Role of Metallothionein in Antigen-Related Airway Inflammation

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Metallothionein (MT) is a protein that can be induced by inflammatory mediators and participates in cytoprotection. However, its role in antigen-related inflammation remains to be established. We determined whether intrinsic MT protects against antigen-related airway inflammation induced by ovalbumin (OVA) in MT-I/II null (MT [-/-]) mice and in corresponding wild-type (WT) mice. MT (-/-) mice and WT mice were intratracheally challenged with OVA (1 µg per body) biweekly four times. Twenty-four hours after the last OVA challenge, significant increases were shown in the numbers of total cells, eosinophils, and neutrophils in bronchoalveolar lavage fluid from MT (-/-) mice than in those from WT mice. The protein level of interleukin-1β (IL-1β) was significantly greater in MT (-/-) mice than in WT mice after OVA challenge. Immunohistochemical analysis showed that the formations of 8-oxy-deoxyguanosine and nitrotyrosine in the lung were more intense in MT (-/-) mice than in WT mice after OVA challenge. These results indicate that endogenous MT is a protective molecule against antigen-related airway inflammation induced by OVA, at least partly, via the suppression of enhanced lung expression of IL-1β and via the antioxidative properties. Our findings suggest that MT may be a therapeutic target for the treatment of antigen-related airway inflammatory diseases such as bronchial asthma. Exp Biol Med 230:75–81, 2005

Key words: metallothionein; ovalbumin; airway inflammation; cytokine; oxidative stress

Metallothionein (MT) is a highly conserved, low-molecular-weight, cysteine-rich protein. Since cysteine residues of MT bind and store metal ions, MT has been proposed to play an important role in homeostasis and detoxication of heavy metals (1). Metallothionein can react with free radicals and electrophiles because of its high sulfhydryl content (1, 2). Also, MT can serve as a sacrificial scavenger for hydroxyl radicals *in vitro* (3) and protects against free radical–induced DNA damage (4–6). In addition, MT is induced by heavy metals or oxidative stress–producing chemicals (7) and exhibits cytoprotection against toxicity of heavy metals or alkylating anticancer drugs (8), as well as against oxidative stress–related organ damage (9, 10). Both the *MT-I* and *MT-II* genes are constitutively expressed and are highly inducible by metals, glucocorticoids, and inflammatory mediators. Proinflammatory cytokines, including tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1), induce *MT* gene expression *in vivo*. There are conflicting reports about the role of MT in inflammatory processes. In brief, MT plays a pivotal role in mediating the harmful effects of TNF (11). In contrast, MT-I/II null (MT [-/-]) mice are more sensitive to lipopolysaccharide (LPS) (12). Also, we have recently demonstrated that MT (-/-) mice are more susceptible than wild-type (WT) mice to acute lung inflammation induced by intratracheal administration of LPS (13). However, further *in vivo* studies that use other inflammatory stimuli are required to establish the roles of MT in inflammatory disorders.

Bronchial asthma is a complex syndrome, characterized by obstruction, hyperresponsiveness, and inflammation of the airways. Inflammation in asthma is characterized by an accumulation of eosinophils, lymphocytes, and neutrophils in the bronchial wall and lumen (14–16). The mechanisms via which inflammatory cells alter airway function in asthmatic conditions include release of chemotactic mediators, such as IL-4 and eotaxin, and various proteases, as well as generation of reactive oxygen species. Although we and others have indicated the protective role of MT in lung inflammation induced by bacterial endotoxin

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(13, 17), there is no evidence about the direct contribution of MT in antigen-related airway inflammation.

The present study was undertaken to determine the role of MT in antigen-related airway inflammation induced by ovalbumin (OVA) using MT (–/–) mice and control WT mice. Also, we investigated the causal mechanisms by which MT protects against antigen-related airway inflammation.

