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

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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 $CD4^+$ 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.



## 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.

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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, Zena, 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 $2^{-\Delta\Delta Ct}$ 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-CTATGG CTTTCAGGGTGCAT-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-TCTCCATGGTGGTGAAGACA-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.