



Effects of thioredoxin on established airway remodeling in a chronic antigen exposure asthma model

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Abstract

The development and treatment of asthma remains a subject of considerable interest in the medical community. Previous studies implicate an important role of cytokines in the pathology of asthma. In this current study, we examined whether redox-active protein thioredoxin 1 (TRX1) could prevent airway remodeling in an ovalbumin (OVA)-driven mouse chronic antigen exposure asthma model. Balb/c mice were sensitized and then challenged nine times with OVA (days 19–45). In this protocol, airway remodeling was established by day 34. Administration of recombinant human TRX1 during antigen challenge (days 18–32) significantly inhibited airway remodeling, eosinophilic pulmonary inflammation, airway hyperresponsiveness and resulted in decreased lung expression of eotaxin, macrophage inflammatory protein-1 α and IL-13. Airway remodeling and eosinophilic pulmonary inflammation was also prevented in chronic OVA-exposed Balb/c human TRX1 transgenic mice. Importantly, TRX1-administration, after the establishment of airway remodeling (days 35–45), resulted in improved airway pathology. Our results suggest TRX1 prevents the development of airway remodeling, and also improves established airway remodeling by inhibiting production of chemokines and Th2 cytokines in the lungs.

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Persistent allergen-induced inflammation is accompanied by structural changes in the airways (i.e. airway remodeling) of patients with asthma. These airway remodeling changes, observed in both children and adults with asthma, include airway wall edema, hyperplasia and hypertrophy of goblet cells, smooth muscle cells and myofibroblasts [1,2]. These structural changes in the airways are suggested to be responsible for thickening of airway walls, the airway flow limitation, and airway hyperresponsiveness (AHR) in patients with severe asthma [3]. A variety of cells in the airway, including inflammatory cells (such as eosin-

ophils) and the cells of the airway epithelium, may participate in regulating this response [4]. These pulmonary inflammatory cells produce large amounts of chemokines and Th2 cytokines including eotaxin, macrophage inflammatory protein (MIP)-1 α , RANTES (regulated on activation normal T cell expressed and secreted), IL-4, IL-5, and IL-13. Overproduction of these chemokines and Th2 cytokines is involved in the pathogenesis of asthma (see this review article [5]).

Thioredoxin 1 (TRX1) is a redox (reduction/oxidation)-active protein. TRX1 is thought to act as a strong scavenger for reactive oxygen species (ROS) and as an antioxidant both *in vitro* and *in vivo* [6–8]. We reported previously that overproduction of TRX1 in mouse models inhibits interstitial

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pneumonia induced by the pro-inflammatory cytokines IL-18 and IL-2 [9] and lung injury induced by the anticancer drug bleomycin [10]. Exogenous TRX1 inhibits AHR and pulmonary inflammation in mouse models of asthma [11]. Recently, we reported that endogenous and exogenous TRX1 prevent the development and progression of porcine pancreatic elastase-induced emphysema and pulmonary inflammation in a mouse chronic obstructive pulmonary disease (COPD) model [12]. These results suggest that TRX1 can inhibit pulmonary inflammation and airway remodeling and may be able to inhibit AHR in an asthma mouse model.

Here, we investigated whether TRX1 prevents the development of airway remodeling or improves established airway remodeling in a mouse chronic antigen exposure asthma model.

Materials and methods

Reagents. Recombinant human TRX1 protein (rhTRX1) was purchased from Oriental Yeast Co., Ltd. (Shiga, Japan).

Generation of Balb/c background transgenic mice constitutively over-producing human TRX1. Balb/c background human TRX1 transgenic mice were generated as we reported [12].

