



# Superoxide dismutase 3 attenuates experimental Th2-driven allergic conjunctivitis☆☆☆



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## ARTICLE INFO

### Article history:

Received 29 November 2016

Received in revised form 29 December 2016

accepted with revision 29 December 2016

Available online 04 January 2017

### Keywords:

Allergic conjunctivitis

SOD3

Eosinophil

IgE

Th2 type immune response

## ABSTRACT

Allergic conjunctivitis is an inflammatory eye disease mediated by Th2 type immune response. The role of extracellular superoxide dismutase 3 (SOD3) in immune response and allergic conjunctival inflammation was examined in a murine model for experimental allergic conjunctivitis (EAC). Allergic conjunctivitis was induced in mice by allergen challenge with ovalbumin in alum via the conjunctival sac. SOD3 was topically applied and allergy indicators were compared. Clinical signs associated with conjunctivitis, such as OVA-specific IgE production, IgG1/G2a ratio and eosinophil infiltration, were drastically reduced in mice treated with SOD3. They also had less dendritic cells and CD4<sup>+</sup> T cells in conjunctiva than controls. Attenuated allergic inflammation was accredited to reduced Th2 type cytokine responses and increased Treg cytokine in draining lymph node. The characteristics of EAC were attributed to the absence of SOD3. Our findings suggest that SOD3 might be considered as a potential target for Th2-driven allergic conjunctival inflammation.

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## 1. Introduction

Ocular surface disorders have seen a dramatic increase over the last 30 years. In particular, the prevalence of allergic conjunctivitis has steadily risen as a common ocular surface disease. Ranges of severity vary for allergic conjunctivitis, from mild forms including seasonal and perennial AC, to severe cases. Examples of severe forms of AC include atopic and vernal keratoconjunctivitis, which is prone to increased complications due to corneal damage leading to permanent visual loss [1].

The etiology and underlying immunopathogenic mechanisms present for allergic disorders such as AC are closely associated with

immunoglobulin E (IgE)-mediated and Th2 type immune responses [2,3]. By specific provocation of the conjunctiva, a reproduction of an IgE-mediated allergic reaction may be induced, which further drives a predominant infiltration of eosinophilic inflammatory cells [4,5]. As such, severe chronic ocular allergy is defined by a significant number of eosinophils, which are the hallmarks of this disease, found in tissues and tears in patients. A complex system of chemotactic factors, such as interleukin 5 (IL-5), eotaxins 1–3 (CCL11, 24, 26), and regulated on activation normal T cell expressed and secreted (RANTES), with the addition of numerous cells drives recruitment of eosinophils. An important role of IL-5 includes stimulating the proliferation, differentiation, and survival of eosinophils in the bone marrow [6,7].

Extracellular superoxide dismutase 3 (SOD3) is unique in that it is a glycoprotein that constitutes much of the extracellular matrix (ECM) of tissues and is anchored to heparin sulfate proteoglycans in the glycocalyx of cell surfaces. It is also an isoform of SOD that scavenges superoxide radicals [8,9]. In murine models, SOD3 inhibits dendritic cell maturation [10] and modulates Th2 immune responses that result in attenuation of allergic asthma and skin inflammation [10–12]. However, the expression and role of SOD3 in allergic conjunctivitis is not clear.

In this current study, we hypothesized that SOD3 may play a role in regulation of Th2 adaptive immune responses and the progression of allergic conjunctival inflammation. We tested this hypothesis by evaluating the role of SOD3 in ovalbumin-induced allergic conjunctivitis in WT and SOD3 KO mice.

☆ The authors have no financial support and no financial or proprietary interests in this study.

☆☆ Grant supports: This study was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT & Future Planning (2014R1A3A052852, S-H.C) and the Bio & Medical Technology Development Program of the National Research Foundation (NRF) funded by the Ministry of Science, ICT & Future Planning (NRF-2013M3A9A9050567, T-Y.K).

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## 2. Methods

### 2.1. Animals

In this current study, the “ARVO Statement for the Use of Animals in Ophthalmic and Vision Research” protocol was followed. C57BL/6 mice and SOD3 KO mice were used as previously described [10]. BALB/c mice (8-wk.-old females) were purchased from Charles River Laboratories (Orient Co., Sungnam, Korea) and placed under pathogen-free conditions at the Catholic University of Korea (Seoul, Korea) in the animal facilities. Mice were maintained and received sterilized food and water ad libitum. The Institutional Animal Care and Use Committee approved all procedures.

