

Thioredoxin suppresses airway hyperresponsiveness and airway inflammation in asthma

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Abstract

Thioredoxin (TRX) is a 12-kDa redox (reduction/oxidation)-active protein that has a highly conserved site (-Cys-Gly-Pro-Cys-) and scavenges reactive oxygen species. Here we examined whether exogenously administered TRX modulated airway hyperresponsiveness (AHR) and airway inflammation in a mouse asthma model. Increased AHR to inhaled acetylcholine and airway inflammation accompanied by eosinophilia were observed in OVA-sensitized mice. Administration of wild-type but not 32S/35S mutant TRX strongly suppressed AHR and airway inflammation, and upregulated expression of mRNA of several cytokines (e.g., IL-1 α , IL-1 β , IL-1 receptor antagonist, and IL-18) in the lungs of OVA-sensitized mice. In contrast, TRX treatment at the time of OVA sensitization did not improve AHR or airway inflammation in OVA-sensitized mice. Thus, TRX inhibited the asthmatic response after sensitization, but did not prevent sensitization itself. TRX and redox-active protein may have clinical benefits in patients with asthma.

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Airways are unique in terms of their exposure to high levels of both environmental oxidants and endogenous extracellular antioxidants. Asthma is a chronic inflammatory disease of the lower airways, characterized clinically by reversible airway obstruction and airway hyperresponsiveness (AHR). The characteristic features of asthma are airway inflammation (including infiltration of inflammatory cells such as eosinophils and lymphocytes), epithelial damage, and airway remodeling [1,2]. Oxidative stress and oxidants have been implicated in the pathogenesis of asthma, and increased release of

reactive oxygen species (ROS) such as superoxide radicals (O_2^-) and hydrogen peroxide (H_2O_2) has been reported in exhaled condensates and from circulating monocytes and neutrophils, and from the bronchoalveolar lavage cells of patients with asthma [3,4]. Activated eosinophils in asthma secrete ROS and granular basic proteins that damage the bronchial epithelium and cause vasodilation and AHR [2,5].

Thioredoxin (TRX) is a 12-kDa ubiquitous protein with a redox (reduction/oxidation)-active dithiol/disulfide at a highly conserved active site (-Cys-Gly-Pro-Cys-) found in both prokaryotic and eukaryotic genomes. TRX was originally identified as an electron donor for ribonucleotide reductase in *Escherichia coli*.

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Human TRX was cloned as adult T-cell leukaemia-derived factor (ADF) produced by human lymphotropic virus type-I (HTLV-I)-transformed T cells [6]. TRX has been shown to catalyze a protein disulfide reduction in combination with thioredoxin reductase and nicotinamide adenine dinucleotide phosphate (NADPH), and is thought to be a strong scavenger of ROS [7–9]. Recent studies have shown that TRX is induced by a variety of stress conditions, including viral infection, ischemic insult, exposure to UV light, X-ray irradiation, and hydrogen peroxide (H_2O_2) exposure [9]. In addition, it has been reported that TRX itself has a protective effect against ROS-induced cellular damage [10–12]. We recently reported that TRX had a protective effect against lung injury induced by the proinflammatory cytokine interleukin (IL)-18 plus IL-2 [13] or bleomycin [14]. Our results suggest that exogenous TRX can be used for the treatment of lung diseases, including lung injury and asthma. The aim of our present study was to evaluate the possible therapeutic effect of TRX in preventing asthma.

Materials and methods

Mice and reagent. Juvenile female BALB/c mice, aged between 7 and 10 weeks were obtained from Charles River Japan (Yokohama, Japan). Recombinant human wild-type (WT) TRX was kindly supplied by Ajinomoto (Kawasaki, Japan). Human 32S/35S mutant TRX was generated as described in our previous report [15], in which two cysteines at positions 32 and 35 of the active site (-Cys-Gly-Pro-Cys-) were replaced with serines.