Study design. The experimental protocol is shown in Fig. 1. Seven- to 10-week-old female Balb/c mice or Balb/c background human TRX1 transgenic mice were treated seven times with an intraperitoneal injection of 10 µg sterile chicken ovalbumin (OVA, grade V, Sigma–Aldrich Chemical, St. Louis, MO) emulsified with 4 mg of sterile aluminum hydroxide (Alu-Gel-S Suspension, Serva Electrophoresis GmbH, Heidelberg, Germany) in a total volume of 200 µL. The injections were given every 2 days from days 0 to 12. These mice were challenged for 48 min with 0.9% saline (vehicle) (group A) or 5% OVA in 0.9% saline (group B), given via the airways by an ultrasonic nebulizer (Omron NE-U07, Tokyo, Japan) amounting to a total of 1, 3, 6, 9, or 12 challenges in a closed box [11]. Mice in groups C and D were sensitized with OVA and then challenged for 48 min with 5% OVA in 0.9% saline as described above. Every second day from days 18 to 44 (14 times in group C) or days 35 to 45 (six times in group D), mice were treated with an intraperitoneal injection of 40 µg rhTRX1 suspended in 100 µL of sterile phosphate-buffered saline (PBS). On day 20, 25, 34, 46, or 54 (24 h after the last OVA aerosol), the mice were euthanized and bronchoalveolar lavage fluid (BALF) analysis, pulmonary histopathologic analysis, and AHR analysis were performed.

Analysis of bronchoalveolar lavage fluid. BALF were collected as per our previous report [11].

Histological analysis. Histological examination was performed as previously reported [9].

Morphometric analysis. The thickening of the peribronchial basal membrane (BM) and the area of smooth muscle (SM) were measured by using a computerized color image analysis software system (Win Roof Version 5.0, Mitani Co., Fukui, Japan) as we previously reported [13]. At least paraffin sections of the right and left lungs of each mouse were made (i.e., at least 16 sections per mice) and stained with Masson's trichrome. The lengths of the BM and SM were measured in at least 10 fields at a magnification of 400× under a light microscope.

Assessment of airway hyperresponsiveness. Airway responsiveness to aerosolized acetylcholine (ACh) as previously reported [14]. We also evaluated Penh in a mouse acute antigen exposure model [11] (see Supplementary Materials).

Statistical analyses. Results are expressed as means ± standard error of the mean. Comparisons among three or more groups were made by ANOVA. Differences were considered significant at $P < 0.05$.

Results and discussion

Establishment of airway remodeling in chronic antigen exposure asthma model

HE staining showed severe airway inflammation in group B but not group A on days 25, 34, and 46 (Fig. 1 of Supplementary Materials). Masson trichrome staining revealed thickened smooth muscle layer in group B (Fig. 2b) but not group A (Fig. 2a) on days 34 and 46 (data not shown). Inflammation and smooth muscle hyperplasia in lungs on day 54 were dramatically decreased in group B mice challenged 12 times with OVA (data not shown). These results show airway remodeling accompanied by airway inflammation and smooth muscle hyperplasia was established in the chronic OVA-exposed mice by day 34.

Exogenous TRX1 inhibits airway smooth muscle hyperplasia

We performed three independent experiments ($n = 6$ per group at each time point) to see whether chronic antigen

