Published in final edited form as:
Toxicol Appl Pharmacol. 2011 February 1; 250(3): 245–255. doi:10.1016/j.taap.2010.10.027.

Role of TNFR1 in lung injury and altered lung function induced by the model sulfur mustard vesicant, 2-chloroethyl ethyl sulfide

Vasanthi R. Sunil[a,*], **Kinal Patel-Vayas**[a], **Jianliang Shen**[a], **Andrew J. Gow**[a], **Jeffrey D. Laskin**[b], and **Debra L. Laskin**[a]

[a]Department of Pharmacology and Toxicology, Rutgers University, Piscataway, NJ 08854, USA

[b]Department of Environmental and Occupational Medicine, University of Medicine and Dentistry of New Jersey, Robert Wood Johnson Medical School, Piscataway, NJ 08854, USA

Abstract

Lung toxicity induced by sulfur mustard is associated with inflammation and oxidative stress. To elucidate mechanisms mediating pulmonary damage, we used 2-chloroethyl ethyl sulfide (CEES), a model sulfur mustard vesicant. Male mice (B6129) were treated intratracheally with CEES (3 or 6 mg/kg) or control. Animals were sacrificed 3, 7 or 14 days later and bronchoalveolar lavage (BAL) fluid and lung tissue collected. Treatment of mice with CEES resulted in an increase in BAL protein, an indication of alveolar epithelial damage, within 3 days. Expression of Ym1, an oxidative stress marker also increased in the lung, along with inducible nitric oxide synthase, and at 14 days, cyclooxygenase-2 and monocyte chemotactic protein-1, inflammatory proteins implicated in tissue injury. These responses were attenuated in mice lacking the p55 receptor for TNFα (TNFR1−/−), demonstrating that signaling via TNFR1 is key to CEES-induced injury, oxidative stress, and inflammation. CEES-induced upregulation of CuZn–superoxide dismutase (SOD) and MnSOD was delayed or absent in TNFR1−/− mice, relative to WT mice, suggesting that TNFα mediates early antioxidant responses to lung toxicants. Treatment of WT mice with CEES also resulted in functional alterations in the lung including decreases in compliance and increases in elastance. Additionally, methacholine-induced alterations in total lung resistance and central airway resistance were dampened by CEES. Loss of TNFR1 resulted in blunted functional responses to CEES. These effects were most notable in the airways. These data suggest that targeting TNFα signaling may be useful in mitigating lung injury, inflammation and functional alterations induced by vesicants.

Keywords

TNFR1; TNFα; CEES; iNOS; COX-2; MCP-1; SOD; Lung function

Introduction

Sulfur mustard and related analogs including the half mustard, 2-chloroethyl ethyl sulfide (CEES) are potent vesicants known to cause severe and debilitating damage to the lung (Ghanei and Harandi, 2007; Ghanei et al., 2010). Toxicity involves alkylation of nucleophilic sites in cells including guanine nucleotides in DNA, a process that can lead to mutations and cell death (Debiak et al., 2009; Papirmeister et al., 1991). Following exposure

*Corresponding author. Department of Pharmacology and Toxicology, Rutgers University, Ernest Mario School of Pharmacy, 160 Frelinghuysen Road, Piscataway, NJ 08854, USA. Fax: +1 732 445 2534.

of animals to vesicants, DNA damage, apoptosis and autophagy are observed in the airways and lower lung within 24 h, along with increased expression of activated caspases and DNA repair enzymes, biochemical markers of these processes (Kehe et al., 2008; Malaviya et al., 2010; Ray et al., 2010). This is associated with inflammatory cell accumulation in the respiratory tract and increased production of proinflammatory mediators, as well as reactive oxygen and nitrogen species (reviewed in Weinberger et al., in press). Findings that vesicant-induced increases in inflammatory cells and mediators correlates directly with altered lung functioning suggest that they play a key role in the toxicity of these agents (Sunil et al., 2010, 2011).

Macrophages release a number of proinflammatory mediators, which have been implicated in lung injury (Laskin et al., 2010a). One notable cytokine is tumor necrosis factor (TNF)-α, which is rapidly generated in the lung in response to injury induced by vesicants and other pulmonary toxicants (Chatterjee et al., 2003; Emad and Emad, 2007; Laskin et al., 2010a, 2007; Malaviya et al., 2010; Mukhopadhyay et al., 2006). TNFα is unique among proinflammatory cytokines in that it has the capacity to directly induce necrosis and apoptosis, which may be important in its cytotoxic actions. TNFα also promotes oxidative metabolism in phagocytic leukocytes resulting in increased production of cytotoxic reactive oxygen and nitrogen species, and it stimulates the synthesis of matrix metalloproteinase-9. TNFα generation by alveolar macrophages is also associated with an accumulation of ceramides in the lung, which are thought to contribute to apoptosis and impaired surfactant production (Chatterjee et al., 2003).

The biologic activities of TNFα are mediated by two structurally related, but functionally distinct, receptors: TNFR1 (p55) and TNFR2 (p75) (Aggarwal, 2003; Bradley, 2008). These receptors are co-expressed on the surface of most cell types and both are capable of being released following proteolysis. TNFα signaling through the p55 TNFR1 is the primary pathway leading to inflammatory responses. TNFR1 is expressed at relatively high levels on alveolar macrophages (Dai et al., 1999; Ermert et al., 2003; Gaede et al., 1999). Moreover, expression of TNFR1 has been reported to be increased in various models of inflammatory lung injury and disease, suggesting that TNFα may be a major mediator of the pathogenic response to toxicants (Bradley, 2008; Chen et al., 2005; Dai et al., 2005; Ermert et al., 2003). This is supported by findings that mice lacking TNFR1 are protected from lung injury induced by pulmonary irritants such as ozone, silica, bleomycin and radiation (Cho et al., 2001; Ortiz et al., 1999, 2001; Zhang et al., 2008).

In the present studies, we used knockout mice with a targeted disruption of the gene for TNFR1 to evaluate the role of TNFα in vesicant-induced tissue injury, inflammatory mediator and antioxidant expression, and altered lung functioning using CEES as a model. Our findings that TNFR1−/− mice are resistant to the toxic effects of CEES, when compared to wild-type mice, and that this correlates with reduced production of proinflammatory mediators and dampened functional changes, demonstrate that TNFα signaling through TNFR1 plays a critical role in the pathogenic response to this vesicant. Identification of specific inflammatory mediators released following exposure to vesicants and potential functional consequences may be useful in developing efficacious pharmacologic approaches to mitigating the toxicity of inhaled vesicating agents.

Materials and methods

Animals and treatments

Male specific pathogen-free B6129 wild-type (WT) and TNFR1−/− mice (8–10 weeks) were obtained from Jackson Laboratories (Bar Harbor, ME). Animals were housed in filter-top, microisolation cages and maintained on food and water *ad libitum*. All animals received

humane care in compliance with the institution's guidelines, as outlined in the *Guide for the Care and Use of Laboratory Animals*, published by the National Institutes of Health. Animals were anesthetized by intraperitoneal injection of ketamine (80 mg/kg) and xylazine (10 mg/kg) and then placed on a tilting rodent work stand (Hallowell EMC, Pittsfield, MA) in a supine position and restrained using an incisor loop. The tongue was extruded using a cotton tip applicator and the larynx visualized by a hemi-sectioned 3 mm diameter speculum attached to an operating head of an otoscope (Welch Allyn, Skaneateles Falls, NY). PBS containing 12% ethanol or 3 mg/kg or 6 mg/kg CEES (Sigma-Aldrich, St. Louis, MO), diluted in ethanol, was administered via Clay Adams Intramedic PE-10 (I.D 0.76 mm, O.D. 1.22 mm) polyethylene tubing (Becton, Dickinson and Company, Franklin Lakes, NJ) attached to a $27^{1/2}$ gauge hypodermic needle (0.4 × 13 mm). The tubing was advanced approximately 10 mm past the epiglottis and 0.05 ml of control or CEES instilled into the trachea. The tubing and speculum were withdrawn immediately after instillation. Animals were then removed from the work stand and maintained in a vertical position until normal respiration was observed (less than 1 min). All instillations were performed by David Reimer, D.V.M., Rutgers University Laboratory Animal Services. Preparation and instillation of CEES, which included the use of double gloves, safety glasses and masks, was performed in a designated room under a chemical hood by personnel who followed Rutgers University Environmental Health and Safety guidelines.

Sample collection

Animals were sacrificed 3, 7 or 14 days after exposure by intraperitoneal injection of Nembutal (200 mg/kg). BAL was collected by slowly instilling and withdrawing 1 ml of PBS into the lungs through a cannula in the trachea three times. BAL fluid was centrifuged (300 × *g*, 8 min), supernatants collected, aliquoted, and stored at −80 °C until analysis. Cell pellets were resuspended in 1 ml PBS and viable cells (10 μl) counted on a hemocytometer using trypan blue dye exclusion. For differential analysis, cytospin preparations were fixed in methanol and stained with Giemsa (Labchem Inc., Pittsburgh, PA). A total of 300 cells were counted by light microscopy.

Measurement of BAL protein

Total protein was quantified in cell-free BAL using a BCA Protein Assay kit (Pierce Biotechnologies Inc., Rockford, IL) with bovine serum albumin as the standard. Samples (25 μl) from three mice per treatment group were pooled and analyzed in triplicate at 560 nm using a Vmax MAXline™ microplate reader (Molecular Devices, Sunnyvale, CA).

Immunohistochemistry

Tissue sections were deparaffinized. After antigen retrieval using citrate buffer (10.2 mM sodium citrate, 0.05% Tween 20, pH 6.0) and quenching of endogenous peroxidase with 3% hydrogen peroxide for 15 min, sections were incubated with 10% rabbit serum (room temperature, 1 h) to block nonspecific binding. This was followed by an overnight incubation at 4 °C with rabbit IgG or rabbit polyclonal antibodies to inducible nitric oxide synthase (iNOS, 1:50; Santa Cruz Biotechnology, Santa Cruz, CA), cycloxygenase-2 (COX-2, 1:400; Abcam, Cambridge, MA), CuZnSOD (1:300, Assay Designs Stressgen, Ann Arbor, MI) or Ym1 (1:200, Stem Cell, Vancouver, Canada). Sections were then incubated with biotinylated secondary antibody (Vector Labs, Burlingame, CA) for 30 min at room temperature. Binding was visualized using a peroxidase substrate kit DAB (Vector Labs). Sections from three mice per treatment group were analyzed for each of the antibodies.