Protocol for sensitization and airway challenge in mice. Our previous study [14] revealed that the half-life ($t_{1/2}$) of the recombinant human TRX was 51.3 h in the lungs of mice and 8.5 h in the serum. On the basis of these results, we injected mice intraperitoneally with 40 μ g of WT or mutant TRX suspended in 100 μ L sterile PBS. The experimental protocol, as we previously reported [16], is outlined in Fig. 1. Balb/c mice were divided into six groups (Nos. 1–6). Group 1 mice were not treated (nontreat). Group 2 mice were treated with an intraperitoneal injection of 10 μ g 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 μ L on days 0 and 5. Group 3 mice were sensitized with OVA as described for group 2, and on day 18 they were challenged for 20 min with 5% OVA in 0.9% saline, given via the airways by ultrasonic nebulizer. Groups 4 and 5 were sensitized with OVA as described for

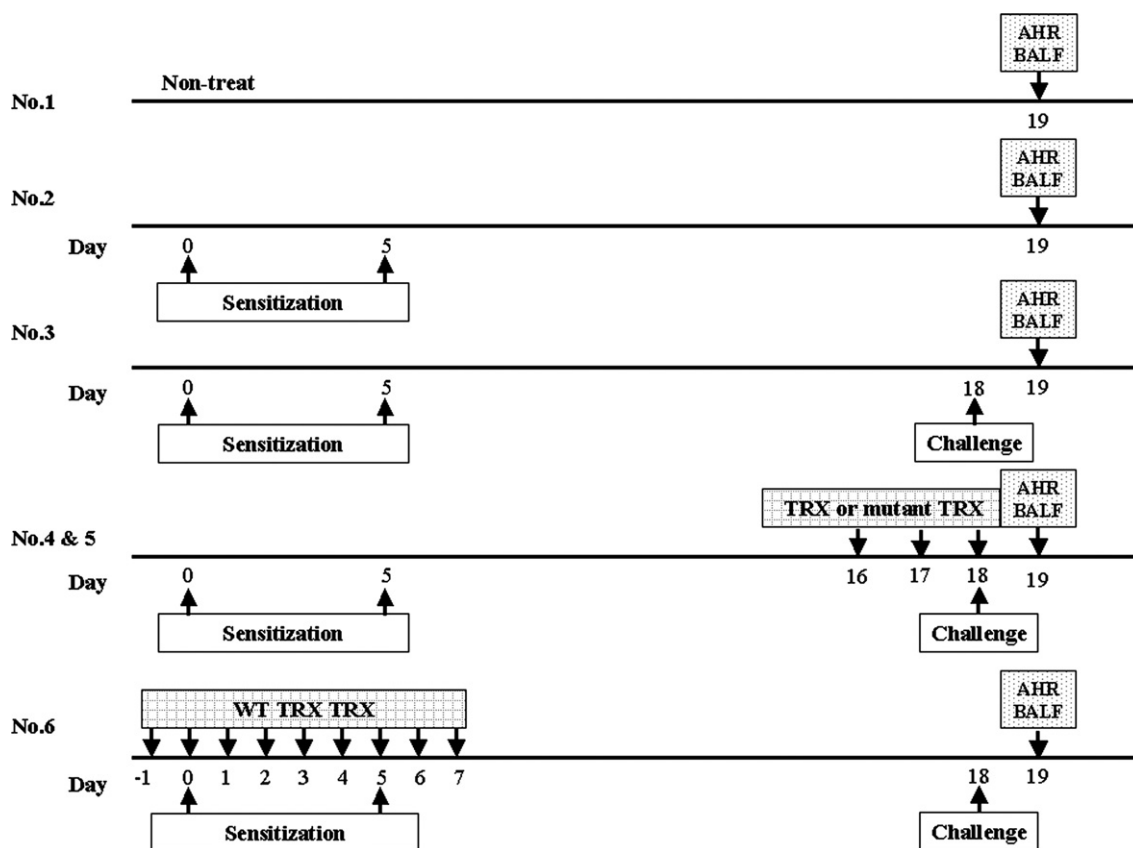


Fig. 1. Schematic summary of experimental protocols used in the study. Group 1: untreated juvenile female Balb/c mice; group 2: ovalbumin (OVA)-sensitized mice intraperitoneally injected with 10 μ g of OVA plus 4 mg of Al (OH)₃ on days 0 and 5; group 3: OVA-sensitized on days 0 and 5 and OVA-challenged on day 18 with 5% OVA (w/v) in 0.9% saline for 20 min; group 4: OVA-sensitized and -challenged mice intraperitoneally treated with 40 μ g of recombinant wild-type (WT) TRX on days 16, 17 and 18; group 5: OVA-sensitized and -challenged mice intraperitoneally treated with 40 μ g of 32 S/35 S mutant TRX on days 16, 17, and 18; group 6: OVA-sensitized and -challenged mice treated daily with 40 μ g of recombinant WT-TRX on days -1 to 7. On day 19, the airway hyperresponsiveness (AHR) and bronchoalveolar lavage fluid (BALF) were analyzed in all mice.