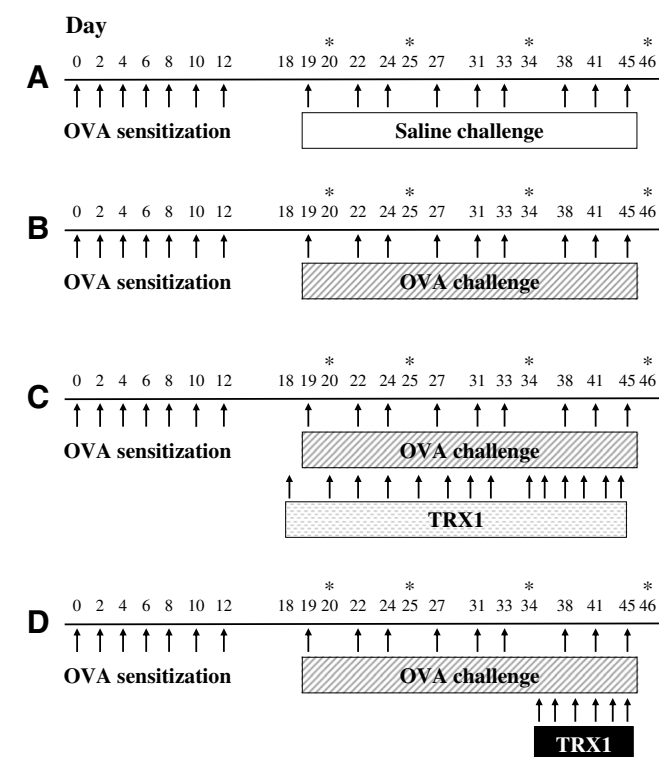


Fig. 1. Schematic summary of experimental protocol. Group A: Mice were sensitized with 10 µg of ovalbumin (OVA) plus 4 mg of Al (OH)₃ on days 0, 2, 4, 6, 8, 10, and 12, and were challenged by inhalation with 0.9% saline on days 19, 22, 24, 27, 31, 33, 38, 41, and 45. Group B: Mice were sensitized with OVA and were then challenged with 5% OVA (w/v) in 0.9% saline on days 19, 22, 24, 27, 31, 33, 38, 41, and 45. Group C: Mice were sensitized, and then challenged with OVA as for group B. The mice were then treated intraperitoneally with 40 µg recombinant human TRX1 protein (rhTRX1) every 2 days from days 18–44. Group D: Mice were sensitized and then challenged with OVA as for group B. The mice were intraperitoneally treated with 40 µg rhTRX1 every 2 days from 35 to 45. *Mice were sacrificed for the analysis of bronchoalveolar lavage fluid, histopathological examination, and airway hyperresponsiveness testing at each time point.

