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# Rapamycin attenuates Tc1 and Tc17 cell responses in cigarette smoke-induced emphysema in mice

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## Abstract

**Objective and design** Chronic exposure to cigarette smoke promotes airway inflammation and emphysema accompanied by enhanced $CD8^+$ interferon (IFN)-$\gamma^+$ T(Tc1) and $CD8^+$ interleukin (IL)-$17^+$ T(Tc17) cell responses. The mammalian target of rapamycin (mTOR) has been involved in the pathogenesis of emphysema. Inhibiting mTOR by rapamycin has been reported to alleviate emphysema, but the mechanism is not fully understood. We aimed to explore the effect of rapamycin on Tc1 and Tc17 cell responses induced by cigarette smoke exposure.

**Materials** Male C57BL/6 mice were exposed to cigarette smoke or room air for 24 weeks. Half of the smoke-exposed mice received rapamycin in the last 12 weeks. The severity of emphysema in those mice was evaluated by mean linear intercept (MLI), mean alveolar airspace area (MAA) and destructive index (DI). Bronchoalveolar lavage was collected and analyzed. Phosphorylated (p-) mTOR in $CD8^+$ T cells, Tc1 and Tc17 cells were detected by flow cytometry. The relative expression of p-mTOR in lungs was determined by western blot analysis. IFN-γ and IL-17A levels were detected by enzyme-linked immunosorbent assays. IFN-γ, mTOR and RAR-related orphan receptor (ROR)γt mRNA levels were evaluated by the real-time polymerase chain reaction.

**Results** Elevated p-mTOR expression in $CD8^+$ T cells and lung tissue was accompanied by the enhanced Tc1 and Tc17 cell responses in lungs of mice exposed to cigarette smoke. Rapamycin reduced inflammatory cells in BALF and decreased MLI, DI and MAA in lungs. Rapamycin decreased p-mTOR expression, and down-regulation of mTOR and RORγt mRNA levels along with the attenuation of Tc1 and Tc17 cell responses in mice with emphysema.

**Conclusions** The mTOR was activated in $CD8^+$ T cells accompanied by the enhanced Tc1 and Tc17 cell responses in cigarette smoke-related pulmonary inflammation. Rapamycin ameliorated emphysema and attenuated Tc1 and Tc17 cell responses probably caused by inhibiting mTOR in cigarette smoke-exposed mice.

**Keywords** Rapamycin · Cigarette smoke · $CD8^+$IFN-$\gamma^+$ T cells · $CD8^+$IL-$17^+$ T cells · Chronic obstructive pulmonary disease · Emphysema

## Introduction

Chronic obstructive pulmonary disease (COPD) is characterized by chronic airway inflammation and airflow limitation that is persistent and irreversible. The progressive pathological process of COPD may be related to exposure of noxious gas or particles, especially cigarette smoke [1–3]. Recently, it has been found that the pathogenesis of COPD is related to dysregulation of the immune system induced by cigarette smoke exposure. T lymphocytes, such as $CD4^+$ interferon (IFN)-$\gamma^+$ T cells (Th1), $CD4^+$ interleukin (IL)-$17^+$ T cells (Th17), $CD8^+$ IFN-$\gamma^+$ T cells (Tc1), and $CD8^+$ IL-$17^+$ T cells (Tc17), may play critical roles in promoting inflammation and lead to immune disorders in emphysema [4–11]. In our previous studies, exposure to cigarette smoke promoted the differentiation of Tc1 and Tc17 cells [12, 13]. Increasing Tc1 and Tc17 cells in COPD/emphysema correlated with limited airflow and associated with the destruction of alveolar walls and inflammatory responses in lungs and

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peripheral circulation [6, 7, 10, 11, 13, 14]. The dysregulation of Tc1 and Tc17 cells is likely to be an intractable pro-inflammatory phenotype and a potential treatment target in COPD/emphysema.

The mammalian target of rapamycin (mTOR) is reportedly involved in the pathogenesis of COPD/emphysema [15–18], but its role and underlying mechanism are poorly understood. mTOR consists of two complexes, mTORC1 and mTORC2, and is critical for the regulation of metabolism, protein synthesis, proliferation, survival, cellular senescence, aging and cell differentiation [15, 17, 19]. It has been demonstrated that increased mTOR activity was shown in patients with COPD, and the increased phosphorylated (p) S6 kinase indicated an activation of the mTORC1 complex [15–18]. Activated mTORC1 could lead to enhanced IFN-γ and TNF-α producing $CD8^+$ T effector cells [20], which is consistent with the pro-inflammatory phenotype in COPD.

Rapamycin is an effective immunosuppressant widely known as a prototypical inhibitor of mTOR, which has been used to treat many conditions such as autoimmune diseases, transplant rejection, and malignant tumors [21–23]. Rapamycin inhibits mTOR activity and modulates T-box expressed in T cells (T-bet), thereby inhibiting $CD8^+$ effector T cell differentiation and decreasing inflammatory cytokines (e.g., IL-6, IL-8, IFN-γ, and tumor necrosis factor (TNF)-α) [24–26]. Rapamycin can also selectively reduce the Th1/Tc1 ratio, and the levels of IL-2 and IFN-γ that contribute to a reduction of lethal graft-versus-host disease after allogeneic bone marrow transplantation [27]. In addition, activating mTORC1 positively modulates IL-17 expression through STAT3, RORγt, HIF-1α, S6K1, and S6K2 pathways [28]. Thus, we believe that rapamycin may serve as a regulating treatment in disorders characterized by dysregulation of Tc1 and Tc17 cells. It has been reported that inhibiting mTOR by rapamycin can alleviate emphysema [18]. However, the anti-inflammatory and immune regulatory effects of rapamycin on cigarette smoke-related emphysema, and its effect on Tc1 and Tc17 cells in COPD/emphysema, are not understood.

In this study, we investigated the expression of p-mTOR in $CD8^+$ T cells in lungs of mice exposed to cigarette smoke and observed the anti-inflammatory and immune regulatory effects of rapamycin on Tc1 and Tc17 cell responses in cigarette smoke-related emphysema model in mice.

## Materials and methods

### Mice

Thirty male C57BL/6 mice (8–10 weeks of age, 18–22 g) were obtained from the Laboratory Animal Centre of Guangxi Medical University. All animals were housed separately in specific-pathogen-free glass cages, and there was free access to tap water and a standard diet. Mice were divided randomly into three groups: air control (AIR), cigarette smoke-exposed (CS), and cigarette smoke-exposed + rapamycin-treated (CS + RAPA) mice. Mice in CS group and CS + RAPA group were placed in a closed 0.75-$m^3$ chamber and were exposed to the smoke from five cigarettes (Nanning Zhen long unfiltered cigarettes: 12 mg of tar and 0.9 mg of nicotine). The smoke-to-air ratio was 1:6. The cigarette smoke exposure takes 40 min per time, four times a day and 5 days a week, for 24 weeks totally, according to the method described previously [12]. Air control mice were placed in a chamber of the same size, but they were exposed to fresh air for 24 weeks. Besides exposure to the same cigarette smoke regimen, mice in CS + RAPA group were also treated with 1 mg/kg rapamycin (Sigma-Aldrich, St. Louis, MO, USA) by gavages for three times a week from 12 to 24 weeks as described previously [8, 29]. All experimental protocols were established and approved by the Animal Research Care Committee for Animal Studies of Guangxi Medical University.

