

## **TITLE**

**Category:** Original Articles

**Title:** Redox-active protein thioredoxin-1 administration ameliorates influenza A virus (H1N1)-induced acute lung injury in mice

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**Key Words:** acute lung injury; cytokine; influenza virus; mouse; oxidative stress; thioredoxin-1

## ABSTRACT

**Objectives:** Influenza virus infections can cause severe acute lung injury leading to significant morbidity and mortality. Thioredoxin-1 is a redox-active defensive protein induced in response to stress conditions. Animal experiments have revealed that thioredoxin-1 has protective effects against various severe disorders. This study was undertaken to evaluate the protective effects of recombinant human thioredoxin-1 (rhTRX-1) administration on influenza A virus (H1N1)-induced acute lung injury in mice.

**Design:** Prospective animal trial.

**Setting:** Research laboratory.

**Subjects:** Nine-week-old male C57BL/6 mice inoculated with H1N1.

**Intervention:** The mice were divided into vehicle-treated group and rhTRX-1-treated group. For survival rate analysis, the vehicle or rhTRX-1 was administered intraperitoneally every second day from Day -1 to Day 13. For lung lavage and pathological analyses, vehicle or rhTRX-1 was administered intraperitoneally on Day -1, 1, and 3.

**Measurements and Main Results:** Lung lavage and pathological analyses were performed at 24, 72, and 120 hr after inoculation. The rhTRX-1 treatment significantly improved the survival rate of H1N1-inoculated mice, although the treatment did not affect virus propagation in the lung. The treatment significantly attenuated the histological changes and neutrophil infiltration in the lung of H1N1-inoculated mice. The treatment significantly attenuated the production of TNF- $\alpha$  and CXCL1 in the lung and oxidative stress enhancement which were observed in H1N1-inoculated mice. H1N1 induced expressions of TNF- $\alpha$  and CXCL1 in murine lung epithelial cells MLE-12, which were inhibited by the addition of rhTRX-1. The rhTRX-1 treatment started 30 min after H1N1 inoculation also significantly improved the survival of the mice.

**Conclusions:** Exogenous administration of rhTRX-1 significantly improved the survival rate and attenuated lung histological changes in the murine model of influenza pneumonia. The protective mechanism of TRX-1 might be explained by its potent antioxidative and anti-inflammatory actions. Consequently, rhTRX-1 might be a possible pharmacological strategy for severe influenza virus infection in humans.

Influenza virus infections cause a broad array of illnesses that are responsible for significant morbidity and mortality both in children and adults on a yearly basis (1). Influenza can cause periodic global pandemics with even higher penetrance of illness. Highly pathogenic avian influenza virus H5N1 emerged in 1996 in Hong Kong, China (2). Although cases of avian influenza infections have decreased since 2006, the emergence of a pandemic strain remains a threat. In 2009, novel swine-origin influenza virus H1N1 was identified in Mexico. It continues to spread globally (3).

Several antiviral compounds have been developed against influenza virus to interfere with specific events in the replication cycle. Under treatment with these drugs, however, influenza virus infection occasionally causes severe pneumonia, necessitating intensive care and mechanical ventilation in the clinical settings. The course of illness and complications might also be affected by coexisting pathologies. The discovery of a novel anti-influenza therapeutic approach would increase the effectiveness of traditional virus-based strategies (4).

Reactive oxygen species (ROS) such as superoxide ( $O_2^-$ ), hydroxyl radical (OH), and nitric oxide (NO) represent an important component of the host's arsenal to combat invading microorganisms, but ROS present significant immunopathology to surrounding tissues because of their toxicity and lack of specificity (5, 6). It is recognized that much of the oxidative injury associated with simultaneous production of  $O_2^-$  and NO is mediated by a strong oxidant: peroxynitrite ( $ONOO^-$ ) (7). The pathogenesis of influenza pneumonia is almost certain to involve not only apoptotic cell death mediated through viral replication in the infected cells, but also the injury of not-infected cells by ROS derived from infiltrating neutrophils and macrophages and respiratory tract epithelium (4). A critical role of ROS as mediators of influenza virus-induced lung injury is supported by previous studies. In murine models of influenza pneumonia, excessive generation of ROS contributed to lung injury in infected animals; treatment with superoxide dismutase, catalase (antioxidative enzymes), N-monomethyl-L-arginine (NO synthase inhibitor) or allopurinol (xanthine oxidase inhibitor) and overexpression of extracellular superoxide dismutase or heme oxygenase-1 suppressed lung injury and inflammation and improved the survival rate (8-13). Moreover, virus-infected selenium-deficient mice developed more severe influenza pneumonia than did selenium-adequate mice, implying the importance of selenium-dependent glutathione peroxidase and thioredoxin reductase (antioxidative enzymes) for protection against influenza virus-induced inflammatory processes (14).

Thioredoxin-1 (TRX-1), a redox-active small protein that is ubiquitous in the body, is a defensive protein

that is induced in response to various stress conditions (15). In addition to its antioxidative effect by dithiol-disulfide exchange in its active site, TRX-1 has anti-inflammatory and antiapoptotic effects (15, 16). Human TRX-1-overexpressing transgenic mice survive longer and are more resistant to various oxidative and inflammatory conditions than control mice are (15, 17). Of greater importance is the fact that TRX-1 overexpression is also effective in augmenting the host defense against influenza pneumonia, thereby reducing mortality (18). Overexpression of TRX-1 might modulate the ROS generation induced by influenza virus infection and regulate the redox-dependent signal transductions in the host defense responses against influenza virus infection.

These findings prompted us to evaluate the protective effects of recombinant human thioredoxin-1 (rhTRX-1) administration on acute lung injury in mice with influenza pneumonia. If rhTRX-1 administration can produce significant beneficial effects, then its clinical efficacy for treating severe pneumonia will be highly anticipated. This study demonstrated for the first time that rhTRX-1 administration decreases the mortality rate and ameliorates acute lung injury in influenza A virus (H1N1)-infected mice, possibly through its antioxidative and anti-inflammatory actions.

## **MATERIALS AND METHODS**

This study was approved by the Animal Use Committee of Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences and was conducted in accordance with National Institutes of Health Guidelines.

### **Experimental Animals**

Eight-week-old male C57BL/6 mice (21-24 g body weight) were purchased from Charles River Laboratories Japan Inc. (Yokohama, Japan). They were housed in the specific-pathogen-free animal facility at 25°C with a 12-hour light/dark cycle. They were fed a standard diet (Oriental MF; Oriental Yeast Co., Ltd., Tokyo, Japan).

### **Regional Distribution of TRX-1 mRNA**

Nine-week-old mice were anesthetized by intraperitoneal injection of ketamine (50 mg/kg) and pentobarbital (30 mg/kg). The brain, lung, liver, kidney, and spleen were removed and soaked in RNAlater (Applied Biosystems, Foster City, CA). Total RNA was extracted using RNeasy Plus Mini (Qiagen Inc., Hilden, Germany). Two micrograms of total RNA was reverse-transcribed to cDNA using RETROscript

(Applied Biosystems). Reverse-transcribed samples were analyzed for murine TRX-1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-specific cDNA by PCR amplification using the 7500 Real-Time PCR System (Applied Biosystems). The PCR primers were designed according to the protocol described by Sigma-Aldrich Japan (Tokyo, Japan) (Table 1).

### **Production of Influenza Virus Pneumonia**

Influenza virus A/Puerto Rico/8/34 (H1N1) was used throughout the experiments. The virus was propagated in 10-day-old embryonated chicken eggs. The virus titer was quantitated by plaque assay using Madin-Darby canine kidney cells. Nine-week-old mice were anesthetized by intraperitoneal injection of ketamine and pentobarbital, as described above. Then they were inoculated intranasally with H1N1 suspended in 25- $\mu$ l sterile phosphate-buffered saline (PBS). The doses of influenza virus were 300 plaque-forming units for survival rate analysis and 1000 plaque-forming units for lung lavage and pathological analyses. The animals were allowed to recover thereafter. The day of virus inoculation was defined as Day 0.

### **Administration of rhTRX-1**

Recombinant human thioredoxin-1 was supplied by Redox Bio Science Co., Ltd. (Kyoto, Japan). The quality and purity of the rhTRX-1 used for the present experiments were approved by Pharmaceuticals and Medical Devices Agency in Japan, which is equivalent to the FDA in the United States. Its safety and kinetics were established in preclinical studies using animal models (15).

In preliminary experiments, 9-week-old mice were administered rhTRX-1 intraperitoneally (40  $\mu$ g in 100- $\mu$ l PBS) and at 0 hr (untreated), 1, 3, or 6 hr after administration (3-6 mice at each time point) they were anesthetized and blood and the left lung were sampled for measurement of rhTRX-1 using a sandwich ELISA kit (Redox Bio Science Co., Ltd.) (15, 19). The ELISA did not cross-react with murine TRX-1. The lung tissues were homogenized in 1 ml of lysis buffer. The concentrations in the sera and the lung homogenate supernatants at 0, 1, 3, and 6 hr after the rhTRX-1 administration were, respectively, <0.5, 1237 $\pm$ 298, 97 $\pm$ 23, and 18 $\pm$ 3 ng/ml (sera) and <0.5, 62 $\pm$ 21, 20 $\pm$ 10, and 20 $\pm$ 9 ng/ml (lung tissues). In accordance with our previous work (19), a bolus intraperitoneal injection of 40  $\mu$ g of rhTRX-1 led to systemic delivery and lung deposition in mice.

### **Murine TRX-1 mRNA Expression in the Lung after rhTRX-1 Administration**

A group of 9-week-old mice were administered rhTRX-1 intraperitoneally (40 µg in 100-µl PBS). At 0 hr (untreated), 1, 3, or 6 hr after rhTRX-1 administration, the mice were anesthetized and the left lung was sampled for analysis of murine TRX-1 and GAPDH mRNA expression as described above (3-5 mice at each time point).

### **Treatment with rhTRX-1 for H1N1-Inoculated Mice**

The C57BL/6 mice were divided randomly into a vehicle-treated group (control group) and an rhTRX-1-treated group (treatment group).

Survival rate analysis: The vehicle (100-µl PBS) or rhTRX-1 (40 µg in 100-µl PBS) was administered intraperitoneally every second day from Day -1 (the day before H1N1 inoculation) to Day 13 (10 mice per group). Survival was observed until Day 14. No other parameter was measured in these mice.

Lung lavage and pathological analyses: The vehicle (100-µl PBS) or rhTRX-1 (40 µg in 100-µl PBS) was administered intraperitoneally on Day -1, 1, and 3. Lung lavage and pathological analyses were performed on Day 1 (24 hr), 3 (72 hr), and 5 (120 hr) after H1N1 inoculation (5 mice per group at each time point).