Materials and Methods

Animals and Study Protocol. The MT (–/–) mice, whose *MT-I* and *MT-II* genes had null mutation, and corresponding WT mice were provided by Dr. Choo (Murdoch Institute for Research into Birth Defects, Royal Children's Hospital, Parkville, Australia) (18). The mice were of a mixed genetic background of 129 Ola and C57BL/6 strains. The F1 hybrid mice were mated with C57BL/6 mice, and their offspring were back-crossed to C57BL/6 for six generations in the National Institute for Environmental Studies (NIES) database. The MT (–/–) mice and WT mice were obtained by mating of those heterozygous MT (+/–) mice. The MT (–/–) mice and WT mice were routinely bled in the vivarium of the NIES. Microbiological and viral examinations were performed with regular quarantine procedures for a more than 1-year period, and neither pathogenic infections nor significant phenotypical abnormalities were found. Both genotypes of mice were housed in cages in ventilated rooms with a controlled temperature of 23° ± 1°C, a relative humidity of 55% ± 10%, and a 12:12-hr light:dark cycle. They were maintained on standard laboratory chow and tap water *ad libitum*, and they received humane care throughout the experiment according to the guidelines of the NIES.

The MT (–/–) and WT mice were treated with vehicle or OVA. In both genotypes, the vehicle groups intratracheally received 100 μl of phosphate-buffered saline at pH 7.4 (Nissui Pharmaceutical Co., Tokyo, Japan). The OVA groups received 1 μg of OVA (Sigma Chemical, St. Louis, MO) dissolved in the same vehicle. Both vehicle and OVA were administered every 2 weeks for four times. Intratracheal inoculation was conducted using a polyethylene tube under anesthesia with 4% halothane (Hoechst Japan, Tokyo, Japan) as described previously (19, 20). All mice were sacrificed by deep anesthesia using diethylether 24 hrs after the instillation.

Bronchoalveolar Lavage (BAL). The trachea was cannulated after exsanguination. The lungs were lavaged with 1.2 ml of sterile saline at 37°C, instilled bilaterally by syringe. The lavaged fluid was harvested by gentle aspiration. This procedure was conducted two more times. The average volume retrieved was 90% of the 3.6 ml that was instilled; the amounts did not differ by treatment. The fluid collections were combined and cooled to 4°C. The lavage fluid was centrifuged at 300 *g* for 10 mins, and the total cell count was determined on a fresh fluid specimen using a hemocytometer. Differential cell counts were assessed on cytologic preparation. Slides were prepared using a Cytospin (Tomy Seiko, Tokyo, Japan) and were stained with Diff-Quik (International Reagents Co., Kobe, Japan). A total of 500 cells were counted under oil immersion microscopy (n = 8–10 in each group).

Histological Examination. After exsanguinations, the lungs were fixed by intratracheal instillation with 10% neutral phosphate-buffered formalin (pH 7.4) and embedded in paraffin. Sections 4 μm thick were routinely processed with hematoxylin-eosin stain as previously described (n = 5 in each group) (19).

Quantitation of Cytokines in Lung Tissue Supernatants. In a separate series of experiments, animals were exsanguinated, and the lungs were subsequently homogenized with 10 m*M* potassium phosphate buffer (pH 7.4) containing 0.1 m*M* EDTA (Sigma), 0.1 m*M* phenylmethanesuiphonyl fluoride (Nacalai Tesque, Kyoto, Japan), 1 μ*M* pepstatin A (Peptide Institute, Osaka, Japan), and 2 μ*M* leupeptin (Peptide Institute) as described previously (20). The homogenates were then centrifuged at 105,000 *g* for 1 hr. The supernatants were stored at –80°C. Enzyme-linked immunosorbent assays for IL-1β (Endogen, Cambridge, MA), IL-4 (Amersham, Buckinghamshire, UK), and eotaxin (R&D Systems, Minneapolis, MN) in the lung tissue supernatants were conducted using matching antibody pairs according to the manufacturer's instruction. The second antibodies were conjugated to horseradish peroxidase. Subtractive readings of 550 nm from the readings at 450 nm were converted to picograms per milliliter using values obtained from standard curves generated with the limits of detection of 3, 5, and 3 pg/ml for IL-1β, IL-4, and eotaxin, respectively (n = 8–10 in each group).

