

## Original Article

# Inhibitory effects of thalidomide on bleomycin-induced pulmonary fibrosis in rats via regulation of thioredoxin reductase and inflammations

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**Abstract:** In this study, the potential clinical effects of thalidomide on bleomycin-induced pulmonary fibrosis were investigated. A Sprague-Dawley rats' model of pulmonary fibrosis induced by an intratracheal instillation of bleomycin was adopted. The rats in thalidomide treated groups were intraperitoneally injected with thalidomide (10, 20, 50 mg/kg) daily for 28 days, while the rats in control and bleomycin treated groups were injected with a saline solution. The effects of thalidomide on pulmonary injury were evaluated by the lung wet/dry weight ratios, cell counts, and histopathological examination. Inflammation of lung tissues was assessed by measuring the levels of interleukin (IL)-6, IL-8, tumor necrosis factor (TNF)- $\alpha$ , and transforming growth factor (TGF)- $\beta$  in bronchoalveolar lavage fluid (BALF). Oxidative stress was evaluated by detecting the levels of reactive oxygen species (ROS), superoxide dismutase (SOD), total antioxidant capacity (T-AOC), and malondialdehyde (MDA) in lung tissue. The results indicated that thalidomide treatment remarkably attenuated bleomycin-induced pulmonary fibrosis, oxidative stress and inflammation in rats' lung. The anti-inflammatory and anti-oxidative effects of thalidomide were also found in human lung fibroblasts. Thalidomide administration significantly stimulated the activity of thioredoxin reductase, while other enzymes or proteins involved in biologic oxidation-reduction equilibrium were not affected. Our findings indicate that thalidomide-mediated suppression of fibro-proliferation may contribute to the anti-fibrotic effect against bleomycin-induced pulmonary fibrosis. The mechanisms are related to the inhibition of oxidative stress and inflammatory response. In summary, these results may provide a rationale to explore clinical application of thalidomide for the prevention of pulmonary fibrosis.

**Keywords:** Thalidoamide, pulmonary fibrosis, oxidative stress, inflammation, thioredoxin reductase

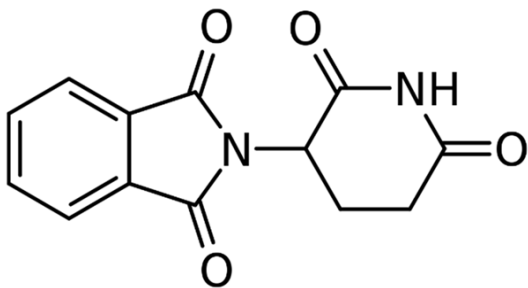
## Introduction

Diffuse interstitial lung disease is characterized by varying degrees of inflammation and fibrosis resulting in derangement of the gas-exchange units of the lung. Around half of interstitial lung diseases are of unknown aetiology and are classified as idiopathic interstitial pneumonias [1]. By far the most common one is idiopathic pulmonary fibrosis, the prognosis of which is worse than that of many cancers [2, 3]. Pulmonary fibrosis has an aggressive course and is usually fatal at an average of 3 to 6 years after the onset of symptoms [3-6]. Pulmonary fibrosis refers to a series of lung diseases characterized by inflammation, fibroblast proliferation and excessive collagen deposition. Although the mechanisms underlying pul-

monary fibrosis are not clearly understood, current evidence suggests that it is associated with the inflammation and oxidative stress [7-9].

Thalidomide ( $\alpha$ -N-phthalimidoglutarimide, as shown in **Figure 1**) was originally introduced in 1954 as a sedative/hypnotic agent but was removed from the market for its teratogenic effects [10]. In contrast, its extra effects such as anti-inflammatory, immunomodulatory, and antiangiogenic activity have been reported [11-13]. Previous studies have demonstrated the efficacy of thalidomide or its analogs in the treatment of erythema nodosum leprosum, rheumatoid arthritis, Castleman's disease, Crohn's disease, renal cell carcinoma, and myelodysplastic disease [14-16]. In recent years, thalido-

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**Figure 1.** Chemical structure of thalidomide.

Thalidomide has been reported efficacious to treat pulmonary fibrosis [17-20]. However, the mechanisms involved were not fully investigated.

A number of key factors were found in the pathogenesis of pulmonary fibrosis including inflammation and oxidative stress. Inflammation is the initial response following lung injury. The inflammatory cells accumulate and release pro-inflammatory cytokines, growth factors, which regulate the proliferation and secretory activity of alveolar fibroblasts [21, 22]. Increasing evidence showed that oxidative stress is involved in the pathogenesis of pulmonary fibrosis. Markers of oxidative stress have been identified in patients and aberrant antioxidant activity has been demonstrated to exacerbate pulmonary fibrosis in animal models. Therefore, anti-inflammation and anti-oxidant strategies are introduced to treat pulmonary fibrosis [23, 24].

Cytosolic thioredoxin (Trx), thioredoxin reductase (TrxR) and nicotinamide adenine dinucleotide phosphate (NADPH) comprise the mammalian Trx system, which plays powerful roles in defense mechanism against oxidative stress, nitrosative stress and in redox regulation [25, 26]. Trx is a small redox-active protein that is ubiquitously present in mammalian and is one of the defense proteins induced in response to various oxidative stress conditions [27, 28]. The reduction of oxidized Trx by NADPH is catalyzed by seleno protein TrxR. TrxR may catalyze the NADPH-dependent reduction of  $H_2O_2$ , lipid hydroperoxides and dehydroascorbate as well [29-31]. In addition to its potent anti-oxidative effect, Trx system also has anti-inflammatory properties, mainly because of its ability to inhibit neutrophil chemotaxis to inflammatory sites and to suppress the expression and activation of the macrophage migration-inhibitory

factors [32, 33]. Because of its desirable anti-oxidative and anti-inflammatory properties, Trx system could be a new and potentially effective therapeutic target for the treatment of pulmonary fibrosis.

