markedly elevated in COPD lung tissues (15). We further examined the changes of these markers in an animal model of COPD induced by chronic CS exposure. As shown in Fig. 2A and 2B, the levels of p-MTOR, MTOR, and p-RPS6 were notably decreased in CS-exposed mouse lungs relative to the air-exposed controls, whereas TSC2 was significantly elevated. Decreased MTOR may in turn activate autophagy, which was evidenced by the increased levels of the autophagy-related hallmark LC3B (Fig. 2A, 2B). In cultured HBE cells, CSE dose-dependently decreased the levels of MTOR and SQSTM1 while inducing the expression of LC3B (Supplemental Fig. 1). Further analysis

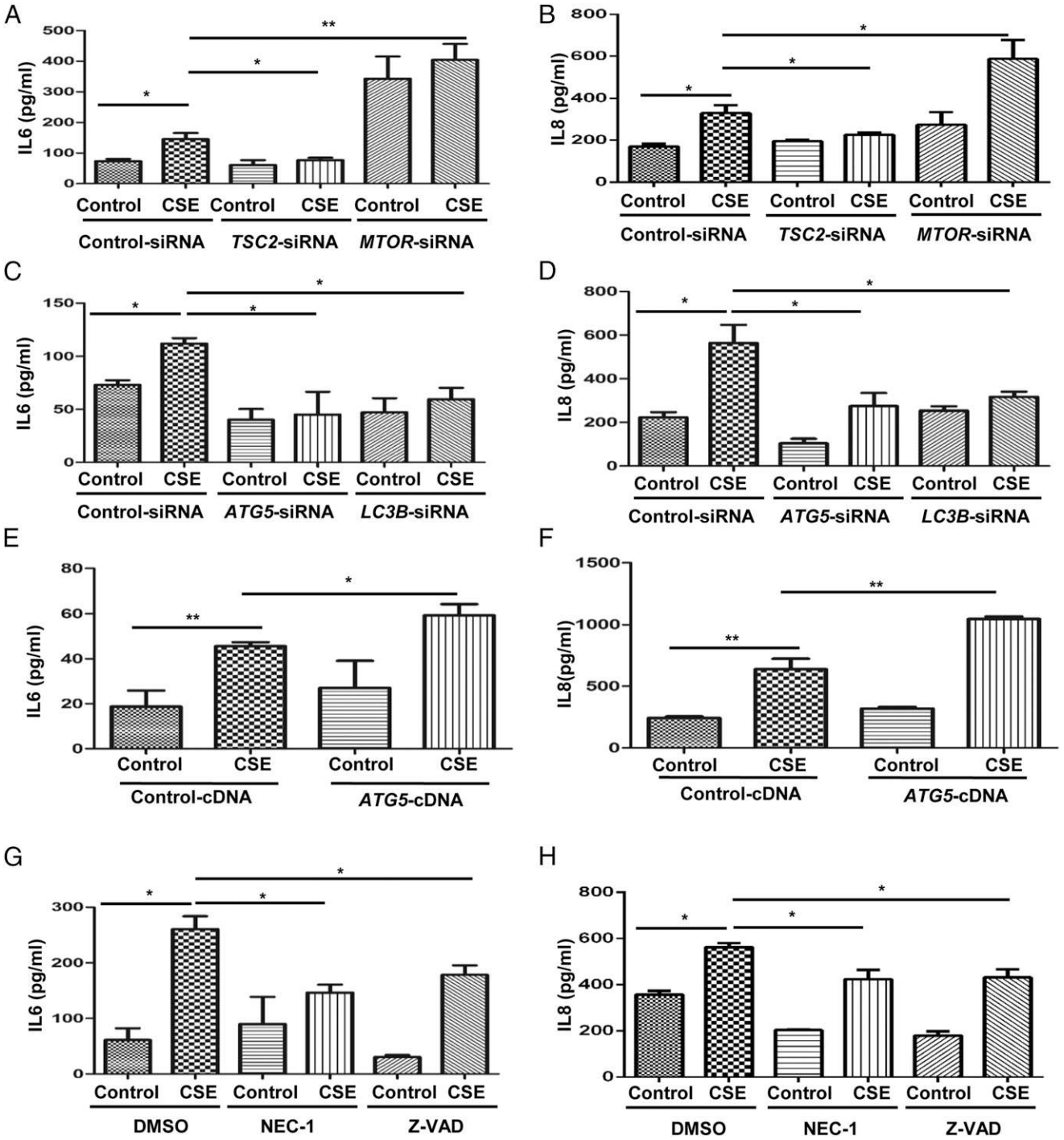

FIGURE 4. TSC2–MTOR axis and subsequent cell death pathways regulate CSE-induced inflammatory cytokine in HBE cells. (**A**–**F**) HBE cells were transfected with the indicated siRNAs or cDNAs for 24 h, followed by 1% CSE treatment for 24 h. Cell culture supernatants were collected for measuring the protein levels of IL-6 or IL-8 by ELISA. (**G** and **H**) HBE cells were cultured with NEC-1 (50 μM), Z-VAD (25 μM), or vehicle (DMSO) together with 1% CSE for 24 h, and the levels of IL-6 or IL-8 in culture supernatants were examined. Data represent mean ± SEM of three independent experiments. $*p < 0.05$, $**p < 0.01$.

revealed that CSE time-dependently activated the upstream p-AMPKα and TSC2, which downregulated MTOR and then induced a marked elevation of autophagy (Fig. 2C). All these data suggest that CS likely inactivates MTOR through induction of TSC2, which in turn induces autophagy in airway epithelial cells.

