

# Thymosin $\beta$ 4 protect against LPS induced lung injury and inflammation and subsequent fibrosis in mice

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## Research Article

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# Abstract

**Background:** Inflammation plays a critical role in the progression of pulmonary fibrosis. Thymosin  $\beta$ 4 (T $\beta$ 4) has antioxidant, anti-inflammatory and antifibrotic effects. Although the potent protective role of T $\beta$ 4 in bleomycin-induced pulmonary fibrosis has been validated, the mechanism is not clear, and its impact on lipopolysaccharide (LPS)-induced lung injury/fibrosis has not been reported.

**Method:** Expression of T $\beta$ 4 in fibrotic lung tissues was assessed by real-time quantitative reverse transcriptase PCR (RQ-PCR), immunohistochemistry (IHC) and Western Blotting. The effects of intraperitoneal adeno-associated virus-T $\beta$ 4 (AAV-T $\beta$ 4) on LPS-induced lung injury and fibrosis were observed through the evaluation of collagen deposition and  $\alpha$ -smooth muscle actin (SMA) expression. In vitro tests with HPAEpiC and HLF-1 cells were performed to confirm the effects of T $\beta$ 4.

**Results:** In this study, we evaluated the role of T $\beta$ 4 in pulmonary fibrosis and explored the possible underlying mechanisms. We found that T $\beta$ 4 was markedly upregulated in human or mouse fibrotic lung tissues. AAV-T $\beta$ 4 markedly alleviated LPS-induced oxidative damage, lung injury, inflammation, and fibrosis in mice. Our in vitro experiments also showed that LPS inhibited mitophagy and promoted inflammation via oxidative stress in HPAEpiC, and usage of T $\beta$ 4 significantly attenuated LPS-induced mitophagy inhibition, inflammasome activation and transforming growth factor- $\beta$  (TGF)- $\beta$ 1 induced epithelial-mesenchymal transition (EMT) in HPAEpiC. Moreover, we found that T $\beta$ 4 suppressed the proliferation and attenuated the TGF- $\beta$ 1-induced activation of HLF-1 cells.

**Conclusions:** In conclusion, T $\beta$ 4 alleviates LPS-induced lung injury, inflammation, and subsequent fibrosis in mice, suggesting a protective role of T $\beta$ 4 in disease pathogenesis of pulmonary fibrosis (PF). T $\beta$ 4 involves in attenuating oxidative injury, promoting mitophagy, and then alleviating inflammation and fibrosis. Modulating of T $\beta$ 4 might be a novel strategy for treating PF.

## 1. Introduction

Pulmonary fibrosis (PF) is a chronic, progressive irreversible and fatal lung disease marked by progressive dyspnea and, ultimately, respiratory failure[1]. Although it is a rare disease, its poor prognosis made it a considerable challenge for clinicians, with a median survival of 2-5 years[2]. Cigarette smoking, exposure to organic and inorganic dust, and genetic factors have been shown to play important roles in disease pathogenesis[3].

Oxidative/antioxidative imbalance and the excessive production of pro-inflammatory and pro-fibrotic cytokines are involved in the pathogenesis of PF. These stimulations then lead to alveolar epithelial injury, followed by proliferation of type I alveolar epithelial cells and myofibroblasts, and deposition of extracellular matrix (ECM) proteins, and then parenchymal remodeling[4]. Anti-oxidative, anti-inflammatory and anti-fibrotic therapies are often used in the treatment of PF. However, none of these treatments has been proven available, and lung transplantation is now the only way to a small minority of PF patients[5, 6].

Thymosin  $\beta$ 4 consists of 43 amino acids and belongs to a highly conserved  $\beta$ -thymosin family[7]. It spreads in nearly all cells and exists in body fluids, including tears, saliva, blood and plasma with important regulatory roles in cell functions[8, 9]. T $\beta$ 4 has been reported participating in wound healing, inflammation, fibrosis and tissue regeneration, and recent studies show that T $\beta$ 4 prevents inflammation and fibrosis in the eye, skin, heart, liver and bleomycin-induced pulmonary fibrosis[8, 10, 11]. T $\beta$ 4 has been shown a protective effect in the long run in the case of scleroderma patients with pulmonary fibrosis[12]. However, the underlying mechanism of T $\beta$ 4 in regulating these fibrotic processes is not fully understood.

Autophagy is a conserved process by which cytoplasmic components, including damaged proteins and organelles, are degraded by lysosomes[13]. An increasing amount of evidence have shown that autophagy limits NLRP3 inflammasome activating by targeting ROS-producing mitochondria, and the process by which mitochondria are degraded by autophagy is called mitophagy[14, 15]. Some recent studies have shown that T $\beta$ 4 limits inflammation via contributing to autophagosome formation and membrane remodeling during autophagy[16], and T $\beta$ 4 could also prevent oxidative stress via upregulating anti-oxidative enzymes Cu/Zn superoxide dismutase (SOD)[17]. However, no studies have examined whether mitophagy regulates inflammation via T $\beta$ 4 during PF.

In the present study, we constructed a recombinant adeno-associated virus (rAAV) to achieve persistent expression of T $\beta$ 4 in LPS-induced PF models, we also explored the possible role of T $\beta$ 4 in regulating mitophagy and inflammation in vitro.

## **2. Materials And Methods**

### **2.1 Histological sampling**

We collected surgical resected paraffin-embedded human fibrotic lung tissues specimens (10 cases) and pathologically normal para-tumor lung tissue specimens (10 cases) from the Department of Pathology, the First Affiliated Hospital of Xi'an Jiaotong University, with the approval of the Institutional Review Board. Immunoreactions were performed on selected lung sections.

### **2.2 Preparation of recombinant AAV**

Self-complementary recombinant adeno-associated virus were constructed by applying an AAV Helper-Free System (Cell Biolabs, San Diego, CA, United States). The coding DNA of human T $\beta$ 4 (GenBank NM\_021109.3) was inserted into pscAAV-MCS to yield the pscAAV- T $\beta$ 4 plasmid. Recombinant AAV containing T $\beta$ 4 (AAV-T $\beta$ 4) was generated via co-transfection of pscAAV-T $\beta$ 4, pHelper and pAAVRC5 into AAV-293 cells using polyethylenimine (PEI). Recombinant AAV carrying LacZ (AAV-LacZ) was constructed as a control virus. 72 hours after transfection, cells were collected for viral particle isolation, purification and quantitative analysis.

TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, United States) was employed to determine the recombinant AAV (rAAV) titers and the abundance of the rAAV in the lung. The primers against the cytomegalovirus promoter region were as follows: 5'-AGACTTGGAATCCCCGTGAGT-3' (forward) and 5'-CGTATTAGTCATCGCTATTACCATGGT-3' (reverse). The sequence of the probe was 5'-6FAM-AACCGCTATCCACGCCATTGATG-TAMRA-3'. The collected data were analyzed by the standard curve method.

