ANTIOXIDANTS & REDOX SIGNALING
Volume 17, Number 10, 2012

DOI: 10.1089/ars.2012.4572

ORIGINAL RESEARCH COMMUNICATION

Superoxide Dismutase 3 Controls Adaptive Immune Responses and Contributes to the Inhibition of Ovalbumin-Induced Allergic Airway Inflammation in Mice

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Abstract

Aims: The extracellular superoxide dismutase 3 (SOD3) is an isoform of SOD. Extensive studies have been focused on role of SOD3 as an antioxidant. However, the role of SOD3 in the immune responses that contribute to the inhibition of allergic lung inflammation has not been investigated. ***Results:*** Here, we report for the first time that SOD3 specifically inhibits dendritic cell maturation. Subsequently, SOD3 controls T cell activation and proliferation, and T helper 2 (Th2) and Th17 cell differentiation. As a consequence, the administration of SOD3 into mice alleviated Th2-cell-mediated ovalbumin (OVA)–induced allergic asthma. In addition, we demonstrated that SOD3 inhibits OVA-induced airway extracellular remodeling and Th2 cell trafficking. Through mass spectrometry analysis, the proteins interacting with SOD3 in the lung of asthma were identified. And it was revealed that signaling molecules, such as transforming growth factor (TGF) and epidermal growth factor (EGF) receptor, adhesion and adaptor molecules, kinases, phosphatases, NADPH oxidase, and apoptosis-related factor, were involved, which were altered by administration of SOD3. Relatively severe asthma was observed in SOD3 KO mice and was ameliorated by both the administration of SOD3 and adoptive transfer of SOD3-sufficient CD4 T cells. Moreover, the expression of endogenous SOD3 in the lung peaked early in OVA challenge and gradually decreased upon disease progression, while both SOD1 and SOD2 expression changed relatively little. ***Innovation and Conclusion:*** Thus, our data suggest that SOD3 is required to maintain lung homeostasis and acts, at least in part, as a controller of signaling and a decision maker to determine the progression of allergic lung disease. *Antioxid. Redox Signal.* 17, 1376–1392.

Introduction

The extracellular superoxide dismutase 3 (SOD3) is an isoform of SOD that scavenges superoxide radicals (22). Unlike other SOD isoforms, SOD3 is a glycoprotein found predominantly in the extracellular matrix (ECM) of tissues and the glycocalyx of cell surfaces, where it is anchored to heparan sulfate proteoglycans (19, 21, 22). SOD3 is highly expressed in normal lung tissue, particularly blood vessels and airways (24, 25), and the antioxidative effects of SOD3 in the lung have been well established (1, 11, 15, 37). Some studies showed that SOD3 directly responds to lung inflammation, including lipopolysaccharide (LPS)–mediated systemic and airflow limited inflammation (5, 29, 30). However, it is unclear whether SOD3 is crucial for protection of allergic asthma. Further, the role of SOD3 in modulating the immune response that contributes to the inhibition of inflammation has not been investigated. Considering that the ECM is essential for tissue homeostasis and that any changes in the ECM microenvironment can be detrimental to cell function during inflammation, SOD3 may have an important role in the protection against lung inflammation.

Innovation

In this study, we report for the first time that superoxide dismutase 3 (SOD3) controls immune response by regulating dendritic cell maturation, T cell activation and proliferation, and T helper 2 (Th2) and Th17 cell differentiation. As a consequence, SOD3 controls Th2-cell-mediated ovalbumin (OVA)–induced allergic asthma by controlling airway extracellular remodeling and Th2 cell trafficking. Interestingly, we found that SOD3 acts, at least in part, as a controller of signaling initiation and strength, and a decision maker in determining the progression of asthma. Therefore, SOD3 may be used as a therapeutic intervention against allergic asthma and other Th2 and Th17 disorders, including atopic skin disease.

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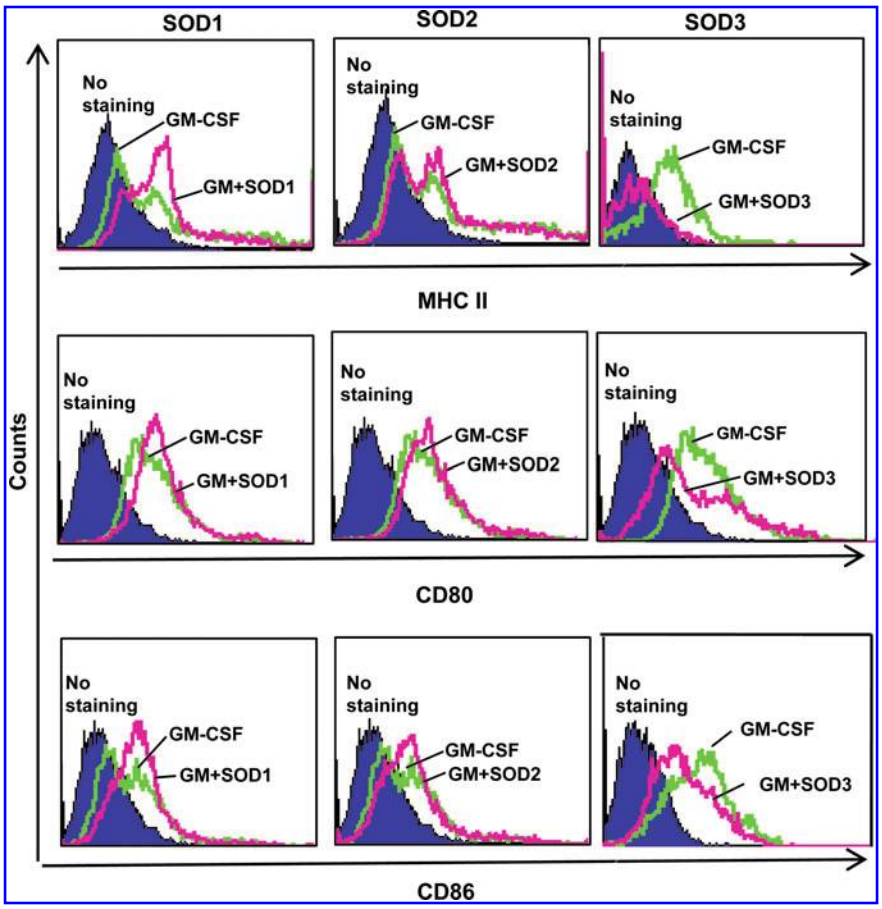
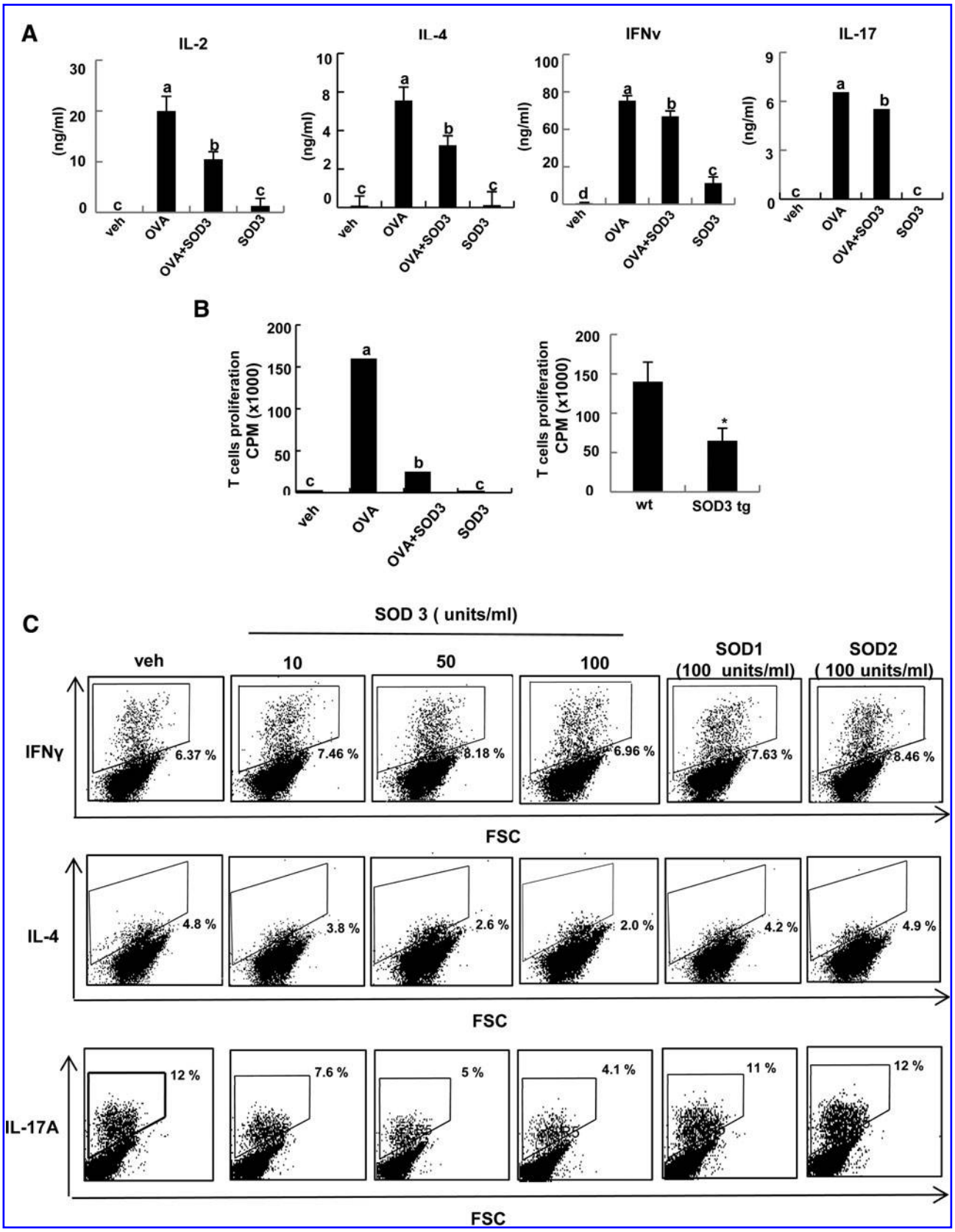


FIG. 1. Superoxide dismutase 3 (SOD3), but not SOD1 and SOD2, inhibits dendritic cell (DC) maturation. MHC II, CD80, and CD86 expression in DCs. Bone-marrow-derived DCs were prepared as described in the Materials and Methods section. DC maturation was accomplished by culturing the cells with recombinant granulocyte-macrophage-colony stimulating factor (rGM-CSF) (10 ng/ml) for 5 days in the presence or absence of SOD3 (100 units/ml). In some experiments, SOD3 was replaced by either SOD1 (100 units/ml) or SOD2 (100 units/ml). MHC II, CD80, and CD86 expression was assessed by flow cytometry. The data shown represent one of three independent experiments. (To see this illustration in color the reader is referred to the web version of this article at www.liebertpub.com/ars).