In the present study, the effects of thalidomide on pulmonary fibrosis in rats and human lung fibroblasts were investigated. Here we show that thalidomide prevents bleomycin-induced pulmonary fibrosis in rats. The underlying regulatory mechanisms associated with the potential anti-inflammatory and anti-oxidant effects of thalidomide were also investigated.

### Materials and methods

#### Reagents

Thalidomide, bleomycin sulfate, DMSO, and chloral hydrate were purchased from Sigma (St. Louis, MO, USA). Fetal calf serum (FCS) and RPMI 1640 medium were purchased from HyClone (USA). The antibodies to TrxR, Trx, GR, GPx, and  $\beta$ -tubulin were purchased from Cell Signaling Technology (USA).

#### Animals

Sprague-Dawley rats were obtained from Shanghai SLAC Laboratory Animal Co. Ltd. (Shanghai, China) and maintained in our specific pathogen-free animal facility. Animals used in the studies were housed in wire-mesh-bottomed cages in a 12 h light/dark cycle and kept in a room at a constant temperature of 22-24°C and a relative humidity of 60%. Food and water were provided ad libitum. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All protocols for animal use and euthanasia were reviewed and approved by the General Hospital of Tianjin Medical University Committee on Animal Resources committee (TMU-2015023). All efforts were made to minimize suffering.

#### Establishment of bleomycin-induced pulmonary fibrosis in rats

The rats were randomly divided into different groups: control group, bleomycin treated group, bleomycin plus thalidomide treated groups. Briefly, all rats were anesthetized with 10% chloral hydrate. Then, the rats were adminis-

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tered a single intratracheal instillation of bleomycin dissolved in saline (5 mg/kg body weight), while an equal volume of saline was instilled into the rats from the control group. The rats of the bleomycin plus thalidomide groups received an intraperitoneal injection of thalidomide at a dose of 10, 20 and 50 mg/kg body weight, respectively, every day for a total of 28 days, while the rats of control and bleomycin treated groups were injected with a saline solution. The rats were sacrificed at the 28th day after the first bleomycin treatment. The bronchoalveolar lavage fluids were collected and the lungs were excised for further study.

### *Immunohistochemistry*

Rats were sacrificed at day 28 after the first instillation of bleomycin, and the lungs were fixed in 4% paraformaldehyde, dehydrated, and embedded in paraffin and sectioned into 5  $\mu$ m slices. Then the sections were stained with hematoxylin and eosin (H&E), and examined under a light microscope (1:100; BD Pharmingen).

### *Detection of lung wet/dry weight ratios*

The lungs of the rats were removed and weighed immediately, and following dried in an incubator at 60°C for 72 h. The pulmonary edema was estimated as a wet/dry weight ratio.

### *Cell counting in bronchoalveolar lavage fluid (BALF)*

The BALF was centrifuged at 12,000 rpm for 10 min at 4°C, and the supernatant was stored immediately at -80°C until analysis. The sediment cells were resuspended in 50  $\mu$ l PBS and 10  $\mu$ l of the cell suspension was made into the smear on a glass slide. The cell smear was air-dried, fixed in methanol for 15 min, and stained with Giemsa solution. Then total cells, neutrophils, macrophages, and lymphocytes were counted with a hemocytometer (Hausser Scientific, Horsham, PA, USA).

### *Biochemical analysis of BALF*

The levels of interleukin (IL)-6, IL-8, tumor necrosis factor (TNF)- $\alpha$ , and transforming growth factor (TGF)- $\beta$  in BALF were measured by Enzyme-linked Immunosorbent Assay (ELISA) kits in accordance with the manufacturer's instructions.

### *Measurement of oxidative stress*

The lung homogenates were centrifuged for 30 min at 12,000 rpm at 4°C. The serum levels of reactive oxygen species (ROS), superoxide dismutase (SOD), total antioxidant capacity (T-AOC), and malondialdehyde (MDA) in lung tissue were measured according to the instructions of detection kits (Jiancheng Bioengineering Institute, Nanjing, China).

### *Cell line and cultures*

Human embryonal lung fibroblastic cell line WI38VA-13 and human fetal lung fibroblasts cell line IMR-90 were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and cultured in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated FCS, incubated in 5% CO<sub>2</sub> incubator at 37°C. Cells were passaged every 2-3 days and used at about 80-90% of the confluence.

To detect the inflammatory mediators and secreted proteins, human fibroblasts were seeded on 6-well plates (3 $\times$ 10<sup>5</sup> cells/well), serum starved for 2 h and then exposed to 10 ng/mL IL-1 $\beta$  for 2 h before treatment with thalidomide. For thalidomide treated groups, cells were treated with thalidomide, which was diluted in DMSO and added to the growth medium to yield the final DMSO solvent concentration 0.05% (v/v). For control group, cells were treated with the same concentration of DMSO. In preliminary experiments, this final concentration of DMSO had no gross effect on WI38VA-13 and IMR-90 cells.