### Tissue processing

After 24 weeks of exposure to cigarette smoke or room air, the mice were killed. The lower left lung was fixed in 10% formalin for 48 h and then the lung tissue was embedded in paraffin. The paraffin was cut into 3–5 μm sections. Then, the slices were stained with hematoxylin and eosin, and examined microscopically. Alveolar airspace enlargement and alveolar destruction were detected by the mean linear intercept (MLI), the mean alveolar airspace area (MAA) and destructive index (DI) by two independent investigators who were blinded to the treatments, as described previously [7, 30–32].

The spleen and right lung were used to determine Tc1 and Tc17 cells by flow cytometry. The upper left lung was stored at − 80 °C for the real-time polymerase chain reaction (RT-PCR), western blot analysis and enzyme-linked immunosorbent assays (ELISAs).

### Bronchoalveolar lavage

To analyze inflammatory cells in bronchoalveolar lavage (BALF), mice were inserted a tracheal cannula after anesthetization. PBS was instilled and recovered gently for four times (0.25 mL per time) via the tracheal cannula. The obtained BALF was centrifuged and then resuspended in PBS containing 10% fetal bovine serum. The total cell count was performed and the differential cell counts (lymphocytes, neutrophils and macrophages) were performed after Wright–Giemsa staining. At least 400 cells per sample were determined. The $CD4^+$ and $CD8^+$ T cells in BALF lymphocytes were identified by flow cytometry.

## Preparation of single cell suspensions

The spleen was cut into small pieces within 30 min of excision. The pieces were ground softly into single cells using a plunger from a 5-mL syringe. Debris was eliminated by filtration through a nylon mesh. Then, spleen cell suspensions were centrifuged at 300×*g* for 10 min at 4 °C. Erythrocytes in the cell suspensions were removed as described previously [13]. Cells were then washed twice with cold phosphate-buffered saline (PBS).

Single cells from mouse lungs were isolated by mechanical fragmentation, enzyme digestion, and centrifugation procedures as described previously [12, 13]. Followed by removing blood and the intravascular pool of cells, lung tissue was dissected into 0.1 cm pieces and digested in RPMI 1640 medium containing 1 mg/mL collagenase type IV (Sigma-Aldrich, St. Louis, MO, USA) for 40 min at 37 °C. To improve the effect of digestion, lung pieces were incubated in a shaker and were pipetted vigorously every 20 min. Subsequently, the remaining intact tissue was triturated with a plunger from a 5-mL syringe. Tissue fragments and dead cell dumps were removed by filtration through a 70-μm strainer. Then, the cells were centrifuged and red blood cells (RBCs) were removed with RBC Lysis Buffer (Solarbio Life Science, China).

## Flow cytometry

Fresh single cell suspensions of lung and spleen cells from each treatment group were prepared. The expression of markers on lung and spleen T cells was determined by flow cytometry after intracellular or surface staining using the following anti-mouse antibodies: peridinin–chlorophyll–protein (PerCP)-Cy5.5-CD8 (BD Pharmingen, San Diego, CA, USA), allophycocyanin (APC)-IFN-γ (BD Pharmingen), phycoerythrin (PE)-IL-17 (BD Pharmingen), and PE-phospho-mTOR (eBiosciences).

For Tc1 and Tc17 staining, single cell suspensions of spleen and lung cells were stimulated for 4 h with 25 ng/mL phorbol myristate acetate (Sigma-Aldrich) and 1 μg/mL ionomycin (Sigma-Aldrich) in the presence of GolgiStop™ (BD Biosciences) at 37 °C in 5% $CO_2$. Then, the cells were washed and stained for surface markers with PerCP-Cy5.5-CD8 for 30 min at 4 °C. After surface staining, cells were fixed/permeabilized with Cytofix/Cytoperm™ Solution (BD Pharmingen) according to a previous method [13] and stained with APC-anti-mouse IFN-γ and PE-anti-mouse IL-17 for 30 min at 4 °C. Then, the cells were washed with 1 × Perm/Wash Buffer (BD Pharmigen) and suspended in PBS. For the p-mTOR staining, single cell suspensions were stimulated for 20 min with 25 ng/mL phorbol myristate acetate (Sigma-Aldrich) and 1 μg/mL ionomycin (Sigma-Aldrich). The following steps of surface staining of CD8, fixed/permeabilized and intracellular staining of PE-anti-mouse p-mTOR were the same to Tc1 and Tc17 staining procedure. The BALF cells were stained with PerCP-Cy5.5-CD8 and fluorescein isothiocyanate (FITC)-CD4 for 30 min at 4 °C and then were fixed with 1% polyformaldehyde. Flow cytometry was performed on an FACS Canto II (BD Biosciences) and analyzed using FlowJo v10 software (Treestar, Ashland, OR, USA).

## Western blot analysis

The lung tissue samples were homogenized and sonicated on ice in RIPA Buffer (Solarbio Life Science) containing a protease inhibitor cocktail (Solarbio Life Science). Following by centrifugation at 12,000 rpm at 4 °C for 15 min, the concentration of total protein in clear cell lysates was measured using BCA protein assay kit (Beyotime, China). Protein extracts were fractionated by SDS-PAGE (8% polyacrylamide) and then transferred to 0.45-μm PVDF membrane. After blocking with 5% (w/v) nonfat milk in Tris-buffered saline (containing 0.1% Tween-20), the membrane was incubated with primary antibodies against p-mTOR (Ser2448, 1:1000, Cell Signaling Technology, Danvers, MA, USA) and β-actin (1:1000, Zsbio, China) overnight at 4 °C, and then incubated with corresponding secondary antibodies (1:800, EarthOx Life Sciences, USA) for 1 h. The blots were visualized with an Odyssey® Imaging System. The analysis of blots was performed with Image J software.

## Cytokine measurements

The concentrations of IFN-γ and IL-17A in mouse lung tissue homogenates were measured by ELISA kits (Cusabio, Wuhan, China) according to the manufacturer's protocols. All samples were assayed in duplicate.

## RT-PCR

Total RNA was extracted from lung cells of individual mice with gDNA Eraser Spin and RNA Spin Columns according to the manufacturer's instructions (TaKaRa Bio, Kusatsu, Japan). The quality and quantity of total RNA were analyzed spectrophotometrically. RNA samples were reverse transcribed into cDNA using the PrimeScript™ RT reagent kit with gDNA Eraser according to the manufacturer's instructions (TaKaRa). Relative levels of mTOR and RAR-related orphan receptor (ROR) γt mRNAs to control β-actin in individual samples were determined by quantitative RT-PCR using SYBR Green I (SYBR®Premix Ex Taq™, TaKaRa) and an Applied Biosystems 7500 (ThermoFisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. The forward and reverse primer sequences were, respectively:

β-actin, 5′-CATCCGTAAAGACCTCTATGCCAAC-3′ and 5′-ATGGAGCCACCGATCCACA-3′; RORγt, 5′-GCTCCATATTTGACTTTTCCCACT-3′ and 5′-GATGTTCCACTCTCCTCTTCTCTTG-3′; and mTOR, 5′-CAAGGCCGAATCGTCTCCA-3′ and 5′-ATTTCACAATCGGAGGCAACAA-3′. DNA was amplified for 40 cycles under the following conditions: denaturation at 95 °C for 30 s, extension at 95 °C for 5 s, and then 62 °C for 30 s. mRNA levels were evaluated by the $2^{-\Delta\Delta Ct}$ method.

### Statistical analyses

Data are expressed as mean ± SD. Differences between two groups were analyzed by the parametric Student's *t* test or nonparametric Mann–Whitney *U* test. The correlation analysis was performed using Spearman's rank correlation coefficient. Analyses were completed with SPSS 24.0 software (IBM, Chicago, IL, USA). $P < 0.05$ was considered to indicate statistical significance.