Allergic asthma is a complex disorder characterized by variable degrees of airway bronchioconstriction, hyperresponsiveness, inflammation, and remodeling (26, 34). T-cell priming to an inhaled allergen is critical in the initiation and orchestration of the inflammatory response in the asthmatic airway (12, 35). An increase in the number of Th2 cells in the lung triggers the recruitment and activation of IgE-producing B cells, eosinophils (2, 26), and fibroblasts, which produce chemokines, leukotrienes, and adhesion molecules that lead to inflammation and airway remodeling (8, 13). Highly produced IgE binds to the surface of mast cells and activated lung fibroblasts, leading to the further activation and differentiation of mast cells and the release of bronchoconstrictors, such as histamine and serotonin, and inflammatory mediators, including cytokines and chemokines (10, 34). Th17 cells are potent inducers of neutrophil inflammation (20, 32, 36), which can lead to severe asthma.

Previously, we reported that SOD3 attenuates dendritic cell (DC) maturation (17). In this study, we found that the inhibition of DC maturation is SOD3 specific. Thus, we hypothesized that SOD3 may function in regulation of adaptive immune response, which may control immunological disorders. To test this hypothesis, T cell priming to antigen for T cell activation and proliferation was performed in the presence or absence of SOD3 for *in vitro* and *in vivo* using SOD3 transgenic (tg) mice. In addition, T cell differentiation was assessed by treatment with SOD3 *in vitro* culture. To test its biological consequences, allergic asthma model was used by airway allergen sensitization in

FIG. 2. SOD3 inhibits T-cell activation and proliferation and T helper 2 (Th2) and Th17 differentiation. (A, B) SOD3 inhibited T-cell activation and proliferation. For *in vitro* T-cell priming, matured DCs that were cultured from immature DCs ($CD11c^+MHC\ II^-$) with GM-CSF for 5 days were cocultured with CD4 T cells purified from OT-II TCR transgenic (tg) mice and the antigen $OVA_{332–339}$ peptide (10 μg/ml) in the presence or absence of SOD3 for 5 days. The supernatants were collected for measurement of the production of interleukin (IL)–2 as an indication of T-cell activation, and Th2, Th1, and Th17 cytokines **(A)**, and the cells were incorporated with [H^3]-thymidine to assess T-cell proliferation **(B)**. For *in vivo* T-cell priming, purified CD4 T cells from OT-II TCR tg mice were adoptively transferred into C57/BL6 wild-type (wt) and SOD3 tg mice. After 24 h, the mice were challenged with ovalbumin (OVA; 75 μg) plus lipopolysaccharide (LPS; 10 μg). Three days later, the mice were sacrificed and their spleens were isolated. To assess T-cell proliferation, the splenocytes were cultured *in vitro* with $OVA_{332–339}$ peptide (10 μg/ml) for 3 days **(B,** *right panel***)**. **(C)** SOD3 inhibited Th2 and Th17 differentiation. Splenic CD4 T cells were cultured in Th1 (*upper panel*), Th2 (*middle panel*), or Th17 (*lower panel*) differentiation conditions. The indicated concentration of SOD3 was added to the cultures 30 min earlier. Some cultures were pretreated with either SOD1 (100 units/ml) or SOD2 (100 units/ml). The data represent the mean ± standard deviation (SD) of three independent experiments. Statistical analysis was performed by analysis of variance (ANOVA) at $p < 0.01$ level and grouped a, b, c, and d as Schiffe's *post hoc* test or *t*-test (*$p < 0.001$ *vs.* wt control). The dot plots shown represent one of three independent experiments.


A
IL-2
IL-4
IFNv
IL-17
(ng/ml)
veh
OVA
OVA+SOD3
SOD3
B
T cells proliferation CPM (x1000)
wt
SOD3 tg
C
SOD 3 (units/ml)
10
50
100
SOD1 (100 units/ml)
SOD2 (100 units/ml)
IFNγ
6.37 %
7.46 %
8.18 %
6.96 %
7.63 %
8.46 %
FSC
IL-4
4.8 %
3.8 %
2.6 %
2.0 %
4.2 %
4.9 %
IL-17A
12 %
7.6 %
5 %
4.1 %
11 %
12 %

SOD3 administered mice or in SOD3 deficient or overexpressed mice. Some experiments were performed by adoptively transferred SOD3 sufficient CD4 T cells to the SOD3 knockout (KO) mice. We then evaluated the roles of SOD3 in the adaptive immune response and allergic asthma. In addition, the proteins interacting with SOD3 in the lung of allergic asthma were identified through mass spectrometry (MS) analysis.

Results

SOD3, but not SOD1 or SOD2, inhibits DC maturation

We previously observed that SOD3 inhibits DC maturation through the inhibition of MHC II, CD80, and CD86 expression (17). Further, as shown in Figure 1, neither SOD1 nor SOD2 inhibited DC maturation, suggesting that the inhibition of DC maturation is SOD3 specific.

SOD3 controls T-cell priming, and consequently alters T-cell activation, proliferation, and differentiation

Because DC maturation affects T-cell priming in an immune response, we tested the role of SOD3 in T-cell priming. As shown in Figure 2A and B, SOD3 greatly reduced the ability of mature DCs to prime T cells, leading to decreased interleukin-2 (IL-2) production for T cell activation, T helper (Th) cytokines (IL-4, interferon γ [IFNγ], and IL-17), and T cell proliferation *in vitro* (Fig. 2B, left panel) and *in vivo* (Fig. 2B, right panel). Based on this result, we further tested the role of SOD3 in T-cell differentiation. Recombinant SOD3 was added in Th1, Th2, and Th17 differentiation conditions *in vitro* and measured the following cytokines by intracellular staining: IFNγ for Th1, IL-4 for Th2, and IL-17A for Th17 cell. As shown in Figure 2C, SOD3 decreased Th2 and Th17 differentiation but barely affected Th1 differentiation. However, both SOD1 and SOD2 could not replicate these effects. Collectively, these data indicate the role of SOD3 in the control of Th2- and Th17-cell-mediated immune responses.

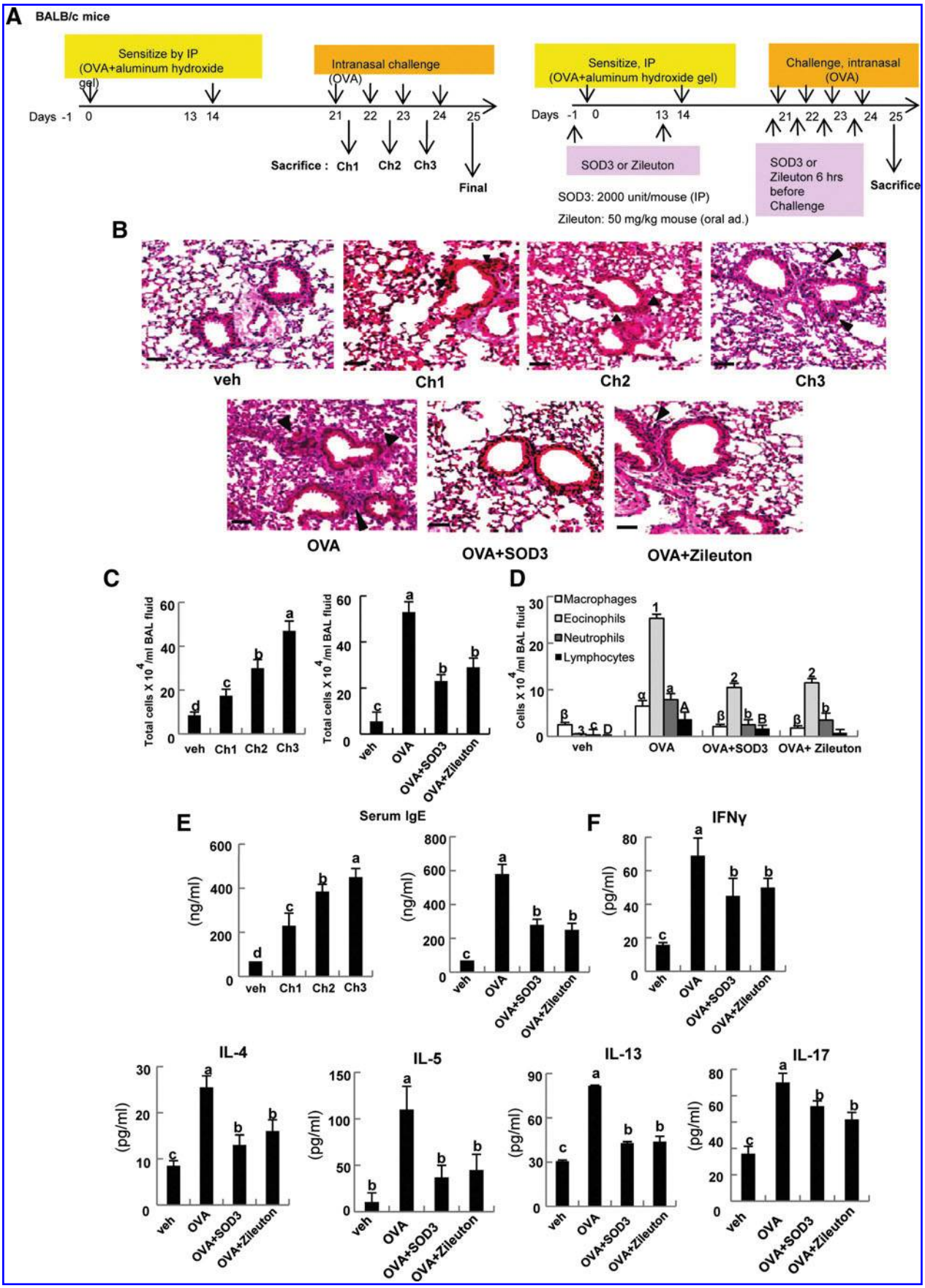
SOD3 alleviates ovalbumin-induced allergic asthma

To understand the potential biological relevance of SOD3, we utilized an allergic asthma model. An inappropriate Th2-mediated immune response is the hallmark of allergic asthma and leads to the infiltration of inflammatory cells (eosinophils, neutrophils, and mast cells) and the production of IgE by B cells, which establishes a chronic inflammatory cycle that eventually leads to airway obstruction caused by mucus overproduction (33). Further, collagen-mediated extracellular remodeling occurs during the progression of asthma. In addition, Th17 cells lead to severe asthma by recruiting neutrophils to inflammatory sites (18, 20, 32). Based on that information, we explored the role of SOD3 in allergic asthmatic inflammation. As shown in Figure 3A, ovalbumin (OVA)–induced allergic asthma was established in the BALB/c mouse model. Intraperitoneally (IP) administrated SOD3 (2000 units) to mouse enhanced SOD3 expression in the lung, spleen, and peripheral lymph node (LN) (Supplementary Fig. S2A–C; Supplementary Data are available online at www.liebertpub.com/ars). Moreover, SOD3 activity was enhanced in SOD3-administrated bronchoalveolar lavage fluid (BALF) and serum (Supplementary Fig. S2F). Based on that information, recombinant SOD3 was IP injected a day before sensitization and 6 h before each nasal challenge. Before injection, recombinant SOD3 was filtered to eliminate endotoxin. Zileuton, an antiasthma therapeutic agent that inhibits the production of leukotrienes, was utilized as a positive control and was orally administered to mice at the same times that of the experimental group.