### 2.2. Preparation of recombinant SOD3

The recombinant SOD3 was prepared as mentioned above [10]. In short, SOD3 was used to transiently transfect 293 cells for 48 h. A column containing Ni-NTA agarose (Qiagen, Hilden, Germany) and dialysis were utilized to purify the collected supernatants. Subsequently, a SOD assay kit (Dojindo, Rockville, MD) was used to measure the activity of the purified SOD3 and then filtered to eliminate possible endotoxins prior to injecting into the mice or treatment in vitro.

### 2.3. Experimental allergic conjunctivitis (EAC) models

BALB/c mice were sensitized i.p. with 10 µg of ovalbumin (OVA; Sigma-Aldrich, St. Louis, MO) and 200 µl of 1.5% aluminum hydroxide (Alum; Pierce, Rockford, IL), on days 0 and 7 for the generation of experimental allergic conjunctivitis (EAC). Starting from day 15 to 18, mice were then challenged with 250 µg of OVA in the conjunctival sac. The same dose of alum was injected to control mice for sensitization, replacing OVA for PBS in challenge stage. For 4 days from day 15 to 18, recombinant SOD3 (1000 unit/mouse, 5 µl) was given at 30 min before OVA challenge in the conjunctival sac.

C57BL/6 and SOD3 KO mice were sensitized i.p. with 10 µg of OVA, 300 ng of cholera toxin (Sigma-Aldrich) and 200 µl of 1.5% alum, on days 0 and 7. Referencing models from past studies, the mice were then challenged daily with 250 µg of OVA in the conjunctival sac from day 9 to 18 [13]. The same dose of cholera toxin and alum was injected to control mice for sensitization, replacing OVA for PBS in challenge stage.

### 2.4. Analysis of clinical scoring

A clinical verification on the occurrence and severity of conjunctivitis was achieved by examination of the mice on day 18 after 20 min of OVA challenge. A total of four clinical signs were examined in a blind fashion and scored referencing Magone et al. [14]. The four signs were conjunctival hyperaemia, discharge and tearing, chemosis and lid edema and each was graded from a scale of 0 to 3 (0 = absent; 1 = mild; 2 = moderate and 3 = severe). Therefore, the range of clinical scoring that could be received for each mouse was from 0 to 12. The data was expressed as the mean ± standard error of the mean (SEM) for each group.

### 2.5. Histologic analysis of conjunctiva

The eyelids and conjunctivas were isolated from the mice and fixed with 10% formalin on day 19. Then sections were cut 4 µm-thick and detected for signs of eosinophils by staining with acid-Giemsa. The areas observed were of the central portion of the eye, including the pupil and the optic nerve head. Two masked observers counted the infiltrating cells in the lamina propria mucosae of the tarsal and bulbar conjunctivas.

Deparaffinizing of the sections was achieved by immersing them in xylene and hydration by serial immersions in differing percentages of

ethanol (100%, 90%, 80%, and 70%) and PBS. After, sections were microwaved in target retrieval solution (DAKO, Santa Clara, CA) for 20 min for antigen retrieval. Subsequently, PBS was used to wash the sections twice for 10 min each time and blocking buffer (10% BSA in PBS) was added for 1 h. Sections were then incubated with anti-CD4, CD11c, or MHC class II Abs, washed with TBS twice, and incubated with Alexa Fluor 488 or 546-conjugated anti-rabbit and mouse IgG Ab, as appropriate. After PBS washing, they were mounted with DAPI (Vector Laboratories, Burlingame, CA). Zeiss LSM 510 confocal microscopy (Carl Zeiss, Jena, Germany) was used to view the sections.

### 2.6. Quantification of OVA-specific IgE and IgG1/G2a ratio in serum

Blood of immunized mouse was collected 24 h after OVA challenge. It was allowed to coagulate at room temperature and the serum was then extracted by centrifugation. The serum was stored at -80 °C until further analysis. An OVA-specific mouse IgE ELISA kit (Cayman, Ann Arbor, Michigan, USA) was used as per the manufacturer's instructions. OVA-specific IgE detection was executed in a 96-well format with the samples analyzed in triplicates.

