Tyrosine Phosphatase SHP-1 in Oxidative Stress and Development of Allergic Airway Inflammation

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Oxidative stress has been implicated in allergic responses. SHP-1 is a target of oxidants and has been reported as a negative regulator in a mouse model of asthma. We investigated the effect of oxidative stress on the development of allergic airway inflammation in heterozygous viable *motheaten* (*mev/+*) mice deficient of SHP-1. Wild-type (WT) and *mev/+* mice were compared in this study. Human alveolar epithelial cells (A549) transfected with mutant SHP-1 gene were used to evaluate the role of SHP-1 in lung epithelial cells. Hydrogen peroxide (H₂O₂) and Paraquat were used *in vitro* and *in vivo*, respectively. We also investigated whether *mev/+* mice can break immune tolerance when exposed to aeroallergen intranasally. Compared with WT mice, bronchoalveolar lavage (BAL) cells and splenocytes from *mev/+* mice showed a different response to oxidant stress. This includes a significant enhancement of intracellular reactive oxygen species and STAT6 phosphorylation *in vitro* and increased CCL20, decreased IL-10, and increased number of dendritic cells in BAL fluid *in vivo*. Mutant SHP-1-transfected epithelial cells secreted higher levels of CCL20 and RANTES after exposure to oxidative stress. Furthermore, break of immune tolerance, as development of allergic airway inflammation, was observed in *mev/+* mice after allergen exposure, which was suppressed by antioxidant N-acetylcysteine. These data suggest that SHP-1 plays an important role in regulating oxidative stress. Thus, increased intracellular oxidative stress and lack of SHP-1 in the presence of T helper cell type 2-prone cellular activation may lead to the development of allergic airway inflammation.

Keywords: SHP-1; oxidative stress; allergen; asthma; immune tolerance

Studies have shown increased oxidants in the asthmatic airway, and it is believed that oxidative stress plays a role in the pathogenesis of allergic asthma (1–4). It has been suggested that, as an important underlying mechanism, oxidative stress activates various intracellular signaling pathways leading to secretion of a variety of pro-inflammatory cytokines and chemokines (5).

However, it is not clear whether oxidative stress is a direct contributing factor in the pathogenesis of asthma. The increased oxidative stress observed in asthmatic airways may be a consequence of inflammation. Furthermore, the mechanism by which increased oxidative stress elicits a polarized immune response is

CLINICAL RELEVANCE

Phosphatase SHP-1 plays an important role in regulating oxidative stress. Thus, increased intracellular oxidative stress and lack of SHP-1 in the presence of T helper cell type 2-prone cellular activation may lead to development of allergic airway inflammation.

unclear considering its nonspecific effects, although some observations have suggested that oxidative stress imposed on immune cells can be closely associated with break of immune tolerance (6–8). Also it has been shown that oxidative stress generated by pollen NADPH oxidases augments allergic airway inflammation induced by pollen antigen in a mouse model of asthma (9). Intrinsic defects of some intracellular molecules, which interact with not only reactive oxygen species (ROS) but also signaling pathways involving certain polarized immune responses, may be a possible molecular mechanism explaining the crucial role of oxidative stress in the pathogenesis of specific inflammatory immune responses.

SHP-1, a protein tyrosine phosphatase (PTP), has been recognized as a critical negative regulator in intracellular signaling and is expressed predominantly in hematopoietic cells and epithelial cells (10–13). The function and importance of SHP-1 was recognized upon the discovery of two strains of SHP-1-deficient mutant mice, the *motheaten* (*me/me*) and viable *motheaten* (*mev/mev*) mice (14, 15). Recently, it was reported that SHP-1 has an inhibitory role in the development of allergic airway inflammation in an allergen-induced asthma model using heterozygous *me* (*me/+*) mice (16). It was demonstrated that *me/+* mice showed significantly increased STAT6 phosphorylation and production of T helper cell type 2 (Th2) cytokines after antigen stimulation (16). Furthermore, we observed that SHP-1-deficient homozygous *mev/mev* mice develop a spontaneous allergic inflammation in the lung without known allergen stimulation (S. Y. Oh and Z. Zhu, unpublished observations).