Histologically, an increased number of infiltrating cells in the airways and a thickening of the airway wall were observed after OVA challenge (Fig. 3B, upper panel). Like Zileuton, SOD3 alleviated these changes (Fig. 3B, lower panel). Consistent with these results, greatly increased levels of total number of cells and inflammatory cells were observed in the BALF upon OVA challenge (Fig. 3C, D). The increases in eosinophils, neutrophils, macrophages, and lymphocytes were drastically reduced by the administration of SOD3. Allergic asthma is typically accompanied by increased serum IgE, an immunoglobulin whose synthesis relies heavily on Th2 cytokines (6). Hence, we also assessed the total IgE in the serum. As expected, OVA challenge led to an increase in serum IgE, but this increase was reduced by treatment with SOD3 (Fig. 3E). T-cell infiltration was assessed by measuring cytokines in the BALF. As shown in Figure 3F, all Th cytokines in BALF were greatly increased after OVA challenge. The levels of these cytokines, particularly, the Th2 cytokine, were drastically decreased by treatment with SOD3 (Fig. 3F).

CCR4 is a chemokine receptor expressed by nearly all IL-4-producing CD4 T cells and CCR4-expressing T cells are found

FIG. 3. SOD3 alleviates OVA-induced allergic asthma. (A) The experimental scheme of the OVA-induced allergic asthma in BALB/c mice. Mice were sensitized twice by intraperitoneal (IP) injection of the allergen OVA (50 μg) and the adjuvant aluminum hydroxide gel (1.3 mg) as described in the Materials and Methods section. Mice were intranasally challenged with OVA (150 μg) four times on days 21, 22, 23, and 24. In some experiments, mice were IP injected with recombinant SOD3 (2000 units/mouse) before sensitization and challenge as described in the Materials and Methods section. As a positive control, Zileuton, an antiasthma therapeutic agent that inhibits the production of leukotrienes, was orally administered to BALB/c mice. The mice were sacrificed 24 h after the last challenge (day 25) and evaluated. For a time course evaluation, some mice were sacrificed after one (Ch1), two (Ch2), or three (Ch3) challenges. **(B)** Lung histology. Lung section stained with hematoxylin and eosin (H&E). *Arrowheads* indicate inflammatory cell infiltration along the larger airways and blood vessels. Bars = 100 μm. **(C)** SOD3 reduced total cell number in the bronchoalveolar lavage fluid (BALF). Total cell number in BALF after OVA challenge (*left panel*), and the challenge after administration of SOD3 (*right panel*). **(D)** SOD3 reduced inflammatory cells in the BALF. **(E, F)** SOD3 reduced the level of serum IgE **(E)**; the Th2-type cytokines IL-4, IL-5, and IL-13; and the Th17-type cytokine IL-17 **(F)**. Levels of IgE in the serum and Th1, Th2, and Th17 cytokines in the BALF were measured by enzyme-linked immunosorbent assay (ELISA). The data are representative of three independent experiments, with four mice per group per experiment. Error bars represent SD. Statistical analysis was performed by ANOVA at $p < 0.01$ level and Schiffe's *post hoc* test was followed. a–d, A–D, 1–3, α, and β on the figure indicate grouping of Schiffe's *post hoc* test. (To see this illustration in color the reader is referred to the web version of this article at www.liebertpub.com/ars).


A
BALB/c mice
Sensitize by IP
(OVA+aluminum hydroxide gel)
Intranasal challenge
(OVA)
Days -1 0 13 14 21 22 23 24 25
Sacrifice : Ch1 Ch2 Ch3
Final
Sensitize, IP
(OVA+aluminum hydroxide gel)
Challenge, intranasal
(OVA)
SOD3 or Zileuton
SOD3 or Zileuton 6 hrs before Challenge
Sacrifice
SOD3: 2000 unit/mouse (IP)
Zileuton: 50 mg/kg mouse (oral ad.)
B
veh
Ch1
Ch2
Ch3
OVA
OVA+SOD3
OVA+Zileuton
C
Total cells X 10^4 /ml BAL fluid
veh Ch1 Ch2 Ch3
veh OVA OVA+SOD3 OVA+Zileuton
D
Macrophages
Eocinophils
Neutrophils
Lymphocytes
Cells X 10^4 /ml BAL fluid
veh OVA OVA+SOD3 OVA+ Zileuton
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IL-4
IL-5
IL-13
IL-17

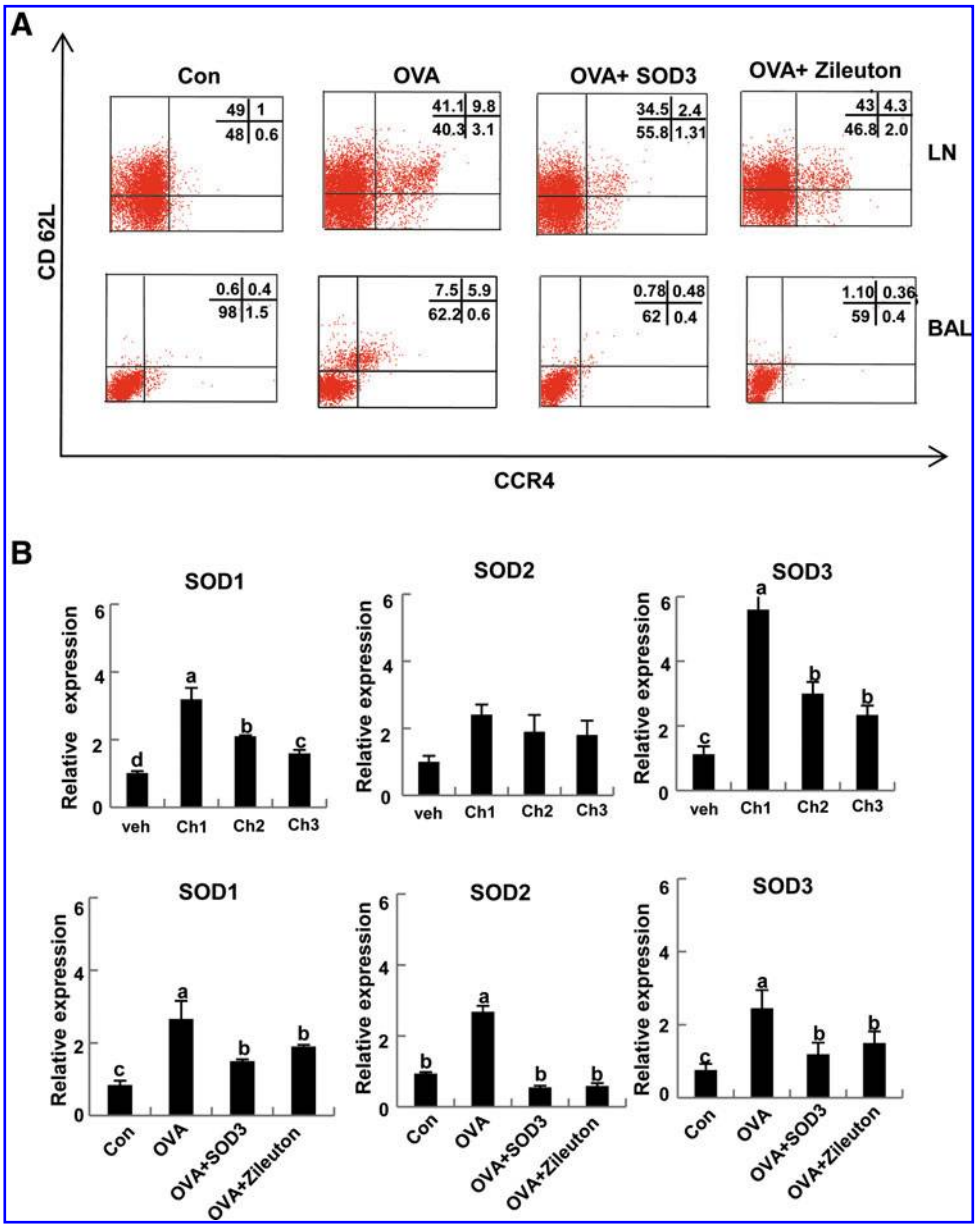
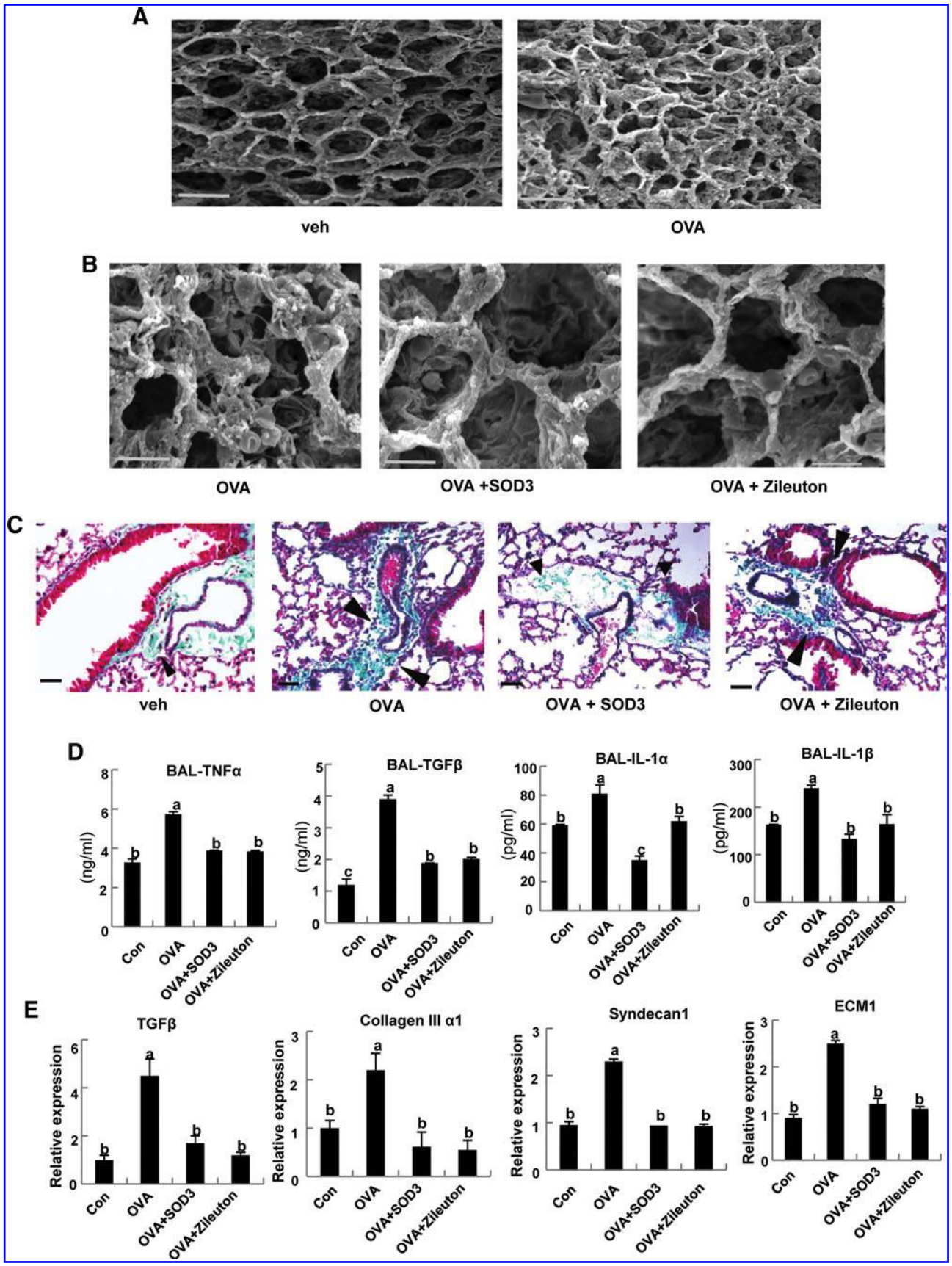