For OVA-specific detection of IgG1 or IgG2a, the immunoplates (Nalge Nunc International, Naperville, IL, USA) were coated with OVA (1 mg/ml) overnight at 4 °C. Then, 1% BSA in PBS was used for blocking for 1 h at room temperature, and serum samples were diluted serially and incubated for 4 h at room temperature. Horseradish peroxidase (HRP)-conjugated rat anti-mouse IgG1 or IgG2a antibodies (Southern Biotech, Birmingham, AL) were used and incubated for 2 h at room temperature. PBST was used for washing and color reaction was developed with 3,3',5, 5'-tetramethyl-benzidine (Moss Inc., Pasadena, CA, USA) and stopped with 0.1 N HCl.

### 2.7. Cytokine analysis from lymphoid cells

Cervical lymph nodes (CLNs) were collected 24 h after OVA challenge and cultured in RPMI 1640 medium supplemented with 50 mM 2-mercaptoethanol, 2 mM L-glutamine, and 10% heat-inactivated fetal calf serum (all from Invitrogen Life Technologies, Carlsbad, CA, USA).  $1 \times 10^6$  cells/ml was cultured with 1 mg/ml OVA for 96 h in 96-well plates (Nunc, Rochester, NY, USA). A commercial ELISA kit (BD Biosciences) was used to detect cytokine levels (IL-4, IL-5, IL-13, and IL-10) of culture supernatants.

### 2.8. RNA isolation and real-time PCR

TRIzol reagent (Gibco-Invitrogen, Grand Island, N.Y., USA) was used in the isolation of total RNA from conjunctiva. Synthesis of strands of complementary DNA (cDNA) including random hexamers was achieved using SuperScript III reverse transcriptase (Invitrogen). A real-time PCR method of SYBR Green I was used. GAPDH was utilized for the internal calibration of the average threshold cycle (CT) values and the  $2^{-\Delta\Delta C_t}$  method was used for relative quantification. The sequences of primers used in PCR were as follows: for eotaxin, forward primer, 5-TCCACAGCGCTTCTATTCCT-3 and reverse primer, 5-CTATGGCTTTCAGGGTGTCAT-3; for RANTES, forward primer, 5-CCCTCACCATC ATCCTCACT-3 and reverse primer, 5-CCTTCGAGTGACAAACACGA-3; and for GAPDH, forward primer, 5-CGTCCCGTAGACAAAATGGT-3 and reverse primer, 5-TCTCCATGGTGGAAGACA-3.

### 2.9. Statistical analysis

One-way ANOVA was utilized, followed by Tukey test using SPSS 17.0 version to examine statistical significance between experimental groups.  $p < 0.05$  was regarded as significant,  $p < 0.01$  highly significant, and  $p < 0.001$  extremely highly significant. The data is representative of the three independent experiments with six mice in each group.

### 3. Results

#### 3.1. SOD3 treatment reduces the clinical symptoms of EAC

To investigate the effect of SOD3 treatment on EAC, BALB/c mice were sensitized intraperitoneally with OVA and alum at days 0 and 7 and then challenged with OVA to conjunctival sac from day 15 to 18. Five microliters of recombinant SOD3 (1000 unit) was given topically in the conjunctival sac at 30 min before OVA challenge for 4 days (Fig. 1A). Four clinical signs were evaluated 20 min after OVA challenge. When compared with the control group, OVA-challenged EAC mice had moderate to severe clinical signs. Of note, SOD3 treatment via conjunctiva sac reduced clinical scores when compared with the untreated group ( $p < 0.01$ ) (Fig. 1B). These observations demonstrate that SOD3 treatment is effective in preventing the clinical signs of allergic conjunctivitis in EAC.