Immunohistochemical Analysis. The production of 8-hydroxydeoxyguanosine (8-OHdG) and nitrotyrosine in the lung was detected by the immunohistochemical localization using anti-8-OHdG polyclonal antibody and antinitrotyrosine polyclonal antibody. Antinitrotyrosine polyclonal antibody was prepared as described previously (21). Polyclonal antibody to recognize 8-OHdG was obtained from the Japan Institute for the Control of Aging (Shizuoka, Japan). Deparaffinized slides were placed in blocking reagent that contained H_2O_2 for 45 secs to quench the endogenous peroxidase. The sections were then blocked with 10% nonimmuno goat serum for 1 hr. After blocking, anti-8-OHdG (0.5 μg/ml) or antinitrotyrosine (0.05 μg/ml) was incubated with the sections for 18 hrs at 4°C in a moist chamber. Incubation was followed by the addition of a biotinylated secondary antibody and streptavidin-peroxidase conjugate. The slides were incubated with 3-amino, 9-ethylcarbazole chromogen and counterstained with hematoxylin using the AutoProbe III kit (n = 5 in each group: Biomeda, Foster City, CA).

Statistical Analysis. Data are reported as mean ± SEM. Differences among groups were determined using

Table 1. Cellular Profiles in Bronchoalveolar Lavage Fluid[a]

Genotype and challenge	Cell counts in BAL fluid ($\times 10^4$)			
	Total cells	Neutrophils	Eosinophils	Macrophages
Wild type				
Vehicle	14.0 ± 1.3	0.3 ± 0.1	0.1 ± 0.0	13.7 ± 1.2
Ovalbumin	20.1 ± 6.4	3.8 ± 1.6	1.2 ± 0.7	15.0 ± 4.3
MT (–/–)				
Vehicle	12.6 ± 1.5	0.4 ± 0.2	0.2 ± 0.2	11.2 ± 1.9
Ovalbumin	45.4 ± 1.5**, ***	16.8 ± 10.1*	3.3 ± 1.1**, ***	25.0 ± 4.7**

[a] Four groups of mice were intratracheally administered vehicle or ovalbumin (OVA) every 2 weeks for 6 weeks. Bronchoalveolar lavage (BAL) fluid was conducted 24 hrs after the last intratracheal instillation. Total cell counts were determined on fresh BAL fluid, and differential cell counts were assessed with Diff-Quik staining (n = 8–10 in each group). Results are presented as mean ± SEM. MT (–/–), MT-I/II null.
*$P < 0.05$ versus vehicle-treated MT (–/–) mice. **$P < 0.01$ versus vehicle-treated MT (–/–) mice. ***$P < 0.05$ versus OVA-challenged wild-type mice.

analysis of variance with post hoc test (StatView, version 4.0; Abacus Concepts, Inc., Berkeley, CA) as previously described (20).

Results

MT Protects Against Airway Inflammation Related to Antigen. To determine the role of MT in airway inflammation related to OVA, we investigated the cellular profile of BAL fluid 24 hrs after the last intratracheal instillation. In both genotypes of mice, OVA treatment induced increases in the numbers of total cells, neutrophils, eosinophils, and macrophages compared with vehicle treatment (Table 1). The differences did not achieve statistical significance in WT mice. In MT (–/–) mice, however, OVA challenge showed significant increases in the numbers of these cells compared with vehicle challenge (Table 1; $P < 0.05$ for neutrophils, $P < 0.01$ for total cells, eosinophils, and macrophages). After OVA treatment, the numbers of total cells and eosinophils were significantly greater in MT (–/–) mice than in WT mice (Table 1; $P < 0.05$).

We next evaluated histopathological assessment of the lungs 24 hrs after the final intratracheal challenge. In the presence of OVA, the number of inflammatory cells, including eosinophils and neutrophils, in the lung was larger in MT (–/–) mice (Fig. 2A) than in WT mice (Fig. 2B). Vehicle treatment caused no histopathological changes in both genotypes of mice (data not shown).