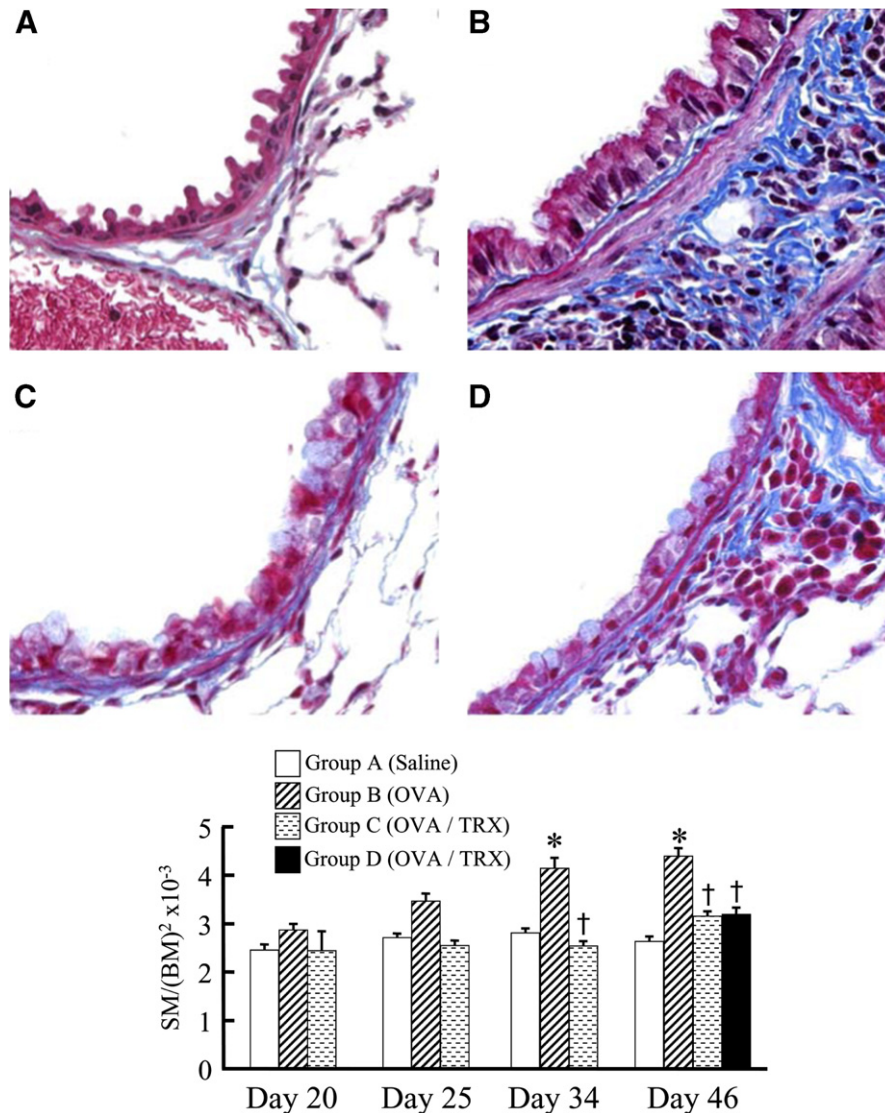


Fig. 2. Exogenous TRX1 prevents pulmonary inflammation and airway remodeling in a chronic antigen exposure model. Lung tissues were microscopically evaluated following Masson's trichrome staining. (a,b,c) Show the lung tissues obtained on day 34 from groups A, B, and C, respectively. (d) Shows the lung tissues obtained from group D on day 46. Original magnification at observation 400 $\times$. The thickening of the peribronchial basal membrane (BM: μm) and the area of smooth muscle (SM: μm^2) were measured by using a computerized color image analysis software system. * $P < 0.05$ versus Group A, † $P < 0.05$ versus Group B ($n = 6$ per group at each time point).

exposure could induce airway smooth muscle hyperplasia in mice. In addition, we performed quantitative analysis to estimate smooth muscle hyperplasia on days 20, 25, 34, 46, and 54 by using the formula $(\text{SM})/(\text{BM})^2$ as previously reported [15]. The value of $(\text{SM})/(\text{BM})^2$ in group B was significantly ($P < 0.05$) greater than that in group A on days 34 and 46 but not on day 20, 25 (Fig. 2), or 54 (data not shown). These results clearly indicate that airway remodeling accompanied by smooth muscle hyperplasia was established in our chronic antigen exposure mouse asthma model by day 34. Next, we performed 3 independent experiments ($n = 6$ per group) to see whether rhTRX1 treatment could inhibit airway smooth muscle hyperplasia in our mouse chronic OVA-exposure model. We previously reported that the half-life of rhTRX1 is 51.3 h in the mouse lung [10]. Thus, 40 μg of rhTRX1 dissolved in PBS

(100 μL) was administered intraperitoneally every other day into OVA-sensitized and -challenged mice (group C). Histological analysis showed that thickened smooth muscle layer and pulmonary inflammation in group C was inhibited on days 34. The value of $(\text{SM})/(\text{BM})^2$ in group C was significantly ($P < 0.05$) lower than that in group B on days 34 and 46 (Fig. 2).

TRX1 prevents pulmonary inflammation and overproduction of eotaxin, MIP-1 α and IL-13 in lungs of a chronic antigen exposure model

We analyzed BALFs in groups A and B on days 20, 25, 34, 46, and 54 ($n = 12$ per group at each time point). On days 20, 25, 34 and 46, but not day 54, the total number of cells and numbers of eosinophils in BALFs were signif-

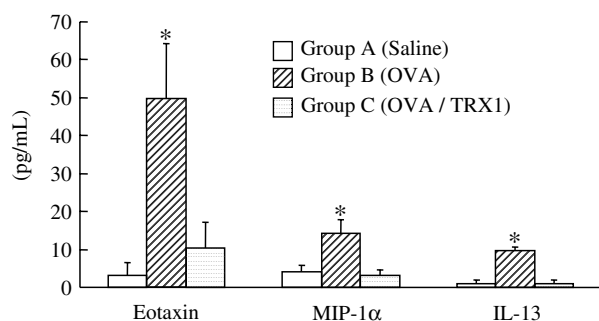


Fig. 3. Exogenous TRX1 decreases overproduction of MIP-1 α and IL-13 in lungs in a chronic antigen exposure model. BALFs were obtained from each group on day 34. Protein levels of eotaxin, MIP-1 α and IL-13 in BALFs were analyzed. * $P < 0.05$ versus group A or group C ($n = 6$ per group).