## 2.3 Animals

Specific pathogen free, 6-week-old male ICR mice, weighing 25-30g were obtained from the Experimental Animal Center, School of Medicine, Xi'an Jiaotong University. The mice were housed under pathogen free conditions under a 12 hours light/dark cycle at constant temperature ( $22\pm 2^{\circ}\text{C}$ ) and humidity, with free access to water and standard laboratory chow. All mice were acclimatized to the abovementioned conditions for one week before initiating experiments. All efforts were undertaken to minimize the suffering of the mice.

To test the transduction efficiency of repeated intraperitoneal (i.p.) rAAV injection, twenty-four mice were divided into 3 groups: PBS, AAV-LacZ and AAV-T $\beta$ 4. Mice in PBS group were injected with PBS, mice in AAV groups were injected were given AAV-LacZ [ $4\times 10^{10}$  viral genome (vg)] or AAV-T $\beta$ 4 ( $4\times 10^{10}$ vg) on day 0. Two mice from each group were randomly euthanized on day 14 and day 28. The remaining mice were injected again with AAV-LacZ and AAV-T $\beta$ 4 on day 28 and were sacrificed on day 42. The lungs of euthanized mice were harvested for further examination.

## 2.4 AAV-mediated T $\beta$ 4 expression upon LPS-induced lung injury and fibrosis

To verify the expression of T $\beta$ 4 in mouse lung after LPS treatment, thirty-five mice were divided into normal saline (NS, n=5) and LPS (n=30) groups. Septic lung injury model was established by i.p. injection of 5mg/kg LPS for five consecutive days[18]. Five mice from the LPS group were euthanized on days 7, 14, 21, 28, 35 and 42, while all the mice in the NS group were euthanized on day 7. Mouse lungs were collected for HE and picosirius red staining, western blotting, and other experiments.

To investigate the effects of T $\beta$ 4 on acute lung injury and fibrosis, forty mice were equally assigned into four groups: NS, NS+LPS, LPS+AAV-LacZ and LPS+AAV-T $\beta$ 4. Mice in AAV groups were i.p. injected with AAV (AAV-LacZ or AAV-T $\beta$ 4,  $4\times 10^{10}$  vg) for the first time, while mice in the other two groups were injected with an equal volume of NS. Two days later (Day 0), the mice were i.p. instilled with NS or LPS. Five mice in each group were sacrificed on day 7. The remaining mice received the second i.p. administration of AAV or NS on day 26 (four weeks after the first adenovirus administration) and were sacrificed on day 42, when the lungs and serum were harvested for subsequent experiments. The mice were weighed during LPS modeling, and their lung coefficient was calculated (lung coefficient=lung wet weight/body weight $\times 100$ ).

## 2.5 Bronchoalveolar lavage (BAL)

BAL was carried out on day 7 following LPS injection. After the mice were sacrificed, their lungs and trachea were extracted immediately, and a 20G intravenous catheter was inserted into their trachea. 1 mL PBS was instilled into the lungs and withdrawn three times via the catheter. More than 85% of the fluid was recovered as bronchoalveolar lavage fluid (BALF), which was then centrifuged at 1000rpm for 10minutes at 4°C. The supernatants were collected and stored at -80°C, and the precipitate was washed with red blood cell lysis buffer and resuspended in 500µL PBS for further tests.

## **2.6 Measurement of malondialdehyde (MDA) and myeloperoxidase (MPO)**

MDA content and MPO activity in mouse lung tissue were detected with commercially available kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's protocols.

## **2.7 Measurement of hydroxyproline content**

Pulmonary hydroxyproline content was detected with commercially available kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's protocols.

## **2.8 Measurement of IL-1β**

IL-1β level was detected by enzyme-linked immunosorbent assay (ELISA) using commercially available kits (eBioscience, San Diego, CA, USA) according to the manufacturer's protocols.

## **2.9 Cell culture, proliferation assay and reagents treatment**

The HPAEpiC were cultured in DMEM, while HIF-1 cells were cultured in F-12K medium supplemented with 10% foetal bovine plasma and 2mM L-glutamine at 37°C in a 95% air, 5% CO<sub>2</sub>-humidified atmosphere.

Cells were trypsinized, and 500 cells were seeded onto 96-well plates and allowed to adhere for 24hours. Cells were then treated with Tβ4 at different concentrations (0, 75, and 150nM) and incubated for another 72hours. Cell viability was assessed using CCK-8 (Dojindo, Kyushu, Japan) assay at 24, 48, and 72hours according to the manufacturer's protocols.

Cells were trypsinized, and 5×10<sup>5</sup> cells were seeded onto plastic dishes and then treated with H<sub>2</sub>O<sub>2</sub> (0, 100, 200 and 400µM), LPS (1µg/mL), NAC (10mM), FCCP (10µM), Oligomycin (10µM) or TGF-β1 (5ng/mL).

## **2.10 Western Blotting**

Protein extracts were prepared from cells and mouse lung tissues by RIPA Lysis Buffer supplemented with Complete EDTA-free protease inhibitor cocktail tablets (Roche Applied Science, Basel, Switzerland) and phosphatase inhibitor cocktails (Sigma-Aldrich). Protein samples (50µg) were loaded onto SDS-PAGE gels and transferred onto PVDF membranes. After blocking in 5% evaporated milk at room temperature for 2hours, the membranes were then incubated with the indicated primary antibodies in 5% evaporated milk in TBS plus 0.1% Tween 20 overnight at 4°C. The following primary antibodies were used: anti-

Thymosin  $\beta$ 4 (ab167650, Abcam, Cambridge, UK), anti- $\alpha$ -SMA (#56856, Cell Signaling Technology, Danvers, MA, USA), IL-1 $\beta$  (#12703, Cell Signaling), PINK1 (#6946, Cell Signaling), anti-Tom40 (H-300, Santa Cruz Biotechnology, Santa Cruz, CA), and  $\beta$ -actin as a loading control (no. 4970; Cell Signaling) and  $\beta$ -Actin as a loading control (#4970, Cell Signaling). Signals were developed using a chemiluminescent substrate and visualized through X-ray films.

## 2.11 Immunohistochemistry

Immunoreactions were performed on selected lung sections. Antigens were detected by the following primary antibody, followed by appropriate secondary antibodies: anti-Thymosin  $\beta$ 4 (ab167650, Abcam, Cambridge, UK) and anti- $\alpha$ -SMA (#56856, Cell Signaling Technology, Danvers, MA, USA). The slides were then observed under a Nikon Eclipse microscope (Tokyo, Japan) coupled to a digital camera.

## 2.12 Statistical analysis

The results are expressed as the means  $\pm$  standard deviation. Statistical analysis was performed using SPSS software 13.0 (SPSS, Inc., Chicago, IL, USA). The Shapiro-Wilk test and Levene statistic were used to evaluate the normality and homogeneity, respectively, of the variance. According to the situation, t-tests or Mann-Whitney U tests were used to evaluate differences between two groups; correlations between two quantitative groups were analysed with Pearson or Spearman correlation tests. The  $\chi^2$  test was used for comparisons between two groups. The reported P-values are two-sided, and P-values <0.05 were considered statistically significant.