## Results

### Chronic cigarette smoke exposure contributes to emphysema and is accompanied by elevated p-mTOR expression in pulmonary CD8$^+$ T cells

The measurements of MLI, DI and MAA were used to compare the lung tissue pathology in mice chronically exposed to cigarette smoke with mice exposed to room air. Alveolar structures in lungs of air control mice were almost complete and exhibited little inflammatory cell infiltration (Fig. 1a). In contrast, lungs from mice exposed to cigarette smoke showed severe emphysema, with enlarged alveolar spaces, thinner alveolar walls, cilia lodging, inflammatory cell infiltration, and significant destruction of alveoli (Fig. 1a). The MLI in cigarette smoke-exposed mice was increased significantly compared with that in air control mice (37.89 ± 5.10 μm vs. 21.89 ± 2.01 μm, $P < 0.001$, Fig. 1b). The DI in cigarette smoke-exposed mice was also increased compared with air control mice (39.82 ± 10.22% vs. 65.29 ± 9.53%, $P < 0.001$, Fig. 1c). The result of MAA was in line with the above

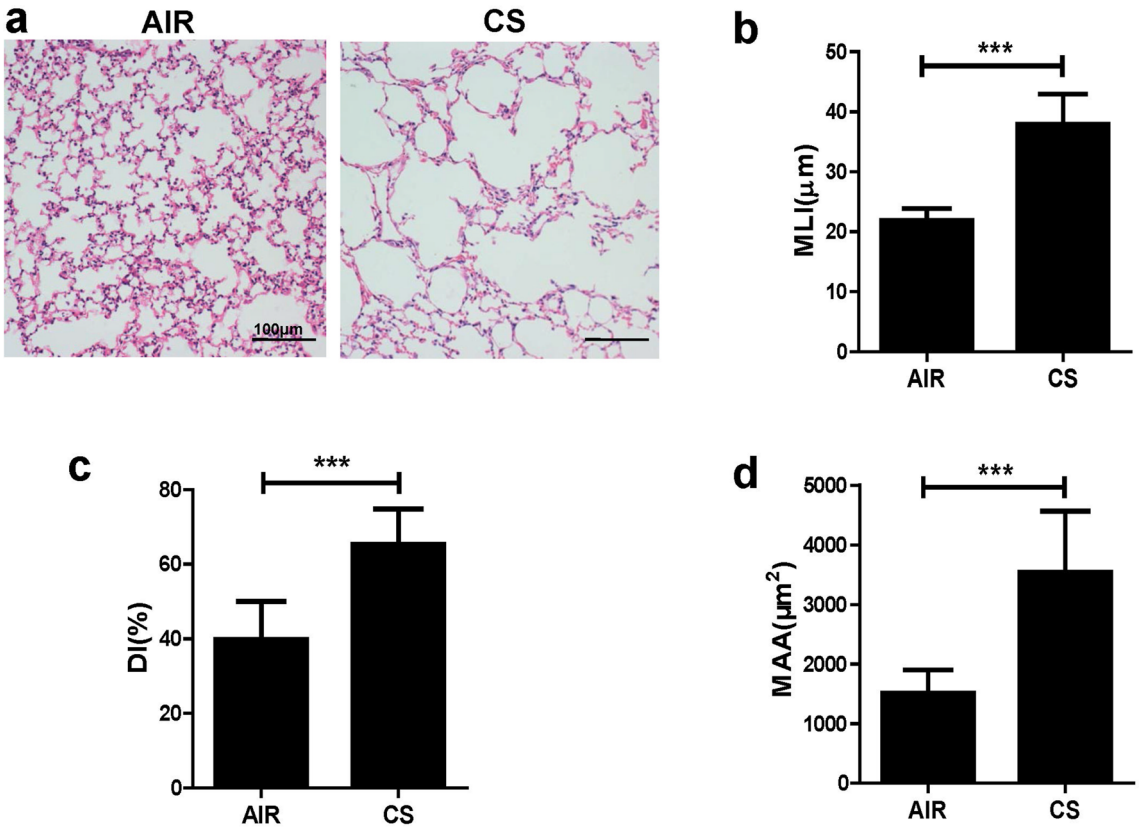


**Fig. 1** Cigarette smoke exposure contributes to pulmonary emphysema. **a** The representative pathology of lung tissue of air control mice (AIR), and cigarette smoke-exposed mice (CS) (hematoxylin and eosin-stained, HE × 200). The comparisons of **b** mean linear intercept (MLI), **c** destructive index (DI) and **d** mean alveolar airspace area (MAA) between mice in AIR group and mice in CS group. Data are expressed as mean ± SD ($n = 10$). $^{***}P < 0.001$

findings (1513.52 ± 389.19 μm$^2$ vs. 3543.18 ± 1022.44 μm$^2$, $P < 0.001$, Fig. 1d). We analyzed the inflammatory cells in BALF of mice. The total cells, macrophages, neutrophils and lymphocytes (including CD4$^+$ lymphocytes and CD8$^+$ lymphocytes) were increased in cigarette smoke-exposed mice (Fig. 2). To test whether mTOR could be activated in CD8$^+$ T cells in smoking-related emphysema, we determined the expression of p-mTOR by flow cytometry (Fig. 3a, b). We observed higher p-mTOR expression in CD8$^+$ T cells of mice with emphysema ($P < 0.01$, Fig. 3c) indicating that the activation of mTOR signaling in CD8$^+$ T cells occurred in the context of cigarette smoke exposure. Consistent with that finding, mTOR mRNA from lung lysates and relative protein expression of p-mTOR were up-regulated in cigarette smoke-exposed mice compared to air control mice ($P < 0.001$ and $P < 0.05$, Fig. 3d, e).

## Increased Tc1 and Tc17 correlate with the severity of emphysema in mice exposed to cigarette smoke

We next investigated the percentages of Tc1 and Tc17 cells in mice chronically exposed to cigarette smoke or room air. Tc1 and Tc17 cells were defined as IFN-γ-producing and IL-17-producing CD8$^+$ T cells, respectively (Fig. 4a). Tc1 and Tc17 cells were increased significantly in spleens and lungs of cigarette smoke-exposed mice compared to air control mice (all $P < 0.01$, Fig. 4b, c), consistent with a previous study [13]. Soluble IFN-γ and IL-17 in lung homogenates were also elevated in smoke-exposed mice ($P < 0.01$ and $P < 0.001$, respectively, Fig. 5a, b). IFN-γ and IL-17 levels correlated with Tc1 and Tc17 in lungs ($R = 0.830$ and 0.649, respectively, all $P < 0.05$, Fig. 5c, d). Furthermore, we found that Tc1 and Tc17 in lungs of cigarette smoke-exposed mice were positively correlated with MLI and MAA (all $P < 0.05$, Fig. 5e–h), but not correlated with DI (data not shown).