The TSC2–MTOR signaling regulates CSE-induced cell death in HBE cells

Multiple patterns of cell death, e.g., apoptosis, autophagic cell death, and necroptosis, are known to contribute to the development of COPD (12–16). We next assessed the role of the TSC2–MTOR axis in regulation of CS-induced cell death. Not surprisingly, *TSC2* siRNA increased the levels of p-RPS6 while suppressing autophagy (Fig. 3A). On the contrary, *MTOR* siRNA decreased p-RPS6 but induced an elevation of autophagy (Fig. 3B). Interestingly, abrogation of *TSC2* evidently protected—while deletion of *MTOR* further augmented—the CSE-induced cell death (including apoptosis and necroptosis) in HBE cells (Fig. 3C–E), as revealed by the cell viability, the flow cytometry analysis, the expression of cleaved caspase-3

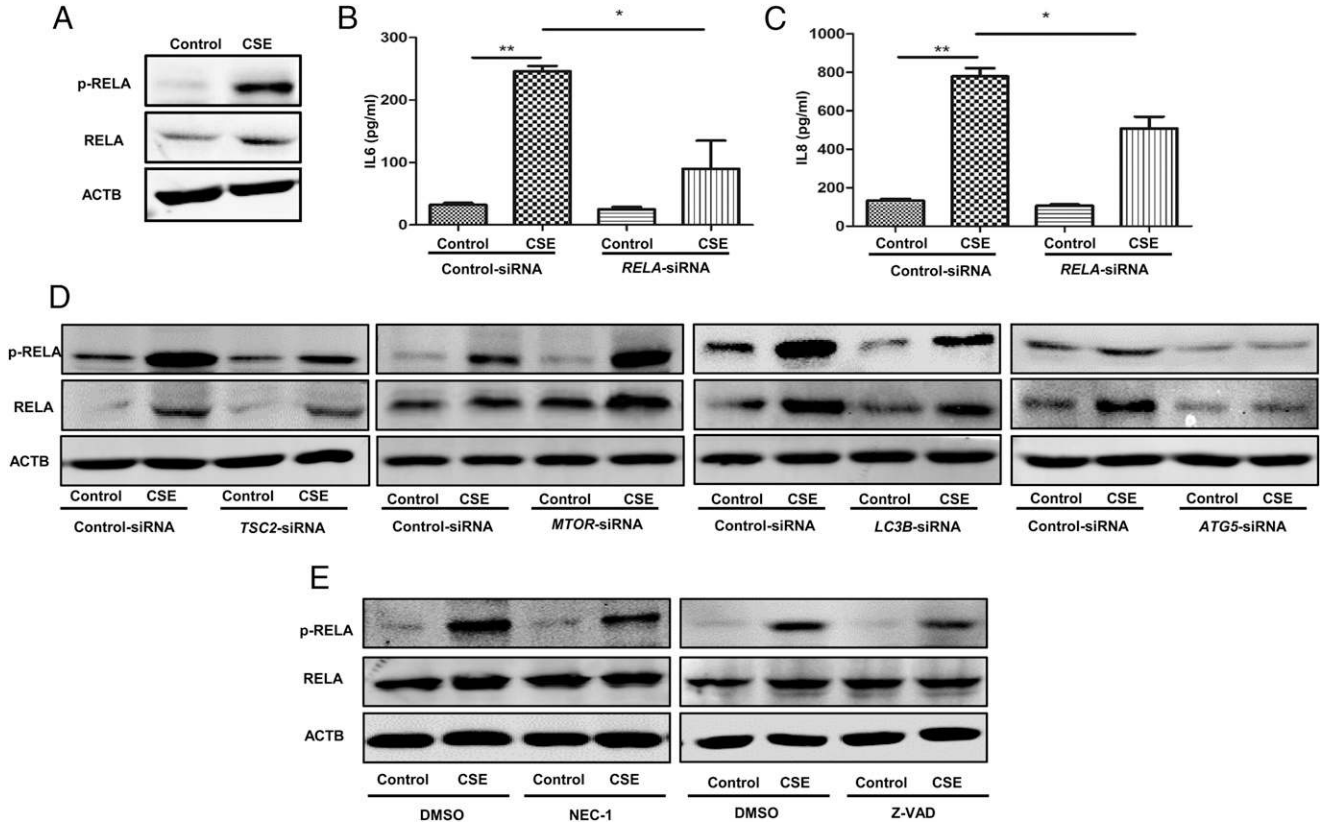

FIGURE 5. MTOR and subsequent cell death pathways modulate NF-κB activation in HBE cells. (**A**) HBE cells treated with 1% CSE for 12 h exhibited increased expression of RELA and p-RELA. (**B** and **C**) HBE cells were treated with control or *RELA* siRNA for 24 h following treatment with 1% CSE for an additional 24 h. Protein levels of IL-6 or IL-8 in culture supernatants were analyzed. Data represent mean ± SEM of three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$. (**D**) After transfection with the indicated siRNAs for 24 h, HBE cells were cultured with 1% CSE for 12 h and the expression of RELA or p-RELA was detected by immunoblotting. (**E**) HBE cells were cultured with NEC-1 (50 μM), Z-VAD (25 μM), or vehicle (DMSO) together with 1% CSE for 12 h, and the expression of RELA or p-RELA was detected by immunoblotting. Blots (A, D, and E) represent three independent experiments.

and -9, and the activation of RIP1, RIP3 (23), and p-MLKL (24).