Meanwhile, intracellular hydrogen peroxide (H₂O₂), generated endogenously upon stimulation and exogenously from environmental oxidants (17), has been known to be involved in the process of intracellular signaling through inhibiting enzymatic activities of various PTPs (7, 8, 18). In general, low levels of oxidative stress imposed on cells result in transient and well-controlled cellular activation with prompt activation of intracellular antioxidant systems without eliciting any abnormal events. In contrast, higher levels of oxidative stress can cause exaggerated inflammatory response and break of immune tolerance (6, 19). Increased intracellular ROS can inhibit the function of SHP-1 (18). In addition, it is also well known that suppressed function of SHP-1 results in increased intracellular ROS levels by permitting the exaggerated and prolonged activation of intrinsic intracellular ROS-generating mechanisms (20–22).

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Thus, it is reasonable to speculate that SHP-1 deficiency can render the cells or host more susceptible to oxidative stress, which may cause exaggerated cellular activation followed by a Th2 immune response in the presence of low levels of oxidant. We deemed that this hypothesis might provide one explanation for direct participation of oxidative stress in the pathogenesis of asthma. To address these questions, we investigated the effect of oxidative stress on the development of allergic inflammation using SHP-1-deficient heterozygous *mev* mice.

MATERIALS AND METHODS

Animals

Heterozygous *mev* (*mev*/+) mice and wild-type (WT, or +/+) mice on C57BL/6 genetic background were purchased from the Jackson Laboratory (Bar Harbor, ME). Mice were maintained under specific pathogen-free conditions and were used at 6 to 8 weeks of age. Genotyping was performed with PCR amplification of tail DNA as described before (23). All animal experiments were approved by the IACUC of the Johns Hopkins University.

SHP-1 Protein Phosphatase Activity in the Lung

The SHP-1 protein phosphatase activity in the lung tissues was determined by immunoprecipitation of SHP-1 followed by a colorimetric assay using the SensoLyte pNPP protein phosphatase assay kit (AnaSpec, San Jose, CA). Lungs were obtained from WT, *mev*/+, and *mev/mev* mice as described below and homogenized in the lysis buffer provided in the kit. SHP-1 protein was immunoprecipitated from 1 mg of protein from each lung sample using anti-SHP-1 (sc-287) and protein A/G Plus-Agarose (Santa Cruz Biotechnology, Santa Cruz, CA) following their protocol and resuspended in 200 μ l of assay buffer. The phosphatase activity in the SHP-1 solution (50 μ l) was measured with pNPP as a substrate using the SensoLyte kit as instructed. The optical density (O.D.) readings were calibrated on the negative control wells containing no protein.

In Vitro Cellular Responses after Exposure to H₂O₂

The tracheas of WT and *mev*/+ mice were cannulated under anesthesia, and bronchoalveolar lavage (BAL) was performed using 2 ml of PBS. The recovered fluid was centrifuged at 400 $\times$ g for 5 minutes to pellet the cells. The supernatant was saved for analysis and the cell pellets were resuspended and incubated in PBS with or without 100 μ M of H₂O₂ (Sigma Aldrich Chemical Co., St. Louis, MO) at 37°C for 30 minutes. Next, the cells were loaded with 20 μ M 2',7'-dichlorofluorescein diacetate (DCFH-DA) (Molecular Probes, Eugene, OR) at 37°C for 20 minutes. After washing and incubation at 37°C for 1 hour, the cells were subjected to flow cytometry for DCF emission.

Similarly, splenocytes were prepared and resuspended in PBS with or without H₂O₂ at 37°C for 1 hour. In some experiments, anti-mouse IL-4 antibody (eBioscience, San Diego, CA) was added (10 ng/ml). Proteins were extracted from cytoplasmic and nuclear compartments using commercial kits (Pierce, Rockford, IL). Total proteins were extracted using protein lysis buffer containing 20 μ l/ml of protease inhibitor cocktail (BD Pharmingen, San Diego, CA) and 5 μ l/ml of Na₃VO₄. For determination of nuclear and cytoplasmic Nrf2, total STAT6, phosphorylated STAT6, I- κ B α , and phosphorylated I- κ B α , Western blot was performed using primary antibodies to Nrf2, I- κ B α , phosphorylated I- κ B α (Santa Cruz Biotechnology, Santa Cruz, CA), STAT6, or phosphorylated STAT6 (Cell Signaling Technology, Danvers, MA).