## Rapamycin suppresses mTOR, decreases Tc1 and Tc17 cells and attenuates pulmonary damage due to emphysema in mice exposed to cigarette smoke

Rapamycin, a sensitive inhibitor of mTOR, can attenuate the airspace enlargement in mice with emphysema [18]. As expected, we found that rapamycin alleviated alveolar damage and decreased the MLI in cigarette smoke-exposed + rapamycin-treated mice, when compared with

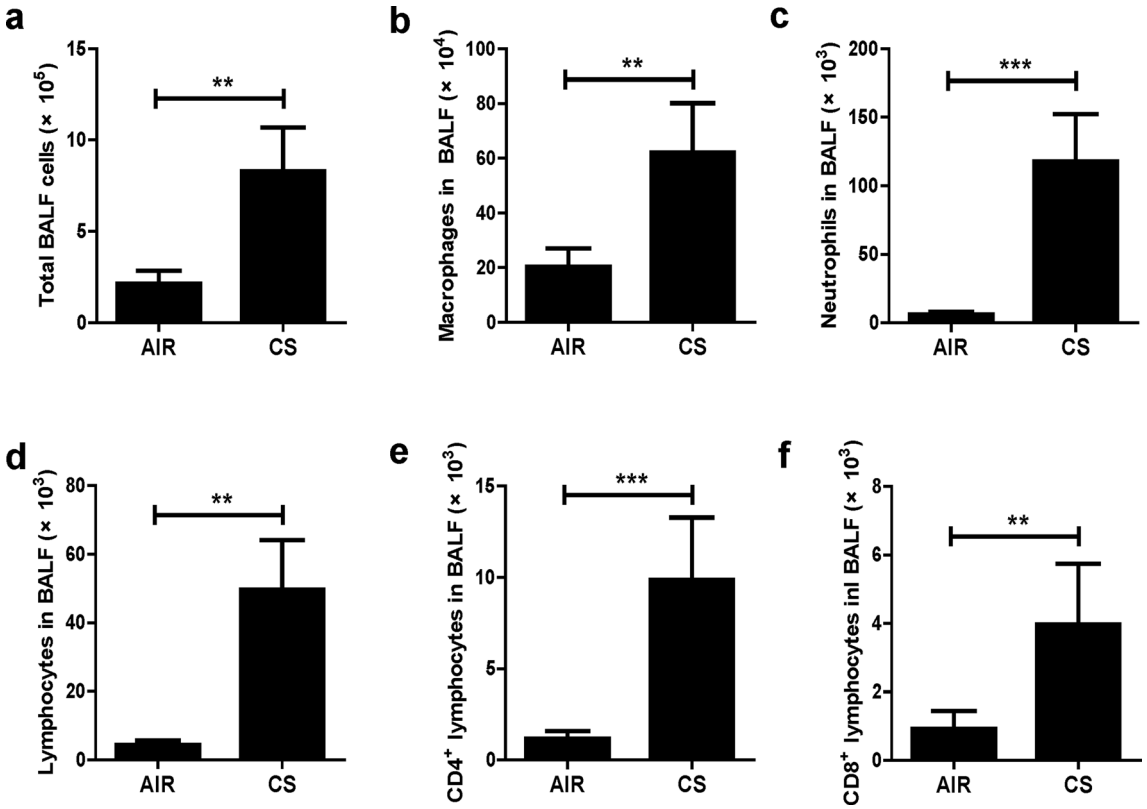


**Fig. 2** BALF analysis in mice exposed to cigarette smoke. The comparisons of **a** total cell, **b** macrophage, **c** neutrophil, **d** total lymphocyte, **e** CD4$^+$ lymphocyte and **f** CD8$^+$ lymphocyte number in BALF of mice in AIR group and CS group. Data are expressed as mean ± SD ($n = 10$). $^{**}P < 0.01$, $^{***}P < 0.001$

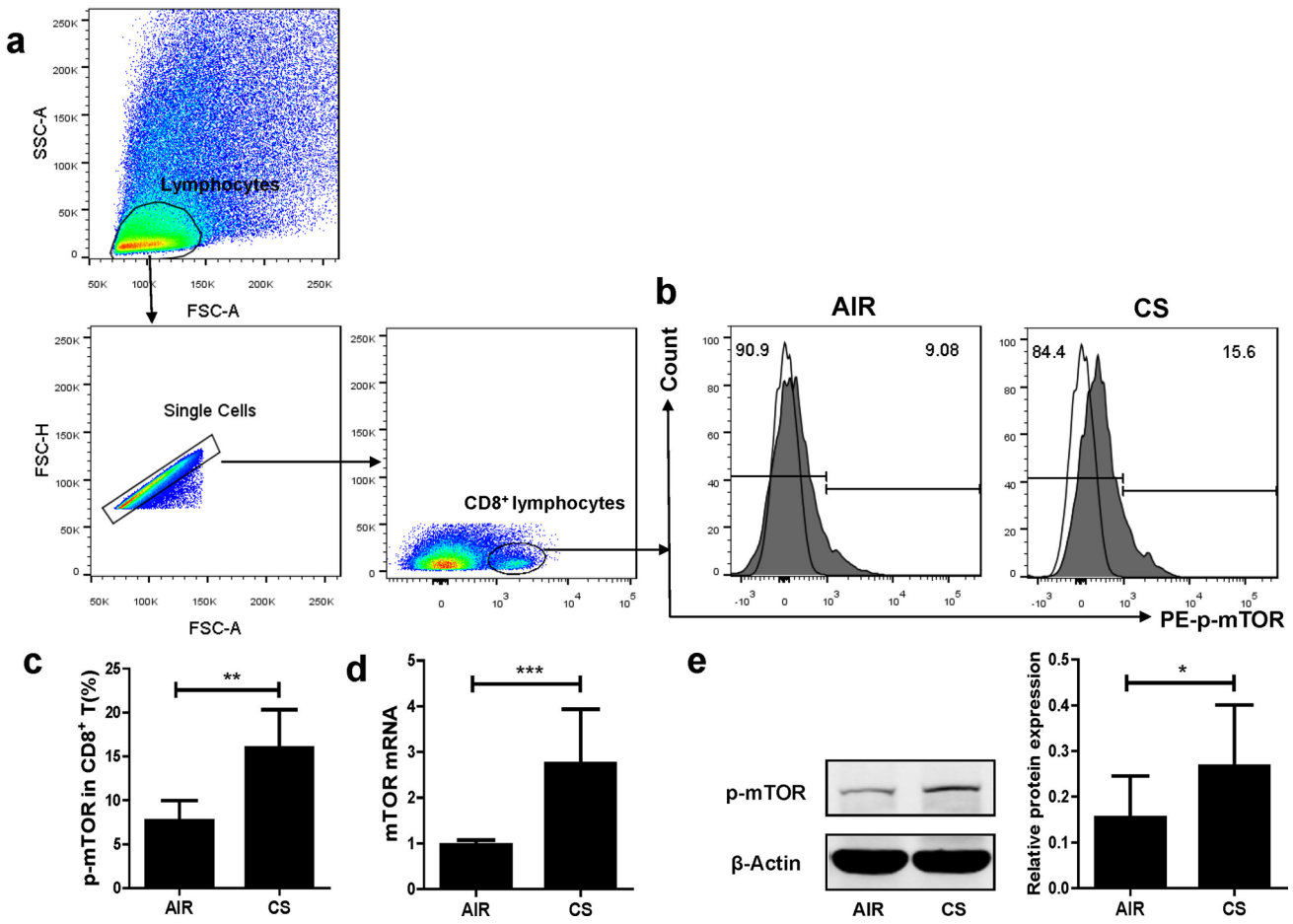


**Fig. 3** Elevated phosphorylated mTOR expression in pulmonary $CD8^+$ T cells in mice exposed to cigarette smoke. **a** Lymphocytes were identified by forward-scattered light (FSC) and side-scattered light (SSC) which were based on the characteristic properties. The adhesion signals of single cells are excluded by gate of FSC-A and FSC-H. Gated from PerCP-cy5.5-$CD8^+$ lymphocytes, the proportion of phosphorylated (p-) $mTOR^+$ cells were analyzed. **b** The representative histogram of p-mTOR in $CD8^+$ lymphocytes in lungs of air control mice (AIR) and cigarette smoke-exposed mice (CS) was shown. White, histogram of fluorescence-minus-one control (FMO); Gray, histogram of p-mTOR. **c** The expression p-mTOR in $CD8^+$ lymphocytes in lungs of air control mice (AIR) and cigarette smoke-exposed mice (CS). Data are expressed as mean ± SD ($n = 10$). $^{**}P < 0.01$. **d** The relative expression of mTOR mRNA in lungs of mice in AIR group and CS group. Data are expressed as mean ± SD. $^{***}P < 0.001$. **e** The relative protein expression of p-mTOR in lungs of mice in AIR group and CS group. Data are expressed as mean ± SD ($n = 10$). $^{*}P < 0.05$