Real time quantitative PCR

Total mRNA was extracted from tissue samples using an RNeasy Mini kit (Qiagen, Valencia, CA). mRNA was reverse-transcribed using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Standard curves were generated using serial dilutions from pooled cDNA samples. Real time PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) on a 7900HT thermocycler according to manufacturer protocol. All PCR primers were generated using Primer Express 3.0 software (Applied Biosystems). Samples from three animals per treatment group were pooled and analyzed in triplicate. Data were normalized to GAPDH mRNA expression and presented as fold change relative to control. Forward and reverse primer sequences were TNFR1: CAG ACT TGC ATG GTG AGC TCT T, AGC CCA GTT ACC CAA CAG ACA; iNOS: CCT GGT ACG GGC ATT GCT, GCT CAT GCG GCC TCC TTT; COX-2: CAT TCT TGC CCA GCA CTT CAC, GAC CAG GCA CCA GAC CAA AGA C; MCP-1: TTG AAT GTG AAG TTG ACC CGT AA, GCT TGA GGT TGT GGA AAA G; CuZnSOD: ACC AGT GCA GGA CCT CAT TTT AA, TCT CCA ACA TGC CTC TCT TCA TC; MnSOD: CAC ATT AAC GCG CAG ATC ATG, CCA GAG CCT CGT GGT ACT TCT C; GAPDH: TGA AGC AGG CAT CTG AGG G, CGA AGG TGG AAG AGT GGG AG.

Measurement of lung mechanics and function

Animals were anesthetized with ketamine (80 mg/kg) and xylazine (10 mg/kg). After 5 min, tracheotomy was performed using an 18 gauge cannula, the animals attached to a SciReq flexiVent (Montreal, Canada) and baseline lung mechanics and function assessed. Animals were then challenged intratracheally with increasing doses of methacholine (0–96 mg/ml) and measurements of lung mechanics and function repeated. Data were analyzed using flexiVent software version 5.2. Measurements were made on five to six mice per treatment group.

Statistical analysis

All experiments were repeated at least 3 times. Data were analyzed using ANOVA and Student's *t*-test; a p value of ≤ 0.05 was considered statistically significant. Changes in gene expression $\geq$ 2-fold were considered biologically significant.

Results

Effects of CEES on TNFR1 expression in the lung; role of TNFR1 in lung injury, oxidative stress and inflammation

Treatment of mice with CEES resulted in a transient dose-related increase in expression of TNFR1 mRNA in the lung of WT mice; maximal effects were noted with 6 mg/kg CEES after 3 days (Fig. 1). To assess the role of TNFR1 in the pulmonary toxicity of CEES, we used TNFR1−/− mice. As expected, TNFR1 was not detectable in TNFR1−/− mice, even after CEES administration. In WT mice, but not TNFR1−/− mice, CEES (3 mg/kg) administration resulted in increased protein in BAL, a marker of alveolar epithelial injury (Bhalla, 1999). This was maximal after 3 days; subsequently protein levels declined toward control (Fig. 2). Protein levels also increased after administration of 6 mg/kg CEES to WT mice, but only after 14 days. No effects were observed in TNFR1−/− mice at this dose of CEES. Low levels of Ym1 protein, a biomarker of oxidative stress and upper airway inflammation (Zhang et al., 2009), were evident in alveolar macrophages and bronchial epithelial cells from both WT and TNFR1−/− mice (Fig. 3). Administration of CEES to WT mice resulted in a persistent increase in expression of Ym1 in alveolar macrophages and bronchial epithelial cells. In contrast, CEES had no effect on Ym1 expression in TNFR1−/−

mice. While CEES had no significant effect on the number of BAL cells recovered from either WT or TNFR1−/− mice, the absolute number of BAL cells was significantly reduced in mice lacking TNFR1, relative to WT mice (Fig. 2). This may be due to increased adherence of these cells to the epithelium making them more difficult to remove by lavage.

Effects of loss of TNFR1 on antioxidant gene expression

Treatment of WT mice with CEES resulted in a transient dose-related increase in expression of CuZnSOD mRNA 3-day post exposure (Fig. 4). This was correlated with increased expression of CuZnSOD protein which was evident in alveolar macrophages and bronchial epithelial cells, and persisted for at least 14-day post-CEES treatment (Fig. 5). MnSOD mRNA also increased transiently in the lungs of WT mice 3 days after administration of the higher dose of CEES (6 mg/kg), with no effect at the lower dose, or at longer times post exposure (Fig. 4). In TNFR1−/− mice, CEES also caused an increase in CuZnSOD and MnSOD mRNA; however this response was delayed for 14 days (Fig. 4). In contrast, expression of CuZnSOD protein was reduced in TNFR1−/− mice, relative to WT mice at all times post-CEES treatment (Fig. 5).

Effects of loss of TNFR1 on inflammatory mediator gene expression

We next analyzed expression of iNOS and COX-2, inflammatory proteins implicated in vesicant-induced injury (Babin et al., 2000; Casillas et al., 2000; Korkmaz et al., 2008; Sunil et al., 2010; Wormser et al., 2004; Yaren et al., 2007). Treatment of WT mice with CEES resulted in a dose-related increase in iNOS mRNA expression, which was evident after 3 days and persisted for 14 days, although at reduced levels (Fig. 6, left panels). iNOS protein expression was also upregulated following CEES administration; this was predominant in alveolar macrophages, as well as Type II cells and bronchiolar epithelial cells at 3 days post exposure (Fig. 7, upper panels). CEES administration to WT mice also resulted in increased COX-2 mRNA expression (Fig. 6, middle panels). However, in contrast to iNOS, COX-2 mRNA was only observed 14 days after administration of the highest dose (6 mg/kg) of CEES. Increased COX-2 protein was also noted in alveolar macrophages, as well as alveolar and bronchiolar epithelial cells after treatment of WT mice with CEES. This was evident 3 days post exposure in alveolar macrophages, Type II alveolar and bronchiolar epithelial cells at both doses (3 mg/kg and 6 mg/kg) of CEES (Fig. 7, lower panels). CEES had no major effect on expression of iNOS or COX-2 mRNA or protein in TNFR1−/− mice (Figs. 6 and 7). Similarly, no significant effects of CEES were observed on iNOS or COX-2 protein expression in either WT or TNFR1−/−mice at 14 days (data not shown).

We also analyzed the effects of CEES on expression of MCP-1 (CCL2), a C-C chemokine that stimulates monocyte and macrophage migration to sites of injury and infection (Crosby and Waters, 2010; Wynn and Barron, 2010). In WT mice, CEES administration resulted in increased MCP-1 gene expression; this was observed 14 days post treatment with the higher dose (6 mg/kg) of CEES (Fig. 6, right panels). In TNFR1−/− mice, CEES had no effect or reduced MCP-1 expression.

Effects of CEES on lung function

To determine if CEES-induced inflammatory changes in the lung were associated with altered lung functioning, mice were examined by a series of perturbations including single frequency and broadband forced oscillation, and pressure-volume loops. Exposure of WT mice to CEES resulted in a significant decrease in total lung compliance with a concomitant increase in tissue elastance (Table 1). Increases in total lung resistance, central airway resistance and tissue damping were also observed in WT mice after CEES; however, these did not reach statistical significance due to animal to animal variability. Whereas CEES-induced alterations in resistance and tissue damping were generally similar in TNFR1−/−

mice, changes in total lung compliance and tissue elastance were blunted. No significant differences in baseline lung function were noted between WT and TNFR1−/− mice in response to control treatment (Table 1).

Consistent with previous reports (Leme et al., 2010), upon challenge of WT mice with methacholine, a dose-related increase in pulmonary resistance was evident, reaching approximately 7-fold at 96 mg/ml, the highest dose examined (Fig. 8). This increase was primarily due to an increase in airway restriction, as demonstrated by the marked elevation in central airway resistance, and was accompanied by a loss of both total and static compliance. Loss of TNFR1 resulted in significant decrease in methacholine responsiveness relative to WT mice; thus, total, as well as central airway resistance responses were reduced (Fig. 8). Blunted methacholine-induced decreases in total lung and static compliance were also noted in TNFR1−/− mice. In WT mice, CEES administration resulted in reduced responsiveness to methacholine with the maximal increase in pulmonary resistance only reaching 4-fold at 96 mg/kg methacholine. This reduced response appeared to result primarily from airway dysfunction, as evidenced by the fact that increases in central airway resistance were almost completely ablated, while there were minimal changes in compliance values. In contrast to WT mice, no major changes in methacholine responsiveness were observed in TNFR1−/− mice following CEES administration. Hence, there was only a marginal loss of the central airway resistance response to methacholine, indicating that the effects of CEES on the airway were reduced in TNFR1−/− mice. Interestingly, in TNFR1−/− mice, but not WT mice, there was a considerable loss of compliance in response to methacholine following CEES administration.

Discussion

TNFα is a proinflammatory cytokine generated mainly by macrophages. It has been implicated in tissue injury induced by a variety of pulmonary toxicants, as well as in the pathogenesis of lung diseases such as asthma, chronic bronchitis, chronic obstructive pulmonary disease (COPD) and acute respiratory distress syndrome (Ding et al., 2002; Laskin et al., 2007; Mukhopadhyay et al., 2006; Shimabukuro et al., 2003). We found that expression of TNFR1, the major receptor mediating the proinflammatory actions of TNFα (Aggarwal, 2003), was transiently upregulated in the lung following exposure of mice to the model sulfur mustard vesicant, CEES. These data, together with reports of increases in TNFα in the lung following vesicant administration to rodents (Chatterjee et al., 2003; Malaviya et al., 2010), prompted us to investigate the role of TNFα signaling via TNFR1 in the pulmonary toxicity of CEES. To accomplish this, we used mice lacking TNFR1. CEES-induced pulmonary toxicity was found to be reduced in TNFR1−/− mice, when compared to WT mice. Thus, BAL protein levels were significantly decreased and oxidative stress ameliorated. Moreover, expression of cytotoxic/proinflammatory proteins was blunted. CEES-induced functional alterations in the airway were also mitigated. These data are novel and provide support for the hypothesis that TNFα is a key mediator regulating pulmonary inflammation and injury following vesicant exposure (Mukhopadhyay et al., 2006).