### *RNA extraction and quantitative real time PCR (RT-PCR)*

Total mRNA was purified using the RNeasy MiniPrep Kit (Qiagen, USA). The gene expression levels of IL-6, IL-8, TNF- $\alpha$ , and TGF- $\beta$  were measured by Superscript II First-Strand Synthesis kit (Life Technologies, USA) in accordance with the manufacturer's instructions. Results were presented as gene expression relative to IL-1 $\beta$  pre-stimulation (100%).

Cell culture medium was collected after 24 h and the level of IL-6 protein expression was analyzed by ELISA according to the producer's protocol (Human IL-6 ELISA set, BD Biosciences, USA). Briefly, 96-well plates were

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coated by kit capture antibody at 4°C overnight, washed and blocked in kit assay diluent. After washing, samples with controls and standards were incubated on the plates for 2 h at room temperature. Then plates were washed, kit detection antibody, streptavidin horseradish peroxidase (HRP, Cell Signaling, USA) and substrate solution were applied according to the producer's protocol. Absorbance was measured within 30 min at 450 nm with 570 nm correction after the addition of a kit stop solution. Results were presented as protein expression relative to IL-1 $\beta$  pre-stimulation (100%).

### *Measurement of TrxR, GR, Trx, and GPx protein expression.*

Immunoblot analysis was carried out by western blot as previously described [34, 35]. Cells were washed three times with ice-cold PBS and harvested in RIPA lysis buffer with 1 mM PMSF, and then lysed on ice for 5 min. The homogenate was centrifuged at 12,000 rpm for 30 min at 4°C and supernatant was collected for analysis. Equal protein extracts were separated by SDS-PAGE, then electrophoretically transferred to PVDF membranes. Incubation with primary and secondary antibodies was performed in Tris-buffered saline containing 5% non-fat dry milk for 2 h or more. After incubation, membranes were washed in Tris-buffered saline containing 0.1% Tween 20. Blots were reprobated with an antibody against  $\beta$ -tubulin as a loading control of protein loading and transfer.

### *Determination of TrxR and Trx activity in cell lysates by insulin reduction assay*

Freshly collected cell lysates were used to determine cellular Trx and TrxR activities as described previously [36]. Briefly, to measure TrxR activity, in each well of a 96-well plate, 25  $\mu$ g of cell lysate was incubated in a final volume of 50  $\mu$ l containing 85 mM Hepes (pH 7.6), 0.3 mM insulin, 10  $\mu$ M Trx, 2.5 mM EDTA and 660  $\mu$ M NADPH for 40 min at 37°C. Controls were also set up. 200  $\mu$ l of 1 mM DTNB in 6 M guanidine-HCl, 200 mM Tris-HCl pH 8.0 solution was added to quench the reaction. The amount of free thiols generated from insulin reduction was determined by DTNB reduction at 412 nm using the VersaMax microplate reader. To measure Trx activity, procedures were carried out

similarly to those for determining cellular TrxR activity, except that the cell lysates were incubated with TrxR in place of Trx. Controls containing lysates and all reaction reagents except recombinant rat TrxR for each lysate sample were also set up. For each sample, Trx or TrxR activity was calculated as the absorbance at 412 nm subtracted from that of the corresponding control and expressed as a percentage of the activity measured in DMSO-treated cells.

### *Determination of GPx activity in cell lysates by GPx activity assays*

For determination of cellular GPx activity, thalidomide (1-50  $\mu$ M) was incubated with lysates of fibroblasts (25  $\mu$ g protein), 20 nM GR, 1 mM GSH and 200  $\mu$ M NADPH in a volume of 100  $\mu$ l phosphate buffer (0.1 M sodium phosphate, 2 mM EDTA pH 7.5) for 1 h at room temperature. H<sub>2</sub>O<sub>2</sub> solution in phosphate buffer was added to initiate the reaction (final concentration 1.5 mM). NADPH consumption was monitored at 340 nm using the VersaMax microplate reader. The results were calculated based on change in absorbance in the initial 3 min and presented as a percentage of GPx activity of drug-treated sample over that of DMSO-treated sample.