The TSC2–MTOR signaling as well as the cell death pathways regulate CSE-induced inflammatory response in HBE cells

Airway inflammation is a cardinal feature of COPD, and the CS-induced inflammatory cytokines IL-6 and IL-8 are associated with the pathogenesis of COPD (25). We next sought to examine the role of TSC2–MTOR as well as the downstream cell death pathways in the CS-induced inflammatory response in HBE cells. CSE exposure induced the expression of *IL-6* and *IL-8* transcripts (Supplemental Fig. 2) as well as the release of these proteins (Fig. 4). Consistent with the cell death results, knockdown of *TSC2* significantly decreased, and depletion of *MTOR* remarkably enhanced, CSE-induced production of IL-6 and IL-8 (Fig. 4A, 4B, Supplemental Fig. 2A, 2B).

As the TSC2–MTOR axis regulates CSE-induced autophagy, apoptosis, and necroptosis; we next asked whether these cell death processes could modulate the CSE-induced inflammatory response in HBE cells. The CSE-induced IL-6 and IL-8 were markedly reduced in the cells treated with siRNAs of *LC3B* or *ATG5* (Fig. 4C, 4D, Supplemental Fig. 2C, 2D), which was in agreement with our previous finding that autophagy is essential for particular matter-induced airway inflammation (22). On the contrary, treatment with *ATG5* plasmid eventually enhanced the production of IL-6 and IL-8 (Fig. 4E, 4F, Supplemental Fig. 2E, 2F). Moreover, pretreatment of HBE cells with the necroptosis inhibitor NEC-1 or the pan caspase inhibitor Z-VAD also significantly attenuated the CSE-induced production of IL-6 and IL-8 (Fig. 4G, 4H, Supplemental Fig. 2G, 2H). These data suggest that the TSC2–MTOR axis together with the downstream cell death pathways cooperatively regulate the CS-induced inflammatory response in airway epithelium.

The TSC2–MTOR signaling and the cell death cascades regulate CSE-induced inflammation through modulation of NF-κB activation

It has been well recognized that NF-κB signaling is associated with the inflammatory response in airway epithelial cells (22), so we asked whether MTOR and the cell death pathways regulated CSE-induced airway inflammation through NF-κB signaling. We observed that CSE significantly induced the expression of RELA or p-RELA in HBE cells (Fig. 5A), and knockdown of *RELA* by siRNA attenuated the levels of IL-6 and IL-8 induced by CSE (Fig. 5B, 5C); demonstrating that NF-κB signaling is indeed involved in CSE-induced inflammation in airway epithelium. Interestingly, knockdown of *TSC2* significantly attenuated, and depletion of *MTOR* notably augmented, the levels of RELA and p-RELA, and they were also markedly inhibited by the siRNAs of *LC3B* or *ATG5* (Fig. 5D). Moreover, inhibition of necroptosis or apoptosis by NEC-1 or Z-VAD also remarkably attenuated the CSE-induced activation of NF-κB signaling (Fig. 5E). These data were in complete agreement with the CSE-induced production of IL-6 and IL-8 shown in Fig. 4.