group 2. On days 16, 17, and 18, mice were treated daily with an intraperitoneal injection of 40 μ g WT (group 4) or mutant (group 5) TRX suspended in 100 μ L sterile PBS. On day 18, 6 h after the last dose of WT or mutant TRX, mice were challenged with OVA as described for group 3. Group 6 mice were treated daily with an intraperitoneal injection of 40 μ g WT TRX from days –1 to 7. These mice were sensitized with OVA as described above, and on day 18 they were challenged for 20 min via the airways with 5% OVA in 0.9% saline. On day 19 (24 h after the OVA aerosol), airway hyperresponsiveness (AHR) and bronchoalveolar lavage fluid (BALF) were analyzed. All procedures were approved by the Ethics Committee for Animal Experiments, Kurume University (Approval Nos. 1144–1148, 2004). Animal care was provided in accordance with the procedures outlined in “Principles of Laboratory Animal Care” (National Institutes of Health Publication No. 86–23, revised 1985).

Assessment of airway hyperresponsiveness. Pulmonary mechanics were determined noninvasively in conscious, freely moving, spontaneously breathing mice by whole body plethysmography (Model Bio-System XA, Buxco Electronics, Troy, NY) before and after inhalation of acetylcholine chloride (ACh, catalog no. A-6625, Sigma–Aldrich Chemical) in 0.9% saline. The degree of bronchoconstriction was expressed as enhanced pause (Penh), as previously reported [17]. The dose of acetylcholine (mg/mL) required to cause a 2-U increase in Penh above the baseline value (EC_2 Penh) was determined by log-linear interpolation between the two doses bounding the point at which a 2-U increase occurred.

Analysis of bronchoalveolar lavage fluid. The trachea was inserted with a tubing adaptor, and the lungs were washed three times with 3 mL PBS. Aliquots of cells were centrifuged onto glass slides, dried in air, and stained with Wright-Giemsa. Cell populations were then calculated.

Histological analysis. For the histological analysis, mice were euthanized by intraperitoneal injection of pentobarbital sodium (2.5–5 mg per mouse). After the thorax had been opened, the trachea was dissected free from the underlying soft tissues, and a 0.8-mm tube was inserted through a small incision in the trachea. The lung tissues were immediately fixed by intratracheal instillation of 20% buffered formalin (pH 7.40) for 15–20 min at a constant pressure of 25 cm H_2O . After gross examination, the extracted tissues were placed into 20% buffered formalin and further fixed for at least 24 h, as previously reported [13,14]. The tissues were embedded in paraffin, cut into 4- μ m-thick sections, and then stained such as with hematoxylin and eosin (HE). Semi-quantitative assessment of eosinophilia was performed as previously reported [18]. Briefly, each HE section was viewed under low-power magnification (40 $\times$). One square field (2.5 mm $\times$ 3.5 mm; 8.75 mm²) where eosinophilia appeared to be most marked, designated as the observation field (OF), was selected from each HE section at 40 $\times$ magnification. Five random high-power fields (hpfs, 0.25 mm $\times$ 0.35 mm; 0.0875 mm²) were randomly selected at 400 $\times$ magnification within the OF. The total numbers of eosinophils in the alveolar wall, airway, and general interstitium were then hand-counted in these hpfs.

Cytokine and chemokine assays. Total RNA was isolated from the lungs with an RNeasy Midi Kit (Qiagen, Valencia, CA). mRNA expression was analyzed by RNase protection assay (RPA) using a RiboQuant kit (PharMingen, San Diego, CA) and a ³³P-UTP-labeled riboprobe set. mRNA levels were quantitated by a densitometer (Typhoon 8600, Amersham Biosciences, Piscataway, NJ). Mouse GAPDH was used as the control for quantitation in the RPAs as previously described [19].