### *Determination of GR activity in cell lysates by glutathione reduction assay*

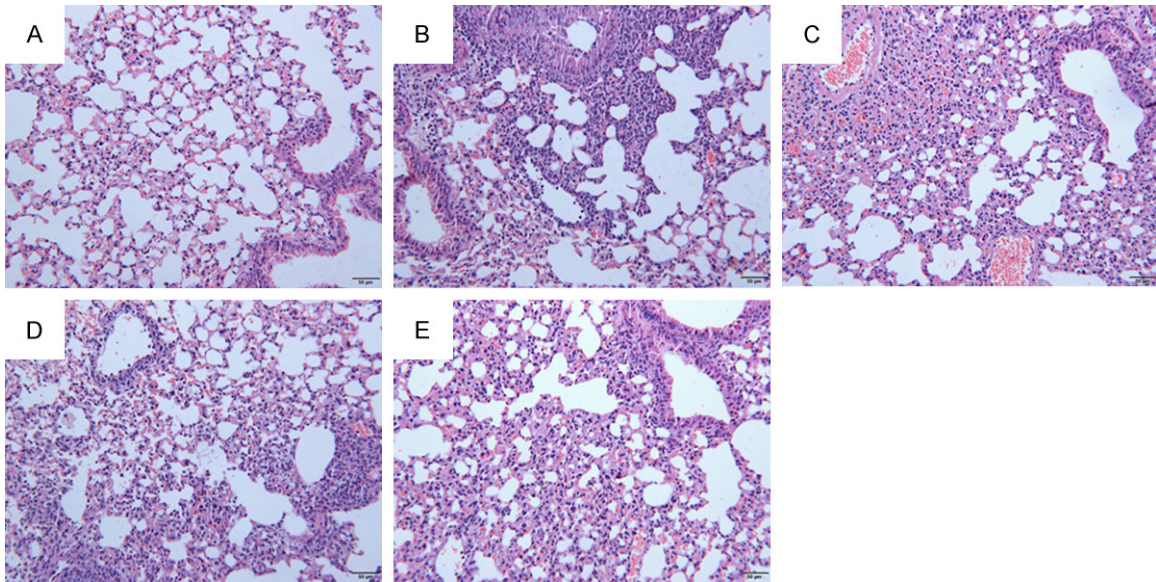
To determine cellular GR activity, 25  $\mu$ g of cell lysate was mixed with a solution of GSSG and NADPH in phosphate buffer to a final volume of 200  $\mu$ l (final GSSG and NADPH concentrations 1 mM and 200  $\mu$ M respectively). The enzyme activity was determined by measuring the decrease in absorbance at 340 nm for 10 min at 37°C and expressed as a percentage of the enzyme activity of that of the DMSO-treated sample.

### *Statistical analysis*

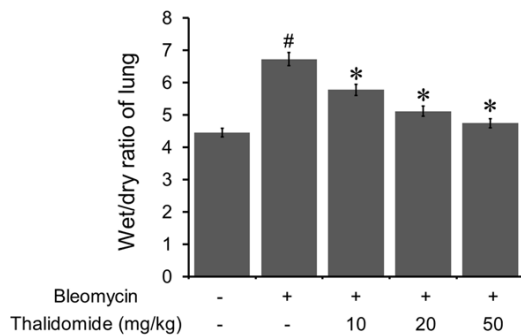
The data were expressed as means  $\pm$  standard deviation (SD). T-test was used to compare the difference between the two groups. Statistical analyses between three or more groups were analyzed by one-way analysis of variance (ANOVA) by using SPSS software version 16.0 (IBM, Armonk, NY). Values for *P* less than 0.05 were considered statistically significant.



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**Figure 2.** Representative photographs of the effects of thalidomide against bleomycin-induced pathological changes (HE stain). At the 28th day interstitial fibrosis was evident in rat's lung from the bleomycin treated group (B), compared with control group (A). Fibrotic areas were obviously reduced in lungs of the thalidomide treated group (C, D, E, respectively) relative to those of bleomycin treated groups at day 28. Scale bar = 50  $\mu$ m.



**Figure 3.** Thalidomide ameliorated bleomycin-induced wet/dry ratio changes in the lung. Bleomycin treatment induced a significant increase of the wet/dry ratio in the lung. However, thalidomide administration reduced the wet/dry ratio in a dose dependent manner. All data were shown as mean  $\pm$  SD of eight rats. <sup>#</sup>P < 0.05 vs. control group, <sup>\*</sup>P < 0.05 vs. bleomycin treated group.

### Results

#### *Thalidomide attenuated bleomycin induced pulmonary fibrosis*

The effect of thalidomide on bleomycin-induced lung fibrosis in rats were examined. The histological changes of the rats' lung tissues at the 28th day were shown in **Figure 2**. In the lung tissues from the bleomycin-treated rats, pulmonary interalveolar septa became thickened

and infiltrated by inflammatory cells, with collagen depositions in the interstitium disclosed by H&E staining. Administration of thalidomide resulted in inhibiting the collagen deposition in the bleomycin-treated rat lung tissues. The pathological changes including damaged lung structure and lung interstitium were attenuated by the administration of thalidomide in a dose-dependent manner.

#### *Thalidomide reduced wet/dry weight ratios of bleomycin-treated rat's lung*

The lung wet/dry ratios were evaluated to indicate the pulmonary edema. As shown in **Figure 3**, compared to the control group, the lung wet/dry ratio significantly increased in the bleomycin-treated group, and thalidomide administration significantly decreased the lung wet/dry ratios.