#### 3.2. SOD3 treatment attenuates OVA-induced allergic conjunctival inflammation in EAC mice

To investigate the notion that SOD3 might be involved in conjunctival inflammation severity triggered by allergens, we evaluated the infiltration of eosinophils in the conjunctiva. As expected, OVA-challenged mice showed significant infiltration of eosinophils in the conjunctiva after systemic priming, as could be observed with histology findings. Interestingly eosinophil counts in the conjunctiva after OVA challenge were reduced by  $62.1 \pm 12.2\%$  in SOD3 treated mice compared to non-treated mice ( $592.5 \pm 44.2$  vs.  $264.1 \pm 32.4$ ,  $p < 0.01$ ) (Fig. 2A). Moreover, SOD3 treated mice showed higher levels of eotaxin mRNA in the conjunctiva compared to mice that were not treated with SOD3 (folds of mRNA expression/GAPDH: no SOD3 =  $28.7 \pm 0.97$  vs. SOD3 =  $15.0 \pm 0.66$ ,  $p < 0.01$ , respectively). Similarly, the impact of SOD3 on RANTES mRNA expression was also evaluated in OVA-challenged mice conjunctiva and it could be observed that SOD3 decreased folds of RANTES mRNA expression levels by  $1.37 \pm 0.29$ ,

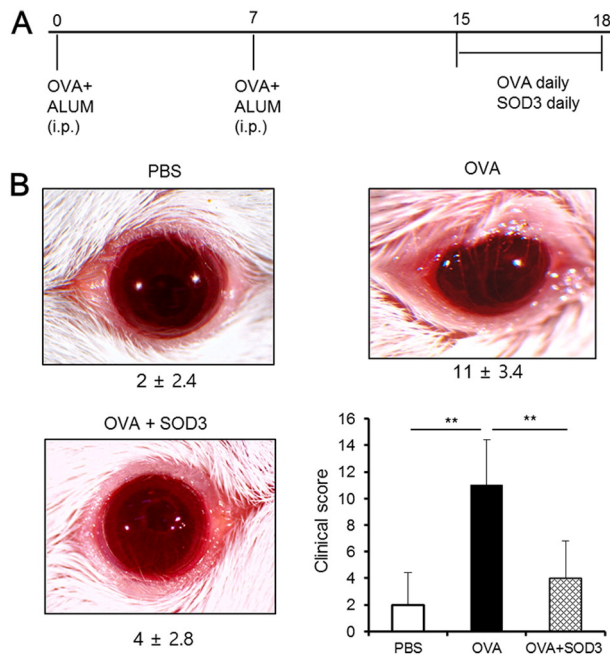


**Fig. 1.** Topical SOD3 treatment reduced clinical scoring in SOD3 treated EAC mice. (A) Experimental protocol. SOD3 was applied topically via conjunctival sac for 4 days at 30 min before OVA challenge. (B) Representative photo and clinical scoring (mean ± SEM) at 20 min after OVA challenge. \*\*,  $p < 0.01$  ( $n = 6$  in each group).