### **Lung Lavage Analysis**

The mice were anesthetized by intraperitoneal injection of ketamine and pentobarbital as described above. Blood was sampled for measurement of hydroperoxides (described below). Then the left lung hilus was ligated and the right lung was lavaged twice with 500-µl cold Hanks' balanced salt solution through a 20-gauge cannula. The recovered lavage was collected and centrifuged at 2000 rpm for 10 min at 4°C and the supernatant was stored at -80°C for measurement of cytokines (described below). The total cell number in the lavage fluid was calculated from the cell number in the 200-µl sediment. Cell differentiation was examined using Diff-Quick staining (Dade Behring Inc., Newark) for at least 200 cells on a smear prepared from the sediment. The percentage of neutrophils was determined. The total neutrophil number in the lavage fluid was calculated and expressed per animal.

### **Lung Pathological Analysis**

The upper portion of the left lung was fixed in buffered 4% paraformaldehyde solution and then embedded in paraffin. Sections (4-µm slices) including a cut at the hilus were stained with hematoxylin and eosin for light microscopy. The histological examination procedure was similar to that described by Xu et al.

(20). Four readily identifiable pathological processes were graded semiquantitatively on a scale of 0 to 4: alveolar and interstitial edema, hemorrhage, margination and infiltration of inflammatory cells, and formation of bronchiolitis: a score of 0 represented normal lung; 1 represented mild; 2 was moderate; 3 was severe; and 4 denoted very severe changes. For each mouse, the lung injury score was calculated by adding the individual grades (the mean value for four sections) for each category. The histology was reviewed by two of the authors (MY and AM) in a blinded manner. The middle portion of the left lung was excised and soaked in RNAlater. Total RNA was extracted and 1 µg of total RNA was reverse-transcribed to cDNA according to the procedure described above. The samples were analyzed for virus copies using the 7500 Real-Time PCR System. The PCR primers were designed according to the protocol of Sigma-Aldrich Japan (Table 1).

### **Immunohistochemical Study**

Immunohistochemical analysis was performed using the other upper lung sections (4-µm slices) that were obtained from vehicle-treated and rhTRX-1-treated mice on Day 3 (72 hr) after H1N1 inoculation. The lung sections obtained from 9-week-old non-virus-inoculated mice served as controls. Antibodies against granulocyte-differentiation antigen (Gr-1) (BioLegend, San Diego, CA) (21) and 8-hydroxy-2'-deoxyguanosine (8-OHdG) (Japan Institute for the Control of Aging, Shizuoka, Japan) (22) were used respectively for detection of neutrophil infiltration and cellular DNA oxidation according to the manufacturers' instructions. The results were evaluated by two of the authors (MY and AM) in a blinded manner.

### **Measurement of Cytokines**

TNF- $\alpha$ , a proinflammatory cytokine, participates in important processes involved in the inflammatory response. CXCL1, a member of the CXC chemotactic cytokine family, plays a pivotal role in the activation and extravasation of neutrophils (23). Concentrations of TNF- $\alpha$  and CXCL1 were measured using sandwich ELISA methods in lung lavage fluids obtained from vehicle-treated and rhTRX-1-treated mice on Day 1 (24 hr), 3 (72 hr), and 5 (120 hr) after H1N1 inoculation (5 mice per group at each time point). Those concentrations were also measured in the right lung homogenate supernatants obtained from the same mice on Day 3 (72 hr) (5 mice per group). The ELISA procedure was referred to that described by Matsukawa et al. (23). The captured antibodies, detection antibodies, and recombinant cytokines were purchased from

R&D Systems (Minneapolis, MN). The ELISAs used for this study did not cross-react with other available murine cytokines. They consistently detected cytokine concentrations higher than 10 pg/ml.

### **Measurement of Serum Hydroperoxides**

Blood was sampled from vehicle-treated and TRX-treated mice on Day 1 (24 hr), 3 (72 hr), and 5 (120 hr) after H1N1 inoculation (5 mice per group at each time point). Blood was also sampled from another group of 9-week-old non-virus-inoculated mice given vehicle (100- $\mu$ l PBS) intraperitoneally 24 hr before (10 mice). Serum was prepared. The serum concentration of hydroperoxides (whole oxidant capacity of serum against N,N-diethylparaphenylene-diamine in acidic buffer) was measured using the Free Radical Analytical System (Diacron International, Grosseto, Italy) (24). The measurement unit was CARR U. It has been established that 1 CARR U corresponds to 0.08 mg/dl hydrogen peroxide (25).

### **Cell Biological Study**

MLE-12 cells (ATCC, Manassas, VA), a SV40-transformed murine lung epithelial cell line, were grown to confluence in RPMI-1640 medium (Sigma-Aldrich Corp., St. Louis, MO) containing 10% fetal bovine serum (Gibco-BRL, Grand Island, NY). The MLE-12 cells were not inoculated or were inoculated with H1N1 (at multiplicity of infection of 10) for 6 hr. The H1N1-inoculated cells were not treated or were treated with rhTRX-1 (at doses of 10 or 100 ng/ml) simultaneously. Expression of TNF- $\alpha$  and CXCL1 mRNA was analyzed using reverse transcription and real-time quantitative PCR (ABI 7700 Sequence Detector System; Applied Biosystems), as described previously by Ito et al. (26).

### **Treatment with rhTRX-1 after H1N1 Inoculation (Therapeutic Protocols)**

Other groups of 9-week-old mice were used in the therapeutic protocols. The dose of influenza virus was 300 plaque-forming units. The intraperitoneal administration of vehicle (100- $\mu$ l PBS) or rhTRX-1 (40  $\mu$ g in 100- $\mu$ l PBS) was started 30 min (14 mice per group) or 4 hr (11 mice per group) after H1N1 inoculation (Day 0) and repeated every second day until Day 12. Survival was observed until Day 14.

### **Statistical Analysis**

All data are expressed as mean $\pm$ SEM and compared using unpaired *t*-test, or one-way ANOVA or two-way ANOVA followed by Bonferroni's post-test where appropriate. Survival curves were analyzed using the Kaplan-Meier log-rank test. Differences for which  $p < 0.05$  were considered significant. All statistical calculations were performed using GraphPad Prism 4.0 (GraphPad Software Inc., San Diego, CA).



## **RESULTS**

### **Regional Distribution of mRNA Expression of TRX-1**

Figure 1A shows the mRNA expression of TRX-1 in normal mice for various organs. The level of TRX-1 expression in the lung was the highest: several times higher than that in the other organs including the brain, liver, kidney, and spleen. Results suggest that TRX-1 plays some physiologically important regulatory role in the animal lung.

### **Murine TRX-1 mRNA Expression in the Lung after rhTRX-1 Administration**

Figure 1B presents changes of mRNA expression of TRX-1 in the lung of normal mice after rhTRX-1 administration. The intrinsic mRNA expression of TRX-1 was suppressed in the lung after a bolus intraperitoneal injection of rhTRX-1 (40 µg). The suppressive effect was statistically significant at 1 hr and 3 hr after the administration compared with the pretreatment level.

### **Effects of rhTRX-1 on Survival Rate and Viral Load in the Lung after H1N1 Inoculation**

Comparison of survival curves using the Kaplan-Meier log-rank test showed a significant difference between the vehicle-treated group and the rhTRX-1-treated group (Fig. 2A). The rhTRX-1 treatment significantly improved the survival rate of H1N1-inoculated mice. All 10 control mice died from Day 7 to Day 9, but 4 (40%) of 10 rhTRX-1-treated mice survived over 14 days after H1N1 inoculation. Of note, all animals in both groups survived over 5 days (120 hr) after inoculation. All the mice that survived until Day 14 recovered health thereafter.

The viral load in the lung increased significantly from 24 hr to 72 hr after inoculation, decreasing thereafter in control mice (Fig. 2B). A similar tendency was observed in rhTRX-1-treated mice. The viral load in rhTRX-1-treated mice was almost comparable to that in control mice at each time point. These results indicate that rhTRX-1 treatment improves the survival rate of H1N1-inoculated mice significantly, although the treatment does not affect propagation of the influenza virus in the lungs of these animals.

### **Effects of rhTRX-1 on Lung Histology after H1N1 Inoculation**

H1N1-inoculated control mice presented diffuse edema and inflammatory cellular infiltration in alveoli and interstitium of the lung, hemorrhage, and thickened airways at 72 hr after inoculation. The rhTRX-1 treatment attenuated the histological changes in the lung (Fig. 3A). The lung injury score increased significantly from 24 hr to 72 hr and 120 hr in the control group ( $p < 0.01$  at 24 hr vs. 72, 120 hr, one-way

ANOVA with Bonferroni's post-test) (Fig. 3B). In the treatment group, the score increased significantly from 24 hr to 72 hr ( $p < 0.05$ ), but the score at 120 hr was not significantly different from that at 24 hr. The lung injury score remained significantly lower in rhTRX-1-treated mice than in control mice. These histological analyses indicate that rhTRX-1 treatment significantly attenuates the degree of acute lung injury in H1N1-inoculated mice.

#### **Effects of rhTRX-1 on Neutrophil Infiltration in the Lung after H1N1 Inoculation**

The neutrophil number in lung lavage fluid increased significantly from 24 hr to 72 hr after virus inoculation in control group ( $p < 0.01$ , one-way ANOVA with Bonferroni's post-test). The neutrophil number at 120 hr was not significantly different from that at 72 hr (Fig. 4A). In the treatment group, the neutrophil number increased significantly from 24 hr to 72 hr and decreased significantly thereafter ( $p < 0.01$  at 72 hr vs. 24, 120 hr). The neutrophil number remained lowered in rhTRX-1-treated mice compared with control mice. The difference reached statistical significance at 72 hr and 120 hr after inoculation. Histologically, influenza virus inoculation increased neutrophil infiltration in the lung at 72 hr after inoculation. The rhTRX-1 treatment almost reversed this effect (Fig. 4B). These results indicate that rhTRX-1 treatment significantly attenuates the neutrophil infiltration in the lung of H1N1-inoculated mice.

#### **Effects of rhTRX-1 on Cytokine Production in the Lung after H1N1 Inoculation**

The TNF- $\alpha$  and CXCL1 concentrations in lung lavage fluid were significantly lower in rhTRX-1-treated mice than in control mice at 72 hr after inoculation (Figs. 5A, 5B). In lung tissue analyses, both cytokine concentrations were lowered significantly by rhTRX-1 treatment at 72 hr after inoculation (Figs. 5C, 5D). These results indicate that rhTRX-1 treatment significantly attenuates the inflammatory cytokine production in the lung of H1N1-inoculated mice.