FIG. 4. SOD3 modulates the expression level of chemokine receptor 4 (CCR4) in the lung draining lymph nodes and BALF, and endogenous SODs in lung of OVA-induced mice. (A) The expression of $CD4^+CCR4^+$ $CD62L^+$ cells from the lung draining lymph nodes (*upper panel*) and the BALF (*lower panel*). CD4 T cells from the lung draining lymph nodes and BALF were stained with fluorescence-conjugated antibodies against CCR4 and CD62L and analyzed by flow cytometry. The dot plots shown represent one of three independent experiments. The numbers in the top right corners of the panels indicate the percentage of cells in each quadrant. **(B)** The expression level of endogenous SOD3, SOD1, and SOD2. The expression level of SODs in the lung was analyzed by quantitative real-time polymerase chain reaction (qRT-PCR) as described in the Materials and Methods section. qRT-PCR data represent the mean ± SD of three independent experiments. Statistical analysis was performed by ANOVA at $p < 0.01$ level and grouped a–d as Schiffe's *post hoc* test. (To see this illustration in color the reader is referred to the web version of this article at www.liebertpub.com/ars).

in the BALF of both human and mouse asthmatics (4, 38). Accordingly, we investigated whether SOD3 is involved in the regulation of these expression in lung-draining LNs and BALF. Both the LNs and BALF showed increased numbers of $CD62L^+$ $CCR4^+$ CD4 T cell upon OVA challenge, but these increases were reduced by the administration of SOD3 (Fig. 4A), indicating that SOD3 regulates Th2 cell trafficking into the lung when OVA induced asthma. Taken together, these results suggest that administration of SOD3 altered asthmatic features in mice, which, in turn, alleviates allergic asthma.

FIG. 5. SOD3 inhibits OVA-induced extracellular remodeling in the airway. (A, B) Lung ultrastructure. Allergic asthma was induced with OVA as described in Figure 3. After sacrifice, lung tissues were prepared for scanning electron microscopy (SEM). OVA-induced allergic asthma lungs showed thick and tightened airway wall (*right panel*, SEM [×500]), whereas the airway walls of wt mice were of normal depth and size (*left panel*, SEM [×500], scale bars = 50 μm) **(A)**. Bar SOD3 inhibited OVA-induced airway extracellular remodeling (scale bars = 10 μm) **(B)**. **(C)** Lung section stained with Masson's trichrome. *Arrowheads* indicate collagen (*green*) in peribronchovascular area (scale bar = 50 μm) **(C)**. **(D)** SOD3 reduced the level of inflammatory molecules in the BALF. TNFα, TGFβ, IL-1α, and IL-1β in BALF were measured by ELISA. **(E)** SOD3 reduced the level of expression of OVA-induced extracellular matrix molecules. The expression levels of collagen type IIIα1, extracellular matrix protein-1 (ECM-1), and syndecan-1 in the lung were analyzed by qRT-PCR as described in the Materials and Methods section. Image data represent one of three independent experiments. ELISA and qRT-PCR data represent the mean ± SD of three independent experiments. Statistical analysis was performed by ANOVA at $p < 0.01$ level and grouped a, b, and c as Schiffe's *post hoc* test. (To see this illustration in color the reader is referred to the web version of this article at www.liebertpub.com/ars).


A
veh
OVA
B
OVA
OVA +SOD3
OVA + Zileuton
C
veh
OVA
OVA + SOD3
OVA + Zileuton
D
BAL-TNFα
(ng/ml)
BAL-TGFβ
(ng/ml)
BAL-IL-1α
(pg/ml)
BAL-IL-1β
(pg/ml)
Con
OVA
OVA+SOD3
OVA+Zileuton
E
TGFβ
Collagen III α1
Syndecan1
ECM1
Relative expression

The level of endogenous SOD3 is altered by OVA challenge

We next wondered whether the level of endogenous SOD3, as well as that of SOD1 and SOD2, in the lung changes during the progression of asthma. We speculated that although the SOD3 level might be increased early in the disease to protect against OVA-induced allergic inflammation and lead to the ECM staying relatively normal, the expression would eventually fall as the increase in SOD3 would not be great enough in magnitude to protect from the overwhelming allergen level and lead to progression of the disease. Thus, we tested the hypothesis. Indeed, the expression of SOD3 in the lung drastically increased upon the first OVA challenge, but gradually decreased upon the subsequent OVA challenges (Fig. 4B, upper right panel). SOD1 expression showed a similar pattern during the OVA challenge, but the level of expression was substantially lower than that of SOD3 (Fig. 4B, upper left panel). On the other hand, SOD2 expression changed little (Fig. 4B, upper middle panel). Thus, these data indicate that SOD3 acts as a first line of defense against allergens and a decision maker in the control of disease progression. However, the mice that received exogenous SOD3 reduced the level of endogenous SOD3, SOD1, and SOD2 when OVA was challenged (Fig. 4B, lower panel). Taken together, these results suggest that SOD3 is essential to maintain lung homeostasis and controls progression of allergic lung disease.

SOD3 alleviates OVA-induced extracellular remodeling in the airway

We next investigated how SOD3 alleviates OVA-induced allergic asthma. Extracellular remodeling in the airway is a common feature in asthma (9, 14). The remodeling occurs as the result of epithelial cell hyperplasia, subepithelial fibrosis, mucus metaplasia, and increased smooth muscle mass (9, 14). These responses are characterized by the enhanced accumulation of fibronectin and collagen, which leads to thickening of the airway wall. TGFβ plays a critical role in asthmatic extracellular remodeling, particularly in regard to subepithelial fibrosis. The inflammatory molecules tumor necrosis factor (TNF), TGF, and IL-1 induce fibroblast proliferation and matrix production, which lead to lung inflammation and alterations in ECM metabolism that result in the increased collagen deposition associated with fibrogenesis. Hence, we tested whether SOD3 could prevent the destruction of the ECM in the airway.

To do this, we first investigated the ultrastructure of the lung with scanning electron microscopy (SEM). As shown in Figure 5A and B, the structure of the airway wall in the lung was irregular and thick upon OVA challenge, but SOD3 prevented this remodeling (Fig. 5B, middle panel). Consistently, in

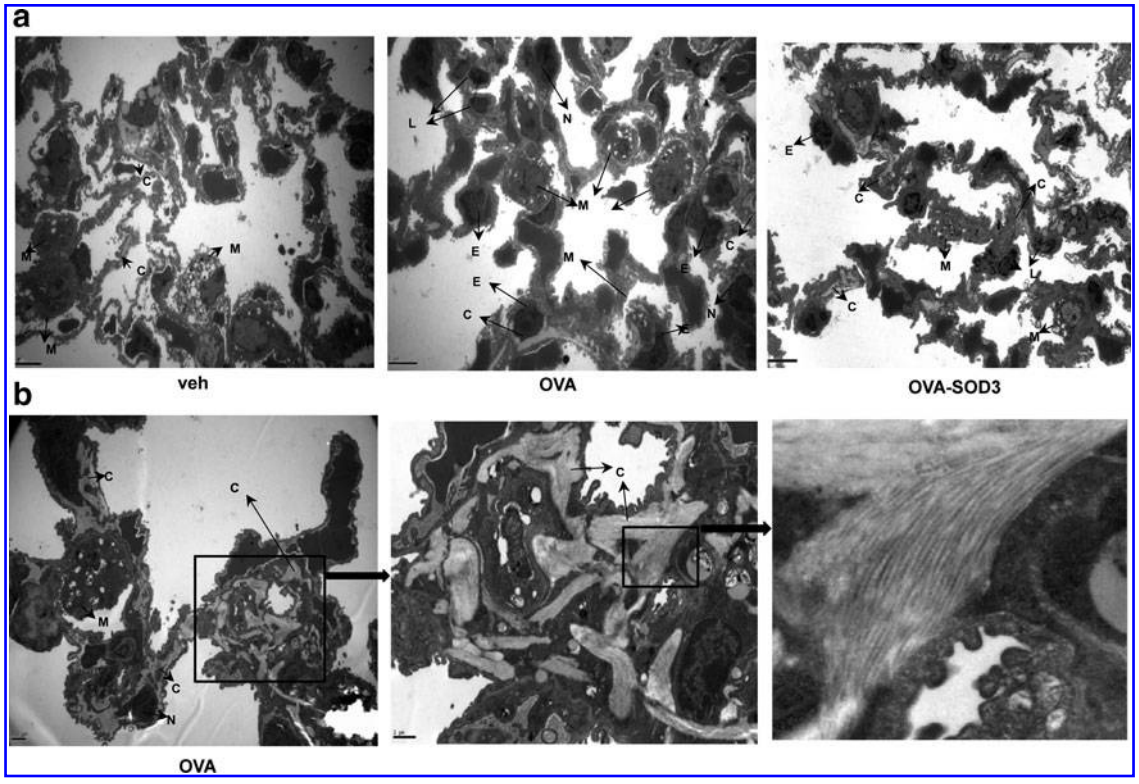


FIG. 6. SOD3 reduces OVA-induced collagen deposition in the airways. (a) Ultrastructure of the lung after the induction of asthma by OVA in the absence (*right panel*) or presence (*lower panel*) of SOD3 **(**the *left panel* of **a** depicts the lung ultrastructure of a control mouse**). (b)** Collagen deposition after OVA challenge. Inflammatory cells infiltrated and localized around airway vessel upon OVA challenge **(a,** *right panel***)** and collagen-mediated fibrosis occurred **(b,** *left panel***)**. But SOD3 reduced this localization as well as the collagen deposition **(a,** *lower panel***)**. BALB/c mice were OVA challenged and SOD3 treated as described in Figure 3. Lung tissues were prepared for transmission electron microscopy (TEM). The abbreviations in the images are indicative of the following: C, collagen; E, eosinophils; M, macrophages; L, lymphocytes; N, neutrophils. The data represent one of three independent experiments.