mice exposed to cigarette smoke alone (37.89 ± 5.10 μm vs. 28.23 ± 3.22 μm, $P < 0.001$, Fig. 6a, b). Consistent with MLI, we found that DI and MAA were also decreased in mice treated with rapamycin compared with mice exposed to cigarette smoke alone (DI: 65.29 ± 9.53% vs. 53.54 ± 8.13%, $P < 0.01$; MAA: 3543.18 ± 1022.44 $\mu m^2$ vs. 1778.84 ± 540.80 $\mu m^2$, $P < 0.001$; Fig. 6c, d). Treated with rapamycin, the total cells, macrophages, neutrophils and lymphocytes in BALF were all decreased (Fig. 6e–h). In addition,there were 3.9-fold and 4.4-fold decreases in $CD4^+$ lymphocytes and $CD8^+$ lymphocytes, respectively (both $P < 0.001$, Fig. 6i, j).

Treated with rapamycin, the pulmonary $CD8^+$p-mTOR was decreased in mice exposed to cigarette smoke ($P < 0.05$, Fig. 7a, b). The relative level of mTOR mRNA and the relative protein expression of p-mTOR was also down-regulated significantly in lung lysates of cigarette smoke-exposed mice given rapamycin ($P < 0.001$ and $P < 0.01$, Fig. 7c, d). Moreover, the percentages of Tc1 and Tc17 cells were decreased significantly in cigarette smoke-exposed mice treated with rapamycin compared with mice without treatment (all $P < 0.05$, Fig. 8a, b). In addition, the levels of IFN-γ and IL-17A in lungs of cigarette smoke-exposed mice treated with rapamycin were lower than those in smoke-exposed mice (all $P < 0.05$, Fig. 8c, d). We also found a decrease in the relative level of RORγt mRNA in lungs of cigarette smoke-exposed mice treated with rapamycin ($P < 0.01$, Fig. 8e).

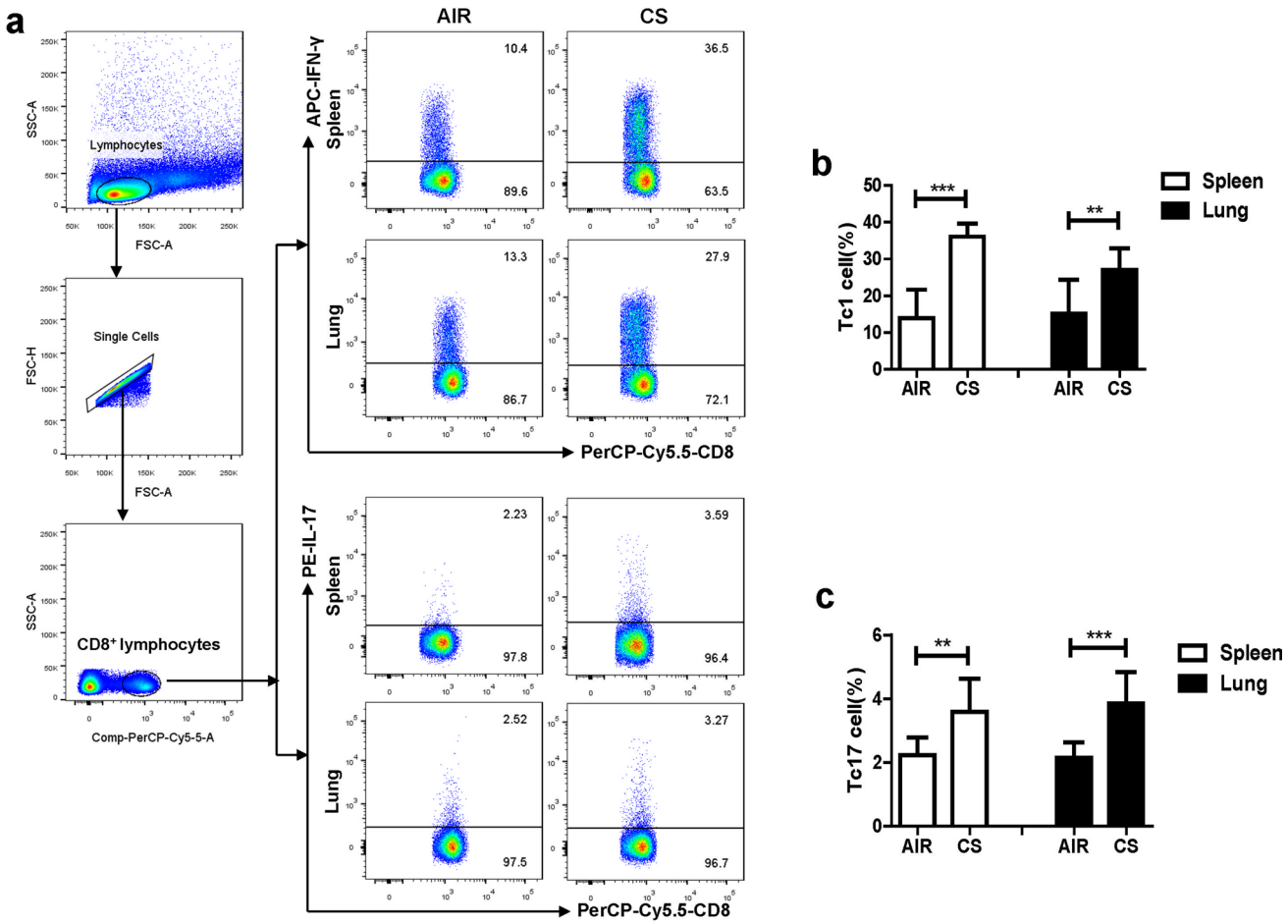


**Fig. 4** Enhanced $CD8^+IFN\text{-}\gamma^+$ T cell and $CD8^+IL\text{-}17^+$ T cell responses in mice exposed to cigarette smoke. Lymphocytes were identified by FSC and SSC which were based on the characteristic properties. The adhesion signals of single cells are excluded by gate of FSC-A and FSC-H. Tc1 cells were identified by $CD8^+IFN\text{-}\gamma^+$ lymphocytes. Tc17 cells were identified by $CD8^+IL\text{-}17^+$ lymphocytes. **a** The representative flow cytometric dot plots of Tc1 and Tc17 cells in spleens and lungs of air control mice and cigarette smoke-exposed mice were shown. The percentages of **b** Tc1 cells and **c** Tc17 cells in $CD8^+$ lymphocytes in spleens and lungs of mice in AIR group and CS group. Data are expressed as mean ± SD ($n = 10$). $^{**}P < 0.01$, $^{***}P < 0.001$

These results indicated that rapamycin decreased Tc1 and Tc17 cell responses along with ameliorating emphysema, while mTOR was suppressed in lungs in vivo.