#### **Effects of rhTRX-1 on Oxidative Stress Markers after H1N1 Inoculation**

Histologically, H1N1 inoculation increased 8-OHdG formation in the lung at 72 hr after inoculation. The 8-OHdG formation was observed primarily in infiltrating cells and occasionally in lung epithelial cells. The rhTRX-1 treatment almost reversed this effect (Fig. 6A). Serum concentration of hydroperoxides was significantly lower in rhTRX-1-treated mice than in control mice at 72 hr after inoculation (Fig. 6B). Concentrations at 24, 72, and 120 hr after inoculation in either group were significantly higher than that ( $122 \pm 6$  CARR U) in non-virus-inoculated mice ( $p < 0.05$  in each, unpaired  $t$ -test). These results indicate that rhTRX-1 treatment significantly attenuates the oxidative stress enhancement that is observed in

H1N1-inoculated mice.

### **Effects of rhTRX-1 on Cytokine mRNA Expression in H1N1-inoculated MLE-12 Cells**

Expression of cytokine mRNA was analyzed by reverse-transcription PCR in MLE-12 cells. The TNF- $\alpha$  and CXCL1 mRNA accumulated significantly at 6 hr after inoculation (Figs. 7A, 7B). Simultaneous rhTRX-1 treatment (at doses of 10, 100 ng/ml) almost abolished this effect. These results indicate that rhTRX-1 treatment significantly attenuates the increase in cytokine mRNA expression in H1N1-inoculated murine lung epithelial cells.

### **Treatment with rhTRX-1 after H1N1 Inoculation (Therapeutic Protocols)**

The rhTRX-1 treatment started 30 min after virus inoculation significantly improved the survival rate of H1N1-inoculated mice ( $p < 0.05$  vs. control) (Fig. 8A). Only one of 14 control mice (7%) survived over 14 days after H1N1 inoculation, but 6 (43%) of 14 rhTRX-1-treated mice survived. However, the rhTRX-1 treatment started 4 hr after inoculation had no effect on the survival rate. None of 11 control mice and only 2 (18%) of 11 rhTRX-1-treated mice survived more than 14 days after H1N1 inoculation (Fig. 8B).

## **DISCUSSION**

Thioredoxin-1 is a ubiquitously expressed, multifunctional protein that has a redox-active dithiol-disulfide within the conserved -Cys-Gly-Pro-Cys- sequence. TRX-1 protects cells against oxidative stress by scavenging ROS in concert with peroxiredoxins and prevents cellular apoptosis by inhibiting apoptosis signal-regulating kinase 1 (15). Furthermore, TRX-1 suppresses inflammation by regulating neutrophil activation and extravasation and exerts the anti-inflammatory effect (15, 16). In the clinical field, extracellular concentrations of TRX-1 have been measured in various conditions characterized by oxidative stress and inflammation, including sepsis, viral infection, autoimmune disease, ischemia-reperfusion injury, and acute lung injury (27-30). These studies document that the TRX-1 concentrations are elevated in patients with these diseases and that they are correlated significantly with the activity of such diseases.

Overexpression of human TRX-1 in transgenic mice induces resistance to harmful conditions, including ischemic brain damage, adriamycin-induced cardiotoxicity, ischemia-reperfusion renal injury, and cerulein-induced pancreatitis (31-34). More importantly, human TRX-1 transgenic mice are more resistant than control mice to proinflammatory cytokine-, bleomycin-, diesel exhaust particle-, or cigarette smoke-induced lung injury (19, 35, 36) and to influenza virus-induced pneumonia (18). Administration of

rhTRX-1 is also effective in animal models, especially for acute lung injury, including proinflammatory cytokine-, bleomycin-, or cigarette smoke-induced inflammatory injury, ovalbumin-induced airway hyperresponsiveness and inflammation, and lipopolysaccharide-induced bronchoalveolar neutrophil infiltration (19, 36-38). All these results demonstrate that TRX-1 has potent protective effects on oxidative stress-associated or inflammation-associated lung disorders in animals.

Influenza virus infections are responsible for numerous pneumonia cases every year. In severe cases, they can cause death (1-3). Influenza virus causes death of infected cells by cytopathology. Furthermore, when the immune system responds to the infection exuberantly, additional lung damage and overwhelming systemic illness are likely to ensue. A marked increase in concern has occurred in relation to the possibility of future severe pandemics. Therefore, it is crucial to identify new therapeutic strategies for influenza (4).

This study demonstrated, for the first time, the protective effects of rhTRX-1 administration in a murine model of acute lung injury induced by influenza virus infection. Intranasal instillation of influenza A virus (H1N1) was used to produce viral pneumonia in this model. Every second day, 40 µg of rhTRX-1 was administered intraperitoneally. At the beginning of the study, we examined the regional distribution of mRNA expression of TRX-1 in normal mice. We found that TRX-1 expression was at the highest level in the lung. Results suggest that the endogenous TRX-1 system plays some physiologically important regulatory role in the lung and that lung disease might be a target for this protein (15). Results also show that the TRX-1 expression in the lung was suppressed significantly after intraperitoneal injection of rhTRX-1. Administration of rhTRX-1 producing elevated blood concentrations (mean: 1237 ng/ml), which are about 10 times higher than those in oxidative and inflammatory disorders in humans (40-140 ng/ml) (15), is likely to suppress the endogenous TRX-1 system in the animal lung.

Intraperitoneal administration of rhTRX-1 significantly improved the survival rate of H1N1-inoculated mice. The viral load in the lung increased from 24 hr to 72 hr after inoculation, confirming the occurrence of influenza pneumonia in both vehicle-treated (control) and rhTRX-1-treated mice. Notably, the viral load in rhTRX-1-treated mice was almost comparable to that of control mice at each time point of the study. The rhTRX-1 treatment did not affect propagation of influenza virus in the animal lung. The result is consistent with previous reports in which administration of N-monomethyl-L-arginine, overexpression of extracellular superoxide dismutase or adenovirus-mediated transfer of heme oxygenase-1 was used as a therapeutic tool against influenza pneumonia in mice (10-12). The occurrence of influenza pneumonia was documented

histologically in mice subjected to H1N1 inoculation in this study. The H1N1-inoculated control mice presented characteristic changes of acute lung injury: diffuse edema and inflammatory cellular infiltration in alveoli and interstitium of the lung, hemorrhage, and thickened airways (20). Particularly, the lung injury score remained at high levels at 24, 72, and 120 hr after inoculation. The rhTRX-1 treatment attenuated the histological changes and the lung injury score, confirming histologically that rhTRX-1 ameliorates H1N1-induced pneumonia in mice.

Previous studies revealed that influenza virus infection caused a marked increase in the cell number in lung lavage fluid, and demonstrated that this increase primarily reflected an increase in neutrophils and macrophages (9-12). In this study, we measured the neutrophil number in lung lavage fluid obtained from the animals. In control mice, the neutrophil number increased from 24 hr to 72 hr and remained high at 120 hr. The rhTRX-1 treatment suppressed the increase in neutrophils. Histological examination revealed much less neutrophil infiltration to lung parenchyma in rhTRX-1-treated mice. Accumulation of activated neutrophils in the lung is an early critical step in the inflammatory process of acute lung injury. According to our previous studies (15, 16, 34, 36, 38), elevated levels of circulating TRX-1 effectively suppress neutrophil extravasation into sites of inflammation. The protective effects of rhTRX-1 on H1N1-induced pneumonia are partly attributed to its antichemotactic action for neutrophils.

Increased synthesis and secretion of inflammatory mediators contribute to the overall pathology of lung injury. Several cytokines apparently play crucial roles in the acute and uncontrollable inflammatory process of influenza pneumonia (1, 2, 4, 39, 40). In this study, we measured TNF- $\alpha$  (a proinflammatory cytokine) and CXCL1 (a CXC chemotactic cytokine) concentrations in lung lavage fluid at 24, 72, and 120 hr after inoculation and lung homogenate supernatants at 72 hr. The rhTRX-1 treatment suppressed the concentrations of these cytokines. Additionally, H1N1 inoculation increased the TNF- $\alpha$  and CXCL1 mRNA expression in MLE-12 cells (a SV40-transformed murine lung epithelial cell line) directly; simultaneous rhTRX-1 treatment almost abolished this effect. The *in vivo* and *in vitro* experimentally obtained results indicate that rhTRX-1 treatment attenuates the increase in cytokine expression and secretion in the lung of H1N1-inoculated mice.

Evaluating oxidative stress status in the experimental animals was crucial because the redox imbalance was regarded as a key lung injury pathway in influenza pneumonia (4, 8-14, 18). The DNA base-modified

product 8-OHdG is a marker for oxidative stress in tissue samples (6, 22, 36). In the present study, 8-OHdG formation was intense in the lung of H1N1-inoculated control mice; 8-OHdG was detected in infiltrating inflammatory cells and lung epithelial cells. Serum concentration of hydroperoxides is a marker for oxidative stress in the whole body (24, 25). A significant increase in serum hydroperoxides occurred in H1N1-inoculated mice, implying the presence of systemic oxidative stress enhancement. The rhTRX-1 treatment suppressed 8-OHdG formation in the lung and serum concentration of hydroperoxides in H1N1-inoculated mice. These results indicate that oxidative stress enhancement is indeed involved in the inflammatory process of H1N1-induced pneumonia and that the protective effects of rhTRX-1 are partly attributed to its potent antioxidative action.

In our murine model, influenza pneumonia is characterized histologically by intense infiltration of inflammatory cells (mainly neutrophils). Activated neutrophils, macrophages, and respiratory tract epithelial cells might release excess amounts of bioactive substances, including cytokines/chemokines, ROS, and tissue degradative enzymes and induce acute lung inflammatory disease (4, 5). Presumably, oxidative injury by the inflamed cells is targeted to the vascular endothelium, leading to lung edema and hemorrhage. Based on these considerations, it is plausible that rhTRX-1 administration ameliorates the lethal effects of influenza A virus (H1N1)-induced pneumonia in mice through antioxidative and anti-inflammatory actions, including the antichemotactic effect for neutrophils.

For potential clinical use, we also examined whether treatment with rhTRX-1 after H1N1 inoculation is efficacious for protection. As a therapeutic protocol, vehicle or rhTRX-1 (40 µg) was given intraperitoneally to mice every second day from 30 min after inoculation (Day 0) to Day 12. Administration of rhTRX-1 started 30 min after H1N1 inoculation exerted a significant survival-promoting effect. Therefore, it is conceivable that rhTRX-1 provided 30 min after virus inoculation ameliorated H1N1-induced lung inflammatory injury in mice. However, administration of rhTRX-1 started 4 hr after virus inoculation had no such therapeutic effect.