Masson's trichrome staining, the collagen was highly deposited in peribronchovascular area upon OVA challenge, but the level was drastically reduced by SOD3 (Fig. 5C). Correspondingly, the levels of the inflammatory molecules TNFα, TGFβ, IL-1α, and IL-1β in the BALF were also increased by OVA challenge but were reduced in the presence of SOD3 (Fig. 5D). Likewise, the levels of the extracellular remodeling molecules TGFβ, collagen IIIα1, syndecan-1, and ECM-1 were greatly increased in the lung upon OVA challenge, but the levels were reduced by SOD3 (Fig. 5E).

Further analysis with transmission electron microscopy (TEM) demonstrated that higher numbers of infiltrating eosinophils, neutrophils, and macrophages were in airway of OVA-challenged lungs (Fig. 6A, right panel). SOD3 administration reduced this infiltration and led to decreased collagen deposition (Fig. 6A, lower panel). In addition, we confirmed that the gradually deposited collagens in the OVA-challenged mice lead to lung fibrosis (Fig. 6B). Collectively, these data suggest that SOD3 negatively regulates extracellular remodeling in OVA-induced allergic asthma.

SOD3 acts, at least in part, as a controller of signal initiation and strength

To further understand how SOD3 inhibits allergic asthma, we investigated which proteins interacted with SOD3 in OVA-induced lung and whether exogenous SOD3 affected these interactions. To test this, we used MS analysis. The lungs of OVA-induced mice and those that also received exogenous SOD3 were lysed for immunoprecipitation with an antibody against SOD3. The immunoprecipitation product was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) on 4%–20% gradient gel, followed by Coomassie staining. Bands around 35–47 and 75–95 kDa were cut out for MS analysis (Supplementary Fig. S1). Mass spectra data were translated with quantitative value using Scaffold proteomic software. Identified proteins were obtained by 85% cut off range, which proteins interacted with SOD3 in OVA-induced lungs in the presence or absence of SOD3 (Table 1). Interestingly, SOD3 interacted with receptors, including EGF and TNF receptors; adaptors; adhesion molecules; kinases; phosphatases; apoptosis-related factors; and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. All of these interactions were altered by the addition of exogenous SOD3. Therefore, these results suggest that SOD3 mediates its effects on the ECM, at least in part, through controlling of the signaling initiation and strength.

SOD3 is necessary to prevent allergic asthma

To confirm the role of SOD3 in allergic asthma, we used SOD3 tg and KO mice that were on a C57BL/6 background.

Table 1. List of Proteins That Interacted with SOD3 in Ovalbumin-Challenged Lung in the Presence or Absence of SOD3

Identified proteins	*Accession number*	*MW (kDa)*	*Control*[a]	*OVA*[b]	*OVA + SOD3*[c]
Protease, serine, 1 precursor	gi|16716569	26	11.26	16.82	28.3
Cadherin EGF LAG seven-pass G-type receptor 3	CELR3_MOUSE	358	3.42	5.71	14.11
Down syndrome cell adhesion molecule-like protein	DSCL1_MOUSE	224	4.33	7.21	2.87
Inter-alpha trypsin inhibitor, heavy chain 2	gi|21707832 (+3)	106	3.42	6.53	3.26
Myosin, light polypeptide 4 (Fragment)	A2A6Q8_MOUSE	21	5.98	2.45	4.34
Tdrd1 protein	gi|125858006 (+10)	103	1.71	6.53	3.26
Zinc finger, MYM-type 3	gi|123230184 (+7)	153	2.6	3.61	5.75
Aryl hydrocarbon receptor nuclear translocator-like protein 2	BMAL2_MOUSE	64	1.73	3.61	4.31
Interleukin-1 receptor-like 1	ILRL1_MOUSE	65	2.6	3.61	4.31
SH3-domain binding protein 1	A2A5V2_MOUSE	65	1.73	4.81	1.44
NADPH oxidase 1	NOX1_MOUSE	68	0	3.61	1.44
Ly6/PLAUR domain-containing protein 3	LYPD3_MOUSE	37	2.6	3.61	0
Gamma-aminobutyric acid receptor subunit theta	GBRT_MOUSE	73	1.71	1.63	0
GRB2-associated binding protein 1	gi|31542871 (+1)	77	0.85	1.63	2.17
Gelsolin, isoform CRA_a, partial	gi|148676699 (+7)	83	1.71	0.82	3.26
Coiled-coil domain-containing protein 7 isoform 1	gi|21312852	43	0.85	1.63	2.17
Phosphoglycolate phosphatase	PGP_MOUSE	35	0.85	1.63	0
Fibroblast growth factor receptor 2 isoform IIIc	gi|116089349 (+25)	94	0.85	1.63	0
Apoptosis-inducing factor 2	AIFM2_MOUSE	41	0.85	0	2.17
Protein phosphatase 1 regulatory subunit 3B	PPR3B_MOUSE	32	0	1.63	1.09
Murine tumor necrosis factor receptor 2	gi|433831 (+2)	49	0.87	1.2	0
Transglutaminase	gi|201941 (+5)	77	0	1.63	0
Nonreceptor tyrosine kinase	gi|23328874	44	0	0.82	0
Collagen alpha-1(XXIV) chain precursor	gi|116326001 (+1)	176	0	0.82	0
Relaxin/insulin-like family peptide receptor 1	gi|148683511	**73**	**0**	**0.82**	**0**
Procollagen type XVI alpha 1	gi|126541148 (+6)	122	0	1.2	0
Alpha-N-acetylglucosaminidase	gi|123210113 (+2)	83	0	0	1.44
Adaptor-related protein complex AP-4, mu 1, isoform	gi|148687263 (+2)	46	0	1.2	0

[a–c]The treatment mice received as depicted in Figure 3. The number shown represents a quantitative value that was determined by normalizing spectra counts through Scaffold software analysis. Proteins were identified with an 85% cut off range and unknown proteins were excluded.

OVA, ovalbumin; SOD, superoxide dismutase.

As shown in Figure 7A, allergic asthma was induced in SOD3 tg, SOD3 KO, and wild-type (wt) C57BL/6 mice. Although the disease severity was milder in C57BL/6 mice than that in BALB/c mice, allergic asthma could be induced reliably in the C57BL/6 mice. A histological examination demonstrated that SOD3 tg mice showed decreased signs of allergic asthma compared with wt mice, whereas SOD3 KO mice showed relatively severe asthma compared with wt mice (Fig. 7B, C). Total number and inflammatory cells in the BALF were increased in antigen-challenged wt mice (Fig. 7D). Further, the levels of serum IgE and T-cell cytokines, including Th2 and Th17, were also significantly increased (Fig. 7E, F). Compared with the levels observed in wt mice, these parameters were decreased in antigen-challenged SOD3 tg mice, but greatly enhanced in SOD3 KO mice (Fig. 7B–E).

Interestingly, SOD3 KO mice that received the vehicle control also showed signs of lung inflammation upon histological examination and a significantly higher number of infiltrated cells in the BALF compared with vehicle-treated wt and SOD3 tg mice (Fig. 7B, upper panel; Fig. 7D), indicating that SOD3 is necessary for maintaining lung homeostasis and preventing lung inflammation. Interestingly, compared with allergic asthma-afflicted BALB/c mice, SOD3 KO mice showed a drastically increased number of lymphocytes. But the increase was not observed in wt and SOD3 tg mice. These results suggest a role for SOD3 in the regulation of lymphocyte infiltration during lung inflammation.

Exogenous SOD3 ameliorates OVA-induced allergic asthma in SOD3 KO mice

To further confirm the preventative role of SOD3 in allergic asthma, we injected recombinant SOD3 into SOD3 KO mice before sensitizing and challenging with OVA (Fig. 8A). As shown in Figure 8, allergic asthma was alleviated by the administration of SOD3 to SOD3 KO mice (Fig. 8B–E). We further verified this by adoptively transferring SOD3-sufficient CD4 T cells into SOD3 KO mice.