In Vivo Responses to Oxidative Stress in the Lung

To generate oxidative stress *in vivo*, Paraquat (Sigma) in PBS was administered intraperitoneally twice a week for 2 weeks at 2.5 μ g/g body weight—10 times less than the usual dose used in the acute lung injury model (24). In one set of experiments, total lung cells were analyzed. After pulmonary perfusion, single-cell suspensions were prepared by digesting the minced lung tissues for 1 hour with disaggregation buffer containing 300 unit/ml collagenase IV (Gibco, Grand Island, NY), followed by gently pushing the tissues through a 100- μ m nylon cell strainer (BD Falcon, Franklin Lakes, NJ). Single-cell suspensions were

analyzed by flow cytometry after staining with fluorescein isothiocyanate-anti-CD11b, APC-anti-CD11c, and PE-anti-B220 antibodies (BD Pharmingen).

In another set of experiments, total BAL cell counts and differential were determined. The levels of C-C chemokine ligand (CCL) 20/macrophage inflammatory protein-3 α (MIP-3 α), CCL5/RANTES, eotaxin, and IL-10 in BAL were determined by enzyme-linked immunosorbent assay (ELISA) (R&D Systems, MN).

Effect of Mutant SHP-1 on Epithelial Cell Responses to Oxidative Stress

A549 human alveolar epithelial cells (ATCC, Manassas, VA) were cultured in Ham's F12K medium (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum and transfected with the pEF-flag plasmid vector containing either wild-type human SHP-1 gene (pEF-flag-SHP-1-wt) or mutant SHP-1 (pEF-flag-SHP-1-C/S) (25) (kindly provided by Dr. Hidekara Yakura, Tokyo Metropolitan Institute for Neuroscience, Tokyo, Japan) using Lipofectamine 2000. H₂O₂ (100 μ M) was added to the culture medium 36 hours after transfection and overnight serum starvation. The supernatant was collected at 1 and 6 hours after H₂O₂ treatment, and the levels of CCL20 and RANTES were determined.

Pulmonary Responses to Intranasal Ovalbumin Challenges and the Effect of Antioxidant on Inflammation

To investigate the effect of specific antigen on SHP-1-deficient *mev*/+ mice, ovalbumin (OVA) allergen was introduced intranasally 100 μ g in 50 μ l PBS once a day for 5 consecutive days. Antioxidant NAC was given intraperitoneally at 200 μ g/g body weight right before administration of OVA. At Day 7, the mice were anesthetized and killed by cardiac puncture. Blood was obtained and serum was collected. Serum OVA-specific IgE was determined by ELISA (BD Bioscience, San Jose, CA).

BAL was performed and total cell counts and differential were determined. In the meantime, splenocytes were prepared and cultured (1 $\times$ 10⁶ cells/ml) in RPMI 1640 medium for 24 hours with or without increasing concentrations of OVA (2.5, 25, and 250 μ g/ml). The MTT cell proliferation assay was performed as instructed (ATCC).

For histopathologic assessment, lungs were removed, fixed in Streck tissue fixative solution (Streck Laboratories, La Vista, NE), embedded in paraffin, sliced, and then stained with hematoxylin and eosin and Alcian Blue.

Statistical Analysis

For comparison between groups, Student's *t* test was used, data were expressed as mean $\pm$ SE, and statistical significance was defined as *P* < 0.05.

RESULTS

SHP-1 Deficiency in *mev*/+ Mice

Using Western blot we found comparable levels of SHP-1 in the lung homogenates of WT, *mev*/+, and *mev/mev* mice (data not shown). This is consistent with previous reports that SHP-1 mRNA transcripts and full-length protein were present in hematopoietic cells of *mev*/+ and *mev/mev* mice, in contrast to *me*/+ and *me/me* mice (23, 26). However, the mutant SHP-1 protein is not functional because there is either a deletion or insertion in the enzyme catalytic region that abrogates its phosphatase activity (26). Using immunoprecipitation and a phosphatase assay we determined the SHP-1 phosphatase activity in the lung of WT, *mev*/+, and *mev/mev* mice. As shown in Figure 1, SHP-1 activity was present in the lung tissues of WT mice. However, *mev*/+ and *mev/mev* mice had only approximately 60% and 10%, respectively, residual SHP-1 activity in the lung tissues.