# 3. Results

## 3.1 T $\beta$ 4 expression was elevated in human and mouse fibrotic lung tissues

Immunohistochemical staining showed a marked increase in T $\beta$ 4 expression in fibrotic human lung tissues, resulted in a significant increase in the average IOD compared with that in normal tissues (Figure 1A). In LPS-treated mice, qRT-PCR and western blot showed markedly elevated expression of T $\beta$ 4 from day 14 after LPS treatment and thereafter (Figure 1B, C). The expression of T $\beta$ 4 was also confirmed by immunohistochemistry (Figure 1D).

## 3.2 Intraperitoneal administration of adeno-associated virus efficiently transduced mouse lung tissue

To verify the transduction efficiency of recombinant adeno-associated viruses in mouse lung, we used qRT-PCR to determine the abundance of vector DNA in mouse lung. As shown in Figure 2A, qRT-PCR revealed the presence of vector DNA in mouse lung after the administration of recombinant adeno-associated viruses. Moreover, western blot showed that expression levels of T $\beta$ 4 following the second injection of recombinant adeno-associated viruses were comparable to those observed following first injection, which indicated that realizing prolonged ectopic expression by repeated injection of recombinant adeno-associated viruses was feasible (Figure 2B).

### 3.3 Tβ4 protected mice from LPS-induced lung injury and inflammation

Body weight continuously decreased, while lung coefficient markedly increased after LPS treatment, AAV-Tβ4 significantly attenuated these changes (Figure 3A, B). We found lower tissue MDA content in AAV-Tβ4 group than in NS+LPS or LPS+AAV-LacZ groups, both in day 7 and day 42 (Table 1). Histological examination showed lung injury and inflammation by interstitial edema, infiltration of inflammatory cells, and hyaline membrane formation, and all these changes were alleviated by AAV-Tβ4 (Figure 3C). Moreover, LPS increases in BALF protein content (Figure 3D), and total cell number (Figure 3E) were significantly attenuated by AAV-Tβ4.

We found tissues MPO activity, an indicator of oxidative injury as well as neutrophil infiltration, was elevated by LPS treatment, and this increase was also attenuated by AAV-Tβ4 (Table 1). To further explore the anti-inflammatory function of Tβ4 in LPS-treated mice, the BALF level of inflammatory mediators, such as TNF-α, IL-1β and IL-6 in fibrotic mouse lungs were tested, and we found AAV-Tβ4 significantly mitigated the increase (Table 2).

### 3.4 Tβ4 attenuated LPS-induced lung fibrosis in mice

42 days after LPS treatment, pulmonary hydroxyproline content was markedly increased (Figure 4A). HE and picro-shaped red staining followed showed that lots of spindle-shaped fibrotic cells clumped together, and collagen fibers accumulated (Figure 4C, D), with increased fibrosis score in LPS-treated mice (Figure 4B). All these above fibrotic changes were significantly alleviated by AAV-Tβ4, while usage of AAV-LacZ showed no significant effect (Figure 4A-D).

The expression of α-SMA was significantly lower in LPS+AAV-Tβ4 group than in NS+LPS and LPS+AAV-LacZ group (Figure 4E), and this result was also verified by western blot (Figure 4F) and immunohistochemistry (Figure 4G).

### 3.5 LPS promoted inflammatory responses and inhibited mitophagy in HPAEpiC

We next investigated the effect of LPS in HPAEpiC. We first confirmed that the ROS donor H<sub>2</sub>O<sub>2</sub> led to decreased mitochondria membrane potential (MMP) and promoted ROS accumulation and inflammatory responses in a dose-dependent manner. Moreover, the antioxidant NAC decreased the LPS/H<sub>2</sub>O<sub>2</sub>-induced inflammatory responses in HPAEpiC, suggesting that ROS plays a central role in LPS-induced inflammation in HPAEpiC (Figure 5A-F).

Recently mitophagy has been shown to alleviate inflammation via inhibiting the NLRP3 inflammasome, we then tested whether ROS induce inflammatory responses through mitophagy. Usage of oligomycin, an ATP synthase inhibitor, promoted LPS-induced IL-1β secretion; moreover, usage of FCCP, a drug dissipates MMP and induces mitophagy by activating PINK1, rescued HPAEpiC from LPS-induced inflammatory responses (Figure 5G, H). Because ROS-induced inflammatory responses in HPAEpiC was modulated by mitophagic inhibitor and inducer, we thus wondered whether ROS regulated mitophagy in HPAEpiC.

As the initiator of mitophagy, PINK1 phosphorylates ubiquitin to active Parkin, which builds ubiquitin chains on mitochondrial outer membrane proteins. We found that incubation with H<sub>2</sub>O<sub>2</sub> led to decreased expression of PINK1 in a dose-dependent manner. Mitophagy inhibition leads to an increase in Tom40 protein level. We found here that usage of H<sub>2</sub>O<sub>2</sub> promoted Tom40 accumulation in a dose-dependent manner (Figure 5I).

### **3.6 Tβ4 attenuated LPS-induced mitophagy inhibition, inflammasome activation and TGF-β1 induced EMT in HPAEpiC**

We firstly tested whether Tβ4 affects mitophagy and inflammatory responses in HPAEpiC, and found that Tβ4 alleviated LPS/H<sub>2</sub>O<sub>2</sub>-induced decreased expression of PINK1, and Tom40 accumulation (Figure 6A), we also revealed that Tβ4 successfully suppressed LPS/H<sub>2</sub>O<sub>2</sub>-induced NLRP3 activation and IL-1β secretion in HPAEpiC (Figure 6B, C). qRT-PCR showed that although Tβ4 did not affect the basal expression levels of vimentin and α-SMA, it markedly opposed the TGF-β1-induced upregulation of vimentin and α-SMA in HPAEpiC (Figure 6D, E).

### **3.7 Tβ4 suppressed the proliferation and attenuated the TGF-β1-induced activation of HLF-1 cells**

The CCK-8 assay showed that Tβ4 significantly inhibited the growth of HLF-1 cells (Figure 7A). qRT-PCR revealed that Tβ4 did not affect basal expression of α-SMA and vimentin, but markedly attenuated the TGF-β1 induced elevation of α-SMA and vimentin in HLF-1 cells (Figure 7B, C).

## **4. Discussion**

Pulmonary fibrosis is a heterogeneous disease with significant global morbidity and mortality. The mechanism of disease pathogenesis of PF is now poorly understood. Recent studies have shown that PF mainly results from inflammation and consequently fibroblast proliferation, which leads to abnormal deposition of extracellular collagen[3]. In the present study, we firstly found increased expression of Tβ4 in human and mouse fibrotic lung tissues. The role of Tβ4 in alleviating hepatic, renal and cardiac fibrosis has been confirmed by some recent researches[9, 19, 20]. The increased production of local Tβ4 in mice serves as an adaptive response to lung injury, and this increased expression of endogenous Tβ4 might not be sufficient enough to alleviate lung injury and fibrosis. Our data revealed protective effect of Tβ4 in pulmonary fibrosis, AAV-mediated dramatic overexpression of in mouse lung successfully alleviated LPS-induced lung injury and fibrosis in mice. Our results also indicated that the protective role of Tβ4 may involve suppressing oxidant damage and inflammasome activity, and then alleviating fibrosis.