## Discussion

Persistent and excessive T lymphocyte responses play an important role in the pathogenesis of COPD/emphysema induced by chronic exposure to cigarette smoke [4, 5, 12, 13, 33]. The infiltrated and accumulated $CD8^+$ T cells in lung tissue of COPD patients or mice with emphysema present an exaggerated IFN-γ- and IL-17-producing $CD8^+$ T cell phenotype which contributes to the persistence of pulmonary inflammation [6, 34, 35]. In this study, we confirmed that enhanced Tc1 and Tc17 cell responses in lungs correlated with emphysema and inflammation in the context of cigarette smoke exposure in mice, consistent with previous observations [7, 11–14]. Moreover, we observed an increased expression of p-mTOR in lung tissue and increased p-mTOR in $CD8^+$ T cells of mice exposed to cigarette smoke. The inhibitor of mTOR, rapamycin, could effectively alleviate the pulmonary and systemic Tc1 and Tc17 cell responses, with down-regulation of p-mTOR in $CD8^+$ T cells in the setting of chronic cigarette smoke exposure in mice.

Emphysema and chronic airway inflammation are major hallmarks of COPD. The long-acting $\beta_2$ adrenergic agonists, long-acting muscarinic antagonists and inhaled corticosteroid are widely used in the treatment of COPD. However, these traditional medications only minimally affect the progression and airway inflammation of COPD [36]. Notably, there is a lack of evidence for these traditional medications in reducing pro-inflammatory cytokines

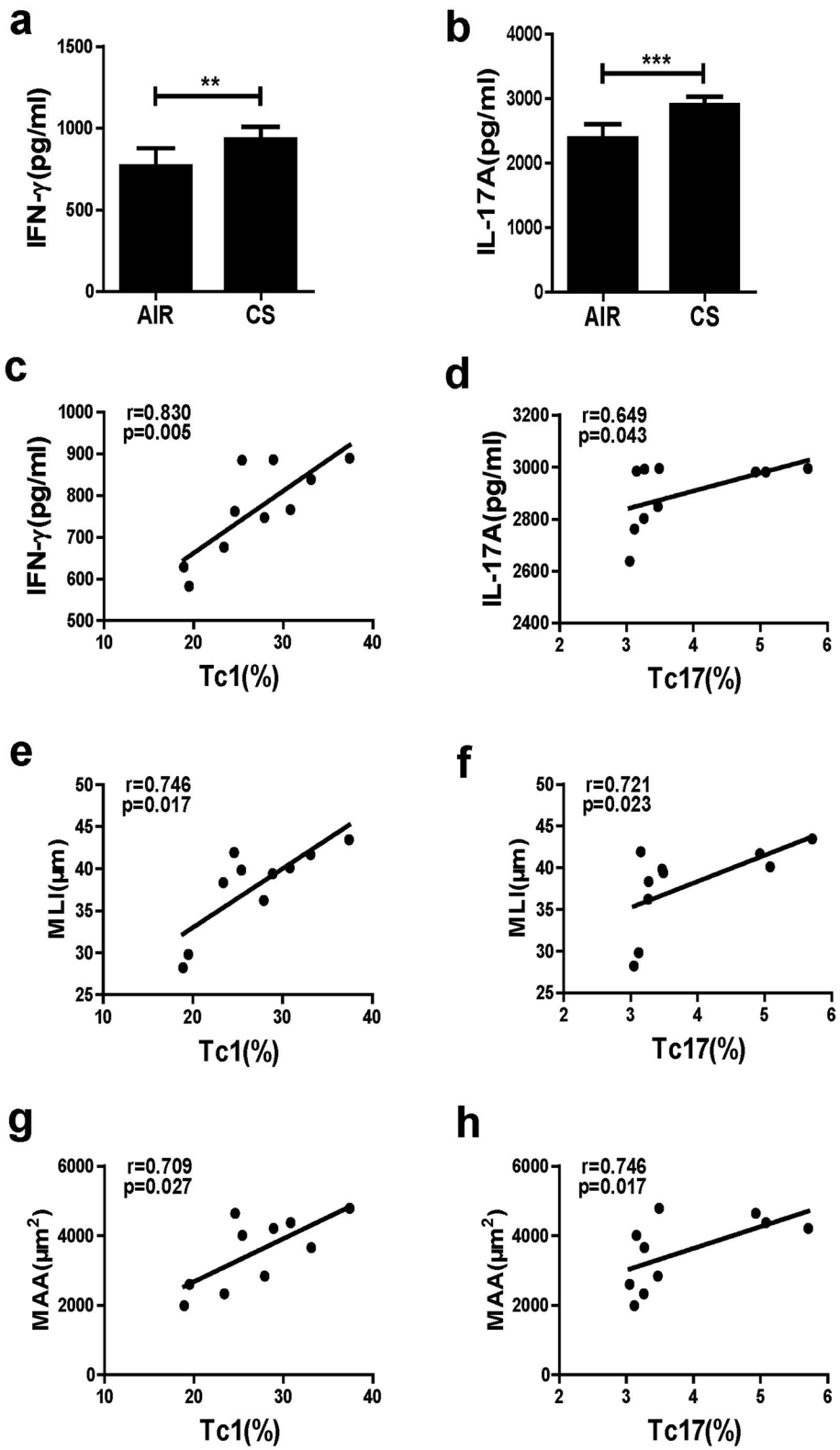


**Fig. 5** Increased $CD8^+IFN\text{-}\gamma^+$ T cells and $CD8^+IL\text{-}17^+$ T cells correlate with the severity of emphysema in cigarette smoke exposure mice. The comparisons of soluble cytokine level of **a** IFN-γ and **b** IL-17A in lung homogenates of air control mice (AIR) and cigarette smoke-exposed mice (CS). Data are expressed as mean ± SD ($n = 10$). $^{**}P < 0.01$, $^{***}P < 0.001$. The correlation analysis between **c** the soluble cytokine level of IFN-γ and the percentage of Tc1 cells, **d** the cytokine level of IL-17A and the percentage of Tc17 cells, **e** the percentage of Tc1 cells and MLI and **f** the percentage of Tc17 cells and MLI, **g** the percentage of Tc1 cells and MAA and **h** the percentage of Tc17 cells and MAA in lungs of cigarette smoke-exposed mice ($n = 10$)

by T lymphocytes [37]. Given dysregulation of T lymphocytes, especially $CD8^+$ lymphocytes, that play crucial roles in the development of COPD, we consider that there is the need for studies focusing on limiting enhanced Tc1 and Tc17 cell responses. This finding may serve as a potential treatment strategy in COPD.