Role of MT in Lung Expression of Cytokines and Chemokines Related to Antigen. To investigate the role of MT in the lung expression of cytokines and chemokines related to OVA, we compared the protein levels of IL-1β, IL-4, and eotaxin in the lung tissue supernatants among the four experimental groups 24 hrs after the last intratracheal instillation. The OVA treatment induced elevations of IL-1β and eotaxin levels compared with vehicle treatment with significance in MT (–/–) mice ($P < 0.05$; Fig. 1A and C) and without significance in WT mice (Fig. 1A and C). In the presence of OVA treatment, the local expression of IL-1β was significantly greater in MT (–/–) than in WT mice ($P < 0.05$; Fig. 1A). On the other hand, the local expression of eotaxin was not significantly different between MT (–/–) and WT mice after OVA challenge (Fig. 1C). The lung expression of IL-4 showed no significant changes among the experimental groups (Fig. 1B).

Roles of MT in 8-OHdG and Nitrotyrosine Formations. The lung specimens obtained 24 hrs after the last intratracheal instillation were immunostained for 8-OHdG or nitrotyrosine. In the vehicle groups of both genotypes, nuclear staining with 8-OHdG was barely detectable (Fig. 3A and B). The OVA challenge induced nuclear staining with 8-OHdG in both genotypes of mice (Fig. 3C and D). The intensity and extent of immunoreactive 8-OHdG were greater in MT (–/–) mice (Fig. 3D) than in WT mice (Fig. 3C) after OVA challenge. Immunoreactive nitro-

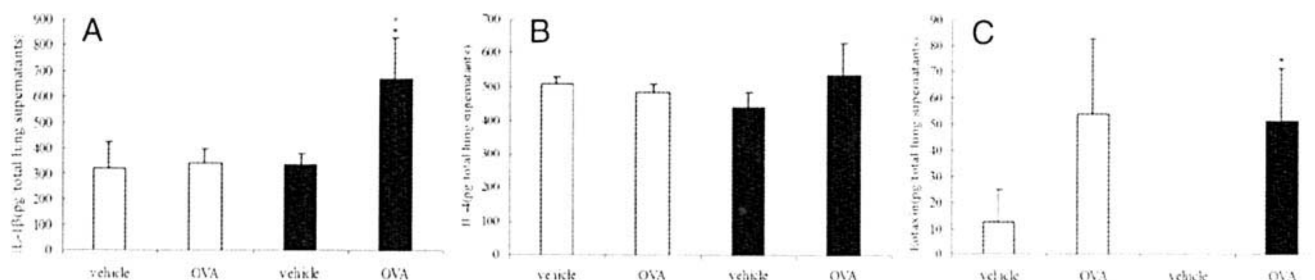

Figure 1. Protein levels of interleukin-1β (IL-1β), IL-4, and eotaxin in the lung tissue supernatants after challenge with ovalbumin (OVA). Lungs from mice (n = 8–10 in each group) were obtained 24 hrs after the last intratracheal administration of vehicle or OVA. The (A) IL-1β, (B) IL-4, and (C) eotaxin levels in the lung tissue supernatants were measured by enzyme-linked immunoabsorbent assays. White bar, wild-type mice; black bar, MT-I/II null (MT [–/–]) mice. Results are expressed as mean ± SEM. *$P < 0.05$ versus vehicle-treated MT (–/–) mice. #$P < 0.05$ versus OVA-challenged wild-type mice.