Measurement of serum mouse OVA-specific IgE levels. Serum OVA-specific IgE concentrations were measured by a modified ELISA procedure that we previously described [20]. Briefly, ELISA plates were coated with 50 μ L of 2 μ g/mL of two different anti-IgE mAbs (clones R35-72 [PharMingen] and LO-ME-3 [Biosource, Camarillo, CA]) in PBS, washed, and blocked with PBS containing 1% bovine serum albumin. Diluted (1:10) serum samples were added and incubated overnight at 4 $^{\circ}$ C. Plates were washed and incubated with 3 μ g of

biotin-labeled OVA in PBS, washed, incubated with streptavidin-horseradish peroxidase conjugate, washed, and developed with ELISA POD substrate TMB kit (Nacalai tesque, Kyoto, Japan). Absorbance was read at 450 nm.

Statistical analyses. Results are expressed as means $\pm$ standard error of the mean (SEM). ANOVA was used to compare differences between groups. $p < 0.05$ was considered to represent statistical significance.

Results

TRX prevents OVA-induced airway hyperresponsiveness

We first analyzed whether exogenous TRX administration could prevent AHR in OVA-sensitized Balb/c mice. Four independent experiments were performed ($n = 10$ per group). The schematic protocol is shown in Fig. 1 and the representative results are shown in Fig. 2. In OVA-sensitized and -challenged mice (group 3 in Fig. 1), airway responsiveness to aerosolized ACh was significantly increased when compared with untreated mice (group 1) or OVA-sensitized mice without challenge (group 2). WT-TRX treatment before OVA challenge (but after OVA sensitization) (group 4) significantly decreased the AHR to aerosolized ACh in OVA-sensitized and -challenged mice (Fig. 2A). In contrast, mutant TRX-treatment before challenge but after OVA sensitization (group 5) did not significantly alter the AHR to aerosolized ACh in OVA-sensitized and -challenged mice (Fig. 2B). Next, we investigated whether WT-TRX treatment at the time of OVA sensitization could modulate the AHR in OVA-sensitized and -challenged mice. TRX-treatment did not significantly suppress the AHR to the aerosolized ACh in group 6 (Fig. 2C).

TRX prevents OVA-induced airway inflammation

Persistent airway inflammation (especially eosinophilic inflammation and mononuclear cells) and AHR have been observed in patients with asthma as well as in asthma animal models [1,5]. As described above, WT, but not mutant, TRX treatment before OVA challenge prevented AHR to the aerosolized ACh in OVA-sensitized mice. We examined whether WT-TRX treatment modulated airway inflammation in OVA-sensitized mice. Three independent BALF analyses were performed ($n = 5$ per group), and the representative results are shown in Fig. 3A. Total cell numbers and numbers of eosinophils and neutrophils were significantly increased in the BALF of OVA-sensitized and -challenged mice (group 3), when compared with untreated mice (group 1) and OVA-sensitized mice without challenge (group 2). Administration of exogenous WT-TRX before OVA challenge significantly reduced the total cell number and the numbers of eosin-