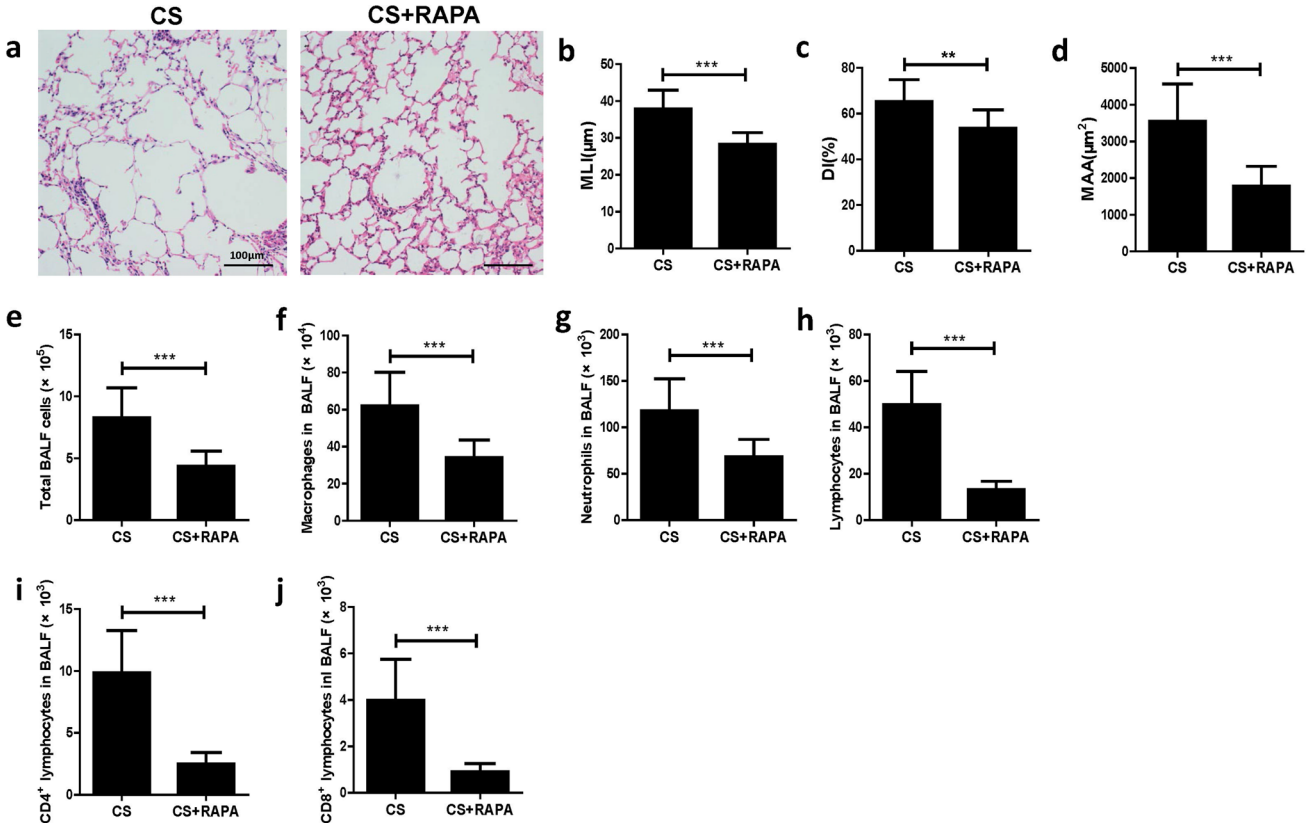


**Fig. 6** Rapamycin attenuates emphysema accompanied by down-regulation of mTOR in $CD8^+$ lymphocytes in lungs of cigarette smoke-exposed mice. **a** The representative pathology of lung tissue of cigarette smoke-exposed mice (CS) and cigarette smoke-exposed + rapamycin-treated mice (CS + RAPA). (HE × 200). The comparisons of **b** MLI, **c** DI, and **d** MAA between mice in CS group and mice in CS + RAPA group. Data are expressed as mean ± SD ($n = 10$). $^{**}P < 0.01$, $^{***}P < 0.001$. The comparisons of **e** total cell, **f** macrophage, **g** neutrophil, **h** total lymphocyte, **i** $CD4^+$ lymphocyte and **j** $CD8^+$ lymphocyte number in BALF of mice in CS group and CS + RAPA group. Data are expressed as mean ± SD ($n = 10$). $^{***}P < 0.001$

Emerging evidence has shown that activation of the mTOR pathway plays a crucial role in the development of COPD [15–18]. Through modulation of mTORC1 and mTORC2, mTOR plays a critical role in cell differentiation [38, 39]. Th1 and Th17 differentiation requires mTORC1 while Th2 differentiation requires mTORC2 signaling [19]. High mTORC1 activity promotes $CD8^+$ effector T cell generation and responses, while mTORC2 activity regulated memory $CD8^+$ T cells [20]. In contrast, rapamycin enhances $CD8^+$ T cell apoptosis, eliminates effector $CD8^+$ T cells, and up-regulates the immunosuppressive function of T regulatory cells [21]. The increased p-mTOR expression in lungs and pulmonary $CD8^+$ T cells is accompanied by the development of emphysema indicating that the activation of mTOR could probably promote the generation of pathogenic $CD8^+$ T cell subsets. In most conditions, rapamycin functions by inhibiting mTORC1, although mTORC2 also can be inhibited by rapamycin [39].Our data indicated that the Tc cell-mediated immune response was attenuated primarily by inhibiting mTORC1, which might contribute to the amelioration of emphysema. In addition, $CD8^+$ T cells are inherently producing IFN-γ but they can also produce IL-17 modulated by RORγt [40]. The decreased expression of RORγt, with the decrease of Tc17 and IL-17 in rapamycin-treated mice, indicating that mTORC1 signaling could play a role in the down-regulation of IL-17 while RORγt was also implicated [28].

mTORC1 can be activated by oxidative and other cellular stresses [15]. In this study, we confirmed the activation of mTOR in lungs of mice exposed to cigarette smoke in vivo, consistent with previous studies [16, 17]. We speculated that exposure to cigarette smoke was the major trigger activating mTORC1 signaling in the setting of COPD/emphysema. However, decreased expression of mTOR (mTORC1) was also reported in the airway epithelium of humans with COPD and in mice with emphysema [41]. This was probably due to cigarette smoke extract activating tuberous sclerosis 2, a suppressive signaling protein, and inducing autophagy, which is protective against in lung damage [41]. Therefore, it seems that cigarette smoke may not only activate mTOR