#### *Thalidomide attenuated the inflammatory responses in bleomycin-induced pulmonary fibrosis*

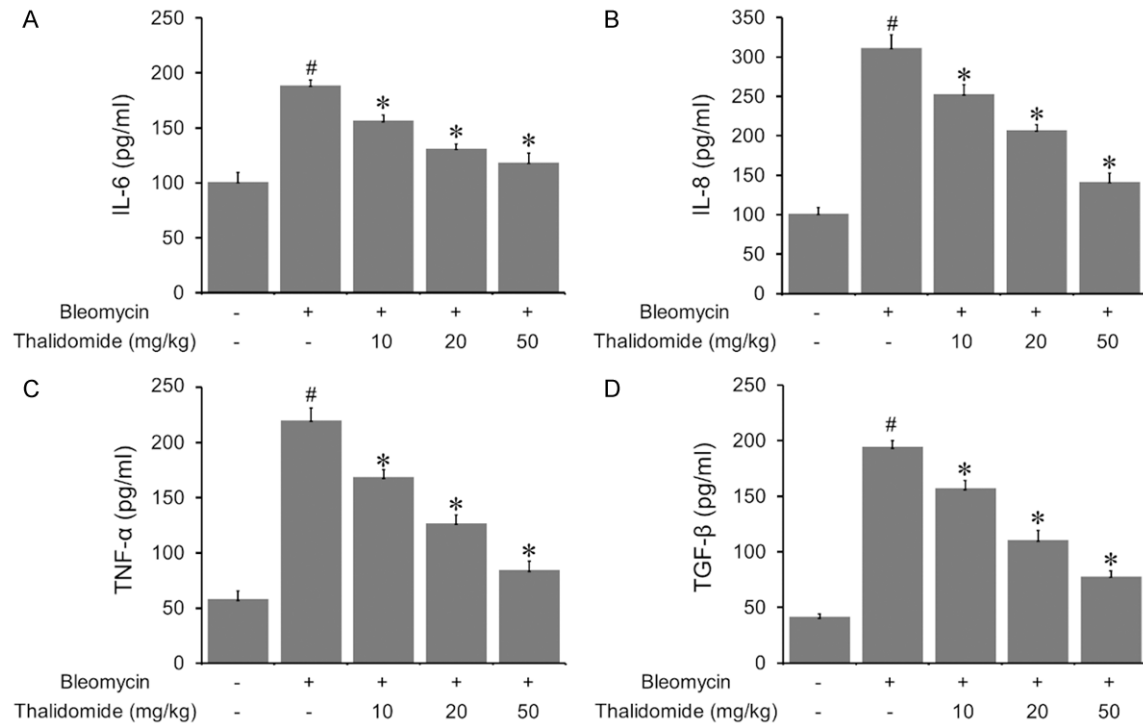
To determine the effects of thalidomide on bleomycin-induced pulmonary inflammation responses in rats, the inflammatory cell counts in BALF were assayed. As shown in **Table 1**, compared with untreated control, the number of total cells, neutrophils, macrophages, and lym-

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**Table 1.** Effect of thalidomide on bleomycin induced changes in total and differential cell counts in the BALF of rats

Group	Total cells ( $\times 10^5$ /ml)	Neutrophils ( $\times 10^4$ /ml)	Macrophages ( $\times 10^5$ /ml)	Lymphocytes ( $\times 10^4$ /ml)
Control	2.21 $\pm$ 0.55	4.93 $\pm$ 0.47	1.40 $\pm$ 0.53	3.24 $\pm$ 0.64
Bleomycin	12.30 $\pm$ 1.38 <sup>#</sup>	22.11 $\pm$ 2.15 <sup>#</sup>	7.92 $\pm$ 1.88 <sup>#</sup>	21.69 $\pm$ 2.43 <sup>#</sup>
Thalidomide (10 mg/kg)	9.23 $\pm$ 1.24*	14.24 $\pm$ 2.81*	6.93 $\pm$ 1.92	17.76 $\pm$ 2.13
Thalidomide (20 mg/kg)	7.82 $\pm$ 0.94*	8.93 $\pm$ 1.11*	5.91 $\pm$ 1.69*	10.17 $\pm$ 2.36*
Thalidomide (50 mg/kg)	5.45 $\pm$ 0.87*	6.16 $\pm$ 1.18*	3.97 $\pm$ 0.95*	8.64 $\pm$ 2.38*

Values are mean  $\pm$  SD. <sup>#</sup>P < 0.05 vs. Control group; \*P < 0.05 vs. bleomycin treated group.



**Figure 4.** Effects of thalidomide on inflammatory mediators in BALF. Cytokines including IL-6, IL-8, TNF- $\alpha$ , and TGF- $\beta$  in lung homogenates and serum were determined using ELISA. All these cytokines were significantly increased by bleomycin treatment. However, thalidomide reduced the values of these cytokines significantly in a dose dependent manner. All the data were shown as mean  $\pm$  SD of eight rats. <sup>#</sup>P < 0.05 vs. control group, \*P < 0.05 vs. bleomycin treated group.

phocytes in BALF were significantly increased after bleomycin treatment, which, however, were rescued by thalidomide administration.

### Effects of thalidomide on the expressions of IL-6, IL-8, TNF- $\alpha$ , and TGF- $\beta$ mRNA in bleomycin-treated rat's lung tissues