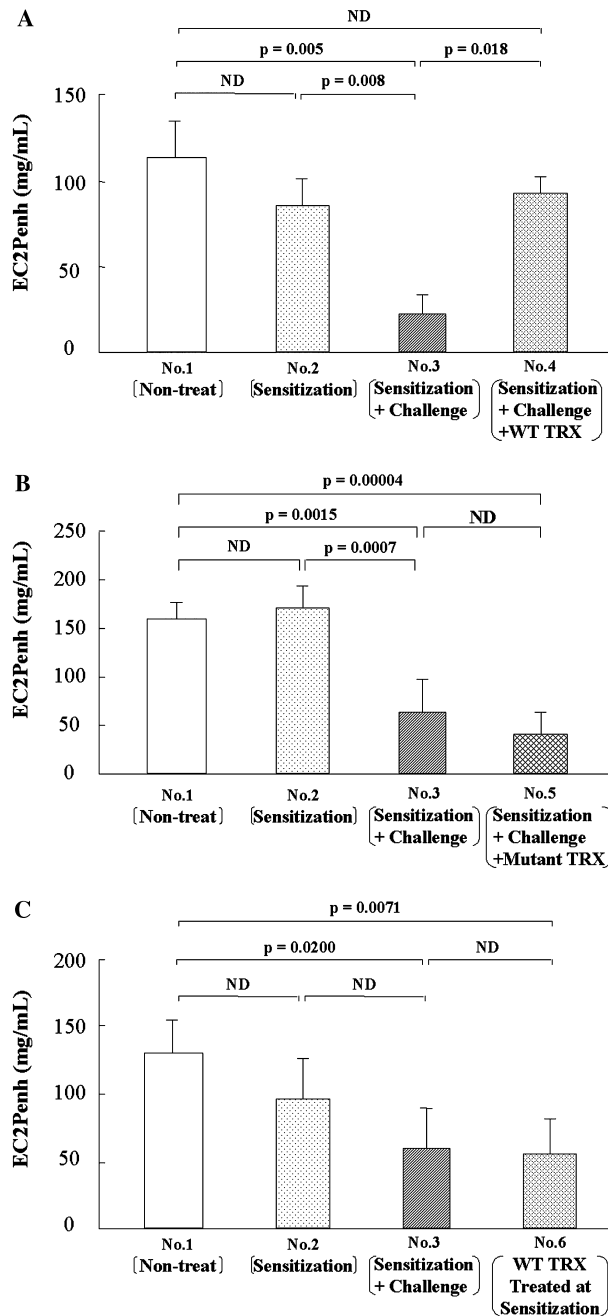


Fig. 2. Wild-type TRX decreased airway responsiveness to aerosolized acetylcholine after OVA challenge. The degree of bronchoconstriction was expressed as enhanced pause (Penh) using noninvasive barometric whole body plethysmography (Model BioSystem XA, Buxco Electronics, Troy, NY), as described under Materials and methods. EC₂Penh (mg/mL), the dose of acetylcholine (ACh) required to cause a 2-U increase in Penh above the baseline value, was determined by log-linear interpolation between the two doses bounding the point at which a 2-U increase occurred. (A) Wild-type (WT) TRX treatment before OVA challenge significantly suppressed AHR to ACh in OVA-sensitized and -challenged mice. (B) Treatment with mutant TRX before challenge did not suppress sensitivity to ACh in OVA-sensitized and -challenged mice. (C) WT-TRX treatment at the time of OVA sensitization did not suppress sensitivity to ACh in OVA-sensitized and -challenged mice.

ophils and neutrophils in the BALF of OVA-sensitized and -challenged mice (group 4). Administration of WT-TRX did not reduce the number of alveolar macrophages and lymphocytes in the BALF of OVA-sensitized and -challenged mice. In contrast, mutant TRX did not suppress total cell numbers or accumulation of eosinophils and neutrophils (group 5). Under light microscopy, infiltration of eosinophils and mononuclear cells was induced in the pulmonary interstitium of OVA-sensitized and -challenged mice (Fig. 3B, panels ii and vi), but not of the untreated (panels i and v) or OVA-sensitized-only mice (data not shown). Administration of WT-TRX significantly decreased the inflammatory infiltration in the interstitium (panels iii and vii), but mutant TRX treatment did not significantly affect the numbers of inflammatory cells of the lung interstitium (panels iv and viii). Semi-quantitative analysis revealed that administration of WT, but not mutant, TRX significantly decreased the numbers of eosinophils in the lung interstitium of OVA-sensitized and -challenged mice (Fig. 3C). It is of note that WT-TRX could not completely suppress airway inflammation and eosinophil accumulation in OVA-sensitized and -challenged mice. Moreover, WT-TRX treatment at the time of sensitization with OVA did not improve airway inflammation in OVA-sensitized and -challenged mice (group 6, data not shown).

Cytokine and chemokine expression in lungs

RPA revealed that mRNAs corresponding to IL-1 α , IL-1 β , IL-1 receptor antagonist (IL-1Ra), and IL-18 were constitutively but weakly expressed in the lungs of untreated mice (Fig. 4, lanes 1 and 2). Quantitation of the RPA revealed IL-1 α , IL-1 β , IL-1Ra, and IL-18 mRNA levels in the lungs of TRX-treated and OVA-sensitized and -challenged mice (Fig. 4, lines 5 and 6) were 2.2-, 2.0-, 1.6-, and 1.6-fold higher than those of OVA-sensitized and -challenged mice (Fig. 4, lanes 3 and 4) when normalized with GAPDH, respectively. WT-TRX treatment before OVA challenge increased the levels of IL-1 α , IL-1 β , IL-1Ra, and IL-18 in the lungs of OVA-sensitized and -challenged mice when compared with those of OVA-sensitized and -challenged mice.