Effect of SHP-1 Deficiency on Cellular Response to H₂O₂

First, we examined the effect of exogenous ROS on cells. After exposure to H₂O₂, BAL cells from *mev*/+ mice showed significantly increased intracellular ROS compared with WT mice

Figure 1. SHP-1 protein phosphatase activity in lung tissues. SHP-1 protein was immunoprecipitated from lung homogenates of wild-type (WT), heterozygous viable *motheaten* (*mev/+*), and viable *motheaten* (*mev/mev*) mice and the phosphatase activity was determined using substrate pNPP in a colorimetric assay. The data are expressed as percent of SHP-1 phosphatase activity relative to that of WT mice. Shown is a representative of two experiments with similar results (WT, $n = 3$; *mev/+*, $n = 4$; *mev/mev*, $n = 4$; * $P < 0.05$ and ** $P < 0.01$).

(Figure 2A), suggesting that SHP-1 deficiency is associated with decreased cellular tolerance to oxidative stress.

Next, we investigated the activation of signaling pathways related to oxidative stress after H_2O_2 stimulation. The amount of Nrf2, a known key transcription factor regulating antioxidant defense, was determined by Western blot in the nucleus and cytoplasm compartments of splenocytes, respectively, 1 hour after H_2O_2 treatment. Interestingly, basal nuclear Nrf2 of *mev/+* mice without H_2O_2 was significantly higher compared with that of WT mice (Figure 2B). However, there was no further increase in Nrf2 after exposure to H_2O_2 (Figure 2B). These results suggest that even in resting state, *mev/+* mice may already suffer from substantial levels of oxidative stress. Whether Nrf2 activation is time dependent in these cells remains to be determined. We investigated the phosphorylation status of STAT6, since it is important in Th2-type immune response and can be regulated by SHP-1. Phosphorylated STAT6 was observed in the *mev/+* mice treated with H_2O_2 , while no band was found in the WT mice or *mev/+* mice without H_2O_2 treatment. Blocking IL-4 pathway by anti-IL-4 antibody did not show any effect on the activation of STAT6, suggesting an intracellular event (Figure 2C). NF- κ B is known to be a pivotal transcription factor related to oxidative stress. We examined the phosphorylation of I- κ B α to determine the activation of NF- κ B. I- κ B α phosphorylation was lower in the splenocytes of *mev/+* mice relative to that in the splenocytes of WT mice when exposed to H_2O_2 (Figure 2C).

Effects of SHP-1 Deficiency on Pulmonary Response to Oxidative Stress

We evaluated the *in vivo* effect of oxidative stress imposed on SHP-1-deficient *mev/+* mice. A significant increase in the total number of BAL cells was found in the *mev/+* mice exposed to Paraquat (Figure 3A). Differential cell count showed that most of the cells were monocytes or macrophages. The subtypes of macrophages and dendritic cells (DCs) were determined by flow cytometry using total lung cells. Interestingly, the portion of CD11b+CD11c+ DC was significantly increased in the lung of *mev/+* mice, while CD11b+CD11c- macrophages were remarkably higher in WT mice when exposed to oxidative stress (Figure 3B, and Figure E1 in the online supplement).

The BAL levels of cytokines and chemokines were measured to see if the microenvironment in the lung was altered after oxidative stress exposure in SHP-1-deficient mice, which may

be able to explain the different profiles of recruited inflammatory cells. The level of CCL20, which is recently reported to play an important role in the recruitment of immature DCs, was found to be significantly enhanced in the BAL fluid of *mev/+* mice exposed to oxidative stress (Figure 3C). The level of CCL5/RANTES, which is also one of the important monocyte/DC chemoattractant factors, was evaluated, but no significant difference was found. The levels of CCL2/monocyte chemoattractant protein-1 (MCP-1) and eotaxin were below detection limit. The level of IL-10 showed a decrease in the BAL fluids of *mev/+* mice treated with oxidative stress (Figure E2), while no significant difference was observed in the transforming growth factor- β level (data not shown).