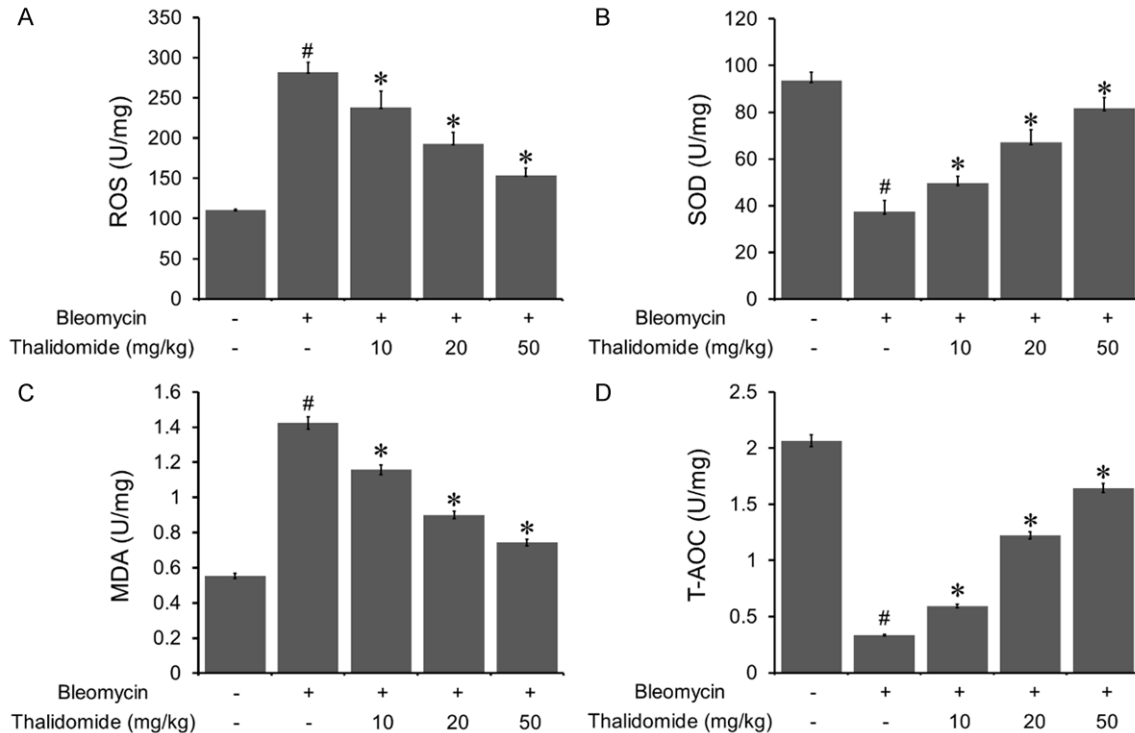
The mRNA levels of IL-6, IL-8, TNF- $\alpha$ , and TGF- $\beta$  from lung tissues of rats after injection of bleomycin were analyzed by RT-PCR and shown to be significantly elevated compared with control rats, which were obviously suppressed by the

administration of thalidomide in a dose-dependent manner **Figure 4**.

### Thalidomide attenuated the oxidative stress in bleomycin-induced pulmonary fibrosis

The levels of ROS, SOD, T-AOC, and MDA in pulmonary tissue were detected to evaluate the oxidative stress of bleomycin-induced pulmonary fibrosis. As shown in **Figure 5**, MDA and ROS levels were significantly elevated in bleomycin treated group, in which SOD and T-AOC levels were decreased. In contrast, after tha-

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**Figure 5.** Effects of thalidomide on oxidative stress induced by bleomycin. Bleomycin treatment increased the values of ROS and MDA significantly. While the administration of thalidomide reduced the values of ROS and MDA in a dose dependent manner. Thalidomide could rescue the decrease of the values of SOD and T-AOC induced by bleomycin treatment. Data were shown as mean  $\pm$  SD. #P < 0.05 vs. control group, \*P < 0.05 vs. bleomycin treated group.

lidomide administration, the levels of MDA and ROS were significantly reduced while the levels of SOD and T-AOC were increased.

### *Thalidomide exhibited anti-inflammatory effect in human fibroblasts*

The effect of thalidomide on the expression of inflammatory mediators in human fibroblasts was examined as while. A significant inhibition of mRNA expressions of IL-6, IL-8, TNF- $\alpha$ , and NGF- $\beta$  was detected upon thalidomide treatment (**Figure 6A**). Due to its strong regulation on the mRNA level and pathological relevance, IL-6 was chosen to be measured in cell culture media by ELISA. Thalidomide treatment significantly decreased IL-6 secretion into the medium, compared to the IL-1 $\beta$  pre-stimulated cells (**Figure 6B**).

### *Thalidomide up-regulated the expression level of TrxR in human fibroblasts*

The effect of thalidomide on oxidation relative proteins were evaluated. Western blotting results showed that thalidomide administration

up-regulated the expression level of TrxR. However, the expression levels of Trx, GR, and GPx were not affected, as shown in **Figure 7A**.

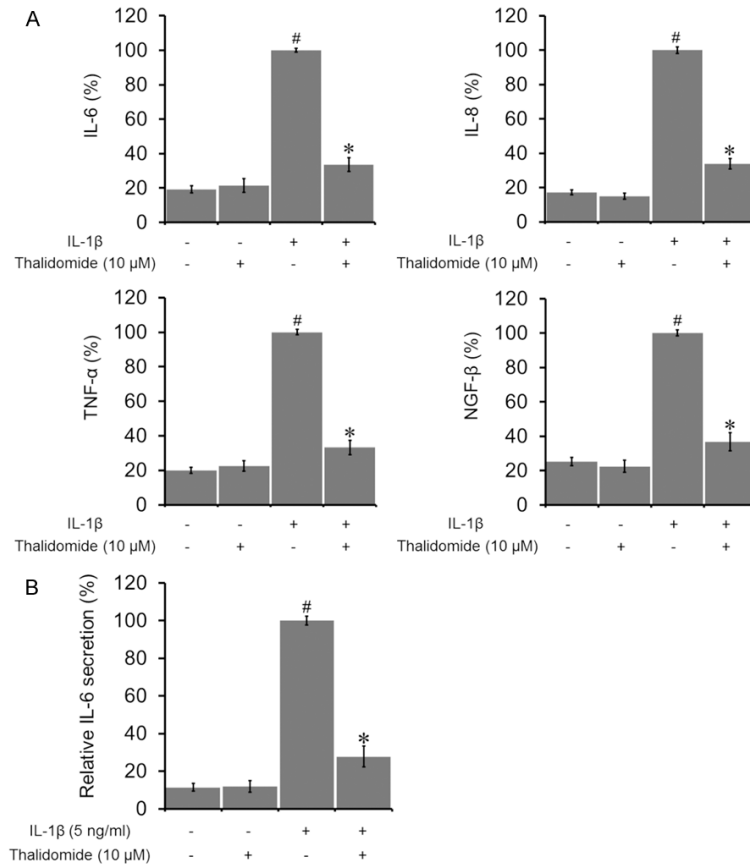
### *Thalidomide activated the activity of TrxR in human fibroblasts*