The lung is susceptible to high oxygen tension, exogenous oxidants and pollutants can increase oxidant production in the lung[21]. Previous studies have revealed that ROS play a role in the pathogenesis of lung inflammation, the generation of mitochondrial ROS is crucial for NLRP3 inflammasome activation, leading to the release of IL-1β[22]. Here, our in vitro data demonstrated that ROS promotes inflammation in alveolar epithelial cells, alveolar epithelial injury leads to the impairment of air exchange function and,

more importantly, the secretion of IL-1 $\beta$ [23]. We also found that treatment of LPS induced ROS generation in HPAEpiC, leading to activation of NLRP3 inflammasome, and this effect was alleviated by NAC, an antioxidant.

Chronic inflammation participates in the pathogenesis of many human diseases, including PF. These diseases are characterized by excessive ROS production, and dysfunctional mitochondria have also been shown implicated in these disorders, act as both a source and a target of ROS[24]. Mitophagy is a special type of autophagy which degrades damaged mitochondrial. In the present study, we found mitophagy was decreased in HPAEpiC, and this phenomenon was alleviated by NAC. Moreover, we found that FCCP, a mitophagy inducer, alleviated LPS/H<sub>2</sub>O<sub>2</sub> induced IL-1 $\beta$  secretion whereas oligomycin, an mitophagy inhibitor, promoted LPS/H<sub>2</sub>O<sub>2</sub> induced IL-1 $\beta$  secretion in HPAEpiC. Defective mitophagy leads to accumulation of damaged ROS-generating mitochondria and then activation of NLRP3 inflammasome, our data revealed for the first time that ROS promotes inflammation via mitophagy inhibition in HPAEpiC.

The anti-oxidative effect of T $\beta$ 4 has been conformed in many previous studies[11, 17], in the present study, we observed that T $\beta$ 4 significantly attenuated LPS-induced elevation of mouse pulmonary MPO activity, MDA content and pro-inflammatory cytokines in vivo and LPS/ H<sub>2</sub>O<sub>2</sub> induced mitophagy inhibition and inflammasome activation in vitro. Inflammation is thought participates in the initial period of pathogenesis of lung fibrosis, dysfunction of alveolar epithelial cells and subsequent inflammation trigger fibrogenic process, resulting in the deposition of matrix and remodeling of lung[25]. Our data demonstrated that T $\beta$ 4 alleviated LPS-induced lung inflammation and fibrosis in mice, and suppressed fibrogenic process in HPAEpiC and HLF-1 cells.

In conclusion, the present study demonstrates that T $\beta$ 4 alleviates LPS-induced lung injury, inflammation, and subsequent fibrosis in mice, suggesting the protective role of T $\beta$ 4 in disease pathogenesis of PF. In addition, this study also indicates that the protective effect of T $\beta$ 4 may involve attenuating oxidative injury, promoting mitophagy, and then alleviating inflammation and fibrosis. Modulating of T $\beta$ 4 may be novel strategies for treating PF.

## Declarations

### **Ethics approval and consent to participate:**

The experimental protocol was established, according to the ethical guidelines of the Helsinki Declaration and was approved by the Human Ethics Committee of Xi'an Jiaotong University.

This study followed the national guidelines and protocols of the National Institutes of Health and was approved by the Local Ethics Committee for the Care and Use of Laboratory Animals of Xi'an Jiaotong University.

**Consent to participate:** Not applicable.

**Consent for publication:** Not applicable.

**Availability of data and materials:** The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Competing interests:** The authors declare that they have no competing interests.

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**Authors' contributions:** Zhen Tian contributed to the study conception and design. Experiments were performed by Naijuan Yao, Zhen Tian. Data analysis were performed by Zhen Tian, Fei Wang. The first draft of the manuscript was written by Zhen Tian and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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## Tables

**Table 1** AAV-Tβ4 alleviates increases in MDA content and MPO activity in lung tissues

		NS (n=5)	NS+LPS (n=5)	LPS+AAV-LacZ (n=5)	LPS+AAV- Tβ4 (n=5)
MDA (nmol/mg prot.)	Time Day 7	0.64±0.68	1.42±0.12	1.46±0.32	0.95±0.17*
	Day 42	0.78±0.35	1.71±0.45	1.69±0.29	1.16±0.29*
MPO (U/g)	Day 7	0.45±0.43	0.89±0.23	0.99±0.34	0.53±0.17*
	Day 42	0.62±0.23	1.07±0.26	1.02±0.19	0.79±0.17*

\*  $P < 0.05$  vs the NS+LPS or LPS+AAV-LacZ group

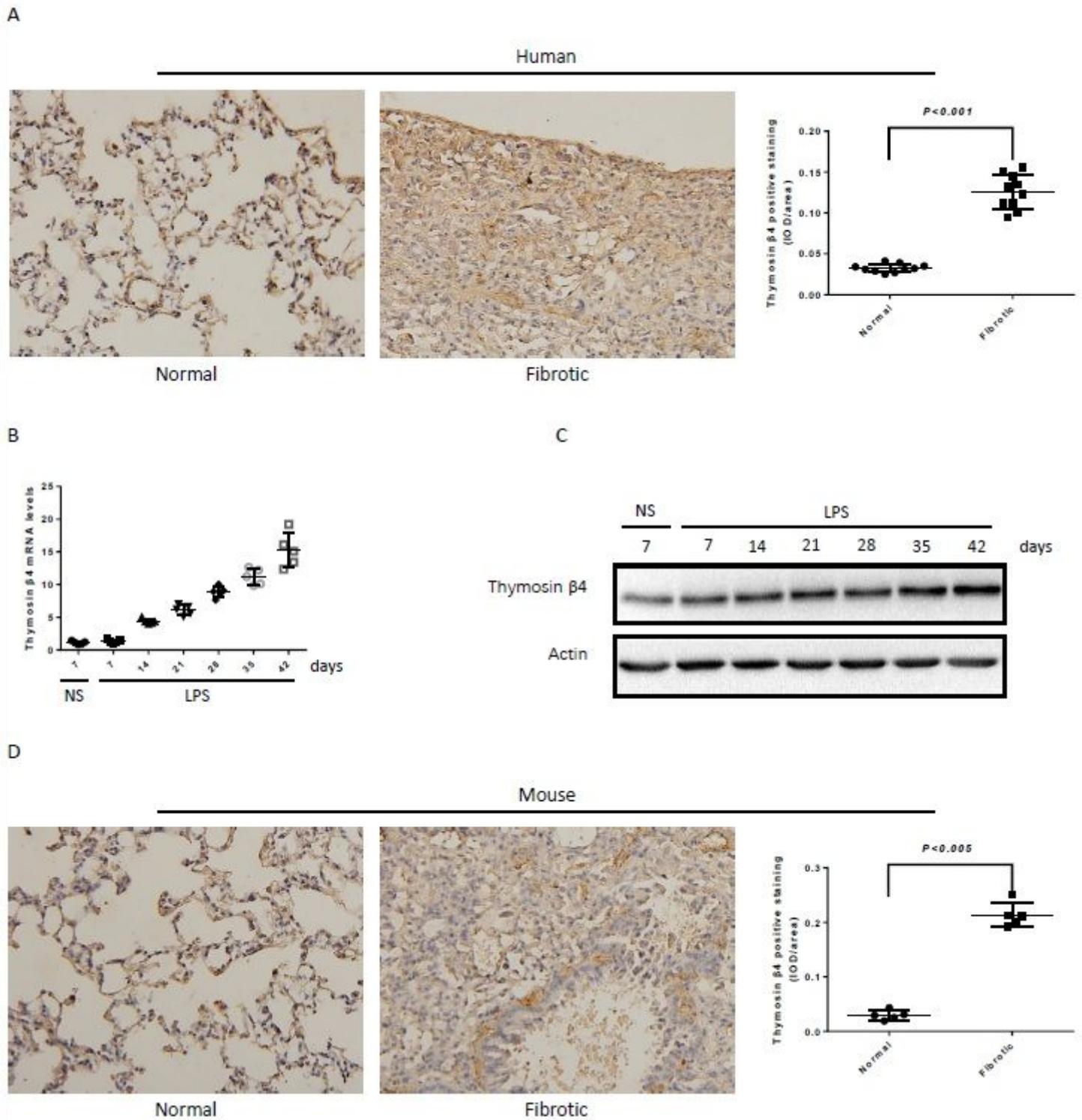
**Table 2** AAV-Tβ4 alleviates LPS-induced upregulation of inflammatory factors in lung tissue