The beneficial effects on H1N1-inoculated mice observed in this study might not be specific to rhTRX-1, but to the other antioxidative agents. The effects of a well-known glutathione precursor, N-acetylcysteine (41), on H1N1-induced pneumonia have been evaluated. The vehicle (100-µl PBS) or N-acetylcysteine (200 mg/kg in 100-µl PBS (42)) was administered intraperitoneally to another group of 9-week-old mice every

second day from Day -1 to Day 13. With this intermediate dose, N-acetylcysteine did not alter the survival rate or lung injury score (at 72 hr) of H1N1-inoculated mice (data not shown). Results of previous studies have indicated that N-acetylcysteine has protective effects in the murine model of influenza pneumonia, especially when administered in combination with anti-viral drugs (4, 43, 44). N-acetylcysteine reportedly has beneficial effects in various clinical conditions characterized by glutathione deficiency or oxidative stress enhancement (41). It is therefore highly possible that higher doses of N-acetylcysteine can efficiently reduce the lethal effects of influenza virus infection in our experimental model. This issue deserves further examination.

Our results raise the possibility that loss of TRX-1 function aggravates influenza virus-induced pneumonia, although complete TRX-1 deficiency results in early embryonic lethality (45). The cecal ligation and puncture septic murine model revealed that neutralization of endogenous TRX-1 impaired mice survival but that treatment with rhTRX-1 enhanced mice survival (30). Although extracellular TRX-1 concentrations were elevated in many oxidative and inflammatory disorders (27-30), plasma TRX-1 concentrations were persistently low in children with meningococcal septic shock, possibly because of a genetic predisposition (46). Plasma TRX-1 concentrations were lower in patients with neutropenia/sepsis than in patients with systemic inflammatory response syndrome/sepsis (47). Although speculative, the low TRX-1 condition might contribute to the devastating nature of sepsis. At present, no information related to the concentration of TRX-1 in blood or lung lavage fluid from patients with influenza pneumonia is available.

We reported that endogenous TRX-1 expression was induced by natural substances including estrogen, prostaglandins and cyclic AMP, geranylgeranylacetone (an anti-ulcer drug derived from a natural plant constituent), and temocapril (a non-sulphydryl-containing angiotensin-converting enzyme inhibitor) (48). We reported also that the cytoprotective action of TRX-1 was augmented after S-nitrosation (49). Moreover, concentrations of TRX-1 (mean: 268 ng/ml) and nitrite/nitrate (mean: 479  $\mu$ mol/L) in early human milk were found to be about 10 times higher than those (mean: 20 ng/ml, 40  $\mu$ mol/L, respectively) in blood of healthy adults (50, 51). The protective effects of such “TRX-1 inducers” or “TRX-1 donors” against infectious disorders, including influenza pneumonia, warrant further study.

The present results suggest that systemic administration of rhTRX-1 can be clinically beneficial for ameliorating acute lung injury in influenza virus-infected mice. However, several concerns persist in terms of

clinical relevance. First, the experimentally obtained results for animals cannot be extrapolated directly to the medical arena. Second, the survival rate of rhTRX-treated virus-inoculated mice is not entirely satisfactory, although the rhTRX-1 treatment proved to exert lung protective effects in the mice. Last, the post-treatment experiments suggest that the therapeutic time window for rhTRX-1 might be narrow. The combination of rhTRX-1 with current anti-influenza drugs might expand the therapeutic window as well as the protective effects of rhTRX-1 against influenza infection. Further investigations are necessary to explore the future clinical application of rhTRX-1 for severe influenza pneumonia in humans.

## **CONCLUSIONS**

Exogenous administration of redox-active protein rhTRX-1 significantly improved the survival rate and attenuated lung histological changes in the murine model of influenza pneumonia. The rhTRX-1 treatment did not affect virus propagation in the animal lung. The protective mechanism of rhTRX-1 might be explained by its potent antioxidative and anti-inflammatory actions. These results suggest that rhTRX-1 represents a possible pharmacological strategy for severe influenza virus infection in humans.

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## FIGURE LEGENDS

### Figure 1. Thioredoxin-1 mRNA expression in mice.

A. Regional distribution of thioredoxin (TRX)-1 mRNA expression. Data are expressed as a percentage relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA and represent the mean (SE) of 5-10 independent experiments.  $*p < 0.05$  vs. brain, kidney, and spleen by one-way ANOVA with Bonferroni's post-test.

B. Changes of TRX-1 mRNA expression in the lung after rhTRX-1 administration. Data are expressed as a percentage relative to GAPDH mRNA and represent the mean (SE) of 3-5 independent experiments.  $*p < 0.05$  vs. 0 hr by one-way ANOVA with Bonferroni's post-test.

### Figure 2. Effects of recombinant human thioredoxin-1 treatment on survival rate and viral load in the lung after H1N1 inoculation.

A. Survival rate. All mice in the vehicle group (10 mice, broken line) died from Day 7 to Day 9. In the thioredoxin (TRX)-1 group (10 mice, solid line), 4 (40%) survived until Day 14.  $*p < 0.05$  vs. vehicle.

B. Viral load in the lung. Data represent the mean (SE) of 5 independent experiments.  $*p < 0.01$  vs. 24 hr, 120 hr by one-way ANOVA with Bonferroni's post-test. Two-way ANOVA was significant for time,  $p < 0.001$ ; but not for treatment or time x treatment. No significant difference was detected between the two groups at any time point by Bonferroni's post-test.

### Figure 3. Effects of recombinant human thioredoxin-1 treatment on lung histology after H1N1 inoculation.

A. Photomicrographs of lung tissue samples stained with hematoxylin and eosin at 72 hr after H1N1 inoculation. They are representative of 5 independent experiments. (a) Vehicle-group lung tissue showed diffuse alveolar and interstitial edema, inflammatory cellular infiltration, hemorrhage, and bronchiolitis. (b) In thioredoxin (TRX)-1 group lung tissue, these features were much less severe. Original magnification, x 200.

B. Lung injury scores. Lung histological changes were graded from 0 to 4 in 4 categories. Lung injury scores were calculated by adding the individual grades for each category. Data represent the mean (SE) of 5 independent experiments. Two-way ANOVA was significant for time,  $p < 0.001$ ; treatment,  $p < 0.001$ ; but

not for time x treatment.  $*p < 0.01$  vs. vehicle by Bonferroni's post-test.

**Figure 4. Effects of recombinant human thioredoxin-1 treatment on neutrophil infiltration in the lung after H1N1 inoculation.**

A. Neutrophil numbers in lung lavage fluid. Thioredoxin (TRX)-1 treatment significantly decreased the neutrophil number in lung lavage fluid at 24 hr and 72 hr after H1N1 inoculation. Data represent the mean (SE) of 5 independent experiments. Two-way ANOVA was significant for time,  $p < 0.005$ ; treatment,  $p < 0.001$ ; but not for time x treatment.  $*p < 0.05$  vs. vehicle by Bonferroni's post-test.

B. Photomicrographs of lung tissue samples stained with granulocyte-differentiation antigen (Gr-1) at 72 hr after H1N1 inoculation. They are representative of 3 independent experiments: (a) No H1N1 inoculation; (b) H1N1 inoculation, no primary antibody; (c) H1N1 inoculation, vehicle group; and (d) H1N1 inoculation, TRX-1 group. Arrowheads indicate positively stained (brown) cells. Scale bars, 100  $\mu\text{m}$ .

**Figure 5. Effects of recombinant human thioredoxin-1 treatment on cytokine production in the lung after H1N1 inoculation.**

A. TNF- $\alpha$  concentrations in lung lavage fluid. Data represent the mean (SE) of 5 independent experiments. Two-way ANOVA was significant for treatment,  $p < 0.05$ ; time x treatment,  $p < 0.05$ ; but not for time.  $*p < 0.01$  vs. vehicle by Bonferroni's post-test.

B. CXCL1 concentrations in lung lavage fluid. Data represent the mean (SE) of 5 independent experiments. Two-way ANOVA was significant for time,  $p < 0.001$ ; treatment,  $p < 0.05$ ; but not for time x treatment.  $*p < 0.01$  vs. vehicle by Bonferroni's post-test.

C, D. Cytokine concentrations in lung tissue samples. Data represent the mean (SE) of 5 independent experiments.  $*p < 0.01$  vs. vehicle by unpaired  $t$ -test.

**Figure 6. Effects of recombinant human thioredoxin-1 treatment on oxidative stress markers after H1N1 inoculation.**

A. Photomicrographs of lung tissue samples stained with 8-hydroxy-2'-deoxyguanosine at 72 hr after H1N1 inoculation. They are representative of 3 independent experiments: (a) No H1N1 inoculation; (b) H1N1

inoculation, no primary antibody; (c) H1N1 inoculation, vehicle group; and (d) H1N1 inoculation, thioredoxin (TRX)-1 group. Arrowheads indicate positively stained (brown) cells. Scale bars, 100  $\mu$ m.

B. Hydroperoxides in blood serum. Data represent the mean (SE) of 5 independent experiments. Two-way ANOVA was significant for time,  $p < 0.05$ ; but not for treatment or time x treatment.  $*p < 0.05$  vs. vehicle by Bonferroni's post-test.