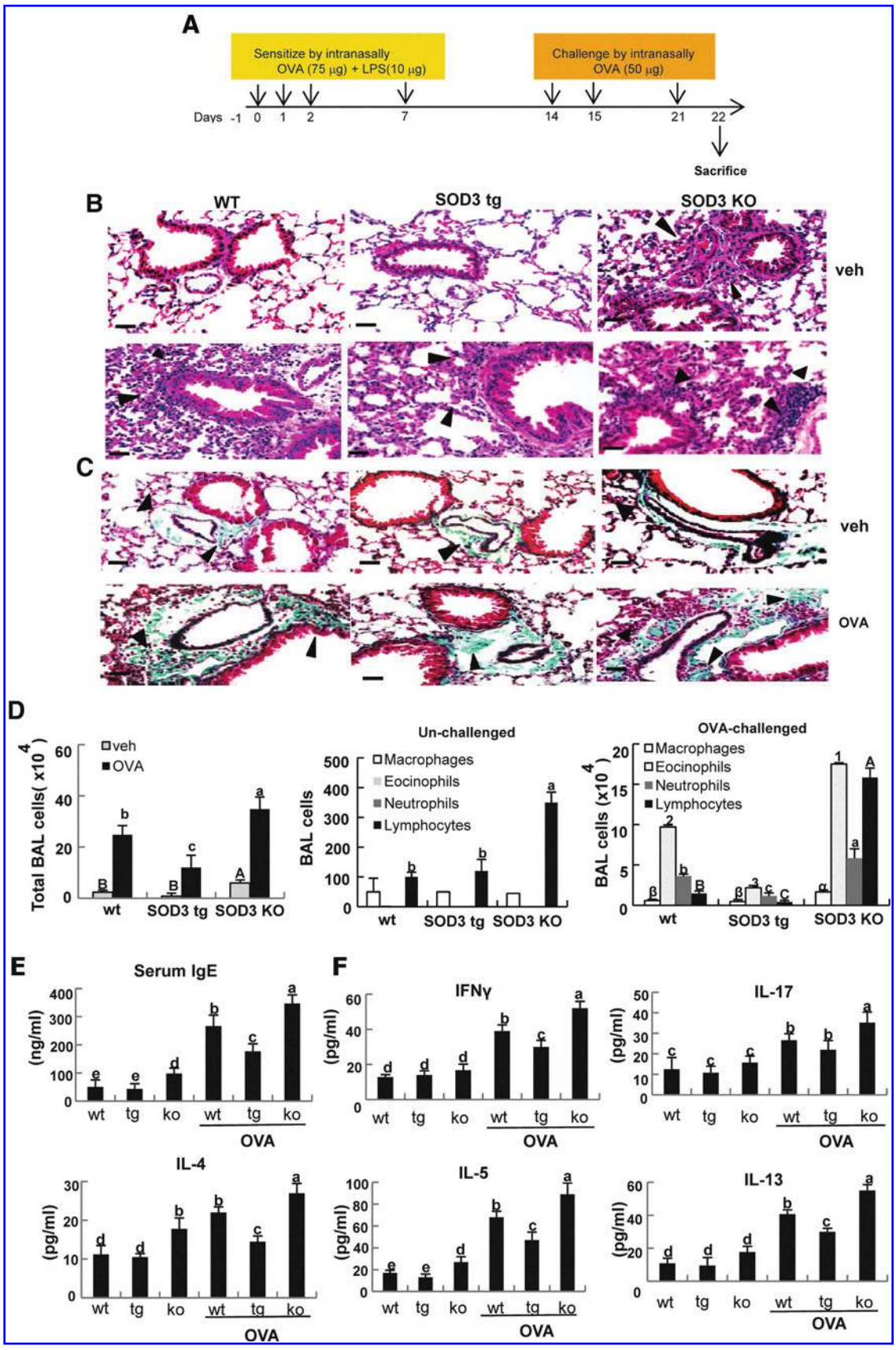
Since we observed that CD4 T cells mediated adaptive immune responses including T cell activation and proliferation, and Th2 and Th17 cell differentiation are inhibited by treatment with SOD3, we speculated that SOD3 KO mice may have improper CD4 T cell–mediated immune response upon allergens, which may cause relatively severe asthma. To test whether SOD3-sufficient CD4 T cells would recover OVA-induced allergic asthma, SOD3-sufficient CD4 T cells were adoptively transferred to the SOD3 KO mice before sensitization (Fig. 8A). As we expected, the allergic asthma in SOD3 KO mice was reduced by the adoptive transfer of SOD3-sufficient CD4 cells (Fig. 8B–E). Further, we observed that administered SOD3 or adoptive transferred SOD3-sufficient CD4 T cells to SOD3 KO mice showed increased SOD3 expression in the lung (Supplementary Fig. S2). Taken together, these data suggest that SOD3 is necessary for the prevention of allergic lung inflammation and maybe used as a therapeutic purpose against allergic asthma.

Discussion

The antioxidative effect of SOD3 is well established (1, 11, 15, 25). However, the role of SOD3 in immune responses remains unexplored. In this report, we demonstrated a unique role of SOD3 in controlling immune response. Unlike other SODs, SOD3 specifically inhibits DC maturation. This is probably because the key events for DC maturation occur extracellularly where SOD3 is mainly located. Indeed, we previously found that SOD3 controls Toll-like receptor 4 trafficking to lipid rafts, which is necessary for the initiation of signaling (17). Therefore, SOD3 may control allergen-mediated signaling at the extracellular level.

SOD3 inhibits Th2 and Th17 differentiation *in vitro* but does not affect Th1 differentiation. Given the importance of the role of SOD3 in Th cell differentiation, it remains unknown how SOD3 selectively blocks Th2 and Th17 differentiation. Perhaps, the different types of Th cells require different types of signaling for differentiation, and SOD3 selectively regulates this signaling. To support this possibility, it has been shown that the kinase mTOR regulates the differentiation of Th cell through the selective activation of signaling by mTORC1 and mTORC2; mTORC1 regulates Th1 and Th17, whereas mTORC2 signaling regulates Th2 cell differentiation (7). Another study showed that inducible co-stimulator (ICOS), a signal mediator to activate T cell with T-cell receptor signal (TCR), selectively controls Th2 differentiation by providing a unique signal (31). Thus, SOD3 may inhibit mTORC2 and/or ICOS signaling and thus, blocks Th2 differentiation. In addition, SOD3 may selectively regulate IL-6-mediated STAT3 or TGFβ signaling that drives Th17 differentiation. Indeed, we have observed that SOD3 inhibits STAT3 phosphorylation (data not shown). Consequently, SOD3 may control their transcriptional factors by regulating the recruitment of basal transcriptional machinery and co-activators, releasing repressors on the promoter, or controlling histone modification. Thus, Th cell differentiation could be dictated by the nature of the initial trigger of an immune response, such as the type of pathogens. Therefore, it is likely that SOD3 is selectively controlled by pathogens.

FIG. 7. SOD3 is required for controlling allergic lung inflammation. (A) The experimental scheme of the allergic asthma in SOD3 tg, SOD3 knockout (KO), and wt C57/BL6 mice. **(B, C)** Severe asthma was observed in SOD3 KO mice, whereas only mild levels of airway inflammation were observed in SOD3 tg mice. Mice were intranasally sensitized with OVA (75 μg) plus LPS (10 μg) and were intranasally challenged three times with OVA (50 μg) as described in the Materials and Methods section. Mice were sacrificed 24 h after the final allergen challenge, and lung sections were stained with H&E **(B)** and Masson trichrome **(C)**. Bars = 50 μm. A representative image of the lung histology is shown. *Arrowheads* indicate infiltrated inflammatory cell along the larger airways and blood vessels, and collagen deposition in peribronchovascular area. Total cell numbers **(D,** *left panel***)** and inflammatory cells **(D,** *right* and *middle panels***)** in the BALF of OVA-challenged or unchallenged mice. The level of serum IgE **(E)** and Th1, Th17, and Th2 cytokines—IL-4, IL-5, and IL-13—in BALF **(F)** was measured by ELISA. BALF and ELISA data represent the mean ± SD of three independent experiments. Statistical analysis was performed by ANOVA at $p < 0.01$ level and Schiffe's *post hoc* test was followed. a–d, A–D, 1–3, α, and β on the figure indicate grouping of Schiffe's *post hoc* test. (To see this illustration in color the reader is referred to the web version of this article at www.liebertpub.com/ars).


A
Sensitize by intranasally
OVA (75 μg) + LPS(10 μg)
Challenge by intranasally
OVA (50 μg)
Days -1 0 1 2 7 14 15 21 22
Sacrifice
B
WT
SOD3 tg
SOD3 KO
veh
C
veh
OVA
D
Total BAL cells(x10^4)
veh
OVA
wt
SOD3 tg
SOD3 KO
Un-challenged
BAL cells
Macrophages
Eocinophils
Neutrophils
Lymphocytes
OVA-challenged
E
Serum IgE
(ng/ml)
wt tg ko wt tg ko
OVA
F
IFNγ
(pg/ml)
IL-17
IL-4
IL-5
IL-13

In contrast with our report, it has been reported that OVA-induced asthma in SOD3 KO mice was not significantly different comparing with wt mice (28). Perhaps, this discrepancy is due to the fact that younger mice were challenged with lower concentration OVA indirectly. Moreover, our study showed that comparing with BALB/c, C57BL/6 mice are less susceptible for OVA-induced allergic asthma. Lower concentration of OVA (25 μg/ml) and challenging with aerosolized OVA (1% w/v) in chamber, instead of direct intranasal challenge, may not be sufficient to induce allergic asthma in C57BL/6 background SOD3 KO mice. Indeed, they showed that mild increased BALF neutrophils and monocytes without affect on the level of IL-6, MIP-2, and TNFα (28). However, although the level was lower, they showed that IL-5 and monocytes in BALF were significantly increased in SOD3 KO mice, which is consistent with our results. Further, perhaps 6-week-old mice in this study may have lower susceptibility for allergen than older mice. Indeed, we have observed that the incidence of asthma in 5-week-old female was comparably lower than that in 8-week-old mice (data not shown). Lower incidence of asthmatic inflammation might be due to lower induced reactive oxygen species (ROS) or reactive nitrogen species (RNS) from eosinophils and neutrophils that were generated by allergens; thus, the symptom of SOD3 KO mice was comparably milder. Therefore, this lower incidence caused by lower ROS signaling would not be able to expect blockade effect by either administration of recombinant SOD3 or adoptive transfer of SOD3-sufficient CD4 T cells to SOD3 KO mice. However, interestingly, the study showed that old SOD3 KO mice (22-month old) showed higher morphology for bronchial and alveolar inflammation, and fibrosis (28), indicating that old SOD3 KO mice might have susceptible amount of ROS or RNS. Indeed, a supportive study showed that younger mice (4 months) have low susceptibility for LPS-induced systemic inflammation than the older mice (24 months), with production of less amount of nitrotyrosine and remaining comparably higher level of SOD3 (29). Therefore, it is likely that the requirement of SOD3 to prevent or alleviate allergic asthma is more susceptible for the older mice than the younger ones.

We found that the addition of exogenous SOD3 prevents the remodeling of the airway ECM when challenged with OVA. In addition, we demonstrated that SOD3 controls the expression of the inflammatory genes TGFβ, TNFα, IL-1α, and IL-1β, all of which are involved in the extracellular remodeling in the lung when asthma was induced. These genes are controlled by nuclear factor kappa B (NFkB) trans-activation, and we earlier reported that SOD3 inhibits these inflammatory genes at the transcriptional level by controlling the recruitment of the NFkB subunit p65 to their promoter regions (17). Further, our study showed that SOD3 controls OVA-induced Th2-associated chemokine receptor CCR4 and adhesion molecule CD62L-expressed CD4 T cells, indicating the role of SOD3 for Th2 cell trafficking. Similar study showed that the transfer of an adenovirus-carrying SOD3 gene reduces inflammatory cell migration by downregulating the expression of adhesion molecules and cytokines in rat ischemic muscle (19). Therefore, it is likely that SOD3 controls the initiation of allergen-mediated signaling that contributes many biological processes, including the regulation of inflammatory genes.