In accord with previous studies (Guignabert et al., 2005; McClintock et al., 2006; O'Neill et al., 2010; van Helden et al., 2004), we found that CEES-induced pulmonary injury in WT mice was associated with damage to the alveolar epithelial barrier, as measured by increases in BAL protein. This was observed 3 days after administration of the lower dose of CEES (3 mg/kg), and after 14 days with the higher dose (6 mg/kg). Delayed injury at the higher dose of CEES may reflect a distinct pathogenic process leading to chronic lung disease. Our findings that expression of MCP-1, a chemokine mediating the recruitment of profibrogenic macrophages (Wynn and Barron, 2010), was upregulated in the lung 14 days after exposure to high dose CEES are consistent with this idea. CEES administration to rodents has

previously been reported to result in acute pulmonary inflammation characterized by neutrophil and eosinophil accumulation in the lung (Mukherjee et al., 2009). In contrast, we found that CEES had no effect on the number of cells recovered in BAL. It may be that increases in BAL inflammatory cell number occur earlier than 3 days and are transient. This is supported by reports of increases in BAL cell number 1–24 h post exposure to vesicants (Malaviya et al., 2010; Mukherjee et al., 2009).

Oxidative stress is known to be important in vesicant-induced lung injury and is thought to be a primary event triggering the inflammatory cascade (Chatterjee et al., 2003; Mukherjee et al., 2009). Markers of oxidative stress, such as glutathione, malondialdehyde, 8-hydroxyguanosine and 4-hydroxynonenal, are increased in the respiratory tract after exposure of animals to vesicants including CEES (Kumar et al., 2001; Mukherjee et al., 2009; O'Neill et al., 2010). Moreover, treatment of animals with antioxidants reduces vesicant-induced lung injury and inflammation (Kumar et al., 2001; McClintock et al., 2002; Ucar et al., 2007; Wigenstam et al., 2009). Ym1 is a chitinase-like protein reported to be a marker of oxidative stress (Zhang et al., 2009). CEES administration resulted in a persistent increase in Ym1 expression in alveolar macrophages and bronchial epithelial cells in WT mice, consistent with induction of oxidative stress. Increases in Ym1 mRNA have been reported in the lung following exposure of mice to diesel exhaust particles, which also induce oxidative stress (Song et al., 2008). These findings suggest that expression of Ym1 may be a sensitive marker of oxidative stress induced by pulmonary irritants.

A characteristic response of cells and tissues to oxidative stress is upregulation of antioxidants such as CuZnSOD and MnSOD, two enzymes critical for limiting tissue injury by reducing oxidative stress (Kinnula and Crapo, 2003). Following CEES administration, we noted a transient induction of both CuZnSOD and MnSOD mRNA in the lung, providing additional evidence that CEES-induced toxicity is associated with oxidative stress. The observation that these antioxidants are present in alveolar macrophages and epithelial cells, suggests that both cell populations are major sources of cytotoxic oxidants. Intratracheal instillation of liposomes containing SOD and catalase has been reported to ameliorate CEES-induced lung injury (McClintock et al., 2006; Mukherjee et al., 2009). Additionally, a catalytic antioxidant possessing SOD and catalase activity was effective in reducing inflammation and oxidative stress following inhalation of CEES (O'Neill et al., 2010). Taken together, these data confirm that CEES-induced pulmonary toxicity involves an imbalance between oxidants and antioxidants.

In response to tissue injury and oxidative stress, macrophages mount an inflammatory response, characterized by the release of early response cytokines such as TNFα. Subsequently, macrophages release chemotactic and cytotoxic mediators, which stimulate inflammatory cell emigration and clearance of pathogens, dead cells and debris. Key proteins involved in the early proinflammatory and cytotoxic response are iNOS and COX-2, enzymes mediating the generation of nitric oxide and prostaglandins, respectively (Laskin et al., 2010c; Park and Christman, 2006). Excessive release of these mediators can exacerbate inflammation and tissue injury. Following CEES administration, expression of iNOS and COX-2 was upregulated in alveolar macrophages, as well as in alveolar and bronchial epithelial cells. These findings are in accord with previous reports on the pulmonary effects of vesicants such as sulfur mustard and nitrogen mustard (Malaviya et al., 2010; Sunil et al., 2011; Ucar et al., 2007). The observation that CEES-induced toxicity is ameliorated in mice lacking iNOS provides support for a role of reactive nitrogen species in the pathogenesis of vesicant-induced lung injury induced by this vesicant (Sunil et al., 2010). Whereas a contribution of COX-2 to the dermal toxicity of vesicants is well established (Babin et al., 2000; Casillas et al., 2000; Wormser et al., 2004), its role in pulmonary pathophysiology is unknown. At early stages of inflammation, COX-2 generates

proinflammatory prostaglandins from arachidonic acid. These eicosanoids are thought to be important in the pathogenesis of diseases such as asthma and COPD (Rolin et al., 2006), which are long-term effects of mustard gas poisoning. It remains to be determined if they play a similar role in acute and chronic pulmonary injury induced by vesicants.

Evidence suggests that macrophages recruited to sites of injury by the C-C chemokine, MCP-1 (CCL2) are alternatively activated and exhibit anti-inflammatory and wound healing activity (Laskin et al., 2010a; Wynn and Barron, 2010). It appears that the development of chronic diseases such as fibrosis involves aberrant response of this macrophage subpopulation. In this regard, a skewing towards an alternatively activated macrophage phenotype has been described in patients with idiopathic pulmonary fibrosis, COPD and cystic fibrosis, and in experimental models of bleomycin or silica-induced pulmonary fibrosis (Hancock et al., 1998; Migliaccio et al., 2008; Misson et al., 2004; Murray et al., 2010; Ortiz et al., 1999). A role for alternatively activated macrophages in the development of chronic lung disease is also supported by findings that pulmonary fibrosis is reduced in animals deficient in CCR2, the receptor for MCP-1 (Okuma et al., 2004). The present studies demonstrate that MCP-1 is upregulated in the lungs of WT mice. The fact that this was not evident until 14 days post-CEES treatment is consistent with a role of MCP-1 and alternatively activated macrophages in the pathogenesis of vesicant-induced pulmonary fibrosis.

Treatment of WT mice with CEES resulted in a rapid induction of TNFR1, suggesting an important role of TNFα signaling through this receptor in the pathogenic response to CEES. This is supported by our findings that BAL protein levels and Ym1 expression, markers of lung injury and oxidative stress, respectively, were at control levels in TNFR1−/− mice, and that there was no evidence of iNOS or COX-2 induction. A similar protective response to loss of TNFα or its receptor has previously been described in animals exposed to the pulmonary irritant ozone, as well as the fibrogenic toxicants bleomycin, silica and asbestos (Cho et al., 2007; Fakhrzadeh et al., 2008; Liu et al., 2001). Thus it appears that TNFα is key to both acute and chronic lung injury. CEES-induced MCP-1 expression was also abrogated in TNFR1−/− mice, which is consistent with reduced injury and, as a consequence, a reduced requirement for tissue repair macrophages.

Protection against CEES-induced lung injury in TNFR1−/− mice was also associated with delayed induction of mRNA for CuZnSOD and MnSOD and a persistent decrease in CuZnSOD protein. As indicated above, oxidative stress is an important early step in vesicant-induced injury not only to the lung, but also the skin (Laskin et al., 2010b). Our results showing delayed expression of SOD in TNFR1−/− mice demonstrate that TNFα also plays a role in regulating oxidant/antioxidant balance in the lung following vesicant exposure, and promoting oxidative stress. We also cannot rule out the possibility that infiltrating macrophages may account for the delayed increase in SOD in TNFR1−/− animals.

CEES administration to WT animals resulted in a number of changes in pulmonary function including significant decreases in total lung compliance. This is consistent with fluid and protein accumulation in alveolar spaces as a consequence of CEES-induced alveolar epithelial barrier dysfunction. We also noted increases in tissue elastance following CEES administration to WT mice, suggestive of the development of restrictive lung disease. Findings that these changes were blunted in TNFR1−/− mice provide additional evidence that these mice are protected from CEES-induced pulmonary toxicity.

Methacholine is a potent bronchoconstrictor used in animal models to examine hyper-responsiveness of the airway, and to assess airway wall stiffness and parenchymal elasticity

(Bates and Lauzon, 2007). As previously reported (Leme et al., 2010), we found that total lung resistance increased while compliance decreased in response to methacholine challenge in WT control animals. The increase in total lung resistance appears to be due to an increase in central airway resistance, indicating that this is primarily an airway effect. CEES administration caused a significant reduction in methacholine-induced increases in total lung resistance and airway resistance, with no major effects on lung compliance suggesting a loss of active airway tone, and the development of obstructive lung disease. These results are similar to our previous findings in animals treated with nitrogen mustard suggesting that these vesicants induce similar types of lung injury (Sunil et al., 2011). Of note, while CEES administration resulted in reduced lung constriction, relative to control, methacholine-induced decreases in compliance were unaltered. This may be explained by parenchymal injury and an accumulation of inflammatory mediators in the lower lung, which results in a reduced static elastic recoil. Loss of TNFR1, while having minimal effects on baseline functional characteristics of standard lung function, resulted in a significant reduction in methacholine-induced changes in lung responsiveness. In particular, the sensitivity of the central airway response to methacholine was considerably reduced; thus the maximal response was reached at a dose of 96 mg/ml in TNFR1−/− mice versus 48 mg/kg in WT mice. Compliance responses were also blunted in TNFR1−/− mice with static compliance, for the most part, unaffected by methacholine, and total compliance reduced in sensitivity, relative to WT mice. These data demonstrate that TNFα signaling through TNFR1 is important for maintaining normal lung responses to stress. This conclusion is supported by our findings that CEES treatment of TNFR1−/− mice, results in little or no changes in the central airway resistance response to methacholine, but increases both total and static compliance responses.

The principal functional effect of CEES in WT mice was a loss of airway responsiveness to methacholine, an effect that was also lacking in TNFR1−/− mice. In contrast, the compliance data indicate an increase in methacholine responsiveness in TNFR1−/− mice relative to WT, following CEES treatment. As airway restriction was not reduced in TNFR1−/− mice, this can be best explained by the persistent accumulation of inflammatory mediators within the parenchyma of WT mice and concomitant injury. Taken together, the functional data suggest that the role of TNFα in CEES-mediated injury was primarily in the airway, rather than within the lung parenchyma.

In summary, the present studies demonstrate that lung injury and oxidative stress induced by CEES is associated with increased expression of the proinflammatory/cytotoxic proteins iNOS and COX-2, as well as the chemokine, MCP-1, each of which has been implicated in lung injury and disease pathogenesis; moreover this is correlated with altered lung mechanics and function. Importantly, CEES-induced injury, oxidative stress, inflammatory mediator expression and functional alterations in the airways are abrogated in mice lacking TNFR1, directly demonstrating a key role of this signaling pathway in vesicant- induced lung injury. These findings suggest that targeting TNFα signaling may be valuable for the development of efficacious treatments of vesicant-induced pulmonary injury.