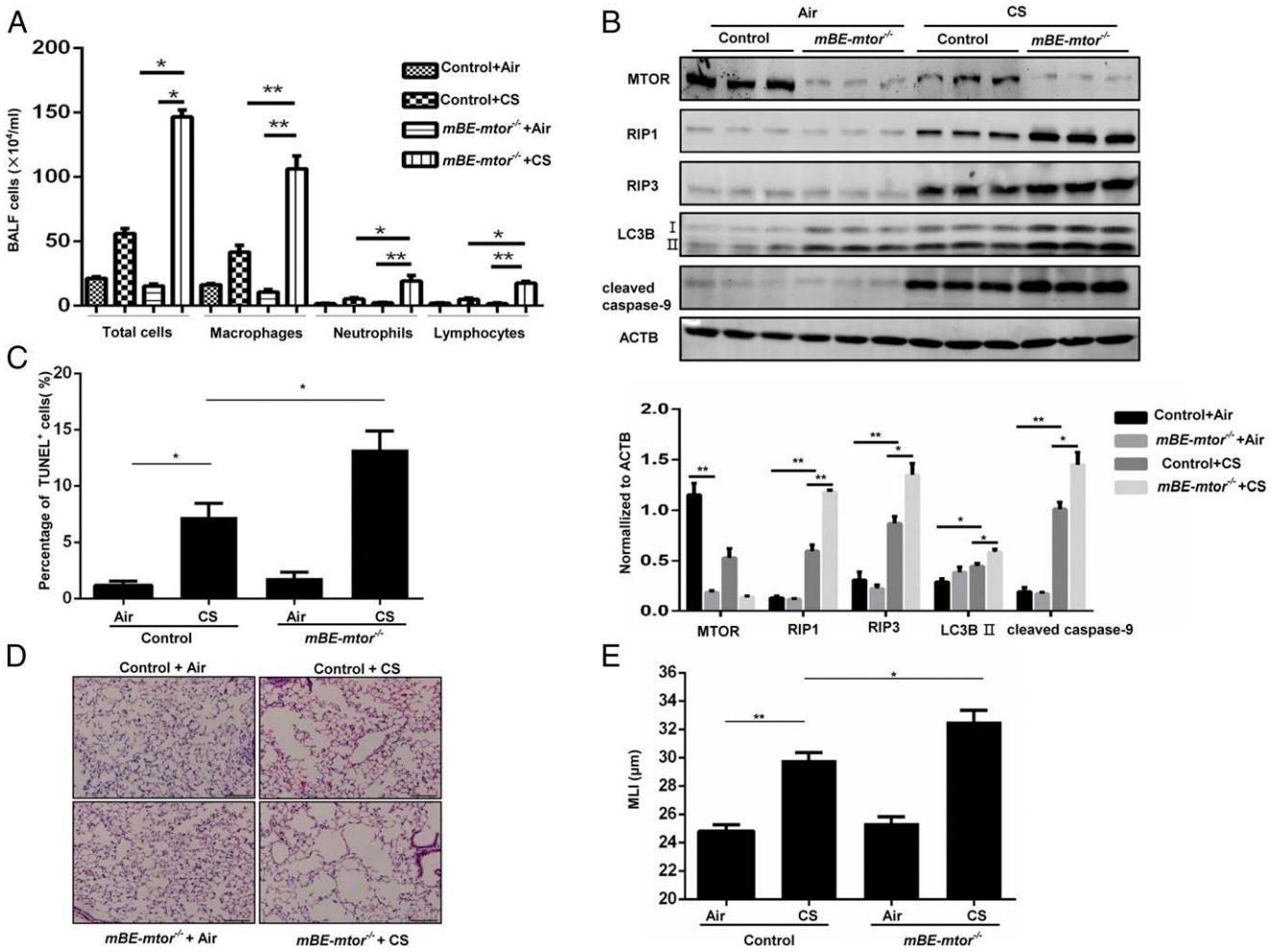

FIGURE 6. Disruption of *Mtor* in bronchial epithelial cells (*mBE-mtor*$^{-/-}$) aggravates CS-induced airway inflammation, cell death, and emphysema in mice. (**A**) Mice were exposed to CS for 6 mo and the number of total inflammatory cells, macrophages, neutrophils, and lymphocytes in the BALF were counted. (**B**) Immunoblot analysis and relative band intensity of MTOR, RIP1, RIP3, LC3B, or cleaved caspase-9 in mouse lung samples following exposed to air or CS for 6 mo. Results were normalized by ACTB and were represented as fold change. (**C**) Percentage of TUNEL-positive cells in mouse lung tissue sections. (**D** and **E**) Representative alveolar morphology of (D) H&E staining or (E) MLI scoring of mouse lungs exposed to air or CS for 6 mo. Scale bar, 100 μm. n = 6–8 for each group. All results represent three independent experiments. Data are presented as mean ± SEM. *$p < 0.05$, **$p < 0.01$.

Selective disruption of Mtor in pulmonary epithelium augments CS-induced airway inflammation, cell death, and emphysema in vivo

To further confirm the role of MTOR in lung epithelium in CS-induced COPD pathogenesis in vivo, we generated *mBE-mtor*$^{-/-}$ BE-μ*ATII-mtor*$^{-/-}$ mice (Supplemental Fig. 3), as described in *Materials and Methods*. After doxycycline treatment, the levels of MTOR were significantly decreased in lung tissues (Supplemental Fig. 3B). Moreover, the immune staining of p-RPS6 revealed that MTOR activity was markedly decreased in either airway or alveolar epithelial cells, as shown in our precious study (26). Chronic CS exposure for 6 mo increased the total number of inflammatory cells, including macrophages, neutrophils, and lymphocytes in BALF, all of which were further exacerbated in the *mBE-* or *ATII-mtor*$^{-/-}$ mice (Figs. 6A, 7A). Moreover, *mBE-* or *ATII-mtor*$^{-/-}$ mice exhibited higher levels of RIP1, RIP3, cleaved caspase-9, and LC3B in lungs in response to CS exposure relative to the *Mtor*-unimpaired controls (Figs. 6B, 7B). TUNEL-positive cells were also further increased in these lung epithelium–specific *mtor*$^{-/-}$ mice with chronic CS exposure (Figs. 6C, 7C). Furthermore, the emphysema-like airspace enlargement, as revealed by the MLI, was also significantly augmented in CS-exposed *mBE-* or *ATII-mtor*$^{-/-}$ mice (Figs. 6D, 6E, 7D, 7E). These results suggest that *mBE-* or *ATII-mtor*$^{-/-}$ mice are more susceptible to CS-induced lung inflammation, cell death, and emphysema.

Discussion

The major findings of this study can be summarized as follows (Supplemental Fig. 4): 1) CS decreases the expression of MTOR and its activity in the lung, which orchestrates the CS-induced cell death and subsequent inflammatory response in airway epithelial cells; 2) the mechanisms of MTOR in regulation of COPD pathogenesis in pulmonary epithelial cells are likely via upstream TSC2 signaling and downstream cell death and NF-κB pathways; and 3) selective disruption of *Mtor* in lung epithelium further exacerbates CS-induced cell death, inflammation, and emphysema in vivo. Collectively, these data provide new insights into the protective role of MTOR in COPD pathogenesis through the suppression of cell death and inflammation in the lung. Moreover, the current study also provides possible mechanistic explanations for the adverse pulmonary effects of MTOR inhibitors in clinical use (20, 21).