Serum OVA-specific IgE levels in mice

Serum OVA-specific IgE levels (optical density at 450 nm) in untreated (group 1), OVA-sensitized and -challenged (group 3), WT-TRX treatment before challenge/OVA-sensitized and -challenged (group 4), and WT-TRX treatment at sensitization/OVA-sensitized and -challenged (group 6) mice were 0.10 ± 0.01 , 0.23 ± 0.06 ($p < 0.05$ vs. group 1), 0.16 ± 0.02 ($p < 0.05$ vs. group 1), and 0.15 ± 0.03 ($p < 0.05$ vs. group 1).

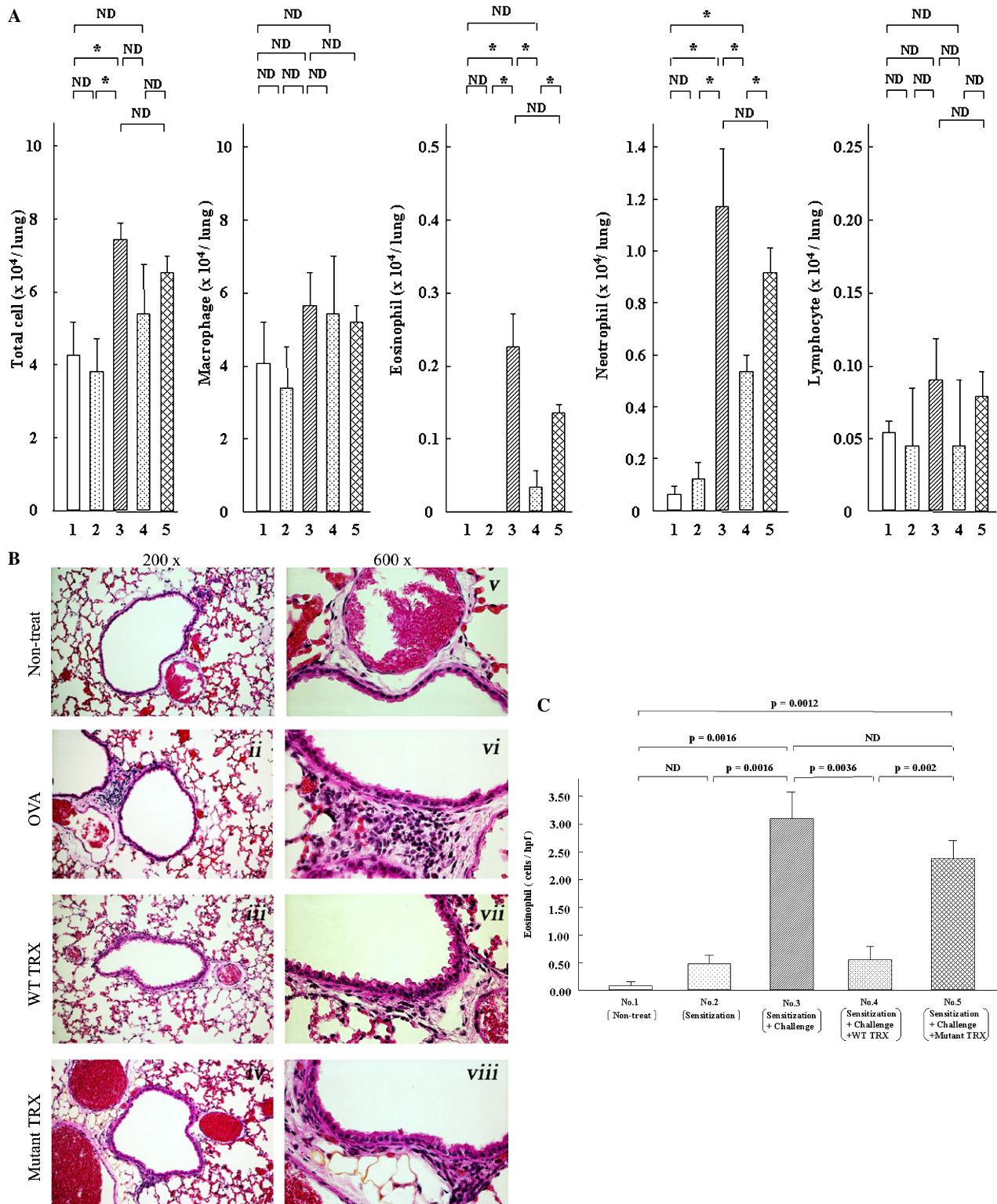


Fig. 3. Wild-type TRX prevented airway inflammation in OVA-sensitized and -challenged mice. (A) Cells in BALF were centrifuged onto glass slides, dried in air, and stained with Wright–Giemsa. Cell populations were calculated as described under Materials and methods. (B) Histological evidence of airway eosinophilia in OVA-sensitized mice. Lung tissues were obtained from untreated mice (group 1, panels i and v), OVA-sensitized and -challenged mice (group 3, panels ii and vi), WT-TRX-treated OVA-sensitized and -challenged mice (group 4, panels iii and vii), and mutant TRX-treated OVA-sensitized and -challenged mice (group 5, panels iv and viii) [original magnification at observation 200 $\times$ and 600 $\times$, HE staining]. (C) Total numbers of eosinophils in the alveolar wall and general