icantly ($P < 0.05$) greater in OVA-sensitized and -challenged mice (group B) than in OVA-sensitized and saline-challenged mice (group A). In contrast, the numbers of macrophages, lymphocytes, and neutrophils in BALFs were not significantly greater in group B on day 20, 25, 34, 46, or 54 than in group A. Total cell numbers and numbers of eosinophils in BALFs were significantly ($P < 0.05$) lower in group C than in group B on days 25 and 34, but not on day 46 (Fig. 2 of Supplementary Materials).

Next, we analyzed protein levels of eotaxin, MIP-1 α , IFN- γ , IL-5 and IL-13 in BALFs in groups A, B, and C on days 34 and 46 by ELISA ($n = 6$ per group at each time point). Protein levels of eotaxin, MIP-1 α and IL-13 in BALFs were significantly ($P < 0.05$) lower in group C than in group B on day 34 (Fig. 3). However, levels of eotaxin, MIP-1 α and IL-13 in BALFs were not significantly increased in group B when compared to group A or C on day 46. In addition, levels of IFN- γ and IL-5 in BALFs were not significantly increased in group B when compared to group A or C on days 34 or 46 in our mouse asthma model (data not shown).

Exogenous TRX1 prevents increase in AHR in a chronic antigen exposure model

Three independent experiments were performed (see Fig. 3 of Supplementary Materials for representative results; $n = 10$ per group). AHR to aerosolized ACh in group B was significantly ($P < 0.05$) higher on days 25, 34, and 46 than in group A. In contrast, hrTRX1 treatment significantly ($P < 0.05$) inhibited AHR in OVA-sensitized and -challenged mice (group C) on days 25, 34, and 46 when compared with group B. Notably, AHR was not significantly greater on days 20 and 54 in group B than in group A.

Endogenous TRX1 prevents airway remodeling and pulmonary inflammation in a chronic antigen exposure model

We examined whether endogenous TRX1 could prevent the smooth muscle hyperplasia and pulmonary inflamma-

tion in a mouse chronic OVA-exposure model. In this study, we established Balb/c background human TRX1 transgenic mice. Littermates were used in our all experiments. Masson's trichrome staining showed that thickened smooth muscle layer was observed in TRX1 transgene (Tg) negative (–) mice in group B. In contrast, airway smooth muscle hyperplasia and pulmonary inflammation was strongly inhibited in TRX1 Tg positive (+) mice in group B. We performed quantitative analysis to estimate smooth muscle hyperplasia as described above. The value of (SM)/(BM)² in group B was significantly ($P < 0.01$) greater than that in group A in control TRX1 Tg (–) littermates. However, in TRX1 Tg (+) mice, the value of (SM)/(BM)² was not significantly increased compared with group A (Fig. 4). In human TRX1 Tg (–) littermates, the total number of cells and numbers of eosinophils in BALFs were significantly ($P < 0.05$) greater in OVA-sensitized and -challenged mice (group B) than in OVA-sensitized and saline-challenged mice (group A) on day 34. In contrast, in TRX1 (+) mice, the total number of cells and numbers of eosinophils in BALFs were not significantly increased in group B when compared with group A (Fig. 4 of Supplementary Materials). The numbers of macrophages, lymphocytes, and neutrophils in BALFs were not significantly greater in group B than in group A in both TRX1 Tg (+) and Tg (–) littermates (data not shown). These results clearly indicate that smooth muscle hyperplasia and eosinophilic pulmonary inflammation was prevented in the OVA-sensitized and -challenged TRX1 Tg (+) mice.