The eventual functions of MTOR in human diseases are likely cell and pathogen dependent and are either deleterious or protective

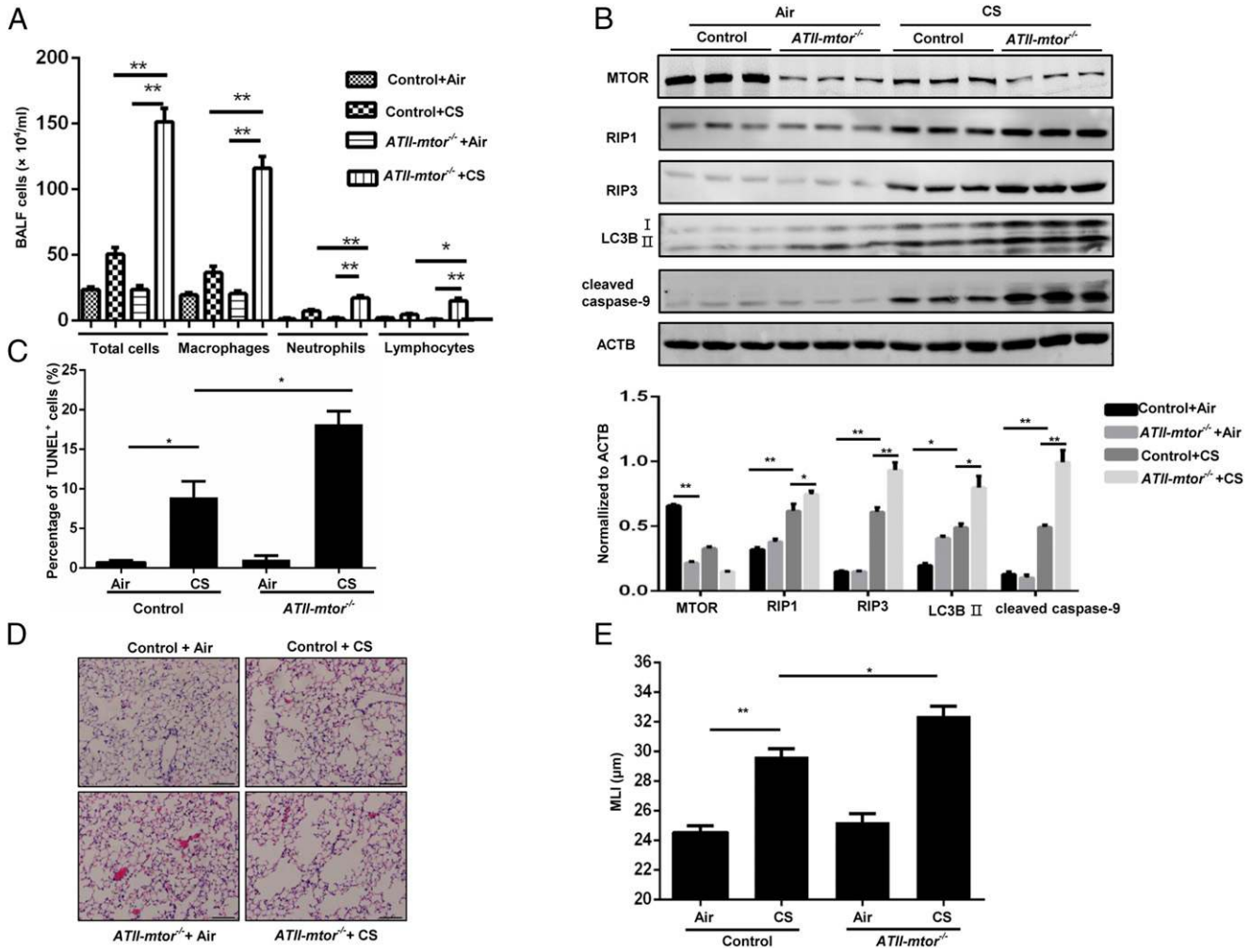

FIGURE 7. Disruption of *Mtor* in alveolar type II epithelial cells (*ATII-mtor*$^{-/-}$) aggravates CS-induced airway inflammation, cell death, and emphysema in mice. (**A**) Mice were exposed to CS for 6 mo, and the number of total inflammatory cells, macrophages, neutrophils, and lymphocytes in the BALF were counted. (**B**) Immunoblot analysis and relative band intensity of MTOR, RIP1, RIP3, LC3B, or cleaved caspase-9 in mouse lung samples following exposure to air or CS for 6 mo. Results were normalized by ACTB and were represented as fold change. (**C**) Percentage of TUNEL-positive cells in mouse lung tissue sections. (**D** and **E**) Representative alveolar morphology of (D) H&E staining or (E) MLI scoring of mouse lungs exposed to air or CS for 6 mo. Scale bar, 100 μm. $n = 6$–8 for each group. All results represent of three independent experiments. Data are presented as mean ± SEM $^{*}p < 0.05$, $^{**}p < 0.01$.

(6, 27). We have recently observed that MTOR is induced in and mediates the LPS-induced airway epithelial injury (26), indicating a deleterious effect of MTOR in certain cases of lung injury. Rapamycin has been shown to specifically increase pulmonary toxicity when used as an immunosuppressive drug for renal transplantation (28), suggesting a protective role of MTOR in chronic pulmonary injury. Accumulating evidence has also suggested that MTOR signaling is altered in airway epithelium or in lung tissues upon CS stimulation, although the results remain controversial. CSE has been shown to inhibit p-MTOR and p-RPS6 in BEAS-2B cells (29) and in aortic endothelial cells (30), and p-MTOR expression is decreased in mouse lung tissues exposed to CS for various periods (31). Our current study is consistent with these results, clearly showing that CS decreases MTOR and its activity in cultured airway epithelial cells, in mouse lungs, and in human COPD lungs. Moreover, we have further shown in this study that mice with epithelium-specific knockdown of Mtor are sensitive to CS-induced airway inflammation and emphysema development, indicating an epithelial-specific protective role of MTOR in COPD. However, Takasaka et al. (32) have reported that CSE treatment slightly enhanced the level of p-RPS6 protein in HBE cells. A more recent study has also shown that MTOR activity was elevated in PBMCs from COPD patients and that MTOR inhibition could help to restore corticosteroid sensitivity (33). These data support our hypothesis that the eventual functions of MTOR signaling in COPD pathogenesis might be cell specific. Meanwhile, there is a study showing that autophagy is impaired in COPD, which may in turn accelerate cellular senescence (34). These discrepancies might be due to the different models or different times and concentrations of CS used, and thus requires further investigation.