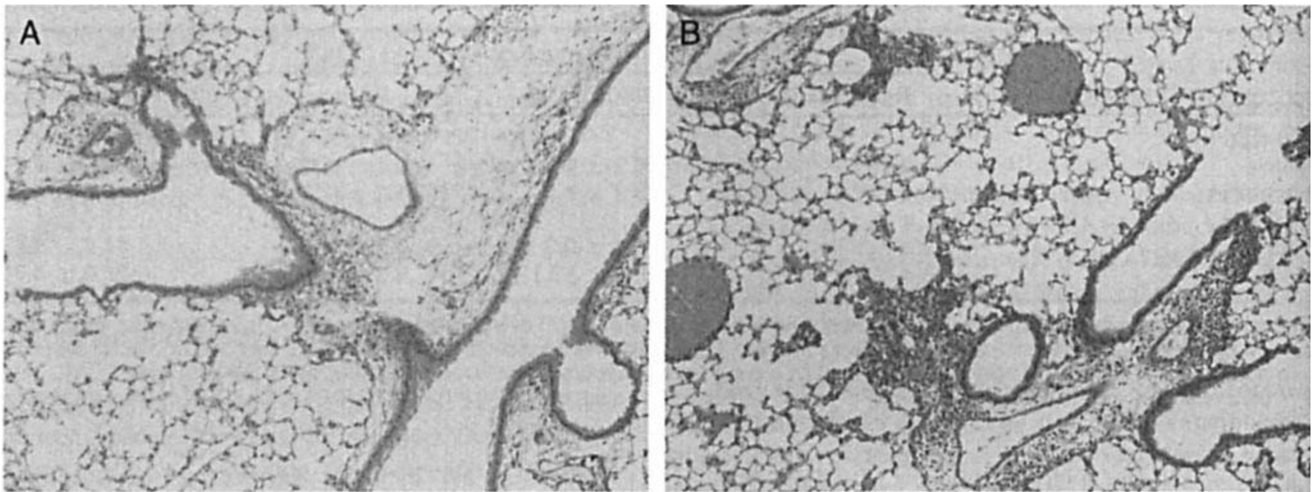

Figure 2. Histopathological findings of the lung obtained from (A) MT-I/II null (MT [-/-]) mice injected intratracheally with 1 μg per body of ovalbumin (OVA) and (B) wild-type mice injected intratracheally with 1 μg per body of OVA . Twenty-four hours after the final challenge with OVA, mice were sacrificed and assessed (*n* = 5 in each group). Magnification: ×200.

Figure 3. Immunohistological staining for 8-hydroxydeoxyguanosine in the lung after challenge with ovalbumin (OVA). Lung sections were obtained from (A) wild-type (WT) mice injected with vehicle, (B) MT-I/II null (MT [–/–]) mice injected with vehicle, (C) WT mice injected with OVA, and (D) MT (–/–) mice injected with OVA (*n* = 5 in each group). Arrows denote positive staining. Magnification: ×400.