In addition, as SOD3 is primarily located in the ECM, we are tempted to speculate that the distinct role of SOD3 is due to its ability to block signaling events at the plasma membrane, such as the formation of receptor–ligand complexes and interactions between signaling molecules. Indeed, through MS analysis, we identified proteins that interact with SOD3 in the OVA-induced lung. The proteins that interacted with SOD3 were receptors, including EGF and TNF receptors; adhesion and adaptor molecules; kinases; phosphatases; apoptosis-related factors; and NADPH oxidase, and these interactions could be affected by the addition of exogenous SOD3. Indeed, our previous study showed that SOD3 inhibited NADPH oxidase activation by interfering with the interaction of NADPH oxidase subunit $p91^{phox}$ and $p47^{phox}$ (17). Thus, these results suggest that SOD3 regulates the initiation and strength of signaling, which, in turn, controls the progress of disease. Therefore, future studies are warranted to unveil the many roles of SOD3 in the pathogen-mediated immune response.

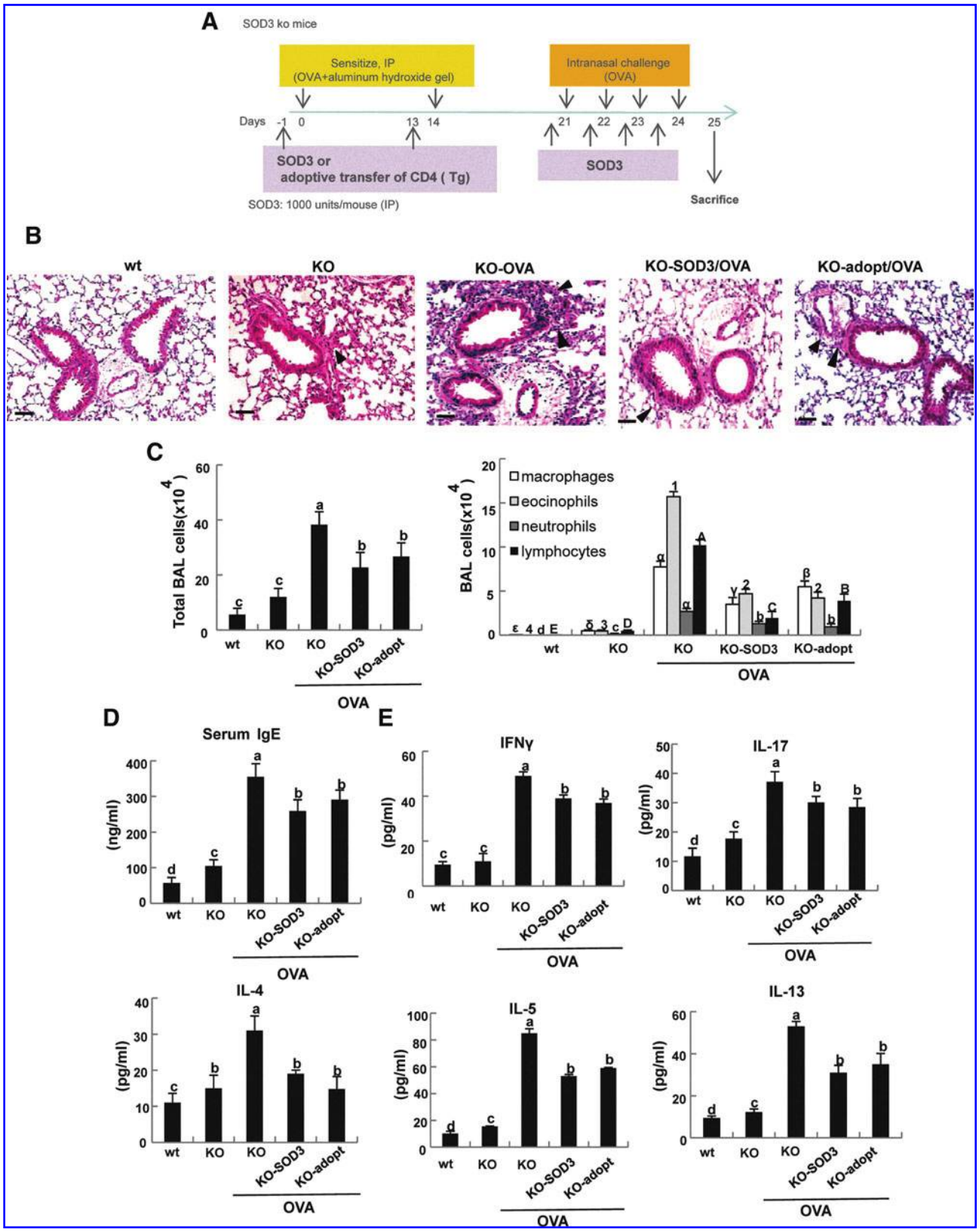
Previously, we observed that SOD3 expression was significantly downregulated in atopic and psoriatic patients (16). In this study, we found that SOD3 plays a critical role in adaptive immune response by modulating DC maturation, T-cell activation and proliferation, Th2 and Th17 cell differentiation, and Th2 cell homing. Considering that atopy and psoriasis are also associated with improper Th2 and Th17 immune response, the ability of SOD3 to regulate Th2- and Th17-mediated immune responses provides a potential clue for determining the specific role of SOD3 in the control of atopic skin disease and other Th2- or Th17-mediated disorder.

Materials and Methods

Animals

C57BL/6 mice, and SOD3 tg and SOD3 KO mice were used as previously described (17). Eight-week-old BALB/c mice were purchased from the Central Lab Animal, Inc. (Woomyun-

FIG. 8. SOD3 ameliorates OVA-induced allergic asthmatic inflammation. (A) The experimental paradigm for OVA-induced allergic asthma in SOD3 KO mice. **(B–E)** OVA-induced allergic asthma was ameliorated by both exogenous injection of SOD3 and the adoptive transfer of SOD3-sufficient CD4 T cells into SOD3 KO mice. Exogenous SOD3 (1000 units/mouse) was IP injected into SOD3 KO mice 1 day before sensitization and 6 h before each challenge, and adoptive transfer of SOD3-sufficient CD4 T cells into SOD3 KO mice was performed as described in the Materials and Methods section. Purified CD4 T cells (2×10^6), obtained from the spleens of SOD3 tg mice, were adoptively transferred into SOD3 KO mice. Twenty-four hours later, the mice were sensitized with OVA. H&E staining of lung sections **(B)**, total and inflammatory cells **(C)** in BALF, the level of serum IgE **(D)**, and cytokines in BALF **(E)**—IFNγ for Th1; IL-17 for Th17; and IL-4, IL-5, and IL-13 for Th2—was measured as described in the Materials and Methods section. Image data represent one of three independent experiments. Scale bars = 100 μm. BALF cells and ELISA data represent the mean ± SD of three independent experiments. Statistical analysis was performed by ANOVA at $p < 0.01$ level and Schiffe's *post hoc* test was followed. a–d, A–D, 1–3, and α–ε on the figure indicate grouping of Schiffe's *post hoc* test. (To see this illustration in color the reader is referred to the web version of this article at www.liebertpub.com/ars).


A
SOD3 ko mice
Sensitize, IP
(OVA+aluminum hydroxide gel)
Intranasal challenge
(OVA)
Days -1 0 13 14 21 22 23 24 25
SOD3 or
adoptive transfer of CD4 (Tg)
SOD3
Sacrifice
SOD3: 1000 units/mouse (IP)
B
wt
KO
KO-OVA
KO-SOD3/OVA
KO-adopt/OVA
C
Total BAL cells(x10 4)
BAL cells(x10 4)
macrophages
eocinophils
neutrophils
lymphocytes
wt KO KO KO-SOD3 KO-adopt
OVA
D
Serum IgE
(ng/ml)
IL-4
(pg/ml)
E
IFNγ
IL-17
IL-5
IL-13
(pg/ml)

dong, Seoul, Korea). OT-II TCR tg mice (H-2^b), originally generated by Barnden *et al.* (3), were obtained from Dr. Yungdae Yun (Ewha Women's University, Seoul, Korea). All mouse care and experimental procedures were performed under specific pathogen-free conditions in accordance with established institutional guidance and approval from the Research Animal Care Committee in Catholic University (Seoul, Korea).

Preparation of recombinant SOD3

The recombinant SOD3 was prepared as described previously (17). In brief, 293 cells were transient transfected with SOD3 construct for 48 h. The supernatant was collected, and purified using a column containing Ni-NTA agarose (Qiagen), and dialysis. The purified SOD3 activity was measured with a SOD assay kit (Dojindo). To inject into the mice or treatment *in vitro*, SOD3 was filtered to eliminate endotoxin.

Maturation of dendritic cells

Bone-marrow-derived DCs (BMDCs) were cultured as described previously (17). In brief, total BM cells from femurs and tibias were flushed out with 2 ml of RPMI 1640 media using a 23-gauge needle. RBCs were depleted with hemolysis buffer (Sigma) and purified using lineage cell depletion kit (Miltenyi Biotec) and cultured in RPMI media with 10 ng/ml murine recombinant granulocite macrophage-colony stimulating factor (rGM-CSF) (R&D Systems) for 5 days. LPS (Sigma) was added for 10 h before harvest. In some experiments, recombinant SOD3 or SOD1 (ATGen) or SOD2 (ATGen) was added to the culture. Additional rGM-CSF, SOD3, SOD1, and SOD2 were added in the middle of culture. CD11c+ cells for DCs were gated and fluorescence-conjugated antibodies—I-A for MHC II, CD80, and CD86 (BD Pharmingen)—were used for flow cytometry analysis.

T cell priming in vitro *and* in vivo

For *in vitro* T cell priming, naive CD4 T cells were enriched from total splenocytes of OT-II TCR tg mice by negative selection using mouse CD4+ cell isolation kit (Miltenyi Biotec). BMDCs from C57BL/6 wt mice were matured with rGM-CSF (10 ng/ml) for 5 days and LPS (100 ng/ml) for 10 h, and extensively washed. The matured DCs (50,000 cells) were co-cultured with enriched CD4 T cells (50,000 cells) plus 10 μg/ml $OVA_{332–339}$ peptide (Genscript) in the presence or absence of SOD3 (100 units/ml) in 96-well plates for 5 days. At day 3, the cells were re-stimulated with $OVA_{332–339}$ peptide with or without SOD3. For *in vivo* T cell priming, purified naive CD4 T cells (5×10^6, 100 μl) from splenocytes of OT-II TCR tg mice were adoptively transferred to the wt or SOD3 tg mice. One day later, the mice were intranasally challenged with 75 μg OVA (Sigma) plus LPS (10 μg). At day 3, the spleen was isolated and splenocytes were prepared, and cultured with $OVA_{332–339}$ peptide (10 μg/ml) for 3 days. Supernatants were collected to assess IL-2 for T cell activation. The cells were used for T cell proliferation assay, as pulsing with 1 μCi ^{3}H-thymidine for 18 h. Cell proliferation was analyzed using a gamma counter (Packard).