MTOR integrates multiple signaling networks in regulating cellular responses, such as cell survival and life span (8, 35), and its downstream autophagy process has been suggested as a crucial mediator of cell fate, regulating apoptosis and necroptosis (15, 16). Inhibition of MTOR could induce apoptosis through the mitochondrial pathway (35, 36). In childhood acute lymphoblastic leukemia, loss of MTOR activity also induces autophagy-dependent cell death with the specific features of necroptosis (37). Consistent with these results, we demonstrate that MTOR acts as a key regulator of not only autophagy but also apoptosis and necroptosis induced by CS.

Neutrophilic inflammation is another cardinal feature of CS-induced lung injury which causes the release of substantial proteases, thus ultimately accelerating emphysematous lung destruction (38). Interestingly, mice treated with the MTOR inhibitor temsirolimus exhibit more neutrophil infiltration and fibrinous exudate into the alveoli, and they develop interstitial lung disease (39), which is aligned with our observations. Our in vitro experiments also suggest that loss of autophagy significantly inhibits CS-induced inflammation in HBE cells, which is in agreement with our previous observations that autophagy is deleterious in CS-induced COPD pathogenesis (13–15) and in particular in matter-induced airway inflammation (22). Moreover, apoptosis or necroptosis inhibitors notably attenuate the CS-induced cytokine production in HBE cells, suggesting that these cell death pathways eventually contribute to the CS-induced airway inflammation. A recent study has also reported that the necroptosis inhibitor NEC-1 relieves the neutrophilic airway inflammation in CS-exposed mice (40). Thus, these data altogether suggest that MTOR exerts a protective role in CS-induced lung inflammation and emphysema, likely through suppressing autophagy, apoptosis, and necroptosis.

Numerous studies have demonstrated that CS induces lung inflammation in COPD through the NF-κB pathway, the intensity of which may be linked with the severity of COPD (41, 42). The typical NF-κB consists of P50/RELA heterodimers and RELA often represents the NF-κB transcriptional activation. Our data suggest that autophagy is essential for CS-induced NF-κB activation in HBE cells. However, the detailed mechanisms of how the MTOR–autophagy axis regulates NF-κB signaling remain unclear. Criollo et al. (43) have also observed that the depletion of essential autophagy modulators, including *ATG5*, *ATG7*, and *BECN1*, by RNA interference inhibits TNF-driven RELA activation in human cancer cell lines. Similarly, in mice lacking *Atg4b*, mechanical ventilation induced less autophagy, resulting in the accumulation of SQSTM1 and ubiquitinated NFKBIA, and less NF-κB activation (44). These studies are in line with our findings that autophagy positively regulates NF-κB activation.

In summary, the current study demonstrates that MTOR suppresses CS-induced airway inflammation and emphysema development, likely through modulating the CS-induced autophagy and cell death in airway epithelium. Thus, activation of MTOR and/or inhibition of autophagy may represent novel therapeutic strategies for CS-induced COPD. Moreover, this study also re-emphasizes the awareness of possible severe adverse effects of increasing the pulmonary sensitivity to CS or other pathogens in ongoing clinical trials of MTOR inhibitors or autophagy upregulators.

Disclosures

The authors have no financial conflicts of interest.