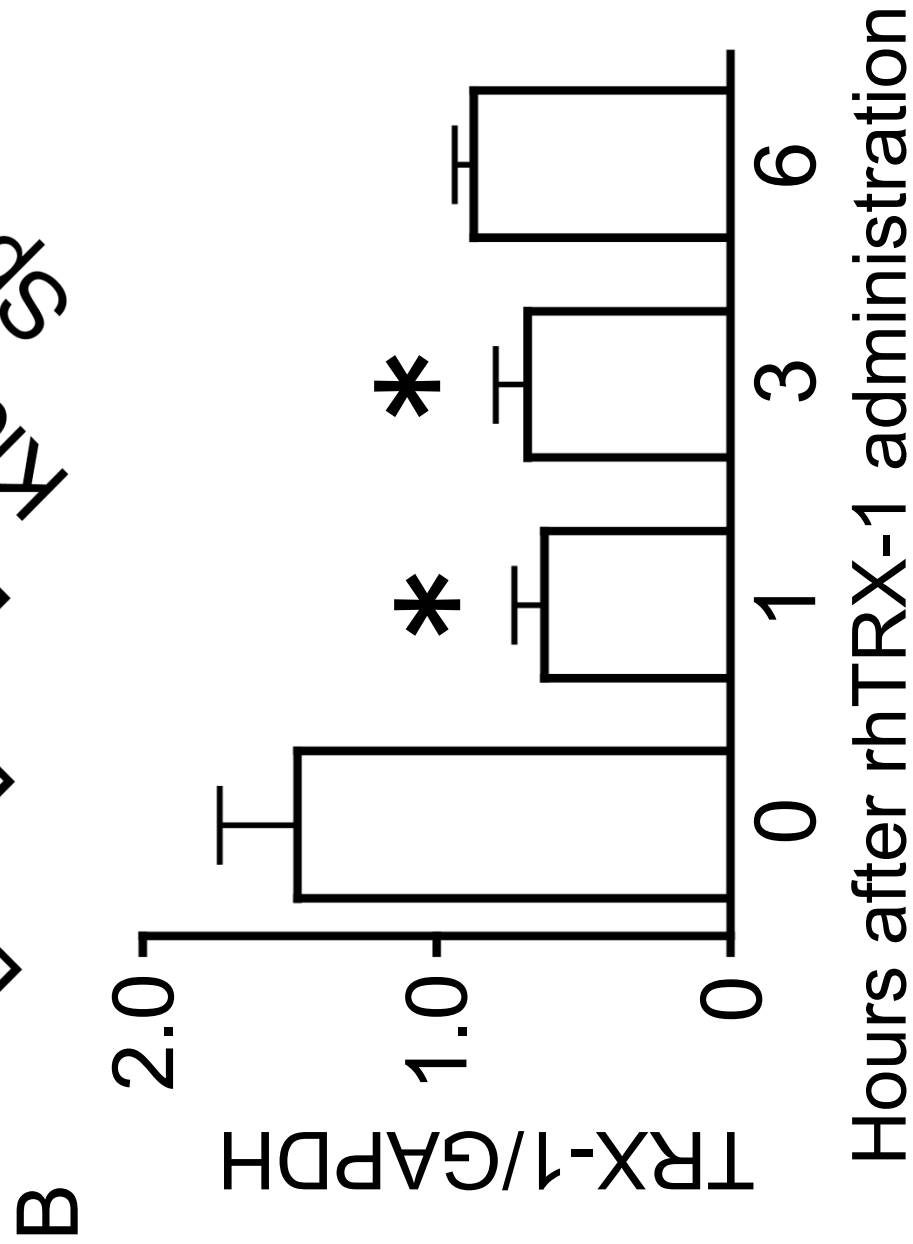
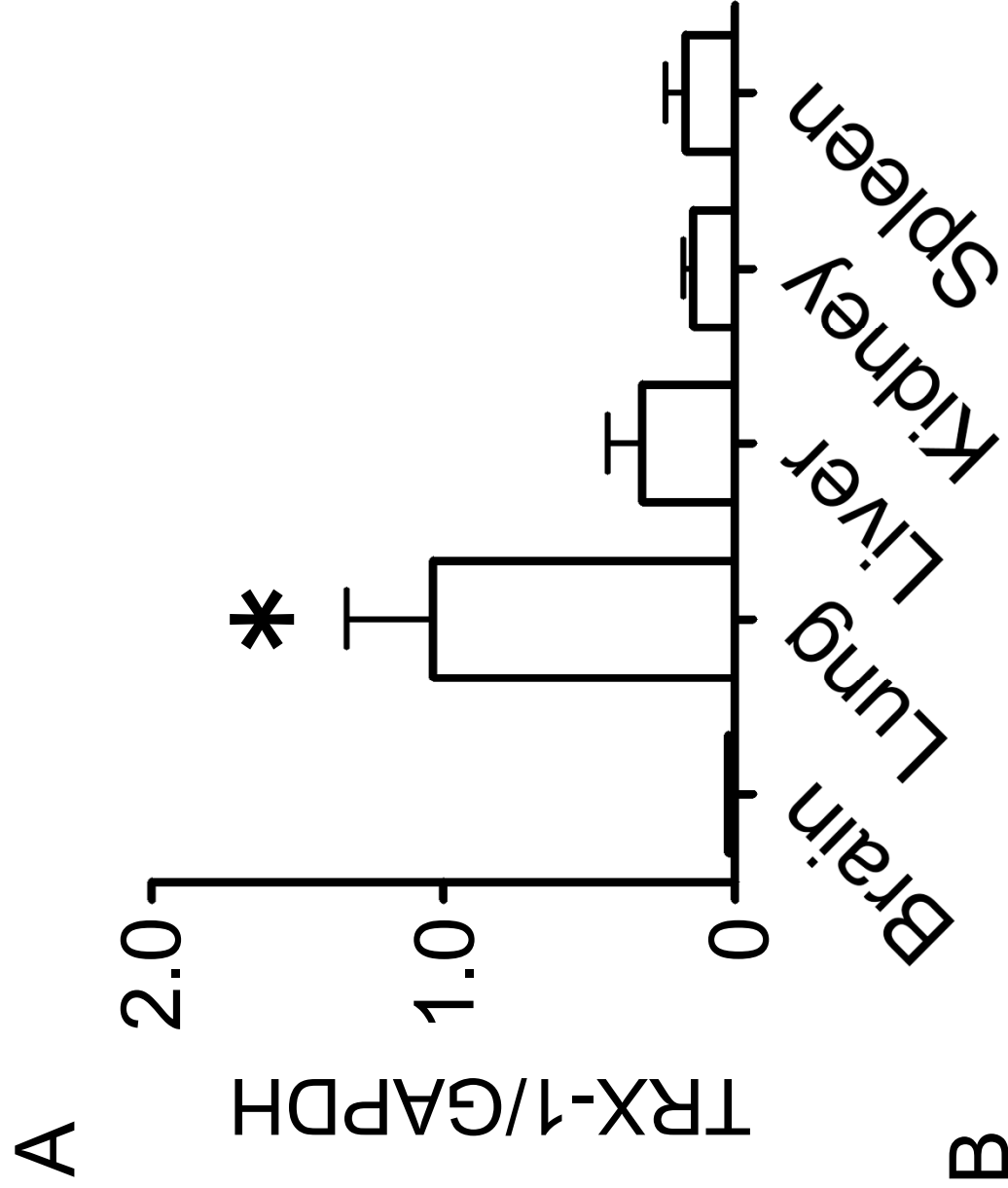
**Figure 7. Effects of recombinant human thioredoxin-1 treatment on cytokine mRNA expression in H1N1-inoculated lung epithelial cells (MLE-12).**

Cells were inoculated with H1N1 (at multiplicity of infection of 10) for 6 hr. They were not treated or were treated with thioredoxin (TRX)-1 simultaneously. Data are expressed as a percentage relative to the basal expression level.  $n = 4$  per group.  $*p < 0.01$  vs. no H1N1 inoculation (medium alone) by unpaired  $t$ -test,  $\#p < 0.001$  vs. H1N1 inoculation, no TRX-1 treatment by one-way ANOVA with Bonferroni's post-test.

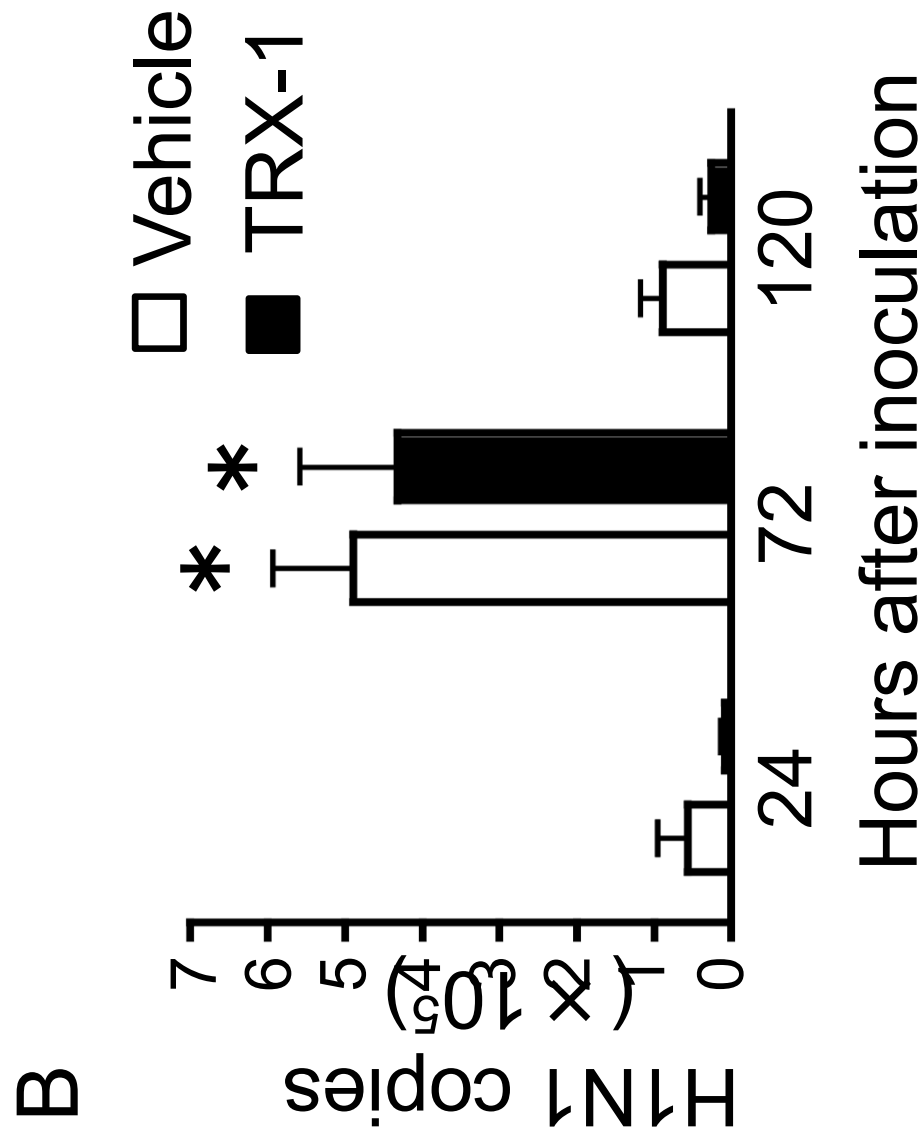
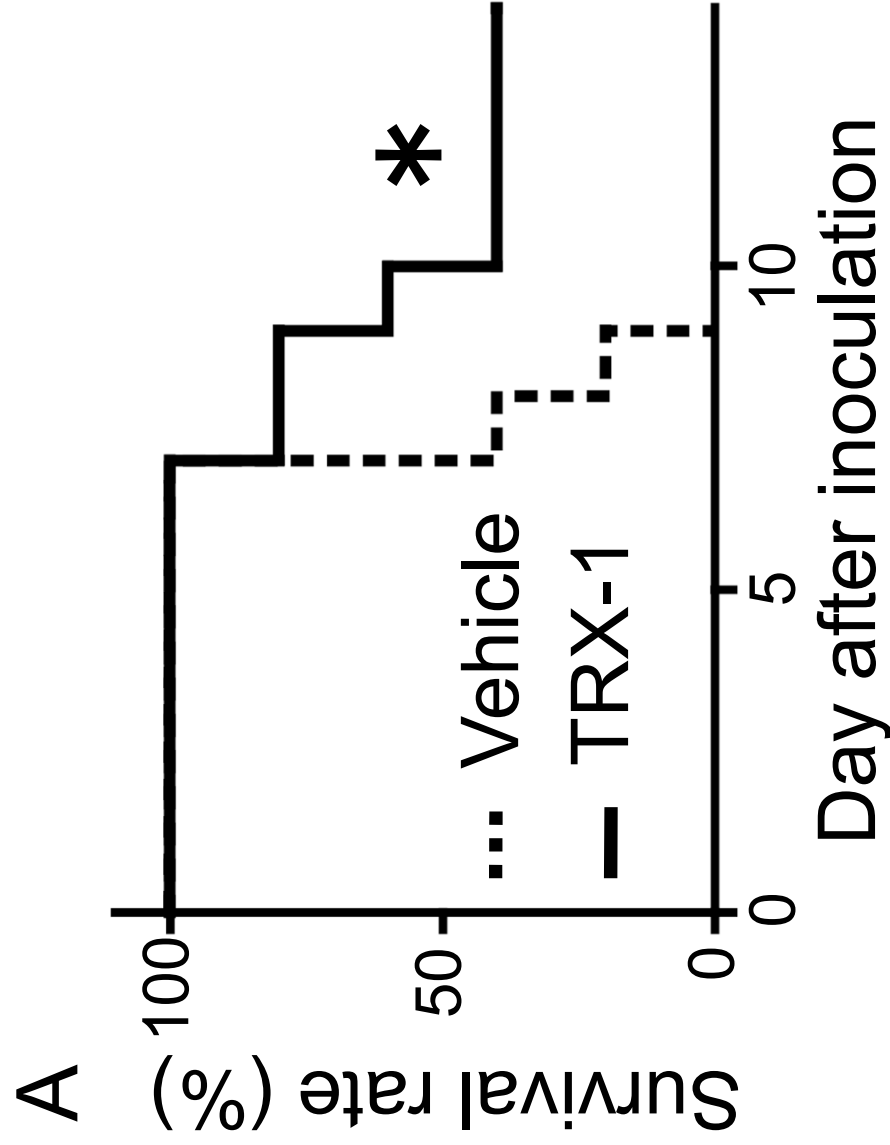
**Figure 8. Therapeutic effects of recombinant human thioredoxin-1 treatment started 30 min or 4 hr after H1N1 inoculation on mouse survival rate.**