Acknowledgments

The authors wish to thank David Reimer, D.V.M., Laboratory Animal Services, Rutgers University, for performing animal instillations and Sherritta Ridgely, D.V.M., Ph.D., for histological evaluation of H&E stained sections.

Funding information

This work was supported by National Institutes of Health grants AR055073, ES004738, ES005022, CA132624, GM034310, and HL086621.

References

Aggarwal BB. Signalling pathways of the TNF superfamily: a double-edged sword. Nat. Rev. Immunol. 2003; 3:745–756. [PubMed: 12949498]

Babin MC, Ricketts K, Skvorak JP, Gazaway M, Mitcheltree LW, Casillas RP. Systemic administration of candidate antivesicants to protect against topically applied sulfur mustard in the mouse ear vesicant model (MEVM). J. Appl. Toxicol. 2000; 20(Suppl 1):S141–S144. [PubMed: 11428627]

Bates JH, Lauzon AM. Parenchymal tethering, airway wall stiffness, and the dynamics of bronchoconstriction. J. Appl. Physiol. 2007; 102:1912–1920. [PubMed: 17204575]

Bhalla DK. Ozone-induced lung inflammation and mucosal barrier disruption: toxicology, mechanisms, and implications. J. Toxicol. Environ. Health B Crit. Rev. 1999; 2:31–86. [PubMed: 10081525]

Bradley JR. TNF-mediated inflammatory disease. J. Pathol. 2008; 214:149–160. [PubMed: 18161752]

Casillas RP, Kiser RC, Truxall JA, Singer AW, Shumaker SM, Niemuth NA, Ricketts KM, Mitcheltree LW, Castrejon LR, Blank JA. Therapeutic approaches to dermatotoxicity by sulfur mustard. I. Modulaton of sulfur mustard-induced cutaneous injury in the mouse ear vesicant model. J. Appl. Toxicol. 2000; 20(Suppl 1):S145–S151. [PubMed: 11428628]

Chatterjee D, Mukherjee S, Smith MG, Das SK. Signal transduction events in lung injury induced by 2-chloroethyl ethyl sulfide, a mustard analog. J. Biochem. Mol. Toxicol. 2003; 17:114–121. [PubMed: 12717745]

Chen B, Tong Z, Ye Q, Nakamura S, Costabel U, Guzman J. Expression of tumour necrosis factor receptors by bronchoalveolar cells in hypersensitivity pneumonitis. Eur. Respir. J. 2005; 25:1039–1043. [PubMed: 15929959]

Cho HY, Zhang LY, Kleeberger SR. Ozone-induced lung inflammation and hyperreactivity are mediated via tumor necrosis factor-alpha receptors. Am. J. Physiol. Lung Cell. Mol. Physiol. 2001; 280:L537–L546. [PubMed: 11159038]

Cho HY, Morgan DL, Bauer AK, Kleeberger SR. Signal transduction pathways of tumor necrosis factor-mediated lung injury induced by ozone in mice. Am. J. Respir. Crit. Care Med. 2007; 175:829–839. [PubMed: 17255564]

Crosby LM, Waters CM. Epithelial repair mechanisms in the lung. Am. J. Physiol. Lung Cell. Mol. Physiol. 2010; 298:L715–L731. [PubMed: 20363851]

Dai H, Guzman J, Costabel U. Increased expression of apoptosis signalling receptors by alveolar macrophages in sarcoidosis. Eur. Respir. J. 1999; 13:1451–1454. [PubMed: 10445626]

Dai H, Guzman J, Chen B, Costabel U. Production of soluble tumor necrosis factor receptors and tumor necrosis factor-alpha by alveolar macrophages in sarcoidosis and extrinsic allergic alveolitis. Chest. 2005; 127:251–256. [PubMed: 15653992]

Debiak M, Kehe K, Burkle A. Role of poly(ADP-ribose) polymerase in sulfur mustard toxicity. Toxicology. 2009; 263:20–25. [PubMed: 18602966]

Ding M, Chen F, Shi X, Yucesoy B, Mossman B, Vallyathan V. Diseases caused by silica: mechanisms of injury and disease development. Int. Immunopharmacol. 2002; 2:173–182. [PubMed: 11811922]

Emad A, Emad Y. Levels of cytokine in bronchoalveolar lavage (BAL) fluid in patients with pulmonary fibrosis due to sulfur mustard gas inhalation. J. Interferon Cytokine Res. 2007; 27:38–43. [PubMed: 17266442]

Ermert M, Pantazis C, Duncker HR, Grimminger F, Seeger W, Ermert L. In situ localization of TNFalpha/beta, TACE and TNF receptors TNF-R1 and TNF-R2 in control and LPS-treated lung tissue. Cytokine. 2003; 22:89–100. [PubMed: 12849708]

Fakhrzadeh L, Laskin JD, Laskin DL. Regulation of caveolin-1 expression, nitric oxide production and tissue injury by tumor necrosis factor-alpha following ozone inhalation. Toxicol. Appl. Pharmacol. 2008; 227:380–389. [PubMed: 18207479]

Gaede KI, Fitschen J, Ernst M, Martinet N, Schlaak M, Muller-Quernheim J. Expression of tumour necrosis factor receptors (CD120a and CD120b) on bronchoalveolar cells. Cytokine. 1999; 11:611–616. [PubMed: 10433809]

Ghanei M, Harandi AA. Long term consequences from exposure to sulfur mustard: a review. Inhal. Toxicol. 2007; 19:451–456. [PubMed: 17365048]

Ghanei M, Naderi M, Kosar AM, Harandi AA, Hopkinson NS, Poursaleh Z. Long-term pulmonary complications of chemical warfare agent exposure in Iraqi Kurdish civilians. Inhal. Toxicol. 2010; 22:719–724. [PubMed: 20560732]

Guignabert C, Taysse L, Calvet JH, Planus E, Delamanche S, Galiacy S, d'Ortho MP. Effect of doxycycline on sulfur mustard-induced respiratory lesions in guinea pigs. Am. J. Physiol. Lung Cell. Mol. Physiol. 2005; 289:L67–L74. [PubMed: 15778244]

Hancock A, Armstrong L, Gama R, Millar A. Production of interleukin 13 by alveolar macrophages from normal and fibrotic lung. Am. J. Respir. Cell Mol. Biol. 1998; 18:60–65. [PubMed: 9448046]

Kehe K, Balszuweit F, Emmler J, Kreppel H, Jochum M, Thiermann H. Sulfur mustard research-strategies for the development of improved medical therapy. Eplasty. 2008; 8:e32. [PubMed: 18615149]

Kinnula VL, Crapo JD. Superoxide dismutases in the lung and human lung diseases. Am. J. Respir. Crit. Care Med. 2003; 167:1600–1619. [PubMed: 12796054]

Korkmaz A, Kunak ZI, Paredes SD, Yaren H, Tan DX, Reiter RJ. The use of melatonin to combat mustard toxicity. Neuro Endocrinol. Lett. 2008; 29:614–619. [PubMed: 18987575]

Kumar O, Sugendran K, Vijayaraghavan R. Protective effect of various antioxidants on the toxicity of sulphur mustard administered to mice by inhalation or percutaneous routes. Chem. Biol. Interact. 2001; 134:1–12. [PubMed: 11248218]

Laskin, DL.; Sunil, VR.; Laumbach, RJ.; Kipen, HM. Inflammatory cytokines and lung toxicity. In: House, R.; Descotes, J., editors. Cytokines in human health: immunotoxicology, pathology and therapeutic applications. Totowa, NJ: Humana Press Inc.; 2007. p. 83-112.

Laskin, DL.; Gardner, GR.; Laskin, JD. Phagocytes. In: Lawrence, D., editor. Comprehensive toxicology, immune system toxicology. UK: Elsevier; 2010a. p. 133-153.

Laskin JD, Black AT, Jan YH, Sinko PJ, Heindel ND, Sunil V, Heck DE, Laskin DL. Oxidants and antioxidants in sulfur mustard-induced injury. Ann. NY Acad. Sci. 2010b; 1203:92–100. [PubMed: 20716289]

Laskin, JD.; Heck, DE.; Laskin, DL. Nitric oxide pathways in toxic responses. In: Ballantyne, B.; Marrs, T.; Syversen, T., editors. General and applied toxicology. UK: Wiley-Blackwell; 2010c. p. 425-438.

Leme AS, Berndt A, Williams LK, Tsaih SW, Szatkiewicz JP, Verdugo R, Paigen B, Shapiro SD. A survey of airway responsiveness in 36 inbred mouse strains facilitates gene mapping studies and identification of quantitative trait loci. Mol. Genet. Genomics. 2010; 283:317–326. [PubMed: 20143096]

Liu JY, Sime PJ, Wu T, Warshamana GS, Pociask D, Tsai SY, Brody AR. Transforming growth factor-beta(1) overexpression in tumor necrosis factor-alpha receptor knockout mice induces fibroproliferative lung disease. Am. J. Respir. Cell Mol. Biol. 2001; 25:3–7. [PubMed: 11472967]

Malaviya R, Sunil VR, Cervelli J, Anderson DR, Holmes WW, Conti ML, Gordon RE, Laskin JD, Laskin DL. Inflammatory effects of inhaled sulfur mustard in rat lung. Toxicol. Appl. Pharmacol. 2010; 248:89–99. [PubMed: 20659490]

McClintock SD, Till GO, Smith MG, Ward PA. Protection from half-mustard-gas-induced acute lung injury in the rat. J. Appl. Toxicol. 2002; 22:257–262. [PubMed: 12210543]

McClintock SD, Hoesel LM, Das SK, Till GO, Neff T, Kunkel RG, Smith MG, Ward PA. Attenuation of half sulfur mustard gas-induced acute lung injury in rats. J. Appl. Toxicol. 2006; 26:126–131. [PubMed: 16252256]

Migliaccio CT, Buford MC, Jessop F, Holian A. The IL-4Ralpha pathway in macrophages and its potential role in silica-induced pulmonary fibrosis. J. Leukoc. Biol. 2008; 83:630–639. [PubMed: 18056481]

Misson P, van den Brule S, Barbarin V, Lison D, Huaux F. Markers of macrophage differentiation in experimental silicosis. J. Leukoc. Biol. 2004; 76:926–932. [PubMed: 15292275]

Mukherjee S, Stone WL, Yang H, Smith MG, Das SK. Protection of half sulfur mustard gas-induced lung injury in guinea pigs by antioxidant liposomes. J. Biochem. Mol. Toxicol. 2009; 23:143–153. [PubMed: 19367648]