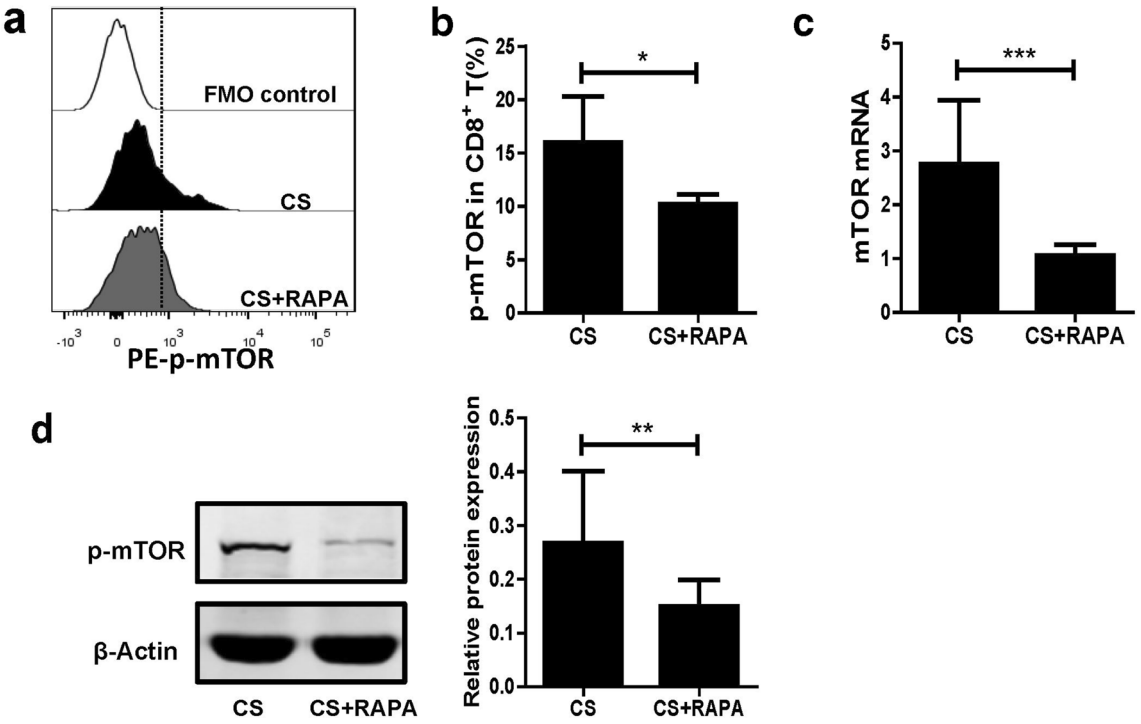


**Fig. 7** Rapamycin attenuates emphysema accompanied by down-regulation of mTOR in $CD8^+$ lymphocytes in lungs of cigarette smoke-exposed mice. **a** The representative histogram of p-mTOR in $CD8^+$ lymphocytes in lungs of CS group and CS + RAPA group was shown. White, histogram of fluorescence-minus-one control (FMO); black, histogram of $CD8^+$p-mTOR of cigarette smoke-exposed mice; gray, histogram of $CD8^+$p-mTOR of cigarette smoke-exposed + rapamycin-treated mice. **b** The p-mTOR expression in $CD8^+$ lymphocytes in lungs of mice in CS group and CS + RAPA group. Data are expressed as mean ± SD ($n = 10$). $^*P < 0.05$. The **c** relative expression of mTOR mRNA and **d** the relative protein expression of p-mTOR in lungs of mice in CS group and CS + RAPA group. Data are expressed as mean ± SD ($n = 10$). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

by oxidative stress, but also can negatively modulate mTOR by activating suppressive signaling protein, indicating a heterogeneous and dynamic pattern in the COPD airway under cigarette smoke exposure.

The absolute cell counts of both $CD4^+$ and $CD8^+$ T cells were decreased in rapamycin-treated mice, especially $CD8^+$ T cells. Additionally, the decreased macrophages and neutrophils in BALF of rapamycin-treated mice suggested that besides T cells, rapamycin can modulate and affect some other immune cells [17, 21, 42–46]. The activation of mTOR in airway epithelial cells and pulmonary vascular endothelial cells has been recognized in pulmonary inflammation including COPD [17, 46]. It has also been reported that rapamycin could inhibit the viability and chemotaxis of macrophages [43], inhibit the maturation of dendritic cells [44], and inhibit the activation of neutrophils [45]. Thus, the attenuation of the inflammation and emphysema may also attribute to the interaction between rapamycin and other immune cells. Nevertheless, in this study, we identify a clinical medicine that effectively inhibits $CD8^+$ T cells in smoke-related emphysema.

This is the first report that p-mTOR is increased in $CD8^+$ T cells in lungs of mice exposed to cigarette smoke and that rapamycin inhibits the Tc1 and Tc17 cell immune responses and ameliorates emphysema. The limitation of this study is the lack of observations of the effect of rapamycin on Tc1 and Tc17 differentiation under cigarette smoke extract stimulation in vitro; this effect requires further study.

Taken together, we confirmed that mTOR was activated in $CD8^+$ T cells in cigarette smoke-related pulmonary inflammation. Moreover, we demonstrated that rapamycin ameliorated emphysema and attenuated Tc1 and Tc17 cell responses which may occur through down-regulating mTOR signaling. These findings may offer a potential opportunity to treat smoking-related emphysema with enhanced Tc1 and Tc17 cell responses.

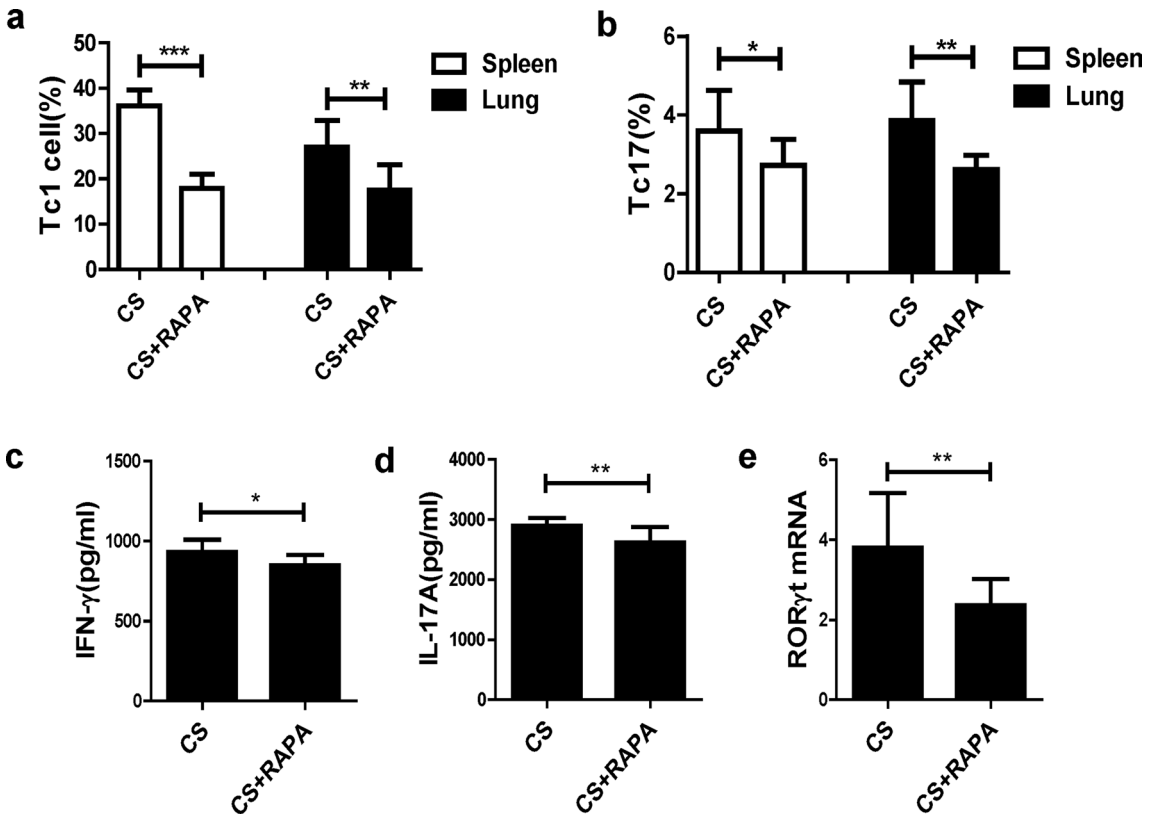


**Fig. 8** Rapamycin alleviates Tc1 and Tc17 cell responses in mice with cigarette smoke-induced emphysema. The percentages of **a** Tc1 cells and **b** Tc17 cells in $CD8^+$ lymphocytes in spleens and lungs of mice in CS group and CS + RAPA group. Data are expressed as mean ± SD ($n = 10$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. The soluble cytokine levels of **c** IFN-γ and **d** IL-17A in lung homogenates of mice in CS group and CS + RAPA group. Data are expressed as mean ± SD ($n = 10$). $^{*}P < 0.05$, $^{**}P < 0.01$. **e** The relative expression of RORγt mRNA in lungs of mice in CS group and CS + RAPA group. Data are expressed as mean ± SD. $^{**}P < 0.01$

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## Compliance with ethical standards

**Conflict of interest** No conflicts of interest, financial or otherwise, are declared by the author(s).

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