A. Thioredoxin (TRX)-1 treatment started 30 min after virus inoculation significantly improved the survival rate of H1N1-inoculated mice (14 mice per group).  $*p < 0.05$  vs. vehicle.

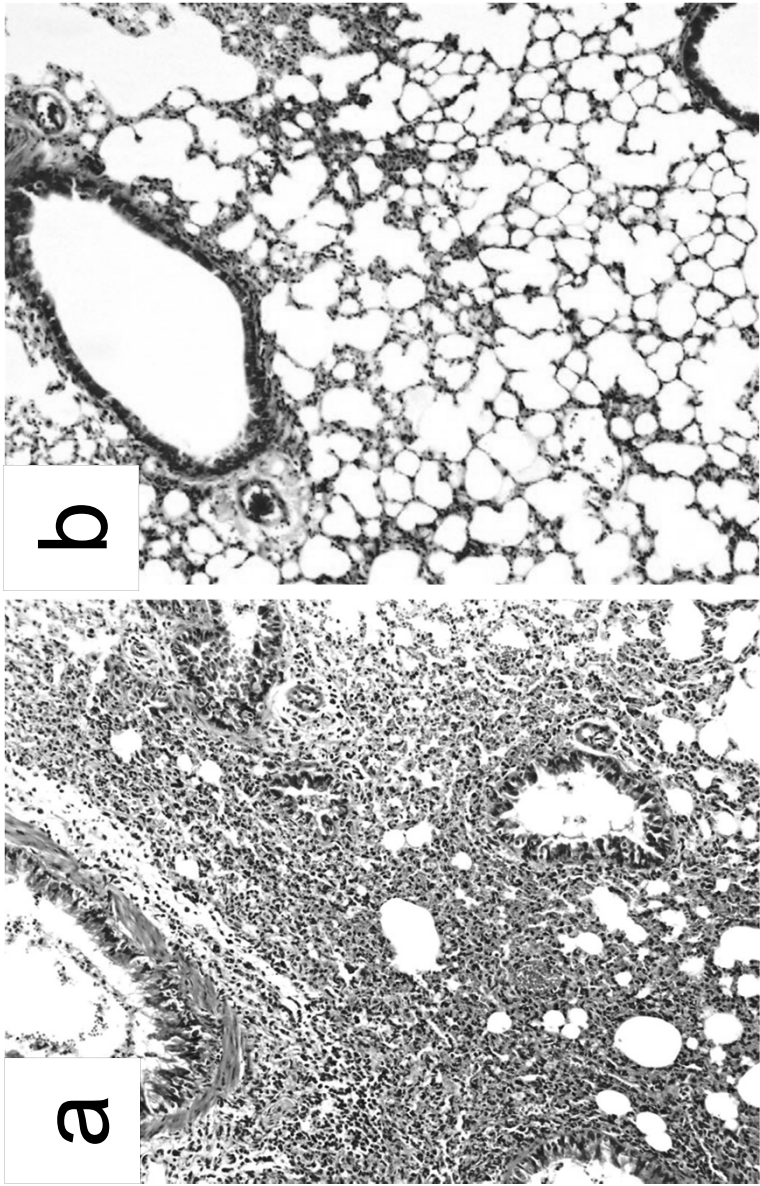
B. The TRX-1 treatment started 4 hr after inoculation had no effect on the survival rate (11 mice per group).