Exogenous TRX1 improves established airway remodeling

We examined whether TRX1 treatment could improve established airway remodeling in a mouse chronic OVA-exposure model. We knew that airway remodeling was established by day 34. Therefore, we treated mice with 40 μ g of rhTRX1 from days 35 to 45 every 2nd day. Histological analysis revealed that airway smooth muscle hyperplasia and pulmonary inflammation was strongly inhibited in group D on day 46 (Fig. 2d; Fig. 1 of Supplementary Materials). Administration of TRX1 from days 35 to 45 also significantly ($P < 0.05$) inhibited AHR (Fig. 3 of Supplementary Materials) on day 46, when compared with the results in group B. In contrast, TRX1 administration from days 35 to 45 did not significantly decrease the total number of cells or the number of eosinophils in the BALF of OVA-sensitized and -challenged mice (Fig. 2 of Supplementary Materials). These results show that exogenous TRX1 administration after the establishment of airway remodeling can improve the airway remodeling in a mouse chronic OVA-exposure model. TRX1 treatment after the establishment of airway remodeling may inhibit AHR by improving airway remodeling but not pulmonary inflammation in our exposure model. However, validation of this hypothesis awaits further testing.

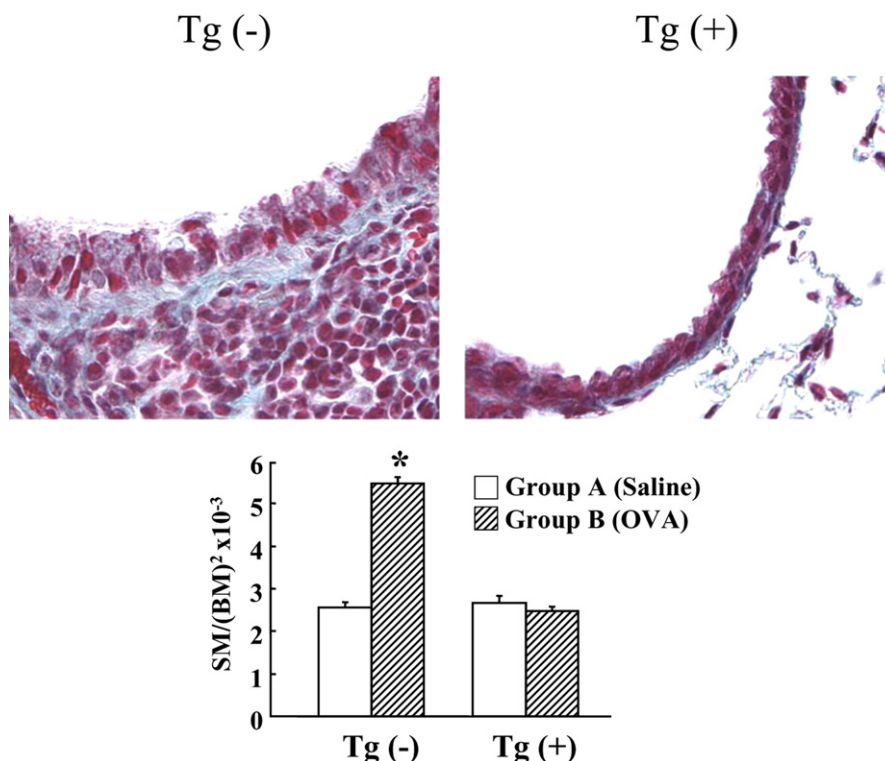


Fig. 4. Airway remodeling and pulmonary inflammation is prevented in a chronic antigen (OVA)-exposed human TRX1 transgenic mice. Lung tissues were obtained from Balb/c human TRX1 transgene (Tg) positive (+) or Tg negative (–) littermates in groups A and B on day 34, and were microscopically evaluated following Masson's trichrome staining. Original magnification at observation 400 $\times$. The degree of smooth muscle hyperplasia was estimated by the formula $(SM)/(BM)^2$ as described in the Methods section. * $P < 0.01$ versus group A ($n = 4$ per group).