SHP-1 and Lung Epithelial Cell Response to H_2O_2

We evaluated the effect of SHP-1 deficiency on the release of certain chemokines by alveolar epithelial cells when exposed to H_2O_2 , since lung epithelial cells are an important source of several critical chemokines, including CCL20 and RANTES. A549 human alveolar epithelial cells transfected with a plasmid vector containing a mutant SHP-1 gene (C/S) lacking catalytic activity showed increased production of CCL20 and RANTES compared with the cells transfected with vector with WT SHP-1 gene, regardless of exposure to H_2O_2 (Figures 4A and 4B). Treatment with H_2O_2 tended to stimulate higher levels of CCL20 and RANTES by A549 cells. The production of CCL2 and IL-10, however, revealed no difference regardless of SHP-1 deficiency (data not shown).

OVA Induced Airway Allergic Inflammation and the Effect of Antioxidant

First, we demonstrated that a single intranasal administration of OVA could induce significantly enhanced levels of intracellular ROS in the BAL cells of *mev/+* mice compared with those of WT mice (Figure E3). We then performed intranasal OVA challenges without adjuvant (Figure E4). Using this protocol, no evidence of airway inflammation was found in the WT mice. However, in *mev/+* mice with OVA challenge, substantial airway inflammation was observed evidenced by increased infiltrating cells, particularly eosinophils, in the BAL and in lung tissue (Figures 5A–5C). Alcian Blue stain was performed to detect mucin-producing cells in the airways. A few positive-stained cells were found in the large airways but not medium or small airways of WT mice challenged with PBS or OVA and *mev/+* mice with PBS. In contrast, increased Alcian Blue positive-stained cells were seen in the medium and small airways of *mev/+* mice with OVA (Figure E6). These changes were accompanied by increased levels of serum OVA-specific IgE (Figure 5D) and enhanced cellular proliferation in the presence of OVA (Figure E5). Furthermore, treatment with antioxidant NAC significantly suppressed all aspects of airway inflammation (Figures 5A–5D), although it did not eliminated the mucin-producing cells in the airways (Figure E6).

DISCUSSION

Epidemiologic studies have demonstrated an association between increased oxidative stress and adverse effects on asthma and allergic inflammation (1, 2, 4). However, it is still unclear if increased oxidative stress plays a causative role in the development of asthma. The study presented herein demonstrated that oxidative stress contributes to the development of inflammation in the lungs of SHP-1-deficient mice through break of immune tolerance to aeroallergen.

SHP-1 is a negative regulator in various signaling pathways (13, 27). Recently, SHP-1 was reported to be able to suppress

Figure 2. *In vitro* cellular responses to oxidative stress. (A) Ratio of mean fluorescent intensity (MFI) for intracellular reactive oxygen species in H₂O₂-treated (100 μ M) bronchoalveolar lavage (BAL) cells over basal conditions. Data were collected from 5,000 events ($n = 3$, $*P < 0.05$). The data are the mean $\pm$ SEM. (B) Nrf2 in nucleus and cytoplasm of splenocytes from WT and *mev/+* mice 1 hour after H₂O₂ treatment. (C) Total and phosphorylated STAT6 and I- κ B α in the splenocytes from WT and *mev/+* mice. Data are representative of three experiments.

intrinsic generation of oxidative stress through counteracting NADH-oxidase and iNOS (20, 21). It is also believed that reversible redox regulation of cysteine-based PTPs plays a dominant role in setting the level of tyrosine phosphorylation in cells and is likely to be an important mechanism for maintaining appropriate cellular responses (18, 22). ROS, one of the critical intracellular second messengers, can modulate intracellular protein phosphorylation by reversible oxidation of the active site cysteine residues of PTPs, leading to transient inactivation of these enzymes (8, 10, 18). With two cysteinyl residues in its catalytic site, SHP-1 is vulnerable to oxidation (10).