interstitium were hand-counted in five random high-power fields (hpf) (observation at 400 $\times$) of the lung HE section of each mouse, as described under Materials and methods. Results are expressed as the mean number of cells $\pm$ SEM/hpf for five mice per group.

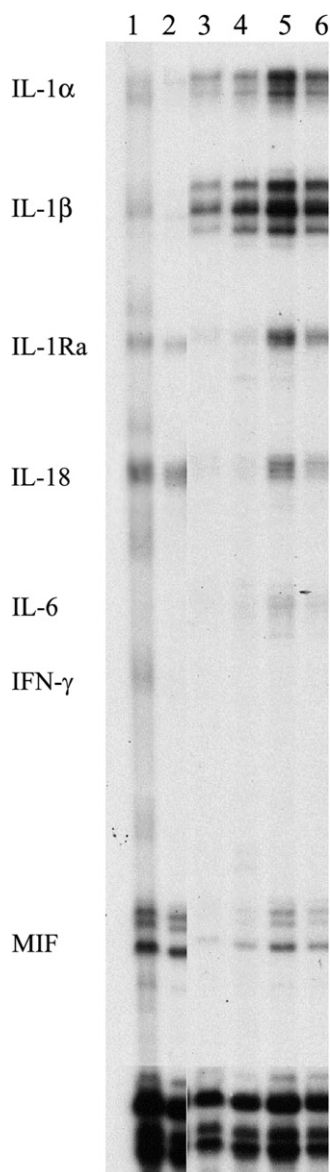


Fig. 4. Wild-type TRX-modulated pro-inflammatory cytokine induction before OVA challenge. Juvenile female Balb/c mice were sensitized with OVA on days 0 and 5. On days 16, 17, and 18, mice were treated daily with 40 μ g WT-TRX. On day 18, 6 h after the last dose of WT-TRX, mice were challenged with OVA as described under Materials and methods. Six hours after OVA challenge the mice were sacrificed, and the lung tissue was immediately harvested. Total RNA (1 μ g) samples, obtained from the individual mice, were used for mRNA analysis using a multi-probe RNase protection assay. Lanes 1 and 2, untreated mice (group 1); lanes 3 and 4, OVA-sensitized and -challenged mice (group 3); and lanes 5 and 6, WT-TRX-treated OVA-sensitized and -challenged mice (group 4).

group 1), respectively. Serum OVA-specific IgE levels in groups 3, 4, and 6 were not significantly different.

Discussion

The histopathological picture of bronchial asthma is one of chronic airway inflammation caused by immune