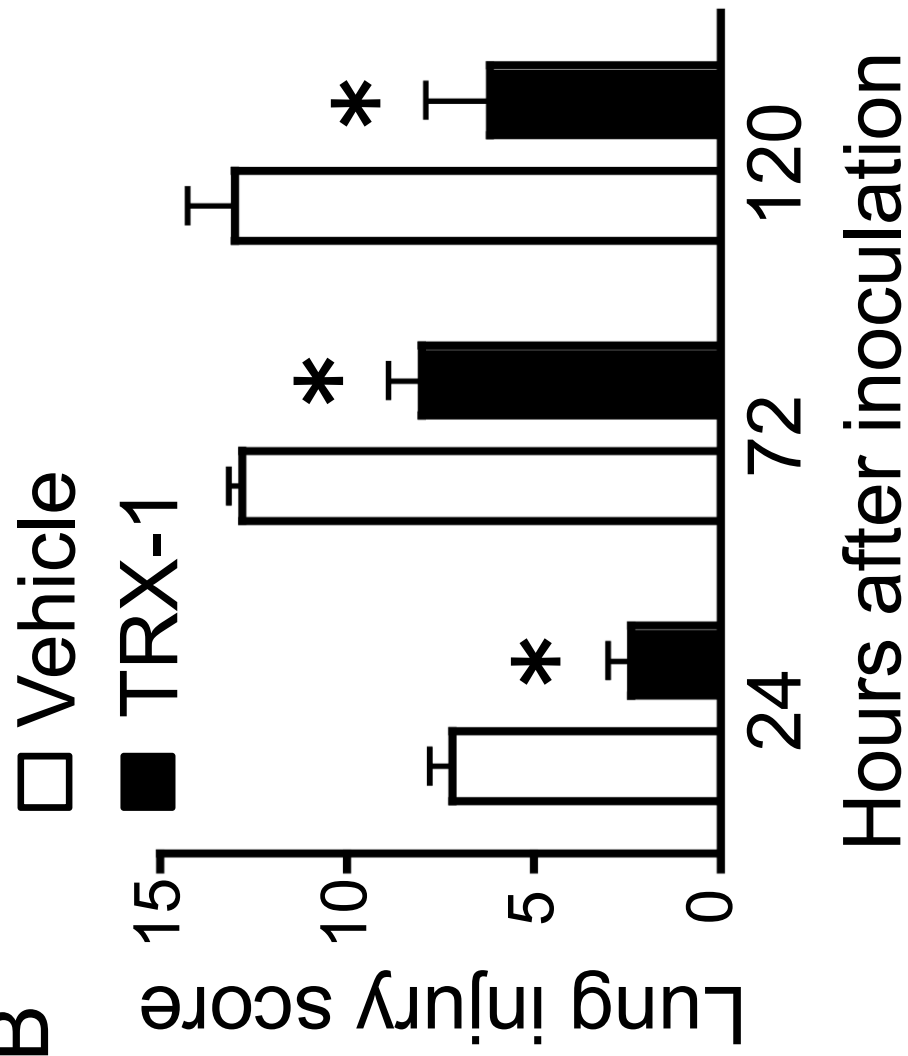


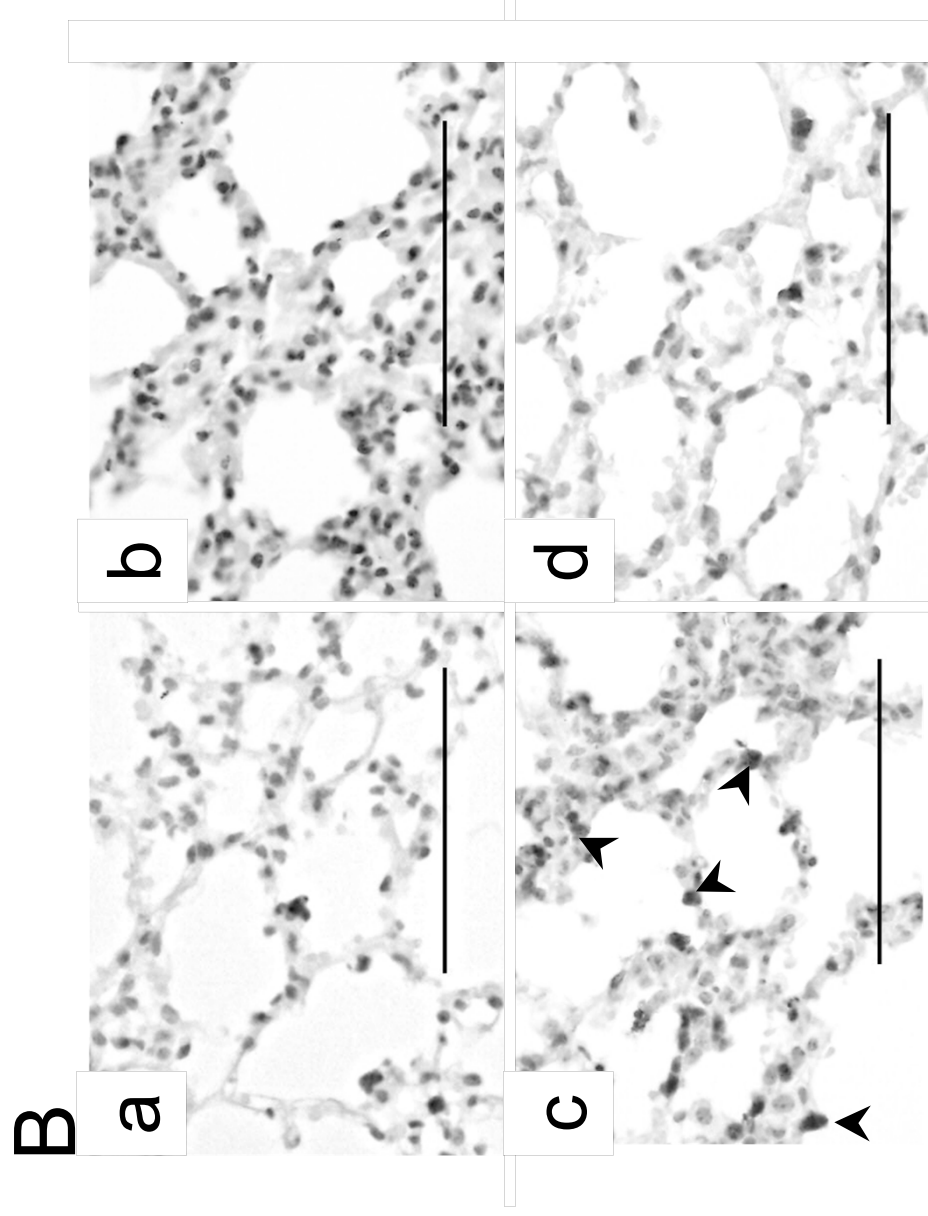
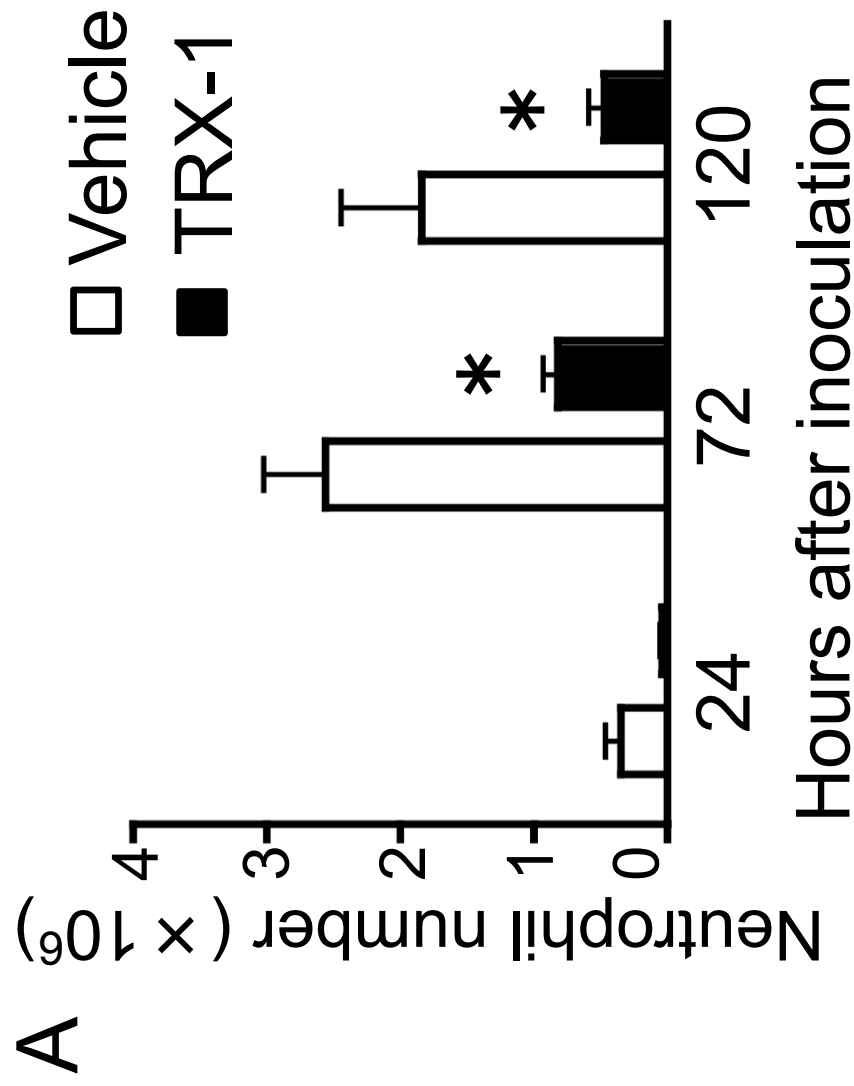