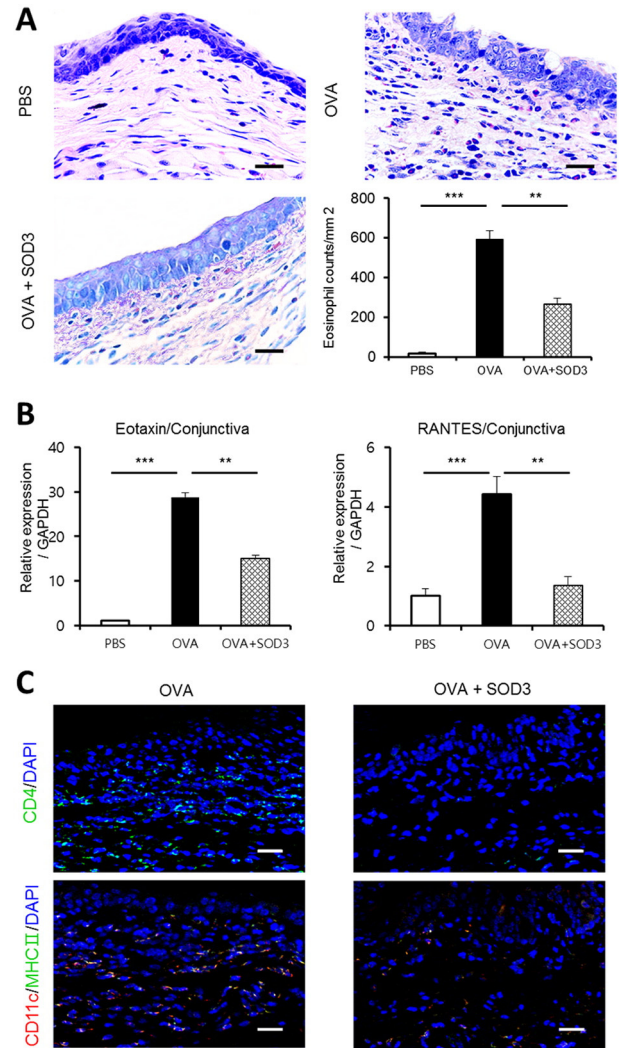


**Fig. 2.** Topical SOD3 treatment attenuated development of OVA-induced allergic conjunctival inflammation. Compared to those in WT EAC mice, conjunctival infiltration of eosinophils decreased in SOD3 treated EAC mice. (A) Representative image for infiltration and number of eosinophils (mean ± SEM). (B) Eotaxin and RANTES mRNA expression levels by qPCR. (C) Representative image of infiltration of CD4<sup>+</sup> cells and CD11c<sup>+</sup>/MHCII<sup>+</sup> cells. Bars = 20  $\mu$ m. \*\*,  $p < 0.01$ , \*\*\*,  $p < 0.001$  ( $n = 6$  in each group, three independent experiments).

$p < 0.01$  (Fig. 2B). The infiltration of CD4<sup>+</sup> T cells and CD11c<sup>+</sup>/MHCII<sup>+</sup> cells in the conjunctiva after OVA challenge was also decreased in SOD3 treated mice compared to those in non-treated mice (Fig. 2C). These results suggest that topical SOD3 treatment inhibited the infiltration of mature dendritic cells, resulting in suppressed infiltration of eosinophil and CD4<sup>+</sup> T cells in the conjunctiva of mice with EAC.

#### 3.3. SOD3 treatment reduces selective OVA-specific IgE antibody and OVA-specific IgG1/G2a in serum of EAC mice

Concentrations of OVA-specific IgE Abs in sera were investigated for additional clarification on the relationship between SOD3 and severity of EAC. The results showed significantly higher levels OVA-specific IgE Ab secretion in EAC when systemic priming and local boosting with OVA ( $p < 0.01$ , Fig. 3A). However, in SOD3 treated mice, the concentrations of OVA-specific IgE Ab in serum after OVA challenge were shown to be greatly reduced by  $48.5 \pm 14.3\%$  ( $p < 0.01$ , Fig. 3A). As expected, SOD3 treated mice showed decreased levels of OVA-specific IgG1/IgG2a in sera compared to non-treated mice ( $p < 0.05$ , Fig. 3B).

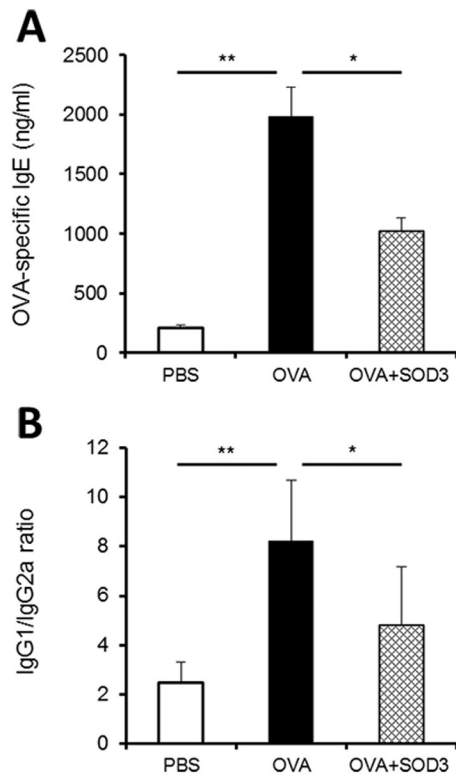


**Fig. 3.** Topical SOD3 treatment reduced OVA-specific IgE Ab secretion in sera of EAC mice. (A) Ag-specific IgE Ab secretion in sera drastically decreased in mice treated with topical SOD3 treatment. (B) Compared to WT EAC mice, those treated with SOD3 displayed a decline in OVA-specific IgG1/IgG2a in antibody levels in sera. \*,  $p < 0.05$ , \*\*,  $p < 0.01$  ( $n = 6$  in each group, three independent experiments).