References

1. Vestbo, J., S. S. Hurd, A. G. Agustí, P. W. Jones, C. Vogelmeier, A. Anzueto, P. J. Barnes, L. M. Fabbri, F. J. Martinez, M. Nishimura, et al. 2013. Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease: GOLD executive summary. *Am. J. Respir. Crit. Care Med.* 187: 347–365.
2. Pauwels, R. A., and K. F. Rabe. 2004. Burden and clinical features of chronic obstructive pulmonary disease (COPD). *Lancet* 364: 613–620.
3. Decramer, M., W. Janssens, and M. Miravitlles. 2012. Chronic obstructive pulmonary disease. *Lancet* 379: 1341–1351.
4. Barnes, P. J. 2000. Chronic obstructive pulmonary disease. *N. Engl. J. Med.* 343: 269–280.
5. Fischer, B. M., E. Pavlisko, and J. A. Voynow. 2011. Pathogenic triad in COPD: oxidative stress, protease-antiprotease imbalance, and inflammation. *Int. J. Chron. Obstruct. Pulmon. Dis.* 6: 413–421.
6. Laplante, M., and D. M. Sabatini. 2012. mTOR signaling in growth control and disease. *Cell* 149: 274–293.
7. Zhang, Y., F. Vasheghani, Y. H. Li, M. Blati, K. Simeone, H. Fahmi, B. Lussier, P. Roughley, D. Lagares, J. P. Pelletier, et al. 2015. Cartilage-specific deletion of mTOR upregulates autophagy and protects mice from osteoarthritis. *Ann. Rheum. Dis.* 74: 1432–1440.
8. Appenzeller-Herzog, C., and M. N. Hall. 2012. Bidirectional crosstalk between endoplasmic reticulum stress and mTOR signaling. *Trends Cell Biol.* 22: 274–282.
9. Kim, Y. C., and K. L. Guan. 2015. mTOR: a pharmacologic target for autophagy regulation. *J. Clin. Invest.* 125: 25–32.
10. Wullschleger, S., R. Loewith, and M. N. Hall. 2006. TOR signaling in growth and metabolism. *Cell* 124: 471–484.
11. Levine, B., and G. Kroemer. 2008. Autophagy in the pathogenesis of disease. *Cell* 132: 27–42.
12. Henson, P. M., R. W. Vandivier, and I. S. Douglas. 2006. Cell death, remodeling, and repair in chronic obstructive pulmonary disease? *Proc. Am. Thorac. Soc.* 3: 713–717.
13. Chen, Z. H., H. C. Lam, Y. Jin, H.-P. Kim, J. Cao, S.-J. Lee, E. Ifedigbo, H. Parameswaran, S. W. Ryter, and A. M. Choi. 2010. Autophagy protein microtubule-associated protein 1 light chain-3B (LC3B) activates extrinsic apoptosis during cigarette smoke-induced emphysema. *Proc. Natl. Acad. Sci. USA* 107: 18880–18885.
14. Zhou, J. S., Y. Zhao, H. B. Zhou, Y. Wang, Y. F. Wu, Z. Y. Li, N. X. Xuan, C. Zhang, W. Hua, S. M. Ying, et al. 2016. Autophagy plays an essential role in cigarette smoke-induced expression of MUC5AC in airway epithelium. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 310: L1042–L1052.
15. Chen, Z. H., H. P. Kim, F. C. Sciurba, S. J. Lee, C. Feghali-Bostwick, D. B. Stolz, R. Dhir, R. J. Landreneau, M. J. Schuchert, S. A. Yousem, et al. 2008. Egr-1 regulates autophagy in cigarette smoke-induced chronic obstructive pulmonary disease. *PLoS One* 3: e3316.
16. Mizumura, K., S. M. Cloonan, K. Nakahira, A. R. Bhashyam, M. Cervo, T. Kitada, K. Glass, C. A. Owen, A. Mahmood, G. R. Washko, et al. 2014. Mitophagy-dependent necroptosis contributes to the pathogenesis of COPD. *J. Clin. Invest.* 124: 3987–4003.
17. Lam, H. C., S. M. Cloonan, A. R. Bhashyam, J. A. Haspel, A. Singh, J. F. Sathirapongsasuti, M. Cervo, H. Yao, A. L. Chung, K. Mizumura, et al. 2013. Histone deacetylase 6-mediated selective autophagy regulates COPD-associated cilia dysfunction. *J. Clin. Invest.* 123: 5212–5230.
18. Yoshida, T., I. Mett, A. K. Bhunia, J. Bowman, M. Perez, L. Zhang, A. Gandjeva, L. Zhen, U. Chukwueke, T. Mao, et al. 2010. Rtp801, a suppressor of mTOR signaling, is an essential mediator of cigarette smoke-induced pulmonary injury and emphysema. *Nat. Med.* 16: 767–773.
19. Hou, H. H., S. L. Cheng, K. P. Chung, M. Y. Kuo, C. C. Yeh, B. E. Chang, H. H. Lu, H. C. Wang, and C. J. Yu. 2014. Elastase induces lung epithelial cell autophagy through placental growth factor: a new insight of emphysema pathogenesis. *Autophagy* 10: 1509–1521.
20. Kennedy, B. K., and J. K. Pennypacker. 2016. Mammalian target of rapamycin: a target for (lung) diseases and aging. *Ann. Am. Thorac. Soc.* 13: S398–S401.
21. Študentová, H., D. Vitásková, and B. Melichar. 2016. Safety of mTOR inhibitors in breast cancer. *Expert Opin. Drug Saf.* 15: 1075–1085.
22. Chen, Z. H., Y. F. Wu, P. L. Wang, Y. P. Wu, Z. Y. Li, Y. Zhao, J. S. Zhou, C. Zhu, C. Cao, Y. Y. Mao, et al. 2016. Autophagy is essential for ultrafine particle-induced inflammation and mucus hyperproduction in airway epithelium. *Autophagy* 12: 297–311.
23. Vanden Berghe, T., W. J. Kaiser, M. J. Bertrand, and P. Vandenabeele. 2015. Molecular crosstalk between apoptosis, necroptosis, and survival signaling. *Mol. Cell. Oncol.* 2: e975093.
24. Sun, L., H. Wang, Z. Wang, S. He, S. Chen, D. Liao, L. Wang, J. Yan, W. Liu, X. Lei, and X. Wang. 2012. Mixed lineage kinase domain-like protein mediates necrosis signaling downstream of RIP3 kinase. *Cell* 148: 213–227.
25. Su, B., T. Liu, H. Fan, F. Chen, H. Ding, Z. Wu, H. Wang, and S. Hou. 2016. Inflammatory markers and the risk of chronic obstructive pulmonary disease: a systematic review and meta-analysis. *PLoS One* 11: e0150586.
26. Hu, Y., J. Lou, Y.-Y. Mao, T.-W. Lai, L.-Y. Liu, C. Zhu, C. Zhang, J. Liu, Y.-Y. Li, F. Zhang, et al. 2016. Activation of MTOR in pulmonary epithelium promotes LPS-induced acute lung injury. *Autophagy* 12: 2286–2299.
27. Hu, Y., J. Liu, Y. F. Wu, J. Lou, Y. Y. Mao, H. H. Shen, and Z. H. Chen. 2014. mTOR and autophagy in regulation of acute lung injury: a review and perspective. *Microbes Infect.* 16: 727–734.
28. Pham, P. T., P. C. Pham, G. M. Danovitch, D. J. Ross, H. A. Gritsch, E. A. Kendrick, J. Singer, T. Shah, and A. H. Wilkinson. 2004. Sirolimus-associated pulmonary toxicity. *Transplantation* 77: 1215–1220.
29. Zhu, L., E. C. Barrett, Y. Xu, Z. Liu, A. Manoharan, and Y. Chen. 2013. Regulation of cigarette smoke (CS)-induced autophagy by Nrf2. [Published erratum appears in 2014 *PLoS One* 9: 10.1371/annotation/d13c3d06-8eb8-49ec-8326-2db7487a7a8a.] *PLoS One* 8: e55695.
30. Lemaître, V., A. J. Dabo, and J. D'Armiento. 2011. Cigarette smoke components induce matrix metalloproteinase-1 in aortic endothelial cells through inhibition of mTOR signaling. *Toxicol. Sci.* 123: 542–549.
31. Carlos, S. P., A. S. Dias, L. A. Forgiarini Júnior, P. D. Patricio, T. Graciano, R. T. Nesi, S. Valença, A. M. G. Chiappa, G. Cipriano, Jr., C. T. Souza, and G. R. S. Chiappa. 2014. Oxidative damage induced by cigarette smoke exposure in mice: impact on lung tissue and diaphragm muscle. *J. Bras. Pneumol.* 40: 411–420.
32. Takasaka, N., J. Araya, H. Hara, S. Ito, K. Kobayashi, Y. Kurita, H. Wakui, Y. Yoshii, Y. Yumino, S. Fujii, et al. 2014. Autophagy induction by SIRT6 through attenuation of insulin-like growth factor signaling is involved in the regulation of human bronchial epithelial cell senescence. *J. Immunol.* 192: 958–968.
33. Mitani, A., K. Ito, C. Vuppusetty, P. J. Barnes, and N. Mercado. 2016. Restoration of corticosteroid sensitivity in chronic obstructive pulmonary disease by