cells such as eosinophils and lymphocytes. At the site of inflammation, eosinophils release cytotoxic products including eosinophil granule proteins and ROS [1,2,5,21]. In addition, of the various organs in the body, the lung is the richest in oxygen and is always subject to high levels of harmful ROS [3,4]. Our previous studies using an ozone-exposure model showed that exogenous ROS-induced airway epithelium injury causes AHR [22,23] and increased permeability of the blood vessels [24,25]. Thus, oxidative stress, including that caused by both exogenous and endogenous ROS, may play an important role in the pathogenesis of asthma. A previous study reported that the levels of serum TRX in patients with asthma attacks were increased in comparison with those in remission [26], suggesting that endogenous TRX may have a protective effect against oxidative stress in asthma. Moreover, a protective effect of TRX has been reported in lung damage induced by ischemia–reperfusion injury, influenza infection, the anticancer drug bleomycin, or IL-2 plus IL-18 [9,10,14]. Therefore, we examined the effect of exogenous TRX on AHR and airway inflammation in a mouse asthma model. We found that administration of exogenous WT-TRX but not 32S/35S mutant TRX strongly suppressed AHR to inhaled ACh and airway inflammation accompanied by eosinophilia and neutrophils in OVA-sensitized and -challenged mice. This protective effect of TRX can be dependent on the antioxidant activity and the redox-active site (-Cys-Gly-Pro-Cys-), because 32S/35S mutant TRX does not have reducing activity, as our previous study reported [15].

In the antigen-induced airway inflammation of asthma, Th2 cytokines (e.g., IL-4, IL-5, and IL-13) and Th2-like cells have been shown to play crucial roles in the development of IgE production, AHR, and eosinophilic airway infiltration [1,5]. It has been reported that Th1 cell and Th1-type cytokines such as IL-1 β , IL-12, and IFN- γ prevent Th2 development. IL-18, a member of the IL-1 family, also induces IL-12-driven Th1 development, and prevents Th2 development in the presence of IL-12 [27,28]. Here, we showed that administration of exogenous TRX increased the levels of members of the IL-1 family—IL-1 α , IL-1 β , IL-1Ra, and IL-18—in the lungs of OVA-sensitized and -challenged mice. These results suggest that exogenous TRX can prevent Th2 development by up-regulating the expression of Th1-like cytokines, leading to a decrease in AHR and airway inflammation. Besides having a crucial function as an antioxidant and a catalyst, TRX plays important roles in intracellular signal transduction. TRX modulates transcription factors such as nuclear factor (NF)- κ B and apoptosis signal-regulating kinase (ASK)-1 [9,15]. Therefore, it is also possible that TRX modulates the intracellular signal transduction of Th2 cytokines (e.g., IL-4, IL-5, and IL-13) in OVA-sensitized and -challenged mice. However, TRX-treatment did not decrease

OVA-specific IgE production, although TRX increased the levels of IL-1 family cytokines in the lungs of OVA-sensitized and -challenged mice. Further analysis is needed to clarify this issue.

Here we showed that administration of WT-TRX before OVA-challenging strongly suppressed AHR to inhaled ACh and airway inflammation in OVA-sensitized mice. In contrast, treatment of WT-TRX at the time of OVA sensitization did not improve AHR and airway inflammation in OVA-sensitized and -challenged mice. Nor did it significantly prevent OVA-specific IgE production. Our results suggest that administration of exogenous TRX at the time of sensitization to an allergen cannot prevent allergen-specific Th2 cell development and IgE production.

Most patients with asthma respond to standard treatment with a short-acting inhaled β_2 -agonist for symptom control and long-term use of an inhaled corticosteroid. However, approximately 5% of patients do not respond to this regime and require further investigation to establish the reasons for their lack of response [29,30]. As described above, eosinophilic inflammation is the main pathogenesis in asthma. It is thought to be important to suppress eosinophilic inflammation during treatment of the disease. IL-5 blockade was expected to deplete eosinophils and improve symptoms in patients with asthma. However, a humanized mAb against IL-5 (mepolizumab), which effectively depletes eosinophils from the blood and from induced sputum in mild atopic subjects with asthma, has no effect on AHR or on the late asthmatic reaction to inhaled allergen challenge [5]. In addition, a highly specific monoclonal antibody (mAb), which binds to circulating IgE and prevents it from making contact with its receptors on effector cells, has been developed for the treatment of asthma and other allergic disorders. New anti-inflammatory therapies are under investigation for the treatment of severe asthma and steroid therapy-resistant asthma [31,32]. In our study, exogenous TRX improved AHR and prevented eosinophilic airway inflammation in an allergic mouse model. In addition, the half-life ($t_{1/2}$) of recombinant human TRX is relatively long (51.3 h in the lungs of mice and 8.5 h in the serum) [14]. Therefore, the application of recombinant protein, gene therapy, and induction of TRX may be a new therapeutic modality for asthma, especially severe asthma and steroid therapy-resistant asthma.

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