#### 3.4. SOD3 treatment inhibits Th-2 type cytokine responses in CLN of EAC mice

In order to examine the mechanisms underlying allergen-specific decline of IgE response in SOD3 treated mice, Th-2 and Treg type cytokines were analyzed. Mononuclear cells isolated from CLN of EAC and PBS control mice were re-stimulated with OVA in vitro and cytokine levels of EAC and PBS control mice were compared. As shown in Fig. 4, levels of Th2-type cytokines (IL-4, IL-5, and IL-13) were significantly increased whereas Treg type cytokines (IL-10) decreased in EAC mice compared with PBS control group. The concentrations of IL-4, IL-5, and IL-13 were shown to be reduced by  $44.9 \pm 8.2\%$ ,  $83.1 \pm 13.1\%$ , and  $40.9 \pm 6.7\%$  respectively in SOD3 treated mice compared to those from non-treated mice ( $p < 0.05$ ,  $p < 0.01$ , and  $p < 0.05$ , Fig. 4A–C). On the contrary, the concentration of IL-10 from SOD3 treated mice was increased by  $41.5 \pm 10.4\%$  compared to those from non-treated EAC mice ( $p < 0.05$ , Fig. 4D). Taken together, these data demonstrate that topical SOD3 treatment inhibits allergen-specific Th2 type responses and results in decreased serum IgE production.

#### 3.5. SOD3 is necessary to prevent allergic conjunctivitis in EAC mice

We initially investigated the level of endogenous SOD3 gene expression in the conjunctiva in EAC and found that it was about 1.52 fold compared to that of PBS control mice (data not shown). To confirm the role of SOD3 in development of allergic conjunctivitis, we used SOD3 KO mice (C57BL/6 background). As shown in Fig. 5A, allergic conjunctivitis was induced in WT C57BL/6 and SOD3 KO mice from day 9 to 18. Although severity of conjunctivitis was lower in C57BL/6 mice compared to BALB/c mice, the induction of allergic conjunctivitis was shown in C57BL/6 mice. SOD3 KO EAC mice had higher clinical scores and OVA-

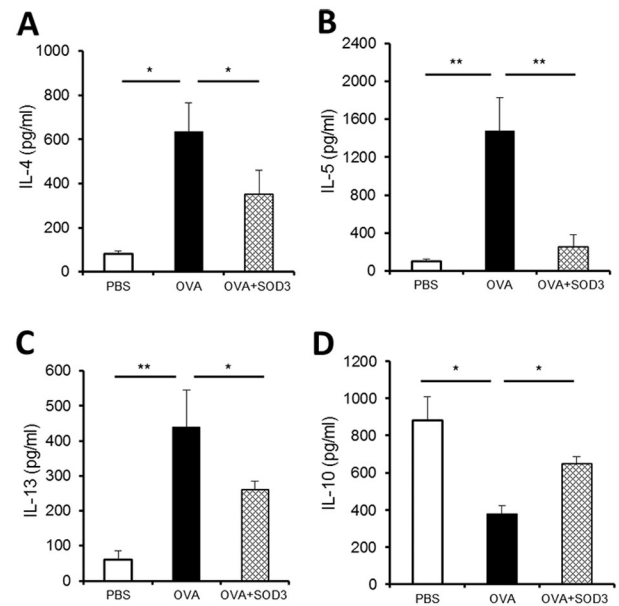


**Fig. 4.** In the cervical lymph nodes (CLNs) of SOD3 treated EAC mice, OVA-induced Th2 cytokine responses are impaired. OVA was used for restimulation of CLN cells. Cytokines such as IL-4, IL-5, IL-13, and IL-10 were measured in culture supernatants. Compared with CLN cells from WT EAC mice, those from SOD3 treated EAC mice produced significantly less IL-4, IL-5, and IL-13 (Th2 cytokines), and more IL-10 (Treg cytokine). \*,  $p < 0.05$ , \*\*,  $p < 0.01$  ( $n = 6$  in each group, three independent experiments).

specific IgE responses than WT EAC mice ( $p < 0.05$ , Fig. 5B–C). Interestingly, SOD3 KO mice also had significantly more eosinophils in the conjunctiva than WT EAC mice ( $p < 0.01$ , Fig. 5D). Furthermore, SOD3 KO EAC mice expressed more Th2-cell cytokines (IL-4, IL-5, and IL-13) than WT OVA control group, whereas Treg type cytokines (IL-10) decreased (Fig. 5E). These data indicate that SOD3 is necessary for preventing Th2-driven allergic conjunctival inflammation.