Concentration in BALF (pg/mL)	NS (n=5)	NS+LPS (n=5)	LPS+AAV-LacZ (n=5)	LPS+AAV- Tβ4 (n=5)
IL-1β	63.15±2.11	84.43±1.67#	85.13±1.76#	70.18±2.55*
IL-6	76.87±1.91	90.12±3.33#	92.23±3.21#	79.38±2.11*
TNF-α	6.76±1.89	15.56±7.23#	16.15±7.15#	9.05±1.97*

#  $P < 0.01$  vs the NS group

\*  $P < 0.01$  vs the NS+LPS or LPS+AAV-LacZ group

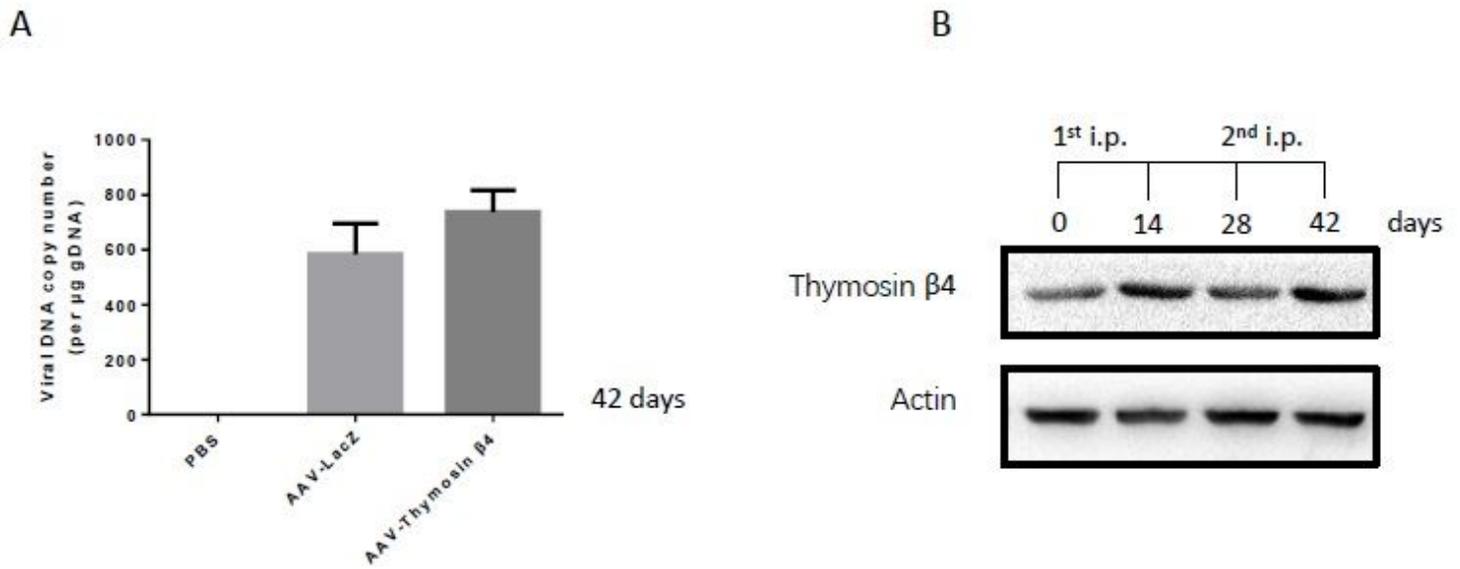
## Figures



**Figure 1**

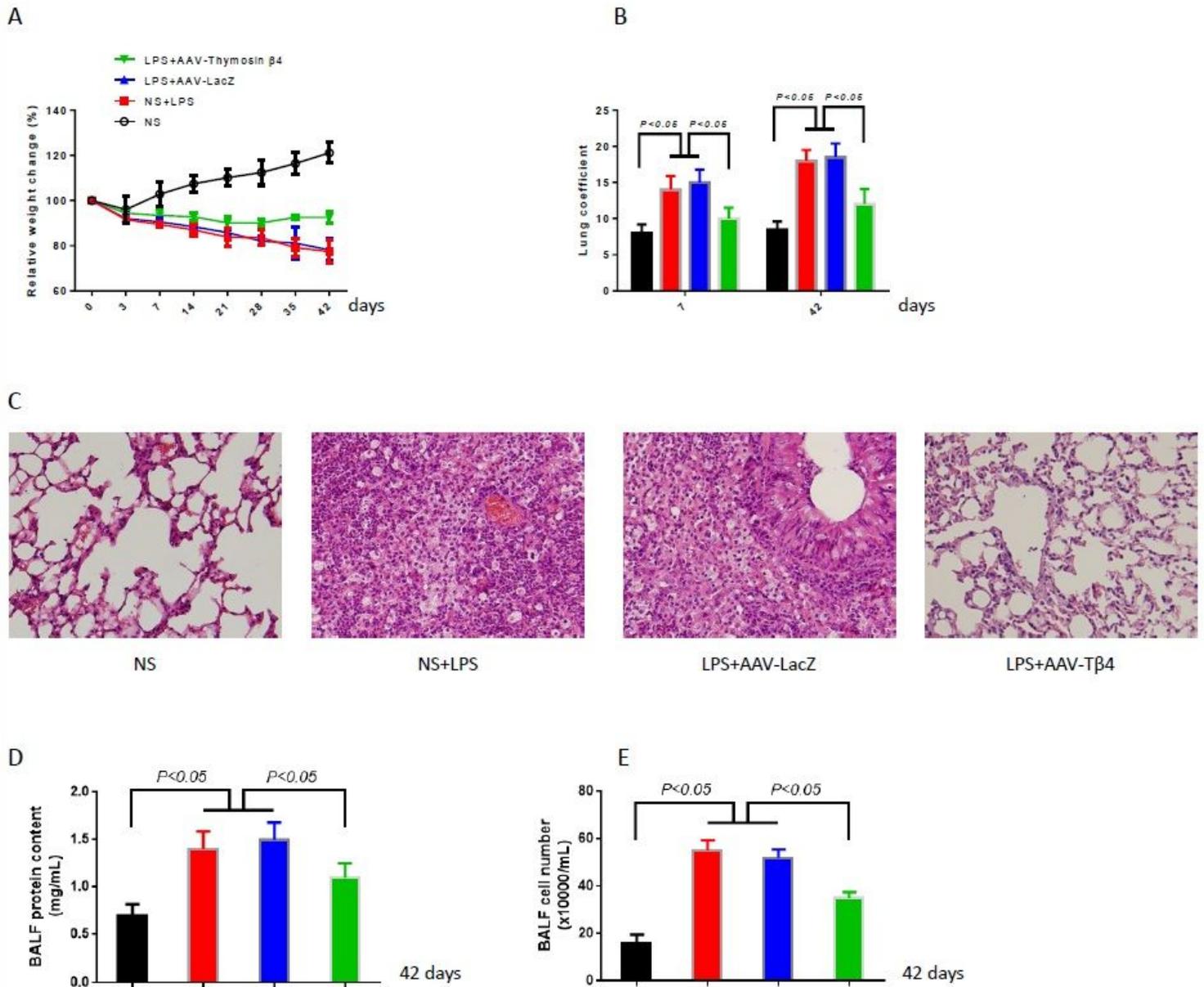
T $\beta$ 4 expression is elevated in human and mouse fibrotic lung tissues. Immunohistochemistry showed that type II alveolar epithelial cells were positively stained with anti-T $\beta$ 4 antibody in normal human lung tissue, and T $\beta$ 4 expression was drastically elevated in fibrotic human lung tissues (A). Expression of T $\beta$ 4 in mouse lung tissues was upregulated at both the mRNA (B) and protein (C) levels. Immunohistochemical