The effect of thalidomide on TrxR in human fibroblasts was evaluated. After 30 min of incubation in the presence of NADPH, the activation of mammalian TrxR by thalidomide was evaluated in the 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) reduction assay. As shown in **Figure 7B**, thalidomide stimulated TrxR activity in both cell lines, and IMR-90 cells were generally more susceptible to thalidomide. These results suggested that TrxR activation could potentially serve as an underlying mechanism for at least part of the anti-oxidant effects of thalidomide.

## **Discussion**

Thalidomide experienced an infamous history, since its application as an antiemetic during the first trimester of pregnancy had caused

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**Figure 6.** Effects of thalidomide on inflammatory mediators in human lung fibroblasts. (A) Thalidomide significantly reduced mRNA expressions of IL-6, IL-8, TNF- $\alpha$ , and NGF- $\beta$  in human lung fibroblastic WI38VA-13 cells. Cells were cultured without any treatment, treated with 10  $\mu$ M thalidomide alone, 5 ng/mL of IL-1 $\beta$  alone, or treated with both thalidomide and IL-1 $\beta$  for 24 h. (B) IL-6 protein secretion in cell culture media was also significantly decreased. Data were presented by the mean  $\pm$  SD. #P < 0.05 vs control. \*P < 0.05 vs IL-1 $\beta$  alone.

serious birth defects [10]. In recent years, thalidomide has been “rediscovered” as a potent immunomodulatory, antiinflammatory, antiangiogenic, and anti-cancer compound [11-13]. Its immunomodulatory properties indicate its potential in therapy for lung diseases [37, 38]. In this study, we investigated the mechanism underlying the effect of thalidomide on pulmonary fibrosis.

Following bleomycin administration, the number of total cells, macrophages, and lymphocytes in BALF were significantly raised, which represented a stimulation of inflammatory responses [39]. Reduction of these cells counts in BALF by thalidomide was also detected in a dose-dependent manner. IL-6, IL-8, TNF- $\alpha$ , and TGF- $\beta$  play important roles in pulmonary fibro-

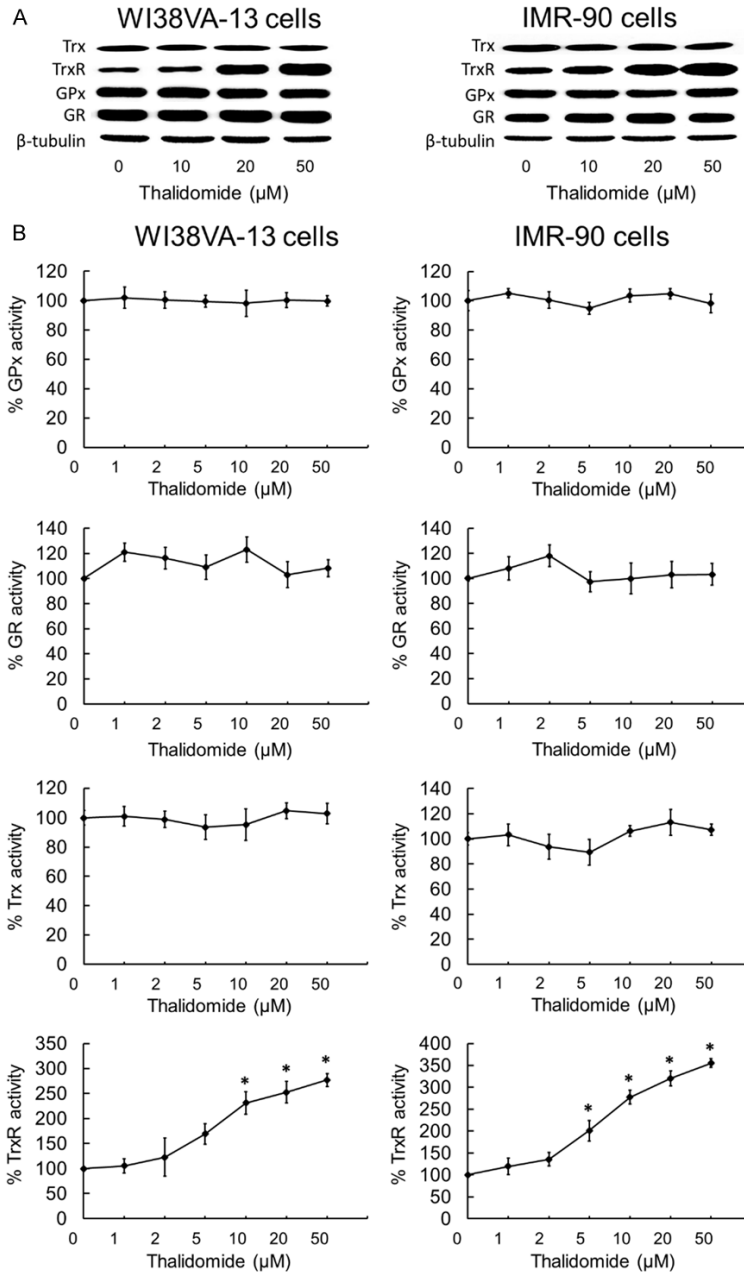
sis, which stimulates the proliferation of fibroblasts, increasing collagen aggregation and synthesis of other cytokines [40, 41]. Our results showed that thalidomide prevented bleomycin-induced elevation of IL-6, IL-8, TNF- $\alpha$  and TGF- $\beta$  in a dose-dependent manner.