We show that airway remodeling was observed in group B on days 34 and 46 but not on day 25 (Fig. 2, Fig. 1 of Supplementary Materials). In contrast, pulmonary inflammation, accompanied by infiltration of eosinophils into the lungs, was most severe on day 25 in OVA-sensitized and -challenged mice (group B). After that, by days 34 and 46, the pulmonary inflammation had gradually decreased (Fig. 2 of Supplementary Materials). AHR was significantly ($P < 0.05$) and strongly greater in group B on days 25, 34, and 46 than in group A (Fig. 3 of Supplementary Materials). These results suggest that airway remodeling, rather than pulmonary inflammation, was the main contributor to the increased AHR on days 34 and 46 in the chronic antigen exposure asthma model. Pulmonary inflammation—characterized especially by eosinophilia—but not airway remodeling may be the main contributor to the increased AHR by day 25 in our asthma model.

We reported previously that exogenous TRX1 modulates the expression of proinflammatory cytokines and chemokines such as IFN- γ , MIP-1 α , IL-1 α , IL-1 β , and IL-18 in an IL-2/IL-18-induced lung injury model [10] and a mouse asthma model [11]. In this study, we show that administration of recombinant human TRX1 protein during antigen challenge significantly ($P < 0.05$) inhibited overproduction of eotaxin, MIP-1 α and IL-13 in lungs in our chronic antigen exposure asthma model (Fig. 3). Previous studies have reported that TRX1 modulates transcription factors such as NF- κ B, AP-1, and p53 [8,11]. Therefore,

TRX1 may inhibit airway remodeling by inhibiting the recruitment of eosinophils into the lungs or by modulating Th2 cytokines such as IL-13, chemokines such as eotaxin, MIP-1 α , TGF- β 1, and/or RANTES. This hypothesis awaits further testing.

The treatment strategy for asthma consists mainly of the use of bronchodilators (such as β -agonists, theophylline, and anticholinergics), corticosteroids, and antileukotrienes. Previous studies have reported that dexamethasone (corticosteroid) treatment during the allergen challenge period inhibits airway remodeling and inflammation in the lungs of mouse [16,17] and rat [18] asthma models. However, dexamethasone treatment initiated after antigen challenge has limited [16] or no effects [18] in reversing the established airway remodeling. A previous study reported that anti-IL-5 antibody inhibited airway subepithelial fibrosis in a mouse asthma model [16]. Recently, established airway remodeling in a mouse asthma model was prevented by administration of the cysteinyl leukotriene (CysLT)1 receptor antagonist montelukast in a mouse asthma model [19]. However, current therapeutic approaches, such as the use of corticosteroids to reverse established airway remodeling, have had very limited success in patients with severe asthma [20] [21]. For these reasons, effective therapies that are targeted at severe asthma and can prevent and/or reverse airway remodeling (structural airway changes) are needed. Our study goal was to determine whether airway remodeling was prevented by exogenous TRX1 in asthma.

We previously showed that exogenous TRX1 inhibits AHR and pulmonary inflammation in mouse acute antigen exposure asthma model [11]. In this study, we demonstrated that exogenous TRX1 administration during the allergen challenge period inhibited airway remodeling and inflammation in the lungs of a chronic antigen exposure mouse asthma model. Moreover, treatment with rhTRX1, after the airway structural changes had become established, improved airway remodeling and thus inhibited AHR. These findings, along with those data in our previous report that the half-life of rhTRX1 (51.3 h) in the lung was much longer than in sera (8.5 h) [10], suggest that TRX1 has potential for use as a new therapy in severe asthmatics who are resistant to treatment with corticosteroids.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.bbrc.2007.06.019](https://doi.org/10.1016/j.bbrc.2007.06.019).

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