#### 4. Discussion

A combination of IgE-mediated and Th2 cell-mediated responses is the main changes that occur in conjunctival allergic reactions that ultimately result in eosinophilic infiltration into the conjunctiva [1–3]. In the early response phase of the allergic inflammation by new contact with an allergen, an immediate response is triggered by the binding of IgE to the membrane of conjunctiva mast cells. Mediators such as IL-4, IL-5, and IL-13 are released [2,3,15]. The activation and recruitment of T-lymphocytes and eosinophils are mediated in the late phase response [15,16]. Antihistamine, mast cell membrane stabilizer and corticosteroids have been used to treat AC, but there is a risk of corticosteroid adverse effects.

Our findings demonstrate strong evidence that pharmacological treatment with antioxidant SOD3 attenuated clinical signs of allergic conjunctivitis in EAC model. Unlike other SODs, SOD3 was known to specifically inhibit DC maturation, through the inhibition of MHCII, CD80, and CD86 expression [10,11]. We believe that this occurs because the key events for DC maturation are extracellular where most SODs are located. In our study topical treatment with SOD3 into conjunctiva sac also inhibited CD11C<sup>+</sup>/MHC class II DC infiltration and Th cell infiltration in the conjunctiva of EAC mice. It can be anticipated that with the inhibition of MHC class II expression, maturation of DC might be inevitably inhibited by SOD3 topical treatment in EAC model.

We evaluated Th2 cytokine responses in draining lymph nodes because in a study of OVA-induced allergic asthma, SOD3 inhibited Th2 differentiation [11]. When mononuclear cells from CLN were cultured in the presence of OVA in vitro, cells from SOD3 treated mice produced significantly fewer Th2-type cytokines such as IL-4, IL-5, and