Mukhopadhyay S, Hoidal JR, Mukherjee TK. Role of TNFalpha in pulmonary pathophysiology. Respir. Res. 2006; 7:125. [PubMed: 17034639]

Murray LA, Rosada R, Moreira AP, Joshi A, Kramer MS, Hesson DP, Argentieri RL, Mathai S, Gulati M, Herzog EL, Hogaboam CM. Serum amyloid P therapeutically attenuates murine bleomycin-induced pulmonary fibrosis via its effects on macrophages. PLoS ONE. 2010; 5:e9683. [PubMed: 20300636]

Okuma T, Terasaki Y, Kaikita K, Kobayashi H, Kuziel WA, Kawasuji M, Takeya M. C-C chemokine receptor 2 (CCR2) deficiency improves bleomycin-induced pulmonary fibrosis by attenuation of both macrophage infiltration and production of macrophage-derived matrix metalloproteinases. J. Pathol. 2004; 204:594–604. [PubMed: 15538737]

O'Neill HC, White CW, Veress LA, Hendry-Hofer TB, Loader JE, Min E, Huang J, Rancourt RC, Day BJ. Treatment with the catalytic metalloporphyrin AEOL 10150 reduces inflammation and oxidative stress due to inhalation of the sulfur mustard analog 2-chloroethyl ethyl sulfide. Free Radic. Biol. Med. 2010; 48:1188–1196. [PubMed: 20138141]

Ortiz LA, Lasky J, Lungarella G, Cavarra E, Martorana P, Banks WA, Peschon JJ, Schmidts HL, Brody AR, Friedman M. Upregulation of the p75 but not the p55 TNF-alpha receptor mRNA after silica and bleomycin exposure and protection from lung injury in double receptor knockout mice. Am. J. Respir. Cell Mol. Biol. 1999; 20:825–833. [PubMed: 10101016]

Ortiz LA, Lasky J, Gozal E, Ruiz V, Lungarella G, Cavarra E, Brody AR, Friedman M, Pardo A, Selman M. Tumor necrosis factor receptor deficiency alters matrix metalloproteinase 13/tissue inhibitor of metalloproteinase 1 expression in murine silicosis. Am. J. Respir. Crit. Care Med. 2001; 163:244–252. [PubMed: 11208652]

Papirmeister, B.; Feister, AJ.; Robinson, SI.; Ford, RD. Medical defense against mustard gas: toxic mechanims and pharmacological implications. Boca Raton, FL: CRC Press; 1991.

Park GY, Christman JW. Involvement of cyclooxygenase-2 and prostaglandins in the molecular pathogenesis of inflammatory lung diseases. Am. J. Physiol. Lung Cell. Mol. Physiol. 2006; 290:L797–L805. [PubMed: 16603593]

Ray R, Simbulan-Rosenthal CM, Keyser BM, Benton B, Anderson D, Holmes W, Trabosh VA, Daher A, Rosenthal DS. Sulfur mustard induces apoptosis in lung epithelial cells via a caspase amplification loop. Toxicology. 2010; 271:94–99. [PubMed: 20226831]

Rolin S, Masereel B, Dogne JM. Prostanoids as pharmacological targets in COPD and asthma. Eur. J. Pharmacol. 2006; 533:89–100. [PubMed: 16458293]

Shimabukuro DW, Sawa T, Gropper MA. Injury and repair in lung and airways. Crit. Care Med. 2003; 31:S524–S531. [PubMed: 12907882]

Song HM, Jang AS, Ahn MH, Takizawa H, Lee SH, Kwon JH, Lee YM, Rhim TY, Park CS. Ym1 and Ym2 expression in a mouse model exposed to diesel exhaust particles. Environ. Toxicol. 2008; 23:110–116. [PubMed: 18214922]

Sunil VR, Patel KJ, Shen JL, Reimer D, Gow AJ, Laskin JD, Laskin DL. Role of reactive nitrogen species in vesicant-induced lung injury and altered lung functioning. Toxicologist. 2010; 114:A336.

Sunil VR, Patel KJ, Shen JL, Reimer D, Gow AJ, Laskin JD, Laskin DL. Functional and inflammatory alterations in the lung following exposure of rats to nitrogen mustard. Toxicol Appl Pharmacol. 2011; 250:10–16. [PubMed: 20883710]

Ucar M, Korkmaz A, Reiter RJ, Yaren H, Oter S, Kurt B, Topal T. Melatonin alleviates lung damage induced by the chemical warfare agent nitrogen mustard. Toxicol. Lett. 2007; 173:124–131. [PubMed: 17765411]

van Helden HP, Kuijpers WC, Diemel RV. Asthma like symptoms following intratracheal exposure of Guinea pigs to sulfur mustard aerosol: therapeutic efficacy of exogenous lung surfactant curosurf and salbutamol. Inhal. Toxicol. 2004; 16:537–548. [PubMed: 15204745]

Weinberger B, Laskin JD, Sunil VR, Sinko PJ, Heck DE, Laskin DL. Sulfur mustard-induced pulmonary injury: Therapeutic approaches to mitigating toxicity. Pulmonary Pharmacology and Therapeutics. in press.

Wigenstam E, Rocksen D, Ekstrand-Hammarstrom B, Bucht A. Treatment with dexamethasone or liposome-encapsuled vitamin E provides beneficial effects after chemical-induced lung injury. Inhal. Toxicol. 2009; 21:958–964. [PubMed: 19572781]

Wormser U, Langenbach R, Peddada S, Sintov A, Brodsky B, Nyska A. Reduced sulfur mustard-induced skin toxicity in cyclooxygenase-2 knockout and celecoxib-treated mice. Toxicol. Appl. Pharmacol. 2004; 200:40–47. [PubMed: 15451306]

Wynn TA, Barron L. Macrophages: master regulators of inflammation and fibrosis. Semin. Liver Dis. 2010; 30:245–257. [PubMed: 20665377]

Yaren H, Mollaoglu H, Kurt B, Korkmaz A, Oter S, Topal T, Karayilanoglu T. Lung toxicity of nitrogen mustard may be mediated by nitric oxide and peroxynitrite in rats. Res. Vet. Sci. 2007; 83:116–122. [PubMed: 17196628]

Zhang M, Qian J, Xing X, Kong FM, Zhao L, Chen M, Lawrence TS. Inhibition of the tumor necrosis factor-alpha pathway is radioprotective for the lung. Clin. Cancer Res. 2008; 14:1868–1876. [PubMed: 18347190]

Zhang L, Wang M, Kang X, Boontheung P, Li N, Nel AE, Loo JA. Oxidative stress and asthma: proteome analysis of chitinase-like proteins and FIZZ1 in lung tissue and bronchoalveolar lavage fluid. J. Proteome Res. 2009; 8:1631–1638. [PubMed: 19714806]

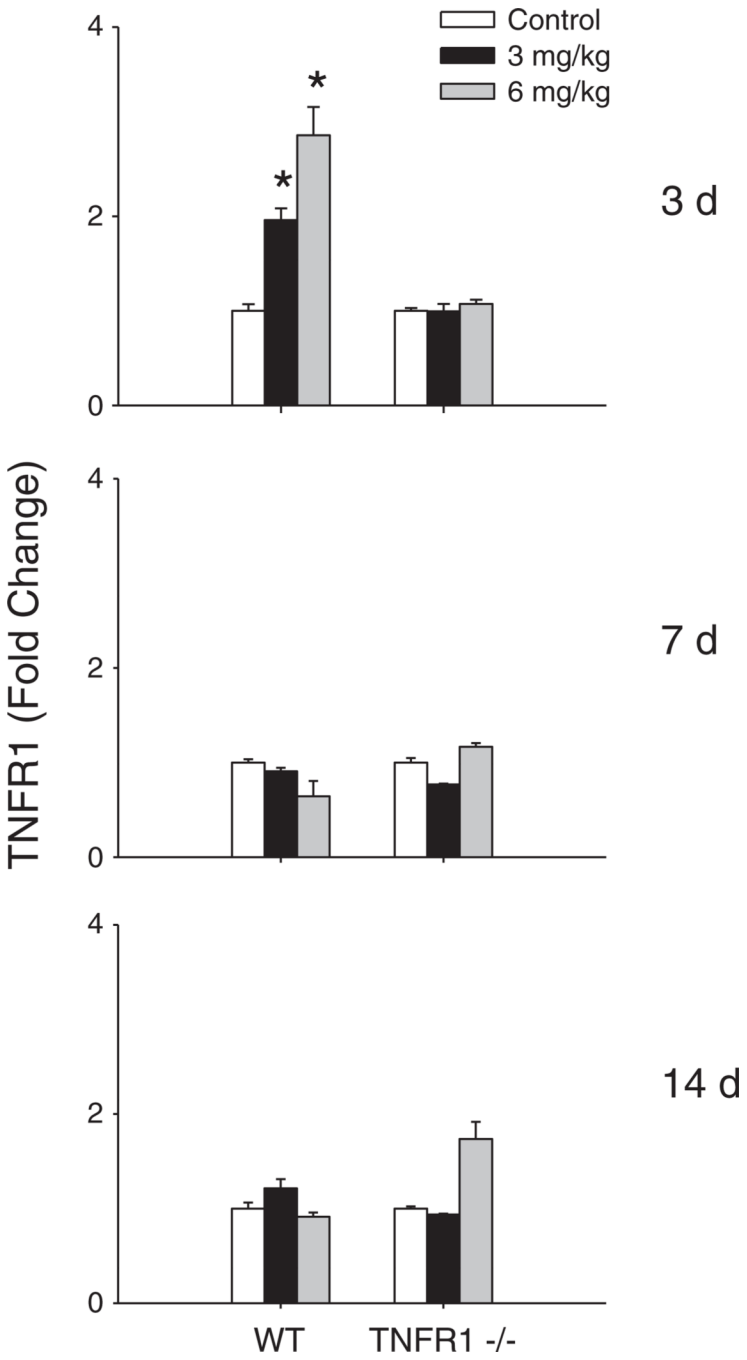

Fig. 1.

Effects of CEES on TNFR1 mRNA expression. Lung tissue was collected 3, 7 or 14 days after treatment of WT and TNFR1−/− mice with control or CEES (3 mg/kg or 6 mg/kg). RNA was extracted from the tissue, pooled and analyzed in triplicate by real time PCR for TNFR1 gene expression. Data were normalized to GAPDH and presented as fold change relative to control. Each bar is the average ± SE (n = 3 mice). *Significantly different ($p \leq 0.05$) from control.