Thus, it is conceivable that functional deficiency in SHP-1 may be related to increased susceptibility to oxidative stress that may cause improper activation of cells. To test this we used the SHP-1-deficient *mev* mice. The genetic mutations, the expression and function of SHP-1 in *me* and *mev* mice, have been well characterized. Severely diminished SHP-1 phosphatase activity was found in hematopoietic cells of homozygous *me/me* and *mev/mev* mice that correlated with their spontaneous inflammatory phenotypes (23, 26). However, it is unclear what levels of SHP-1 activity are present in the heterozygous *mev/+* mice, which show no abnormalities under normal conditions. The SHP-1-specific phosphatase activity in the lung tissues was determined. Compared with WT mice, *mev/+* mice have approximately 60%, and *mev/mev* mice 10%, SHP-1 activity left in the lung (Figure 1), despite the presence of compa-

table levels of full-length SHP-1 protein (data not shown). Our study found that homozygous *mev/mev* mice develop a spontaneous Th2-dominated pulmonary inflammation (S. Y. Oh and Z. Zhu, unpublished data). Thus SHP-1-deficient heterozygous *mev/+* mice are more suitable for studying cellular and immune responses under stress.

Our study revealed enhanced intracellular ROS in BAL cells after exposure to H₂O₂ and activation of Nrf2 even in resting state in *mev/+* mice. These findings strongly suggest that SHP-1-deficient mice even without any stimulation may already have substantially increased burden of oxidative stress, although no phenotypical abnormalities could be observed under normal conditions.

Increased intracellular ROS reaching certain threshold might be a critical common pathway to induce immunity regardless of initial stimuli. In a recent report, it was found that effective T cell responses can occur with adjuvants regardless of the kinds of stimulation through a universal mechanism such as recruitment of DCs (28). Those observations further suggest that various adjuvants might cause a common intracellular event such as increased ROS in the development of immune responses. Enhanced ROS may be closely involved in mounting an immune response through recruitment and activation of DCs. In this study, enhanced ROS in BAL cells and increased recruitment of DCs into the lung were found in *mev/+* mice after exposure to H₂O₂ or OVA antigen. Substantial airway

Figure 3. *In vivo* responses to oxidative stress. (A) Total cell counts of BAL cells from *mev/+* and WT mice with or without Paraquat. (B) Percentage of CD11b+CD11c+ dendritic cells and CD11b+CD11c- macrophages in the lung after exposure to Paraquat. (C) CCL20 in BAL fluids. Dotted line represents detection limit ($n = 3-6$, $*P < 0.05$ compared with other groups). The data are the mean $\pm$ SEM.

inflammation was also induced in these mice by intranasal OVA challenges without adjuvant, a regimen unable to elicit any immune response in WT mice under normal conditions (29, 30). Furthermore, treatment with an antioxidant effectively suppressed the airway inflammation, suggesting a pivotal role of oxidative stress in the development of inflammation in *mev/+* mice.

Airway DCs are essential for the development and maintenance of allergic asthma (31–35). The airway DC system has evolved into a fine-tuned, highly sensitive sentinel network capable of both igniting and shutting down immune responses as appropriate (32, 36). Even in the absence of overt inflammation, DCs are constantly recruited from the blood into the lung to maintain the steady-state deployment of the airway DC network (32). Not surprisingly, various stimuli in the airway have a profound impact on this steady-state dynamics (36–38). Rapid recruitment of DCs into the airway indicates that it is

Figure 4. Levels of (A) CCL20 and (B) RANTES produced by human lung epithelial cells transfected with plasmid vector containing WT or mutant human SHP-1 gene after H₂O₂ exposure. The data are the mean $\pm$ SEM of triplicate samples. $*P < 0.05$ compared with groups transfected with plasmid vectors containing WT SHP-1 gene in each pair of experiments.

a critical part of the early phase of innate immune response, with the potential to progress toward an adaptive immune response (39). Selective depletion of DCs during allergen challenge abolished all cardinal features of asthma (40). In our study, increased recruitment of DCs into the airway occurred only in the *mev/+* mice exposed to oxidative stress. Considering that significantly increased intracellular ROS was also observed only in the H₂O₂-treated BAL cells from *mev/+* mice, enhanced oxidative stress imposed on those mice may be a principal mechanism to recruit airway DCs. Our findings also strongly support the idea that recruited DCs at the site of antigen exposure would play a crucial role in the development of immunity instead of immune tolerance in SHP-1-deficient mice.