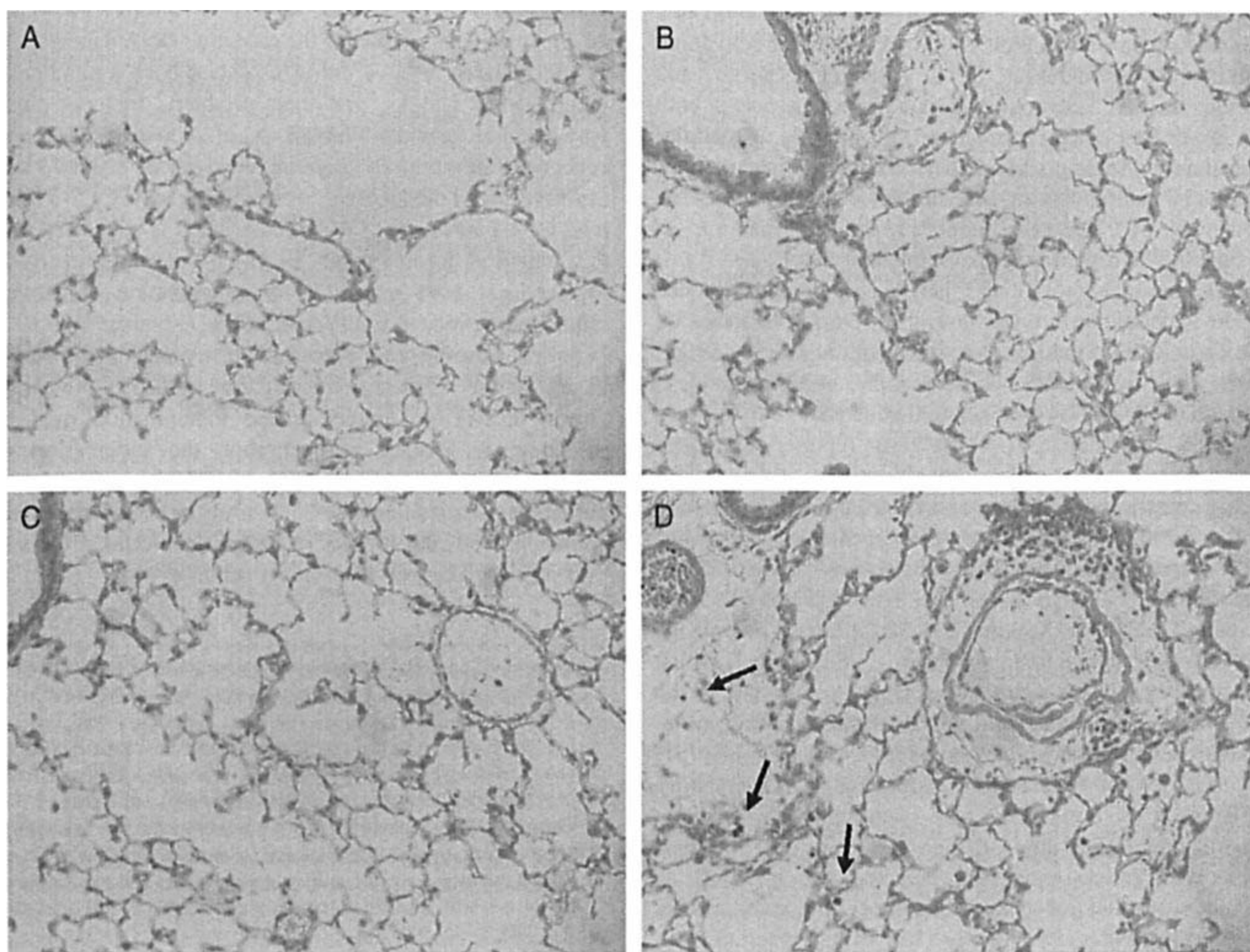

Figure 4. Immunohistological staining for nitrotyrosine in the lung after challenge with ovalbumin (OVA). Lung section was obtained from (A) wild-type WT mice injected with vehicle, (B) MT-I/II null (MT [–/–]) mice injected with vehicle, (C) WT mice injected with OVA, and (D) MT (–/–) mice injected with OVA (n = 5 in each group). Arrows denote positive staining. Magnification: ×200.

tyrosine was also barely detectable in vehicle-challenged mice (Fig. 4A and B) or OVA-challenged WT mice (Fig. 4C). The OVA challenge induced moderate immunostaining with nitrotyrosine only in MT (–/–) mice (Fig. 4D).

Discussion

The present study has demonstrated that MT (–/–) mice are more sensitive to antigen-related airway inflammation than WT mice. In the presence of OVA treatment, the local expression of IL-1β is significantly greater in MT (–/–) than in WT mice. The formations of 8-OHdG and nitrotyrosine are more intense in MT (–/–) mice than in WT mice after the OVA challenge.

Recently, it has been reported that several inflammatory stimuli can induce MT. The induction of MT is mediated by several proinflammatory cytokines, such as IL-1, IL-6, TNF-α, and interferons (22–27). However, the role of MT in inflammation has not been established. The MT (–/–) mice have reportedly been protected from TNF-induced lethality compared with WT mice (11). In addition, MT-I over-expressing mice are more sensitized for the lethal effects of TNF than WT mice (11). In contrast, Kimura *et al.* (12) reported that MT (–/–) mice are more sensitive to LPS-induced lethality in D-galactosamine–sensitized mice.in vivo (17, 24). Furthermore, in the recent study from our laboratory, MT (–/–) mice have been more susceptible to acute inflammatory lung injury induced by LPS than WT mice (13). In the present experiments, significant increases were shown in the numbers of total cells, eosinophils, and neutrophils in BAL fluid from MT (–/–) mice than those from WT mice after OVA challenge. Based on previous reports (13, 17, 24) combined with our present results, MT is suggested to have protective properties against airway inflammation related to antigen and acute lung inflammation induced by bacterial endotoxin. Since we have confirmed that the lung inflammation related to OVA was most prominent 24 hrs after the last challenge with OVA rather than 48 and 72 hrs (unpublished observation), we have estimated BAL and histology 24 hrs after the final OVA challenge as previously reported (19, 28).