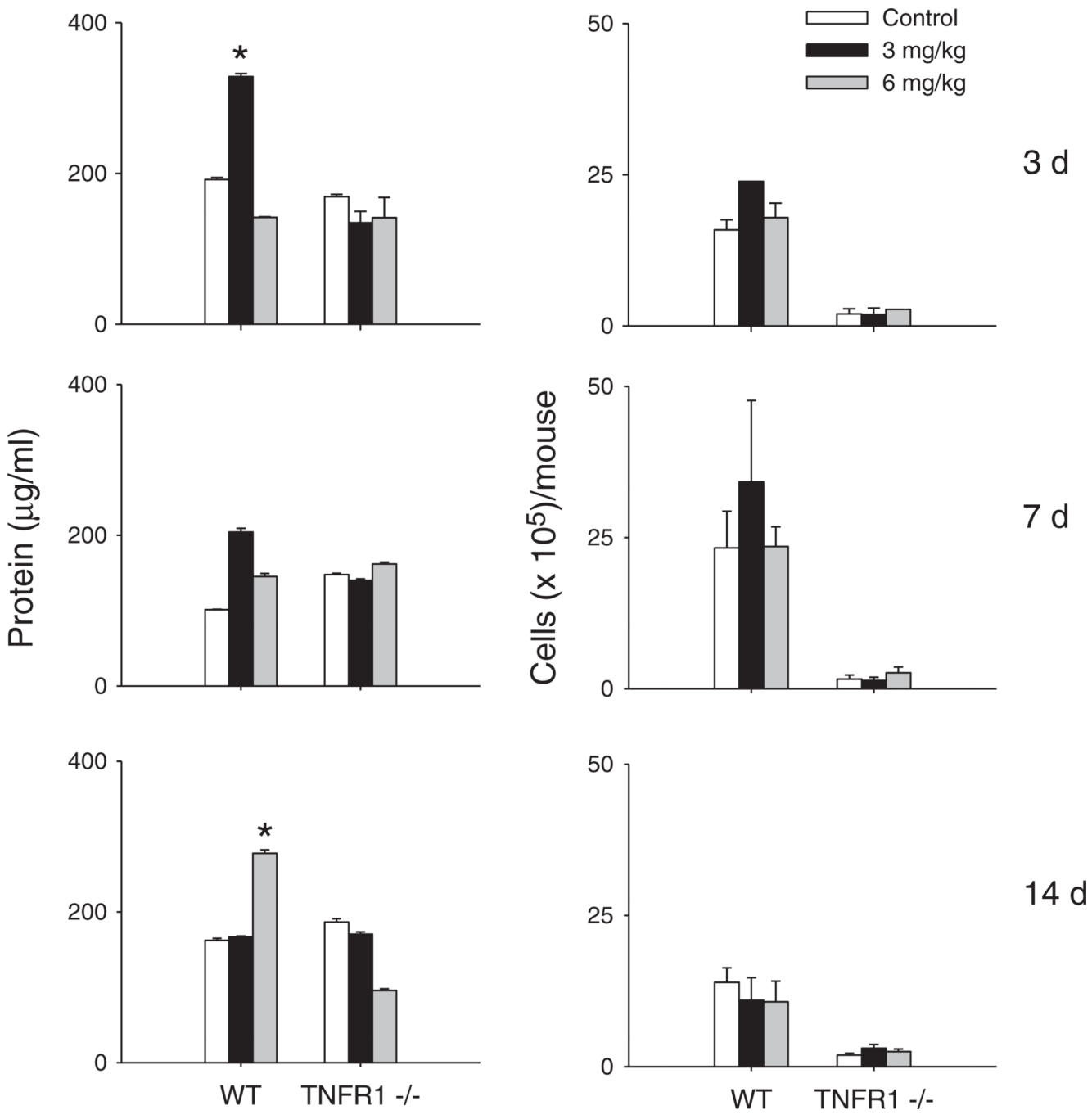

Fig. 2.

Effects of CEES on BAL protein and cell number. BAL was collected 3, 7 or 14 days after treatment of WT and TNFR1−/− mice with control or CEES (3 mg/kg or 6 mg/kg). (Left panels) Cell-free supernatant was analyzed in triplicate for protein using a BCA protein assay kit. (Right panels) Viable cells in BAL were enumerated by trypan blue dye exclusion. Each bar is the average ± SE (n = 3 mice). *Significantly different ($p \leq 0.05$) from control.

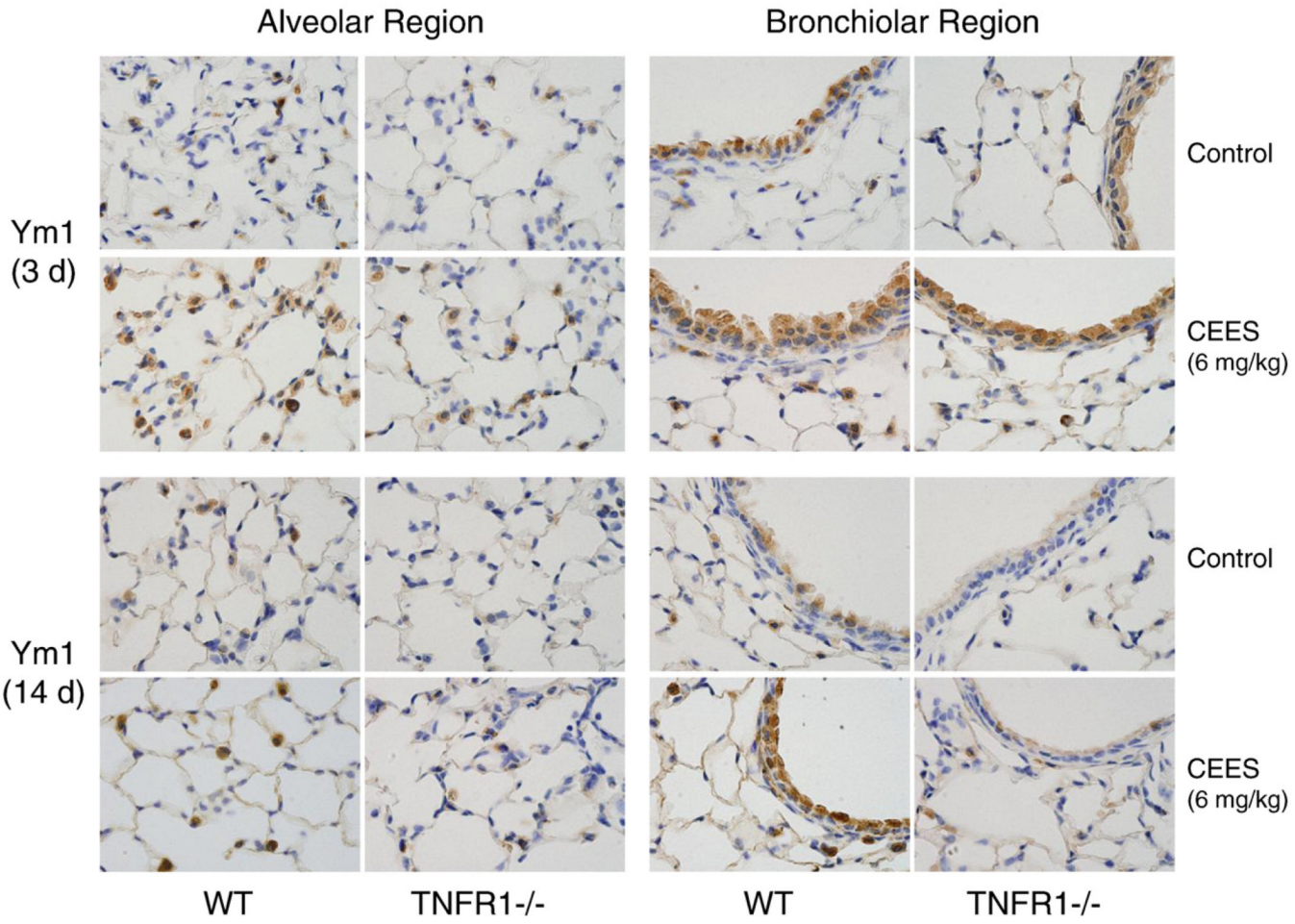

Fig. 3.

Effects of loss of TNFR1 on CEES-induced Ym1 expression. Lung sections prepared 3 day (upper panels) or 14 day (lower panels) after treatment of WT and TNFR1−/− mice with control or CEES (6 mg/kg) were stained with antibody to Ym1. Binding was visualized using a peroxidase DAB substrate kit. One representative section of alveolar and bronchiolar region from three separate experiments is shown (Original magnification, × 1000).