staining showed that the expression of T $\beta$ 4 in normal and fibrotic mouse lung tissues are similar to those in the normal and fibrotic human lung tissues (D).



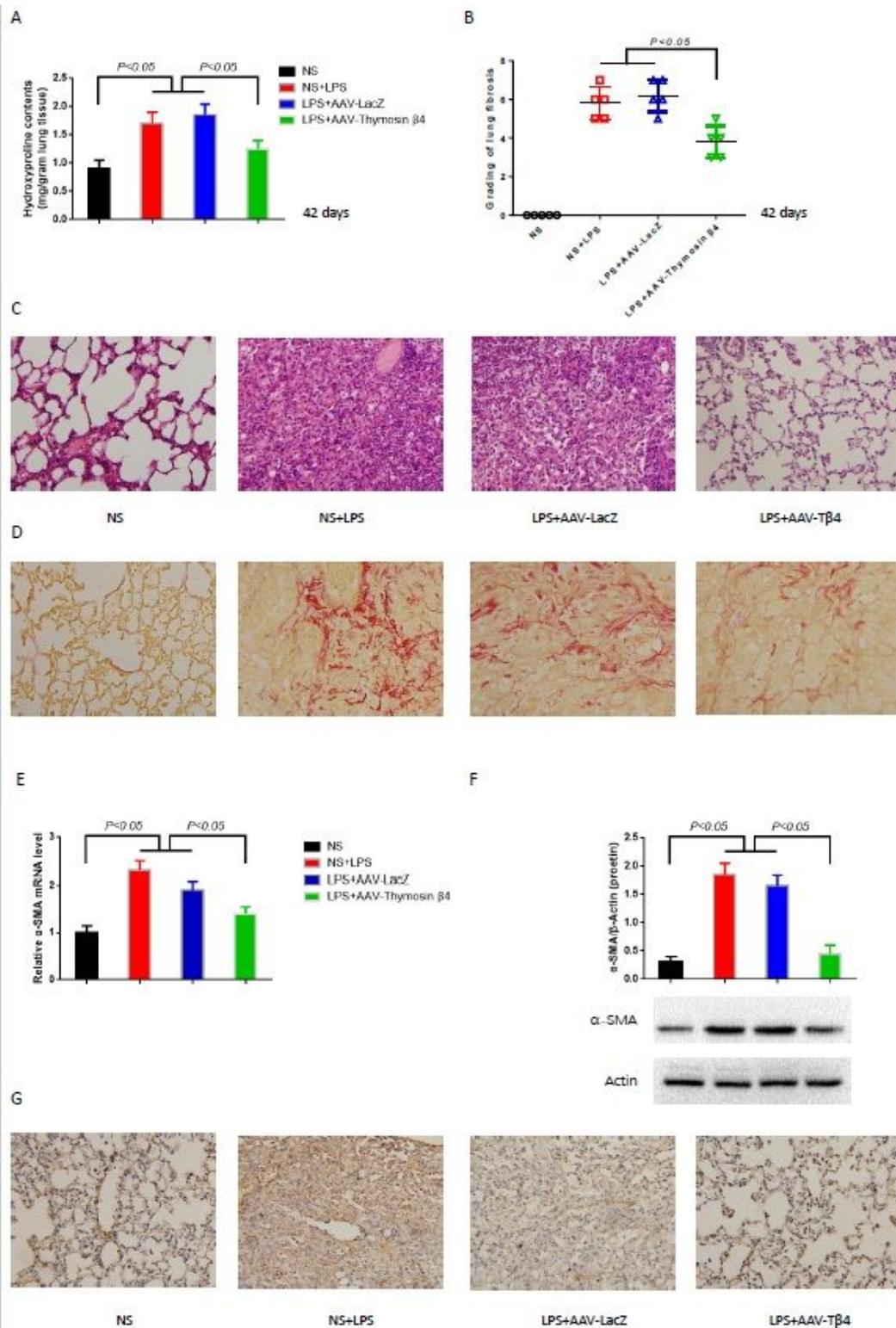
**Figure 2**

I.p. administration of adeno-associated virus carrying Thymosin  $\beta$ 4 mediated expression of T $\beta$ 4 in mouse lung rt-PCR showed the presence of viral DNA in lung tissues of AAV-treated mice 2 weeks after i.p. administration of AAV-T $\beta$ 4 (A). Western blot showed that the expression of T $\beta$ 4 following the second injection of the adeno-associated virus were comparable to those after the first injection in mouse lung (B).