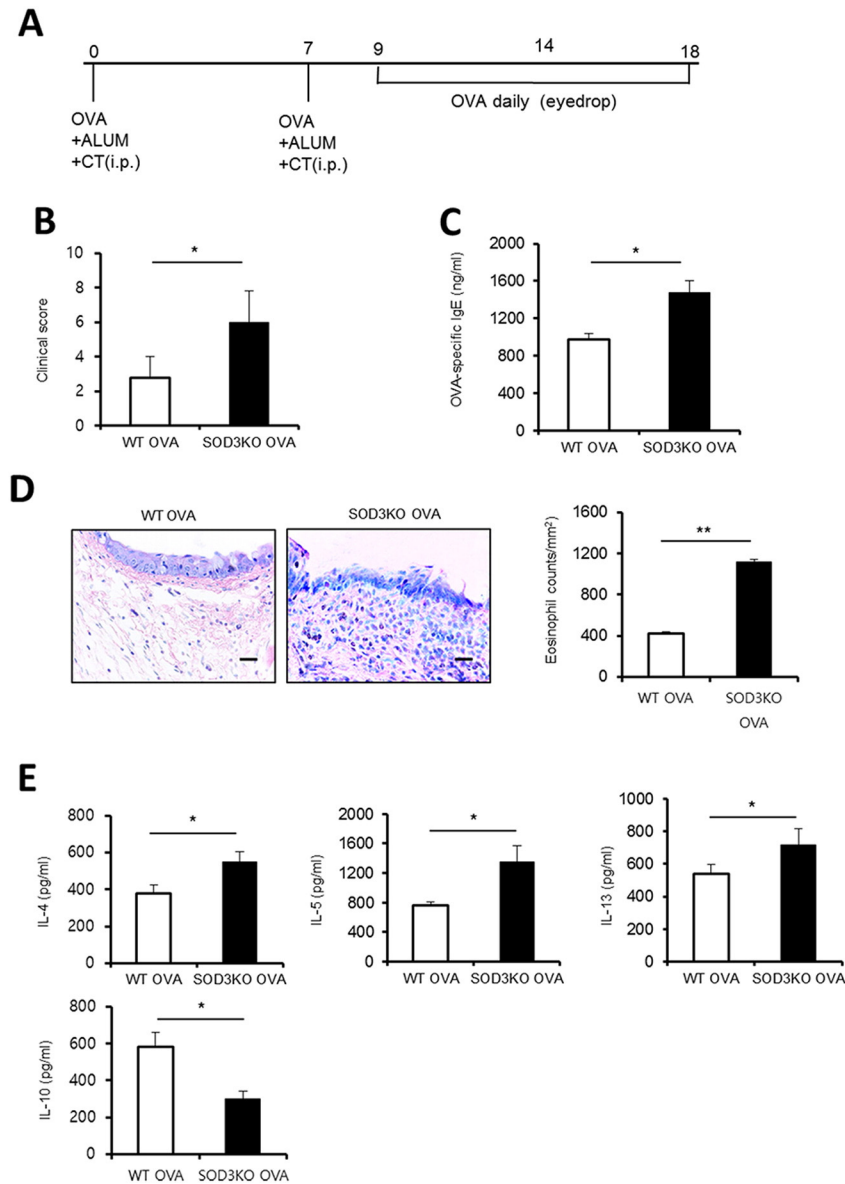


**Fig. 5.** OVA-induced allergic conjunctival inflammation is attenuated by the absence of SOD3. (A) Experimental protocol of C57BL/6 WT and SOD3 KO EAC mice. (B) Clinical scoring and (C) OVA-specific IgE in sera were increased in SOD3 KO EAC mice. (D) In conjunctiva of SOD3 KO EAC mice, infiltration of eosinophils was increased. (E) Th2 cytokine responses in CLN were increased whereas IL-10 in CLN was decreased in SOD3 KO OVA mice. Bars = 20  $\mu$ m. \*,  $p < 0.05$ , \*\*,  $p < 0.01$  ( $n = 6$  in each group, three independent experiments).

IL-13 than those from WT EAC mice. Interestingly, mononuclear cells from CLN of SOD3 treated EAC mice produced more IL-10 than those of non-treated mice.

Extracellular SOD3 controls Th2-driven immune responses by inhibiting DC infiltration and increasing Treg response resulting in inhibition of OVA-specific IgE antibody secretion and eosinophil infiltration in the conjunctiva of EAC model. As a result, topical SOD3 treatment significantly decreased the clinical signs of allergic conjunctivitis (chemosis, conjunctival hyperaemia, lid edema, discharge and tearing) and inhibited the synthesis of Th2 cytokines (IL-4, IL-5, and IL-13) and CC chemokines (eotaxin and RANTES). The suppression mechanism of allergic conjunctivitis by SOD3 is related with decrease in important eosinophil chemoattractants such as eotaxin and RANTES.

Our results also indicate that SOD3 is necessary for prevention of Th2-driven allergic conjunctival inflammation. SOD3 KO EAC mice, unlike WT EAC mice, presented with allergic clinical signs. In SOD3 KO EAC mice, Th2 cytokine responses were increased after OVA challenge. The SOD3 KO EAC mice had increased Th2 cytokine responses and substantially higher circulating levels of OVA-specific IgE antibodies and

eosinophils in the conjunctiva. These results suggest that allergen-induced Th2 cytokine responses are prevented in the presence of SOD3 in EAC model. The exacerbation of allergic responses in SOD3 KO mice was also reported in OVA-induced asthma model as shown by a higher influx of leukocytes in bronchoalveolar lavage and increased Th2-type cytokine responses compared to findings in WT mice [11]. When our results are considered together, it is clear that endogenous SOD3 has a role in the regulation of Th2-driven adaptive immune responses during allergic conjunctival inflammation.

We found that SOD3 plays a role in adaptive immune responses by modulating DC infiltration, Th cell activation, and Th2 cell differentiation. Our results clearly establish the correlation between significantly increased Th2-immune responses in CLN that drives allergen-specific IgE antibody secretion in serum of SOD3CCR6 KO mice and heightened eosinophil infiltration into the conjunctiva. Therefore, a plausible hypothesis is that in order for the prevention of polarized Th2 cell-mediated conjunctival inflammation, SOD3 is required. SOD3 may also have therapeutic potentials for allergic conjunctival inflammations that are Th2-driven.

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