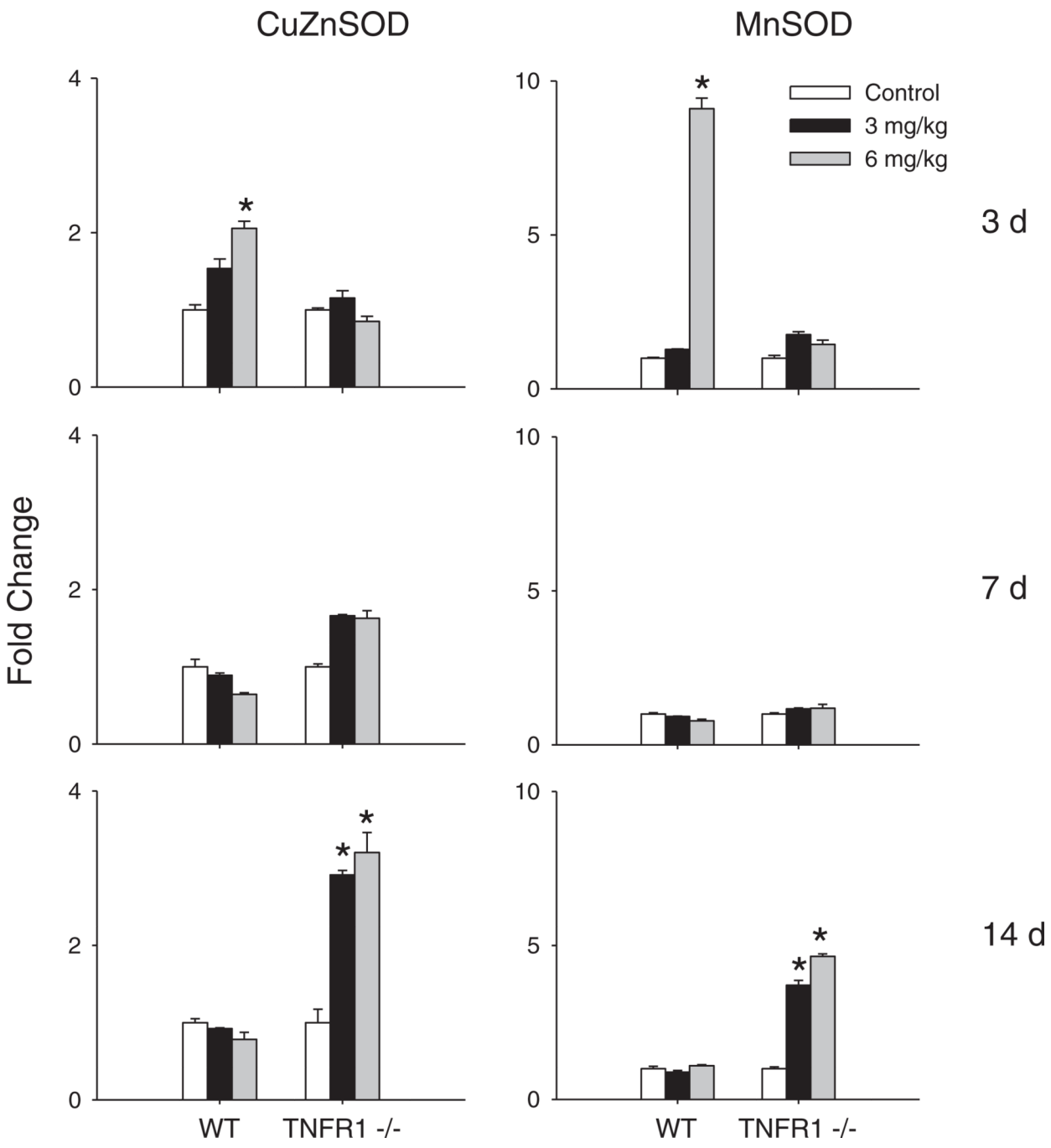

Fig. 4.

Effects of loss of TNFR1 on CEES-induced antioxidant gene expression. Lung tissue was collected 3, 7 or 14 day after treatment of WT and TNFR1−/− mice with control or CEES (3 mg/kg or 6 mg/kg). RNA was extracted from the tissue, pooled and analyzed in triplicate by real time PCR for CuZnSOD (left panels) and MnSOD (right panels) gene expression. Data were normalized to GAPDH and presented as fold change relative to control. Each bar is the average ± SE (n = 3 mice). *Significantly different ($p \leq 0.05$) from control.

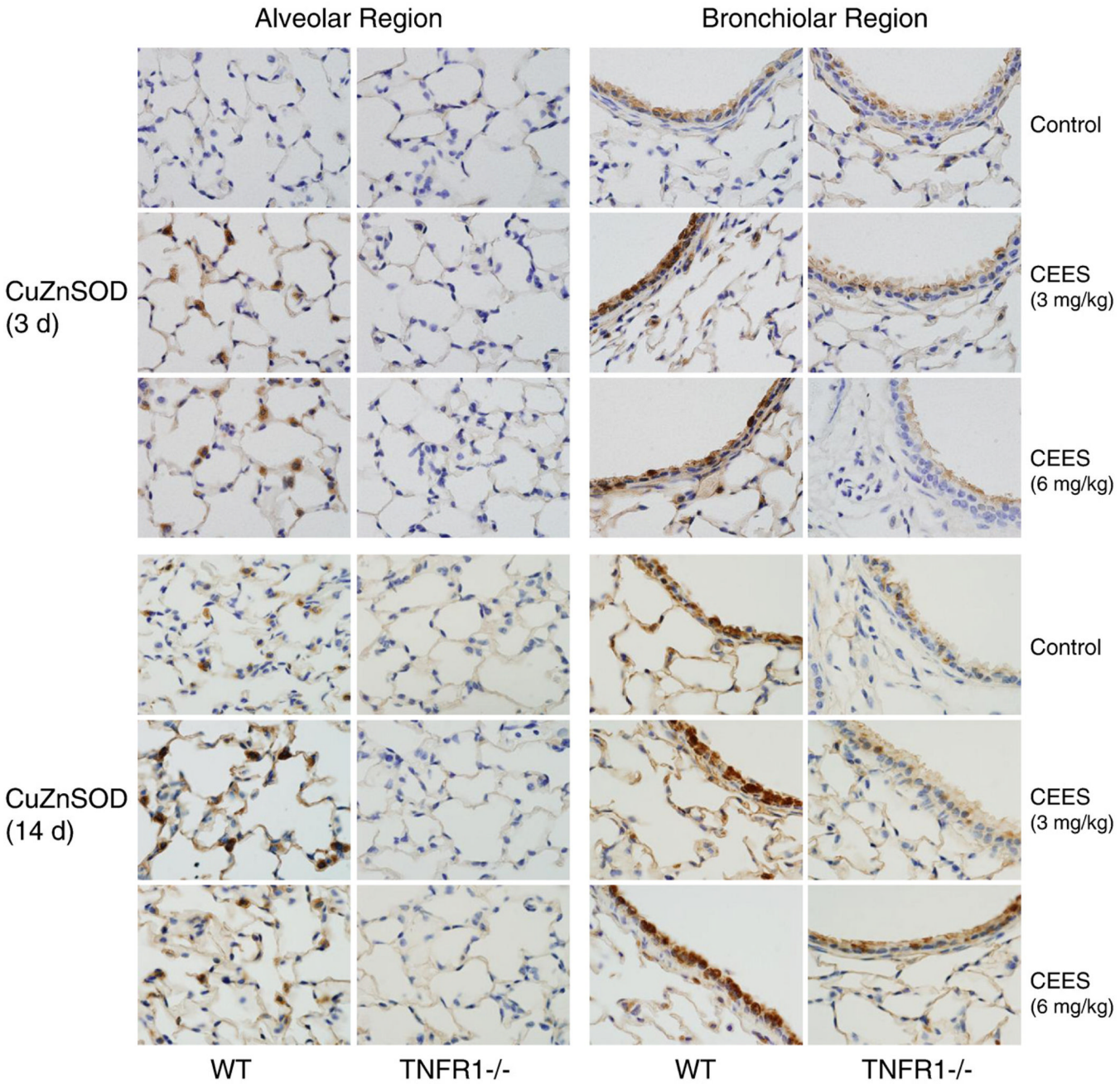

Fig. 5.

Effects of loss of TNFR1 on CEES-induced CuZnSOD protein expression. Lung sections, prepared 3 day (upper panels) or 14 day (lower panels) after treatment of WT and TNFR1−/− mice with control or CEES (3 mg/kg or 6 mg/kg) were stained with antibody to CuZnSOD. Binding was visualized using a peroxidase DAB substrate kit. One representative section of alveolar and bronchiolar regions from three separate experiments is shown (Original magnification, ×1000).

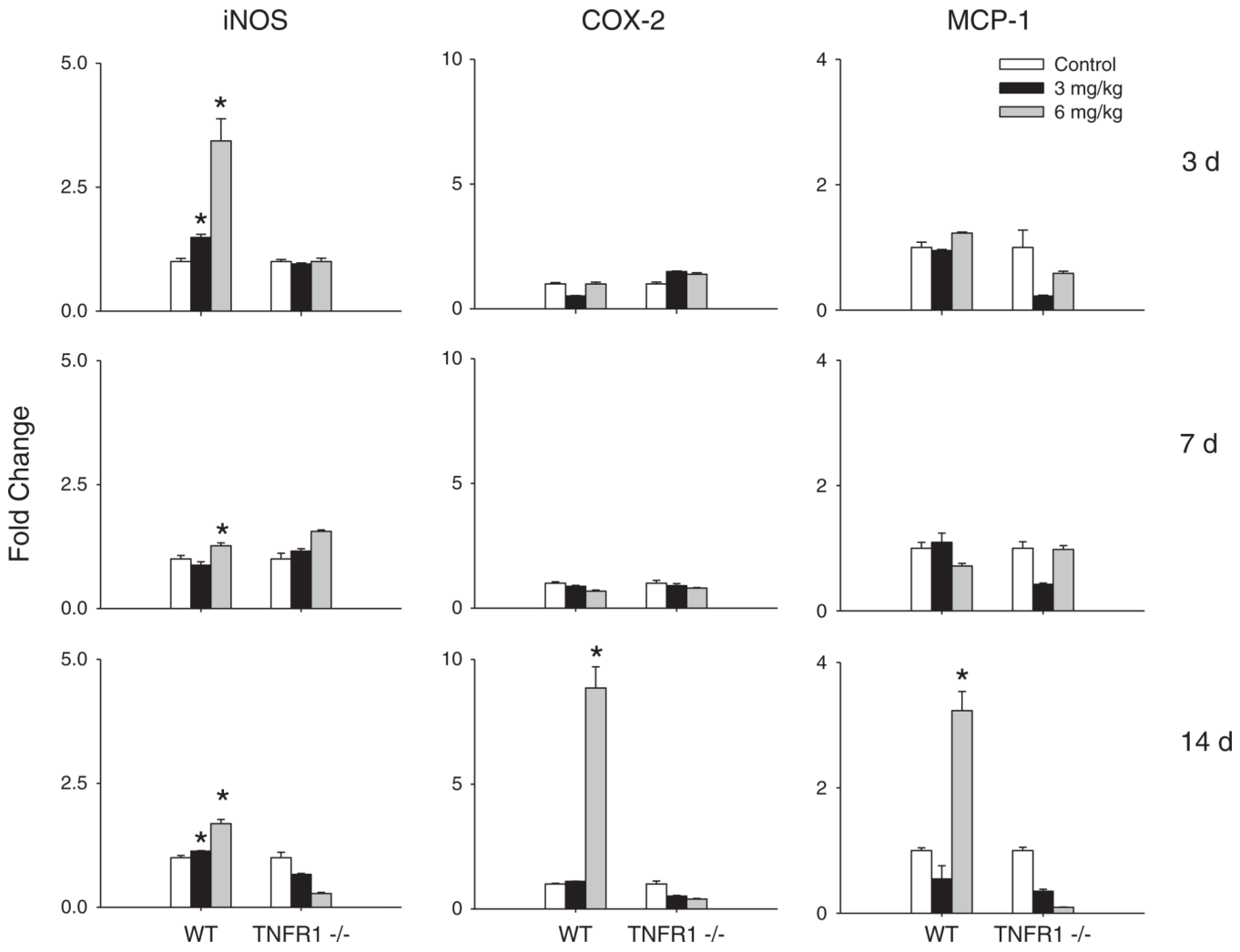

Fig. 6.