**Figure 3**

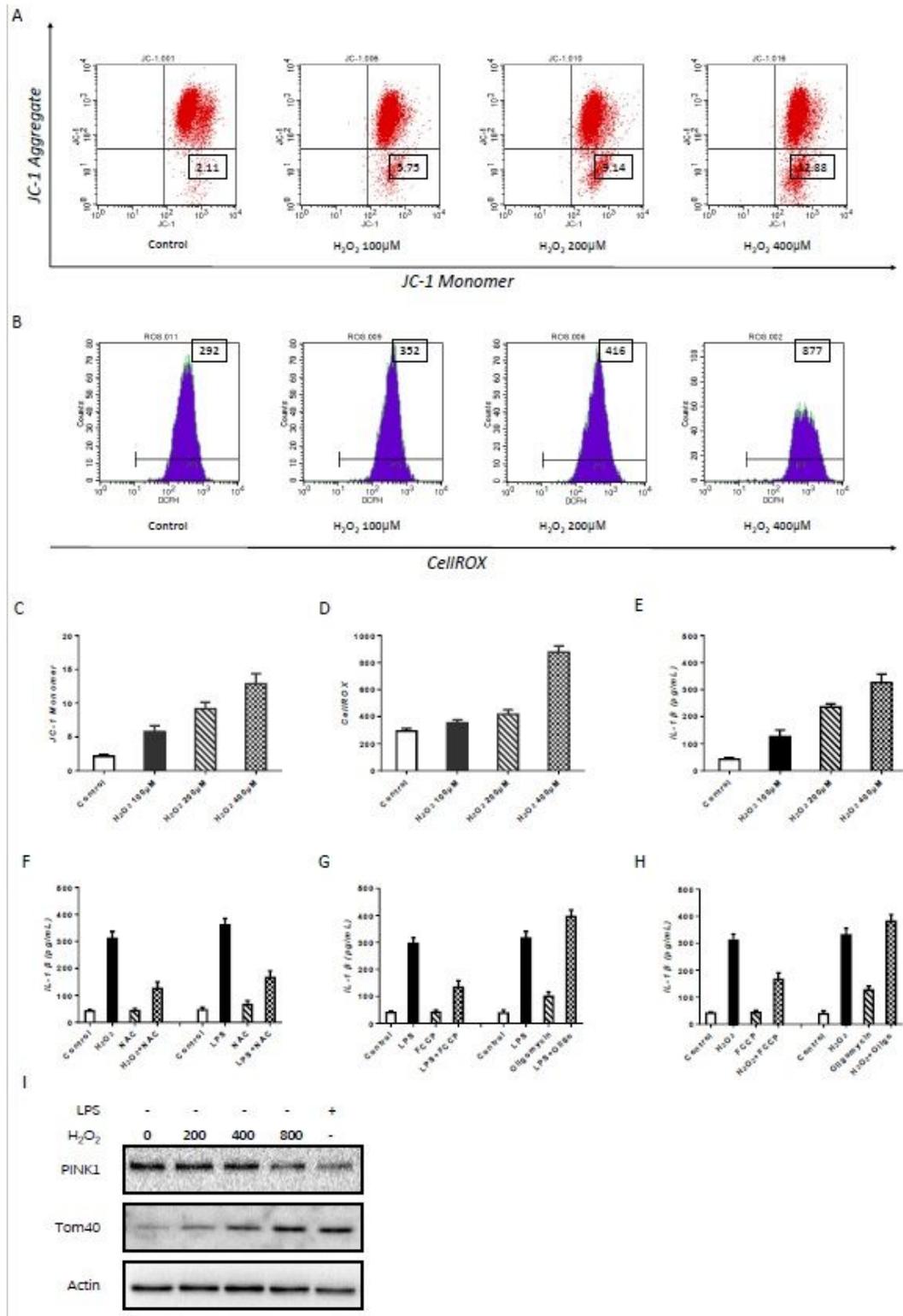
Tβ4 alleviates LPS-induced lung injury and inflammation in mice. Mice in the AAV-Tβ4 group lost less bodyweight than those in the other two LPS groups (A). Similarly, AAV-Tβ4 alleviated increase in the mouse lung coefficient on both day 7 and day 42 after LPS installation (B). AAV-Tβ4 significantly mitigated lung injury and inflammation, as shown by HE staining of mouse lung (C), BALF protein concentration (D), and cell counting (E) at day 42.



**Figure 4**

AAV-Tβ4 alleviates LPS-induced lung fibrosis in mice Tβ4 significantly attenuated LPS-induced lung fibrogenesis in mice, as indicated by lower pulmonary hydroxyproline content (A), milder lung structure destruction (C), less picro-sirius red-positive collagen deposition (D) and lower fibrosis score (B) compared with those in NS+LPS and AAV-LacZ+LPS groups. AAV-Tβ4 significantly alleviated LPS-

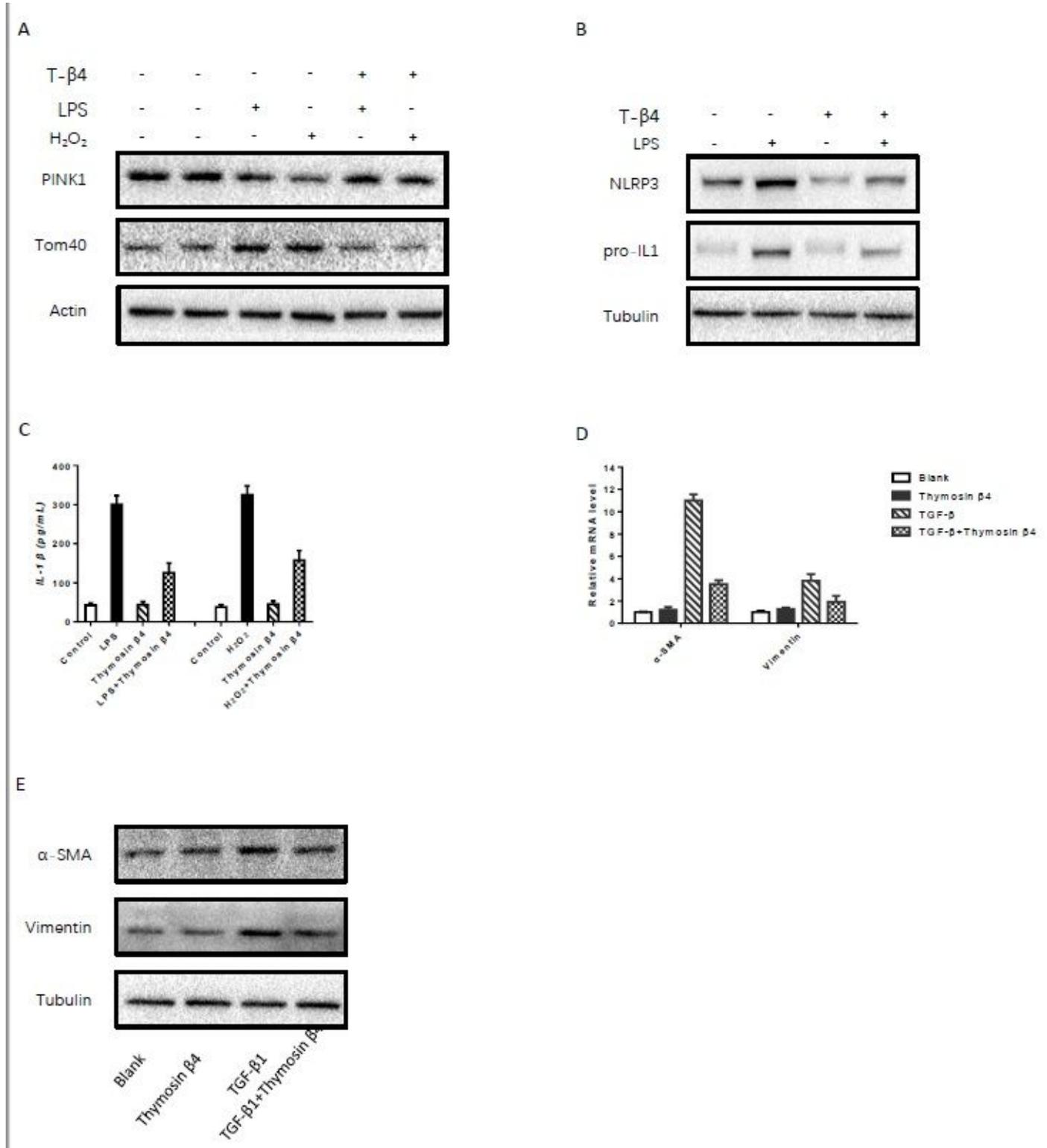
induced excess expression of  $\alpha$ -SMA in mouse lung, as confirmed by rt-PCR (E), western blot (F) and immunohistochemistry (G), compared with those in NS+LPS and AAV-LacZ+LPS groups.