In vitro *Th cell differentiation*

Naive CD4 T cells were purified from spleen of C57BL/6 mice by negative selection using mouse CD4+ cell isolation kit (Miltenyi Biotec) and were stimulated with each Th cell differentiation condition—Th17: anti-CD3 (1 μg/ml), anti-CD28 (1 μg/ml), TGFβ (2 ng/ml), IL-6 (2 ng/ml), anti-IFNγ (2 μg/ml), and anti-IL-4 (2 μg/ml); Th1: anti-CD3 (1 μg/ml), anti-CD28 (1 μg/ml), and anti-IL-4 (2 μg/ml); Th2: anti-CD3 (1 μg/ml), anti-CD28 (1 μg/ml), IL-4 (2 ng/ml), and anti-IFNγ (2 μg/ml). All antibodies and cytokines were obtained from BD Pharmingen. Various doses of recombinant SOD3 were added in these Th cell differentiation conditions. In some experiments, SOD1 (100 units/ml) or SOD2 (100 units/ml) was added. At day 3, PMA (Sigma; 1 ng/ml) and ionomycin (Sigma; 2 n*M*) were added and incubated for 4 h. Golgi stop (BD Pharmingen; 2 ng/ml) was included with the PMA and ionomycin to block transport of secreted cytokines to Golgi, resulting in the accumulation of most cytokines in the endoplasmic reticulum and enhancing cytokine staining signal. After surface staining with fluorescein isothiocyanate (FITC) conjugated anti-CD4, cells were fixed using BD cytofix/cytoperm (BD Pharmingen) for 30 min. The cells were permeabilized using BD Perm/wash buffer. Staining with antibody to detect the production of cytokine of each Th cell—IFNγ for Th1, IL-4 for Th2, and IL-17A for Th17—was performed for 30 min at 4°C, followed by flow cytometry analysis. Fluorescence conjugated antibodies for IFNγ, IL-4, and IL-17A were purchased from BD Pharmingen.

OVA sensitization and airway challenge

BALB/c mice were sensitized on day 1 and day 14 by IP injection with 50 μg OVA (Sigma) emulsified in 1.3 mg aluminum hydroxide gel (Sigma) in a total volume of 200 μl. Nonsensitized control mice were injected with phosphate-buffered saline (PBS) plus aluminum hydroxide gel without OVA. Intranasal challenge with OVA (150 μg in 40 μl PBS) was performed four times starting from day 21 to day 25. Recombinant SOD3 was applied for pharmacological intervention of asthma. SOD3 (2000 units/mouse, 150 μl) was IP injected 1 day before sensitizing and 6 h before OVA challenging. Correspondingly, Zileuton (Sigma; 50 mg/kg mouse), an anti-asthma therapeutic agent that inhibits production of leukotrienes, was orally administered at 1 day before sensitization, and 1 day before each challenge. In some experiments, kinetic study upon OVA challenging was performed to evaluate the disease progression.

To induce allergic asthma in SOD3 tg, KO, and C57BL/6 wt mice, OVA (75 μg) plus LPS (10 μg) were intranasally sensitized on days 0, 1, 2, and 7 and intranasally challenged with OVA (50 μg) on days 14, 15, 21, and 22. To test recovery of allergic asthma by administration of SOD3, exogenous SOD3 (1000 units/mouse) was IP injected before sensitizing and challenging with OVA. As an alternative, SOD3-sufficient CD4 T cells (2×10^6 cells) were purified from splenocytes of SOD3 tg mice and adoptively transferred to SOD3 KO mice before sensitizing with OVA. Allergen-specific adaptive immune responses were examined 24 h after the last allergen challenge.

BALF and inflammatory cells

Mice were killed by IP injection of an overdose of a mixture of zoletil (Virbac) and Rompun (Bayer Health Care). The

lungs were subjected to BAL with 800 μl PBS instilled *via* a tracheal polyethylene catheter twice. BALF was centrifuged at 3000 rpm for 10 min and the supernatant from the first BALF suspension was used to measure cytokines and inflammatory molecules. Collected pellets were resolubilized with 200 μl of PBS. Fifty microliters of cell suspension was counted by hemocytometer. Another 50 μl suspension was subjected to cytospin at 450 rpm for 5 min, followed by Diff-Quick staining (Sysmex Corporation) to detect inflammatory cells. Inflammatory cells were classified as eosinophils, neutrophils, macrophages, or lymphocytes. Types of inflammatory cells were determined by counting 500 cells with a differential cell counter (Hwashin Tech) using standard morphological criteria (27). After lavage, lungs were excised completely from the chest, some parts were fixed for histology or electron microscopy, and other parts were excised for RNA and protein analyses.

Lung histology and ultrastructure examination

The lung was fixed in 4% paraformaldehyde in PBS and embedded in paraffin according to general histochemical procedure. The lung sections were stained with hematoxylin and eosin, and Masson's trichrome. Lung inflammation was assessed by the degree of peribronchiolar and perivascular inflammation as described previously (23).

For examination of ultrastructure, electron microscope analysis was performed. For SEM, lung tissues were fixed in 4% paraformaldehyde and 2.5% glutaraldehyde in 0.1 *M* phosphate buffer for 12 h, and postfixed with 1% osmium tetroxide in PBS for 1 h. The samples were dehydrated in ascending concentrations of acetone from 50% to 100% for 12 h at each concentration, transferred to hexamethyldisilazane, and allowed to air dry. The sample was coated with gold using a sputter coater and examined on a JSM-5410LV (JEOL) with accelerating voltage of 15 kV.

For TEM, lung tissues were fixed using the same procedure as SEM. Then, the sample was dehydrated with a series of graded ethyl alcohol and pure acetone. The sample was embedded in Epon 812 and polymerized at 60°C for 3 days. Ultrathin sections (60–70 nm) were obtained by ultramicrotome (Leica Ultracut UCT). Ultrathin sections collected on grids (200 mesh) were examined in a transmission electron microscope (JEM 1010) at 60 kV. The images in the TEM were recorded by CCD camera (SC 1000; Gatan).

Quantitative real-time polymerase chain reaction

Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using the cDNA. The mouse primers used for qRT-PCR were as follows: collagen, type IIIα1 (5′-GGACCAGCAGGAACTAATGG, 3′-CACCAGGTTCTCCAGGTGAT); TGFβ (5′-CACCATCCATGACATGAACC, 3′-CAACCCAGGTCCTTCCTAAAG); ECM protein-1 (ECM-1) (5′-CTCTGCTGTGACCTGTCTCCT, 3′-GGTGACTCATTCTTCCTTGGAC); Syndecan-1 (5′-TTCTACCACAACCACACCTG, 3′-GGTAAGTGCTATGGCTGGTC); SOD1 (5′-CGGTGAACCAGTTGTGTTGT, 3′-CTCCAACATGCCTCTCTTCA); SOD2 (5′-CCGAGGAGAAGTACCACGAG, 3′-GCTTGGTAGCCTCCAGCAAC); and SOD3 (5′- GACCGGTGCAGAGGAACCTCA, 3′-ATGCGTGTCGCCTATCTTCT).

Enzyme-linked immunosorbent assay

IgE, IL-4, IFNγ, IL-17, TNFα, and TGFβ enzyme-linked immunosorbent assay (ELISA) reagents were purchased from BD Biosciences. IL-5 and IL-13 ELISA reagents were purchased from eBioscience. IL-1α and IL-1β ELISA reagents were purchased from Komabiotech. ELISA was performed according to the manufacturer's instructions.

Flow cytometry

PE-conjugated MHC II (I-A), FITC-conjugated CD80, FITC-conjugated CD86, PE-conjugated CD62L, APC-conjugated IFNγ, APC-conjugated IL-4, PE-conjugated IL-17, FITC-conjugated CD4 T cells, and PE-conjugated CCR7 were purchased from BD Pharmingen. APC-conjugated CCR4 antibody was obtained from Bio-Legend. Cell surface MHC II; co-stimulatory molecules CD80 and CD86; cytokines IFNγ, IL-4, IL-17; and chemokine receptors CCR4 and CD62L were detected by flow cytometry (FACS Calibur; BD Bioscience). The data were analyzed with Cell Quest software (Becton Dickinson).

MS analysis

The lung of OVA-induced mice in the presence or absence of exogenous SOD3 was lysed for immunoprecipitation with antibody against SOD3 (Abcam). The immunoprecipitation products were subjected to SDS-PAGE on 4%–20% gradient gel, followed by Coomassie staining (Supplementary Fig. S1). Bands around 35–47 and 75–95 kDa were cut out for MS analysis. Nono-LC-MS/MS analysis was performed on an Agilent Nano-HPLC 1100 system associated with LTQ mass spectrometer (Thermo Electron). Mass spectra were acquired using data-dependent acquisition with a full mass scan (400–1800 *m/z*) followed by MS/MS scans. Each MS/MS scan acquired was an average of three microscans on the LTQ. The data were translated with quantitative value with Scaffold software (Proteome Software, Inc.). Identified proteins were obtained by 85% cut off range and unknown proteins were excluded.

Acknowledgments

This work was supported by National Research Foundation (NRF) Grant: Future-based Technology Development Project funded by the Ministry of Education, Science and Technology (2010-0002058), Republic of Korea. The authors thank Dr. James D. Crapo (University of Colorado Denver, Denver, CO) for providing SOD3 tg and KO mice.

Author Disclosure Statement

The authors declare no conflict of interest.

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Date of first submission to ARS Central, February 15, 2012; date of final revised submission, May 15, 2012; date of acceptance, May 15, 2012.

Abbreviations Used

ANOVA = analysis of variance
BALF = bronchoalveolar lavage fluid
BMDCs = bone-marrow-derived dendritic cells
CCR4 = chemokine receptor 4
DC = dendritic cell
ECM = extracellular matrix
EGF = epidermal growth factor
ELISA = enzyme-linked immunosorbent assay
FITC = fluorescein isothiocyanate
ICOS = inducible co-stimulator
IFNγ = interferon γ
IL-4 = interleukin-4
IL-17 = interleukin-17
IP = intraperitoneally
KO = knockout
LN = lymph node
LPS = lipopolysaccharide
MS = mass spectrometry
NADPH = nicotinamide adenine dinucleotide phosphate
NFkB = nuclear factor kappa B
OVA = ovalbumin
rGM-CSF = recombinant granulocyte macrophage-colony stimulating factor
ROS = reactive oxygen species
RNS = reactive nitrogen species
SD = standard deviation
SDS-PAGE = sodium dodecyl sulfate–polyacrylamide gel electrophoresis
SEM = scanning electron microscopy
SOD = superoxide dismutase
TCR = T-cell receptor signal
TEM = transmission electron microscopy
tg = transgenic
TGFβ = transforming growth factor β
Th = T helper
TNFα = tumor necrosis factor α
wt = wild-type