Accumulating evidence has indicated that oxidative stress is a potential cause of fibrosis in a variety of organs [42], and there is an imbalance between the generation of ROS and the capacity to detoxify these intermediates [43]. Accordingly, in the present study, oxidative stress was estimated by detecting ROS, SOD, T-AOC, and MDA levels in lung tissue. SOD is an important member of the antioxidant enzymatic defense system and the level of T-AOC reflects the overall cellular endogenous antioxidative capability [44]. And MDA level reflects the degree of organic lipid peroxidation, which indicated the severity of damage to cell membranes [45]. It was shown that in the bleomycin group, MDA and ROS levels were substantially increased while SOD and T-AOC levels were reduced. In contrast, thalidomide remarkably decreased the levels of MDA and ROS, and simultaneously enhanced the levels of SOD and T-AOC. Those results suggested that thalidomide could significantly suppress bleomycin-induced inflammation and oxidative stress, which might explain the protective mechanisms against pulmonary fibrosis induced by bleomycin.

Trx, TR, and NADPH composed Trx system, a very important anti-oxidative system that has been reported many effects, such as regulating cellular reduction/oxidation (redox) status and cell proliferation/cell survival processes [28]. The Trx system has also been reported to regulate the pathologic processes of several



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**Figure 7.** Effects of thalidomide on the expression levels and cellular activities of redox proteins. (A) The lysates of cells treated with thalidomide were analyzed by Western blotting with indicated antibodies for TrxR, GR, Trx and GPx expression. Western blot images are representative of three independent experiments. The results showed that thalidomide administration up-regulated TrxR expression dose-dependently. However, the expression levels of GR, Trx, and GPx were not affected after treating with thalidomide in both WI38VA-13 and IMR-90 cells. (B) Dose-dependent effects of thalidomide on TrxR, GR, Trx and GPx activities in WI38VA-13 and IMR-90 cells. Lysates of WI38VA-13 and IMR-90 cells treated with indicated concentrations of thalidomide for 10 h were assessed for activities of TrxR, GR, Trx and GPx. Enzyme activities were expressed as a percentage of those in DMSO-treated cells. All data points are means  $\pm$  SD of two to four independent experiments. \*Statistically significant difference ( $P < 0.05$ ) in enzyme activity as compared to DMSO control.

kinds of tumors [46], as well involved in cardiovascular disease, heart failure, stroke, inflammation, metabolic syndrome, and other diseases [47]. However, Trx's function in pulmonary diseases has not been thoroughly investigated. Trx expression increased dramatically in asthma and chronic obstructive pulmonary disease, which represents an attempt to protect the lung from injury [48, 49]. Trx has also been proven anti-fibrosis effects in bleomycin-induced fibrosis both in rats and in mice models [50, 51], and suppression of Trx system contributed to silica-induced oxidative stress and pulmonary fibrogenesis in rats [52]. It was also discovered that the expression of Trx increased in the lungs of pulmonary fibrosis patients, compared with the controls [53]. Human serum albumin Trx fusion protein inhibited the increase of inflammatory cells in bronchoalveolar lavage fluid, pulmonary inflammatory cytokines, and oxidative stress markers. In addition, post-treatment of human serum albumin Trx had a suppressive effect against bleomycin-induced fibrosis [54]. All of these results proved that Trx might play a key role in the process of pulmonary fibrosis. However, it has not been revealed the approach through Trx and TrxR exert their affect in pulmonary fibrosis.

In the present study, we found that thalidomide up-regulated the expression level of TrxR without affecting the expression levels of GR, Trx, and GPx. The intracellular TrxR activity was significantly activated by

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thalidomide. Its selectivity towards cellular TrxR activation over related antioxidant enzymes GR, Trx and GPx suggested that thalidomide showed anti-oxidant activity via targeting TrxR. TrxR activation mediated by thalidomide led to cellular Trx reduction, decreased oxidative stress and inhibition of inflammation.

Furthermore, we found that thalidomide exerted direct anti-fibrotic activities in bleomycin-induced pulmonary fibrosis in rats, which was evaluated by the histological changes of the rats' lung tissues, the wet/dry lung weight ratios and by the oxidant stress of lung tissue. This effect was supposed to be associated with decreased expression of inflammatory mediators, and is likely to include both its immunosuppressive and anti-oxidant effects.

### Conclusions

In summary, our study provided direct evidence to prove that in bleomycin-induced pulmonary fibrosis, both inflammatory mediators' production and oxidative stress could significantly inhibited by thalidomide, through an activation of TrxR. Further investigation should be carried out to identify the accurate mechanism of thalidomide's impact on Trx system in pulmonary fibrosis.

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### Disclosure of conflict of interest

None.

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