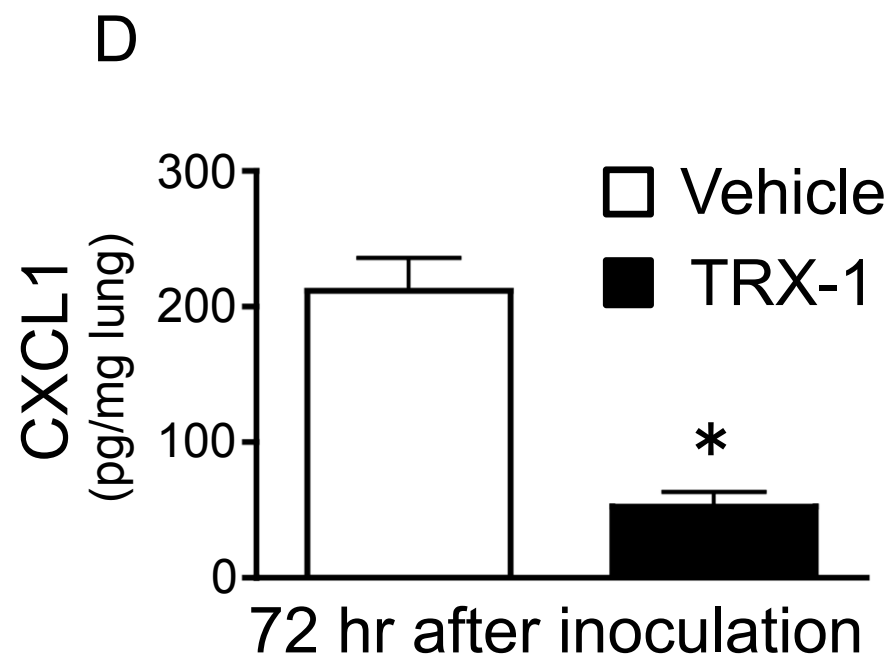
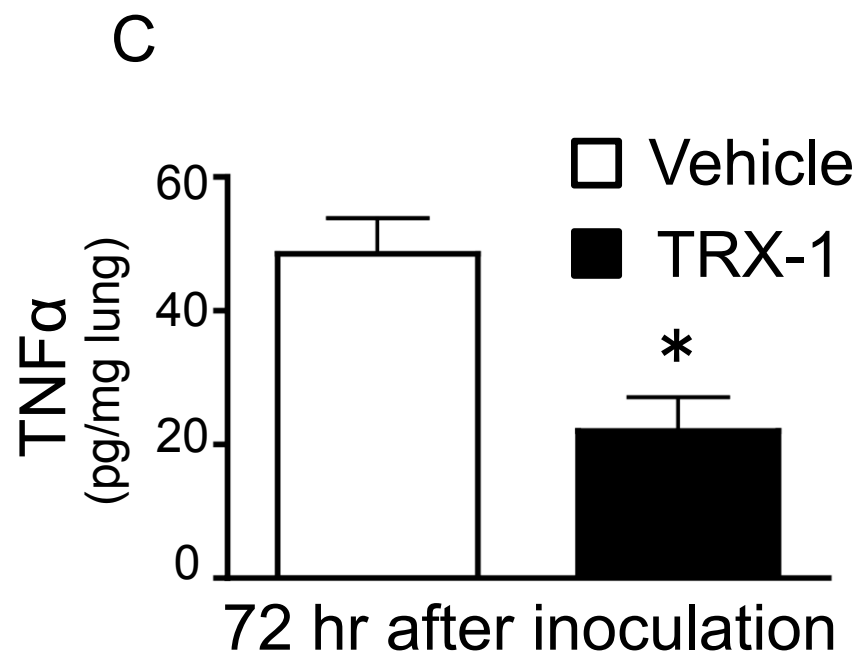
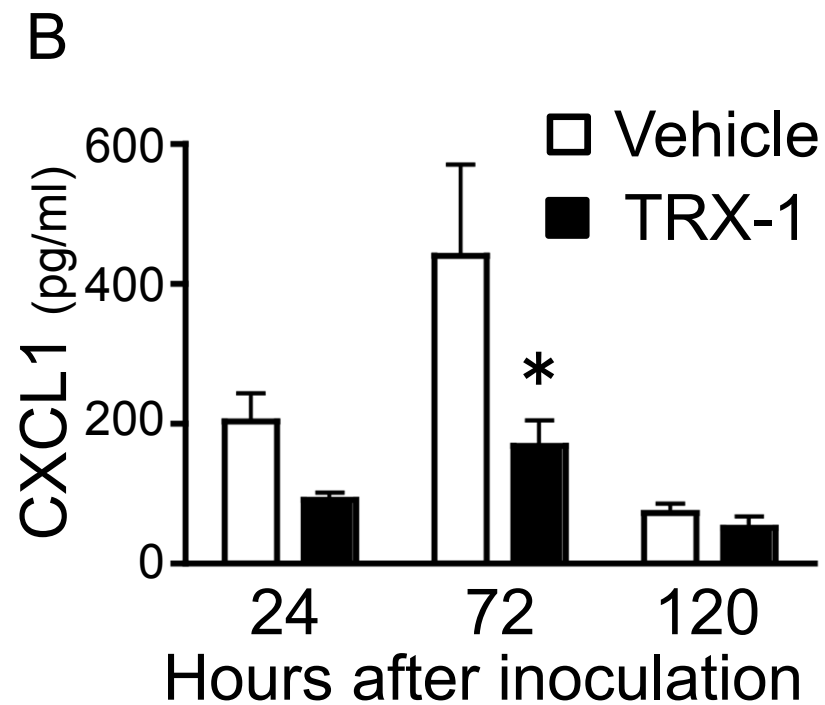
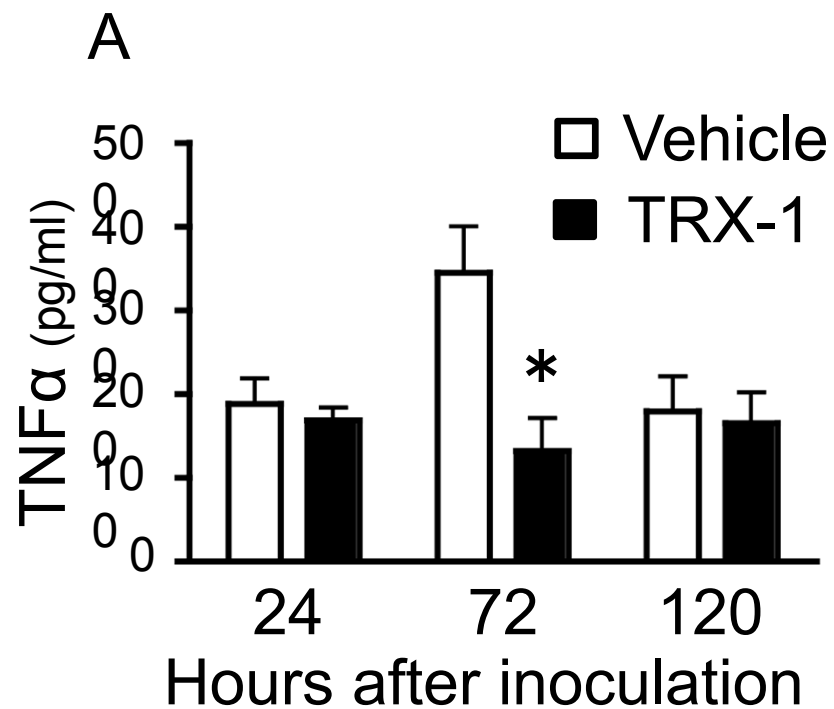
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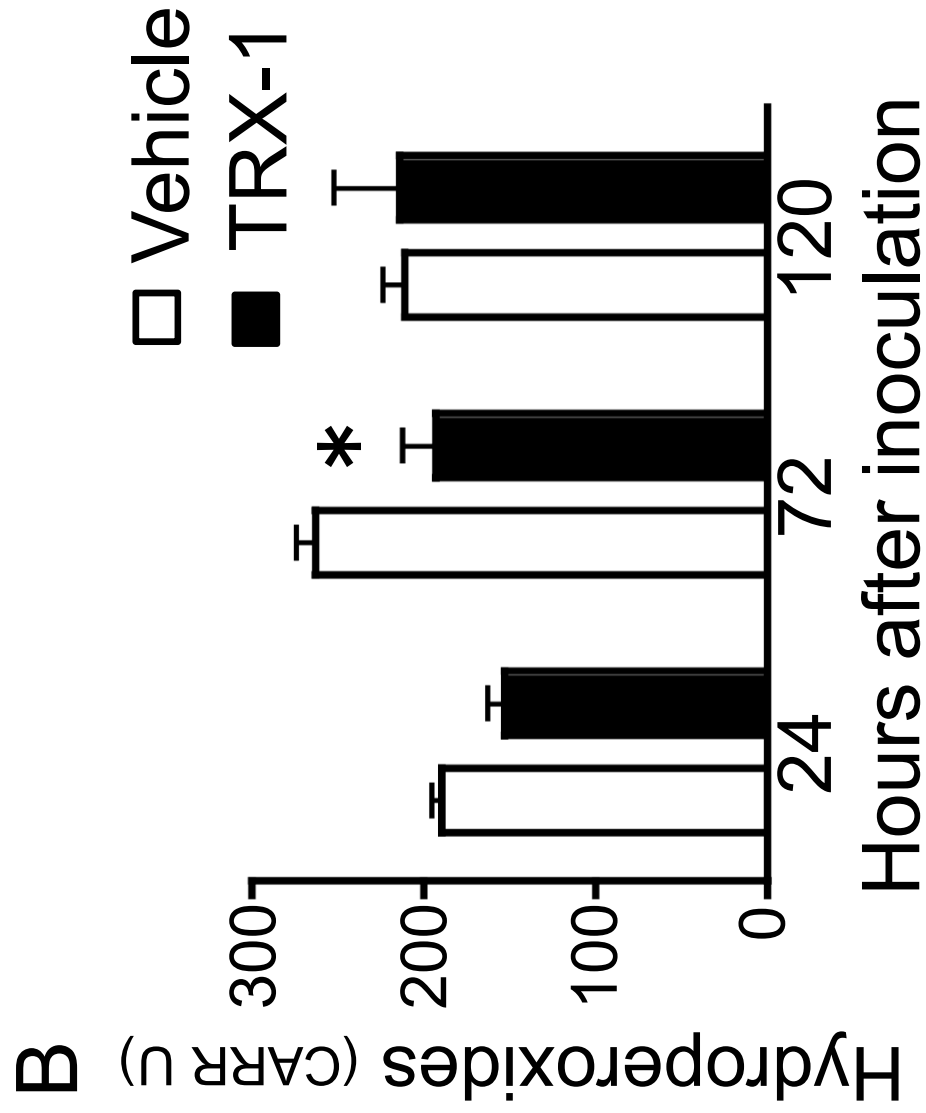
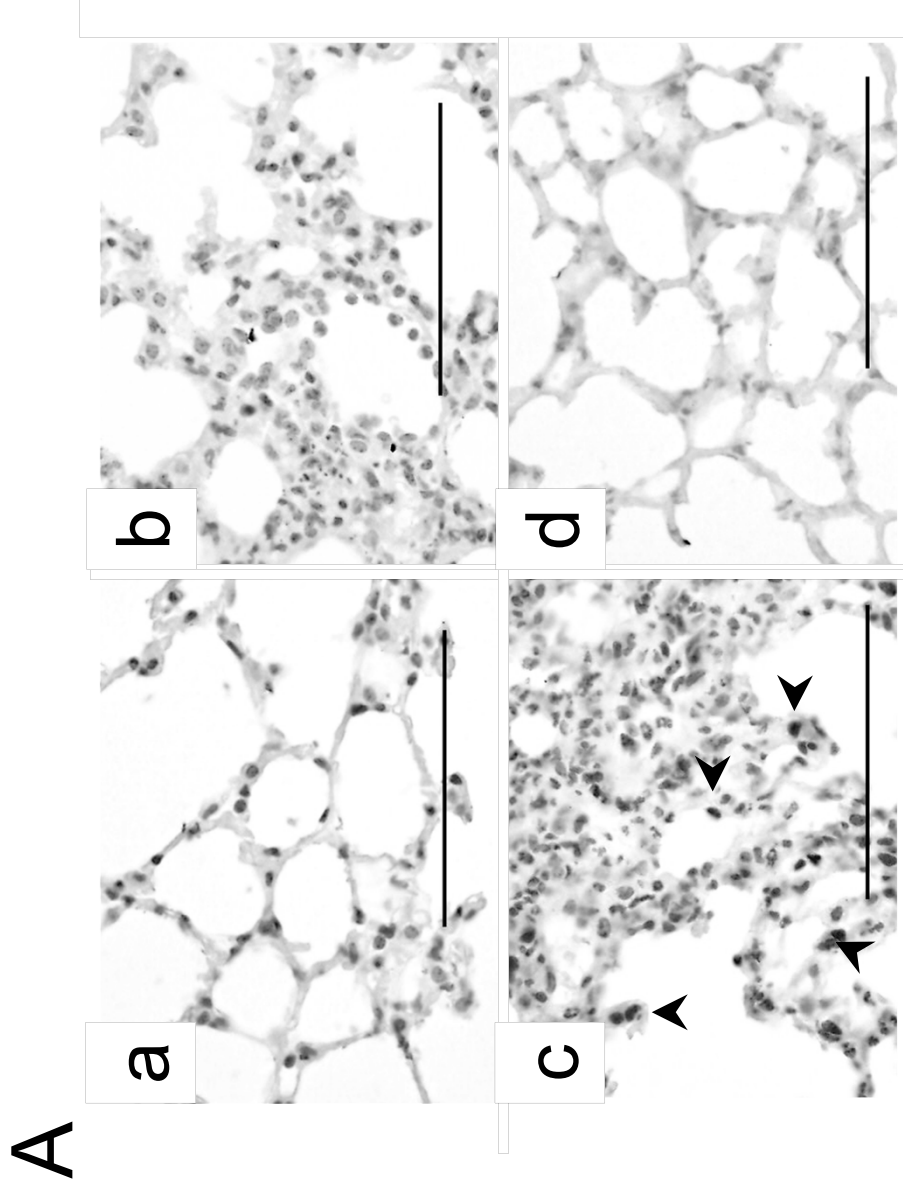


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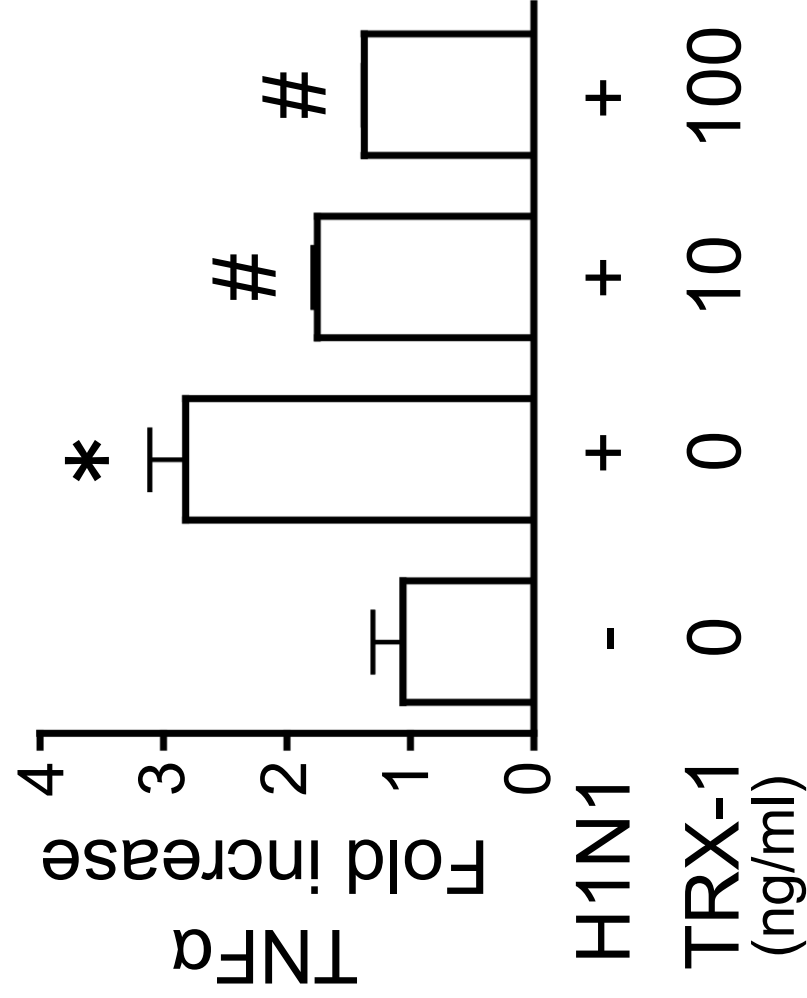








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