The recruitment of cells to sites of inflammation is a complex process that involves cell adhesion and activation, ultimately transmigration of the endothelial barrier, and chemoattraction. Chemokines play an important role in both the activation and chemoattraction phases of cellular recruitment. Among chemokines, eotaxin is essential for eosinophil recruitment in antigen-related airway inflammation (29, 30). Eotaxin regulates the mobilization of eosinophils and their precursors into the blood (29–31). It has also been reported that T helper cell cytokines induce tissue eosinophilia through several different functions (32, 33). Interleukin-4 induces endothelial vascular cell adhesion molecule-1 expression, which together with β1-integrin leads to the activation of eosinophil adherence to the vessels (34, 35). In addition, IL-4 has been reported to induce eotaxin production *in vitro* (36). Therefore, we measured local expression of IL-4 and eotaxin in MT (–/–) mice and WT mice in the presence or absence of OVA. However, there were no significant changes between both genotypes after antigen challenge. In addition, lung expression of IL-5, another T helper 2 cytokine, was not different among the experimental groups (data not shown). Interleukin-1, which is also released after allergen challenge, is important in the induction of endothelial cell adhesiveness, resulting in the recruitment of circulating eosinophils and neutrophils (37, 38). Endothelial cells control trafficking of inflammatory cells, such as eosinophils and neutrophils, through the production of IL-1 after allergen challenge (39). Furthermore, our recent study has demonstrated that MT is protective against endothelial integrity in lung inflammation induced by bacterial endotoxin (13). In our present study, the local expression of IL-1β was significantly greater in MT (–/–) than in WT mice in the presence of OVA. The results suggest that the protective effects of MT on airway inflammation related to antigen may be mediated, at least partly, via the suppression of enhanced lung expression of IL-1β and subsequent protection of endothelial integrity.

The balance between the antioxidative defenses and the production of reactive oxygen species and reactive nitrogen species is a critical factor that determines the extent of tissue injury in various pulmonary diseases, including inflammation such as asthma (40–43). Individuals with asthma demonstrate depressed lavage ascorbate and α-tocopherol and an elevated oxidized-reduced glutathione ratio, suggesting both increased reactive oxygen species and reactive nitrogen species and decreased antioxidant capacity (44). Superoxide dismutase activity is diminished in cells from lavage and brushings of patients with asthma (45). Airway macrophages from individuals with asthma produce more superoxide than those from control subjects (46). On the other hand, MT is one of the potential antioxidants (9, 47). It shares an important similarity with glutathione. One could imply that deteriorated airway inflammation in MT (–/–) mice was due to the loss of antioxidative effects caused by MT deficiency. We, therefore, evaluated the contribution of oxidative stress to the aggravation of antigen-related airway inflammation in the absence of MT. 8-Hydroxydeoxyguanosine is a proper marker of the oxidative DNA damage. On the other hand, reactive nitrogen species have a number of inflammatory actions, and their products, such as nitrotyrosine, are accurate biomarkers of oxidation of amino acids (48). In our study, immunoreactivity of 8-OHdG and nitrotyrosine in the lung was more intense in MT (–/–) mice than in WT mice after OVA challenge. It is suggested that the enhanced formation of 8-OHdG and nitrotyrosine is involved, at least partly, in the aggravation of airway inflammation related to OVA in MT (–/–) mice.

In conclusion, endogenous MT is a protective molecule against antigen-related airway inflammation. The protective effects of MT on antigen-related inflammation may be mediated, at least in part, through the suppression of enhanced lung expression of IL-1β and its antioxidative properties. Our studies may be helpful for establishing new approaches to the treatment of antigen-related airway inflammatory diseases such as bronchial asthma.

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