At the cellular and molecular level, airway DCs were shown to be attracted by a whole array of stimuli including chemokines, complement cleavage products, and bacterial peptides (41). Lung epithelial cells are a front line defense and have been known to actively contribute to the pathogenesis of asthma (42). In this study, we hypothesized that lung epithelial cells would be a major contributor to the recruitment of DCs into the airway through enhanced production of chemokines capable of attracting DCs. It is reasonable to speculate that increased oxidative stress could make SHP-1-deficient epithelial cells produce more chemokines that in turn attract DCs into the airways.

Indeed, after exposure to oxidative stress, increased production of CCL20 and RANTES by lung epithelial cells transfected with a dominant-negative mutant SHP-1 was observed in parallel with enhanced recruitment of DCs in *mev/+* mice *in vivo*. CCL20, a pivotal chemoattractant for CCR6-positive immature DCs, has been reported to be secreted by lung epithelial cells upon stimulation with ultrafine particulate matter

Figure 5. Allergic inflammatory responses to ovalbumin (OVA) challenge. (A and B) Total and differential cell counts of BAL cells. (C) Lung histopathology of *mev/+* mice and WT mice after intranasal OVA stimulation in the presence or absence of antioxidant NAC (hematoxylin and eosin, $\times 100$). (D) Serum OVA-specific IgE. Dotted line represents detection limit. ($n = 4-6$, * $P < 0.05$ and ** $P < 0.05$ compared with groups without NAC). The data are the mean $\pm$ SEM.

(43). However, the mechanism by which the SHP-1-deficient state recruited more DCs into the airway, and induced the immunity instead of immune tolerance in association with enhanced intracellular ROS, has not yet been precisely defined.

In the meantime, airway inflammation observed in the *mev/+* mice is characterized by airway eosinophilia, increased mucin-producing cells, and increased production of OVA-specific IgE, which are features of Th2 immune responses. Although the function of SHP-1 in the development of a specific type of immune response is still largely unknown, it is recently reported that exaggerated allergic inflammation was induced in SHP-1-

deficient motheaten mice (16). In addition, SHP-1 deficiency is known to cause enhanced IL-4-dependent STAT6 activation through suppression of dephosphorylation of phosphorylated STAT6 (44-46). Thus SHP-1 appears to act as a negative regulator of Th2 signaling pathway. Consistent with these observations, our results also showed that when exposed to ROS, enhanced STAT6 phosphorylation was observed in the cells from *mev/+* mice. Interestingly, decreased activation of NF- κ B in the ROS-exposed SHP-1-deficient cells was also observed. This might be caused by enhanced activation of STAT6, which is known to have a strong inhibitory effect on JNK and NF- κ B

pathways (44). Further studies, therefore, are needed to clarify the exact mechanism underlying intracellular signaling of SHP-1. Taken together, these results indicate that break of immune tolerance followed by Th2-dominant airway inflammation can occur when the function of SHP-1 is reduced.

The prevalence of allergic diseases including asthma has increased dramatically over the last half decade (47). Various environmental factors eliciting increased oxidative stress such as air pollution and decreased intake of antioxidant-containing food have been suggested as contributing factors (48, 49). However, it is still not understood by which mechanism the immune disorders could develop through an increased oxidative stress burden that has substantial effect on the host's immune system but without eliciting any clinically apparent host response.

Interestingly, our study revealed that neither phenotypical nor immunological abnormalities could be found in the heterozygous *mev* mice in resting state until mucosa exposure to allergens, which is usually tolerated. Therefore, a defect in certain regulatory molecules could result in altered host microenvironment when exposed to even subtle increase of oxidative stress, such as suboptimal amount of antigens or inert antigens, leading to clinically apparent immune disorders. The effects of oxidative stress on immune response are complex. SHP-1 is probably one of many molecules that participate in the regulation of allergic inflammation through changes in intracellular redox state.

In conclusion, our findings suggest that SHP-1 plays an important role in regulating oxidative stress, and that SHP-1 deficiency plus increased intracellular oxidative stress in the presence of Th2-prone activation may lead to the development of allergic airway inflammation. In addition, this study expands our understanding of molecular mechanisms required to maintain the critical oxidative stress levels in the lung under various conditions and establishes a novel link between oxidative stress, immune response, and lung inflammation.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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