**Figure 5**

H<sub>2</sub>O<sub>2</sub> influences MMP and ROS, and promotes inflammation in HPAEpiC. H<sub>2</sub>O<sub>2</sub> treatment influenced MMP (A, C) and accumulated ROS (B, D) in a concentration-dependent manner over the range of 0-400 $\mu$ M. H<sub>2</sub>O<sub>2</sub> activated inflammation and promoted IL-1 $\beta$  secretion in a dose-dependent manner (E), and

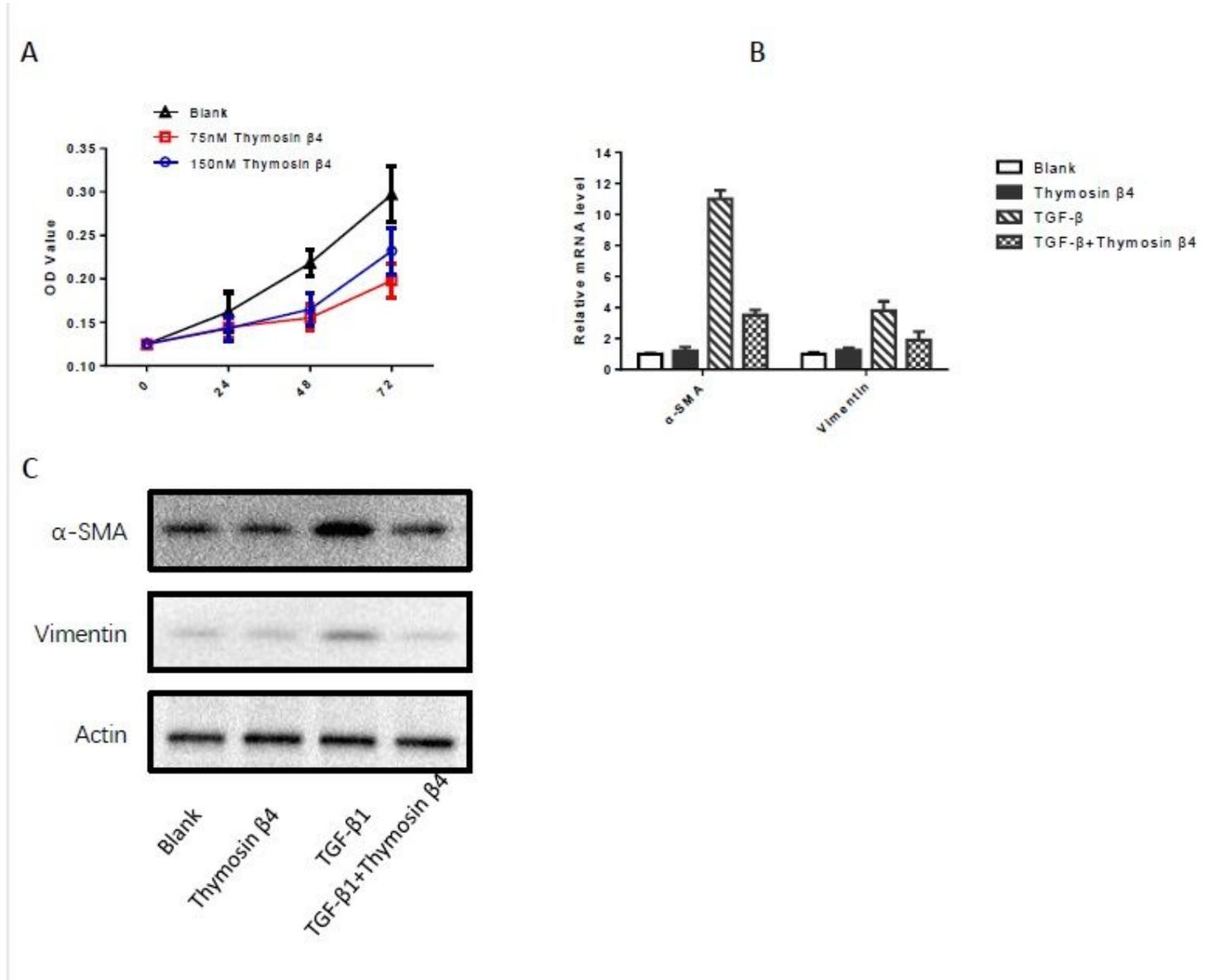
usage of NAC successfully alleviated H<sub>2</sub>O<sub>2</sub>/LPS-induced IL-1 $\beta$  secretion (F). FCCP inhibited and oligomycin promoted H<sub>2</sub>O<sub>2</sub>/LPS-induced IL-1 $\beta$  secretion (G, H) in HPAEpiC. H<sub>2</sub>O<sub>2</sub> inhibited mitophagy in a dose-dependent manner (I).



**Figure 6**

T $\beta$ 4 attenuates LPS-induced mitophagy inhibition, inflammasome activation and TGF- $\beta$ 1-induced EMT in HPAEpiC T $\beta$ 4 alleviated H<sub>2</sub>O<sub>2</sub>/LPS-induced decreased expression of PINK1 and accumulation of Tom40

(A). T $\beta$ 4 suppressed H2O2/LPS-induced NLRP3 inflammasome activation and IL-1 $\beta$  secretion (B, C). T $\beta$ 4 did not affect the basal expression of  $\alpha$ -SMA, vimentin, but markedly attenuated the TGF- $\beta$ 1-induced upregulation of  $\alpha$ -SMA and vimentin (D, E).



**Figure 7**

T $\beta$ 4 suppresses proliferation and TGF- $\beta$ 1-induced activation in HLF-1 cells T $\beta$ 4 suppressed HLF-1 cell growth in a dose-dependent manner (A). T $\beta$ 4 did not affect the basal expression of  $\alpha$ -SMA, vimentin (B), but markedly attenuated the TGF- $\beta$ 1-induced upregulation of  $\alpha$ -SMA and vimentin (C).