inhibition of mammalian target of rapamycin. *Am. J. Respir. Crit. Care Med.* 193: 143–153.
34. Fujii, S., H. Hara, J. Araya, N. Takasaka, J. Kojima, S. Ito, S. Minagawa, Y. Yumino, T. Ishikawa, T. Numata, et al. 2012. Insufficient autophagy promotes bronchial epithelial cell senescence in chronic obstructive pulmonary disease. *OncoImmunology* 1: 630–641.
35. Fulda, S. 2012. Shifting the balance of mitochondrial apoptosis: therapeutic perspectives. *Front. Oncol.* 2: 121.
36. Sun, Y. W., W. J. Huang, C. J. Hsiao, Y. C. Chen, P. H. Lu, and J. H. Guh. 2010. Methoxychalcone induces cell-cycle arrest and apoptosis in human hormone-resistant prostate cancer cells through PI 3-kinase-independent inhibition of mTOR pathways. *Prostate* 70: 1295–1306.
37. Bonapace, L., B. C. Bornhauser, M. Schmitz, G. Cario, U. Ziegler, F. K. Niggli, B. W. Schäfer, M. Schrappe, M. Stanulla, and J. P. Bourquin. 2010. Induction of autophagy-dependent necroptosis is required for childhood acute lymphoblastic leukemia cells to overcome glucocorticoid resistance. *J. Clin. Invest.* 120: 1310–1323.
38. Sharafkhaneh, A., N. A. Hanania, and V. Kim. 2008. Pathogenesis of emphysema: from the bench to the bedside. *Proc. Am. Thorac. Soc.* 5: 475–477.
39. Washino, S., H. Ando, K. Ushijima, K. Hosohata, M. Kumazaki, N. Mato, Y. Sugiyama, Y. Kobayashi, A. Fujimura, and T. Morita. 2014. Temsirolimus induces surfactant lipid accumulation and lung inflammation in mice. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 306: L1117–L1128.
40. Pouwels, S. D., G. J. Zijlstra, M. van der Toorn, L. Hesse, R. Gras, N. H. Ten Hacken, D. V. Krysko, P. Vandenabeele, M. de Vries, A. J. van Oosterhout, et al. 2016. Cigarette smoke-induced necroptosis and DAMP release trigger neutrophilic airway inflammation in mice. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 310: L377–L386.
41. Di Stefano, A., G. Caramori, T. Oates, A. Capelli, M. Lusuardi, I. Gnemmi, F. Ioli, K. F. Chung, C. F. Donner, P. J. Barnes, and I. M. Adcock. 2002. Increased expression of nuclear factor-kappaB in bronchial biopsies from smokers and patients with COPD. *Eur. Respir. J.* 20: 556–563.
42. Marwick, J. A., P. A. Kirkham, C. S. Stevenson, H. Danahay, J. Giddings, K. Butler, K. Donaldson, W. Macnee, and I. Rahman. 2004. Cigarette smoke alters chromatin remodeling and induces proinflammatory genes in rat lungs. *Am. J. Respir. Cell Mol. Biol.* 31: 633–642.
43. Criollo, A., F. Chereau, S. A. Malik, M. Niso-Santano, G. Mariño, L. Galluzzi, M. C. Maiuri, V. Baud, and G. Kroemer. 2012. Autophagy is required for the activation of NFκB. *Cell Cycle* 11: 194–199.
44. López-Alonso, I., A. Aguirre, A. González-López, A. F. Fernández, L. Amado-Rodríguez, A. Astudillo, E. Batalla-Solís, and G. M. Albaiceta. 2013. Impairment of autophagy decreases ventilator-induced lung injury by blockade of the NF-κB pathway. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 304: L844–L852.