Effects of loss of TNFR1 on CEES-induced inflammatory mediator gene expression. Lung tissue was collected 3, 7 or 14 days after treatment of WT and TNFR1−/− mice with control or CEES (3 mg/kg or 6 mg/kg). RNA was extracted from the tissue, pooled and analyzed in triplicate by real time PCR for iNOS, COX-2 and MCP-1 gene expression. Data were normalized to GAPDH and presented as fold change relative to control. Each bar is the average ± SE ($n = 3$ mice). *Significantly different ($p \leq 0.05$) from control.

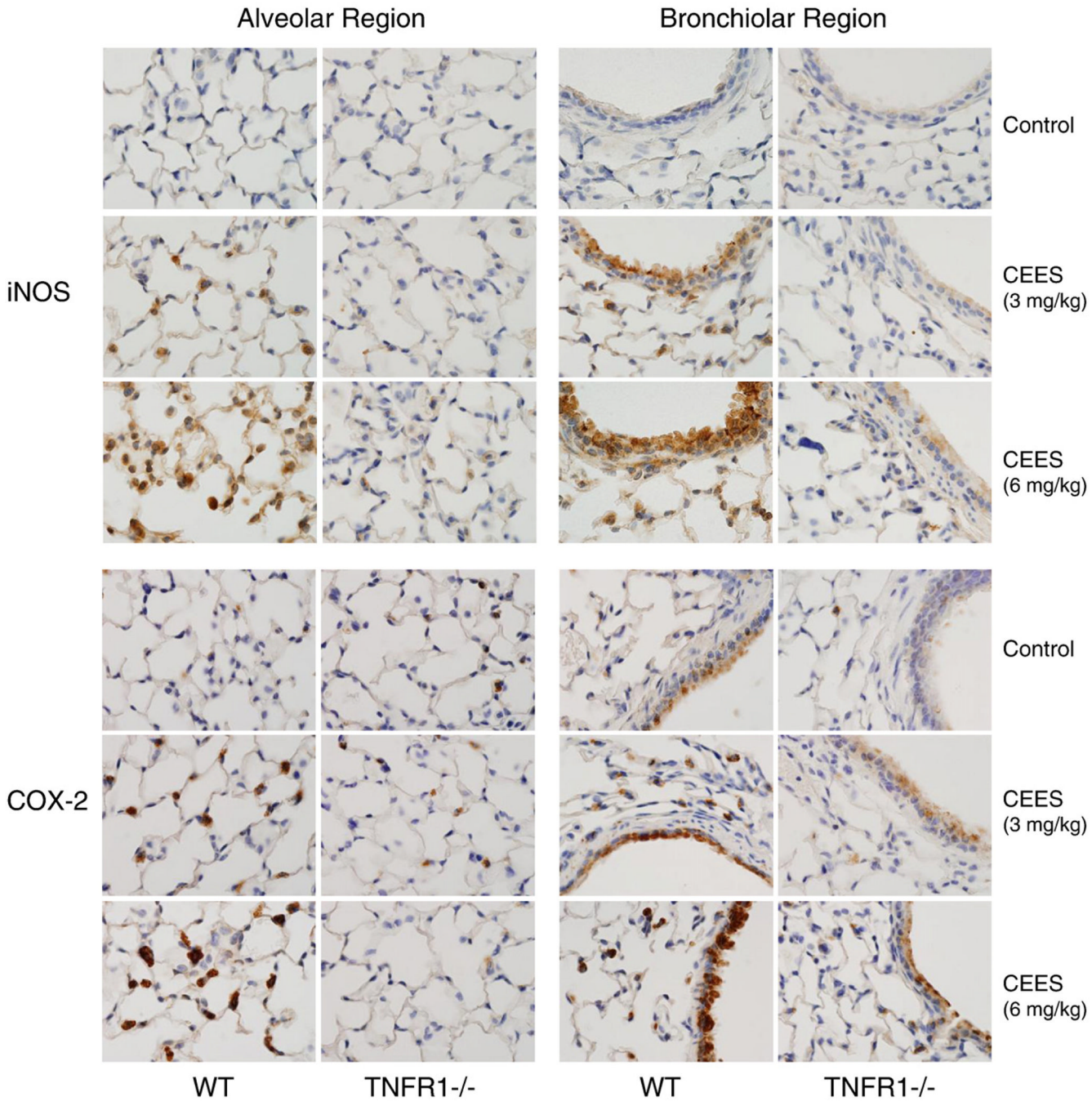

Fig. 7.

Effects of loss of TNFR1 on CEES-induced iNOS and COX-2 protein expression. Lung sections prepared 3 days after treatment of WT and TNFR1−/− mice with control or CEES (3 mg/kg or 6 mg/kg) were stained with antibody to iNOS (upper panels) or COX-2 (lower panels). Binding was visualized using a peroxidase DAB substrate kit. One representative section of alveolar and bronchiolar region from three separate experiments is shown (Original magnification, ×1000).

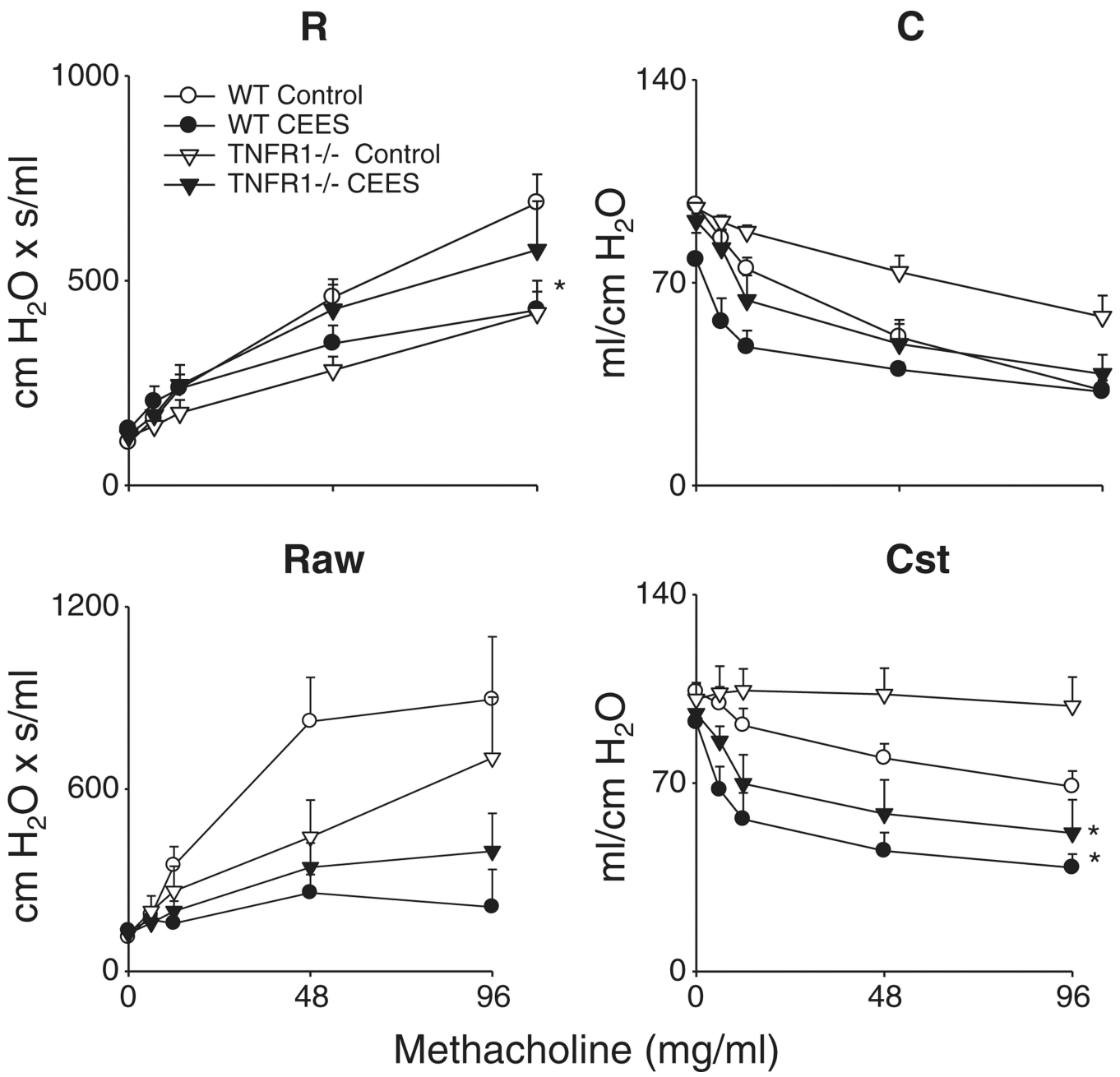

Fig. 8.

Effects of CEES on lung function in response to methacholine challenge. Total lung resistance (R), total lung compliance (C), central airway resistance (Raw) and static compliance (Cst) were evaluated in response to increasing doses of methacholine following exposure of WT and TNFR1−/− mice to PBS (Control) or CEES (6 mg/kg). Values are normalized and expressed as percentage change from baseline. Each point is the average ± SE (n = 5–6 mice). *Significantly different ($p \leq 0.05$) from control.

Table 1

Effects of CEES on baseline lung function. Total lung resistance, central airway resistance, total lung compliance, static compliance, tissue damping and elastance were evaluated following exposure of WT and TNFR1−/− mice to PBS (Control) or CEES (6 mg/kg). Each data point represents the absolute value, mean ± SE (n = 5–6 mice).

	WT		TNFR1−/−	
	Control	**CEES**	**Control**	**CEES**
Resistance	0.7 ± 0.1	1.3 ± 0.4	1.0 ± 0.3	1.8 ± 0.6
Central airway resistance	0.259 ± 0.03	0.771 ± 0.4	0.435 ± 0.1	0.847 ± 0.4
Compliance	0.033 ± 0.003	0.019 ± 0.004*	0.030 ± 0.003	0.023 ± 0.003
Static compliance	0.076 ± 0.008	0.064 ± 0.007	0.056 ± 0.006	0.055 ± 0.010
Tissue damping	6.6 ± 0.4	11.5 ± 2.8	7.0 ± 1.6	13.9 ± 3.7
Tissue elastance	30.3 ± 2.9	50.5 ± 7.3*	33.6 ± 6.3	41.5 ± 6.6

*Significantly different ($p \leq 0.05$) from control.