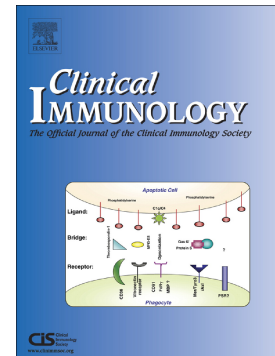


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Rapamycin attenuates Th2-driven experimental allergic conjunctivitis

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Abstract

Allergic conjunctivitis is mediated by eosinophilic infiltration and Th2 type immune responses. This study aims to elucidate the role of rapamycin, mTOR inhibitor, on OVA-induced experimental allergic conjunctivitis (EAC). Rapamycin administration intraperitoneally markedly reduced clinical signs, total and OVA-specific IgE and IgG1/G2a ratio in serum, and conjunctival eosinophilic infiltration. Infiltrations of CD11c⁺ dendritic cells and CD4⁺ T cells, and the expressions of chemokines and adhesion molecules in the conjunctiva were attenuated in rapamycin-treated mice, as well as decreased Th1 and Th2 cytokines in the cervical lymph nodes compared to non-treated mice. The expression of mTOR signaling proteins was increased in EAC and reduced by rapamycin treatment. Topical application of rapamycin was also proved to show reduced clinical signs, eosinophil infiltration, and Th2 type immune responses comparable to those from intraperitoneal injection of rapamycin. These findings suggest the therapeutic implications of rapamycin in the attenuation of allergic conjunctivitis.

Key words: Allergic conjunctivitis, Dendritic cells, Eosinophil, Rapamycin, Th2 type immune response

1. Introduction

The prevalence of ocular allergic diseases has seen a considerable increase in the past several decades. These clinical conditions can range from the acute and mild, such as seasonal allergic conjunctivitis (SAC), to chronic and severe, such as vernal keratoconjunctivitis (VKC) and atopic keratoconjunctivitis (AKC) [1,2]. Symptoms and clinical signs of this disease include chemosis, eye redness, itching, and in severe cases, corneal damage that can develop into impaired visual function [3,4].

The inherent immunopathogenic mechanism of allergic conjunctivitis involves CD4⁺ T helper (Th) cells, of which Th2 plays a prominent role, along with immunoglobulin E (IgE)-mediated responses [5,6]. The reproducibility of an IgE-mediated allergic conjunctivitis response can be accomplished by a specific conjunctival stimulation. It leads to an immediate hypersensitivity reaction followed by a late-phase reaction that prompts a predominant recruitment and infiltration of eosinophils into the conjunctiva [5,7,8]. Dendritic cells that populate the conjunctiva capture allergens that are exposed on the conjunctiva surface, and they are matured and migrate into the cervical lymph nodes [9]. Several chemokine receptors, such as CCR7, are thought to be strongly affiliated with aiding dendritic cell migration to secondary exposures of allergy in ocular allergic diseases. CC chemokines and adhesion molecules play important roles in the recruitment and migration of leukocytes to the sites of inflammation [10,11].

Rapamycin is a bacterial macrolide antibiotic that is a product of *Streptomyces hygroscopicus*. It is an immunomodulatory agent that binds with high affinity to the FK506 binding protein (FKBP12). The Rapa-FKBP12 complex is an allosteric inhibitor of mammalian target of rapamycin (mTOR), a kinase that plays a critical role in modulating both innate and adaptive immune responses. mTOR also regulates the functions of antigen-presenting cells [12]. Rapamycin has been shown in several studies of its effectiveness and

potential in treatment of allergic asthma and cancers [13-17].

Thus, this current study aims to elucidate the role of rapamycin on severity of allergic conjunctivitis in a murine model. We hypothesized that the rapamycin mTOR inhibitor might play a pivotal role in attenuating experimental allergic conjunctivitis (EAC).

2. Materials and Methods

2.1. Animals

In all experiments, 7- to 8-week-old BALB/C female mice obtained from Charles River Laboratories (Orient Co., Sungnam, Korea) were used. All mice were housed under pathogen-free conditions with a 12-hour/12-hour light-dark cycle at the animal facilities in the Catholic University of Korea (Seoul, Korea). They received sterilized food and water *ad libitum*. All protocols for mouse use were approved by the Institutional Animal Care and Use Committee.

2.2. Experimental allergic conjunctivitis (EAC)

BALB/c mice were sensitized intraperitoneally (IP) 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 a span of 4 days from day 15 to 18, the control PBS group and non-treated OVA groups were injected with vehicle intraperitoneally and the rapamycin-treated group were injected with 2mg/kg of rapamycin (Tocris Bioscience, Bristol, UK) IP 30 min before OVA challenge (Fig. 1A). For topical application of rapamycin, an eye drop concentration of 0.04% was administered 30 mins before OVA challenge in the conjunctival sac (Fig. 8A). All the mice were sacrificed after the final OVA challenge and analyzed to evaluate the severity of EAC.

2.3. Analysis of clinical signs

Severity of EAC was evaluated by clinical signs at 20 mins after the final inoculation of

OVA or PBS into the conjunctiva sac. The range of severity of EAC was assessed by utilizing clinical scorings of four signs including redness, discharge, chemosis, and lid edema graded on a scale of 0~3 by two masked observers [18,19]. Zero was for no severity, 1 was for mild, 2 was for moderate, and 3 was for severe. The scores of four clinical signs were added for a total ranging from 0~12. The data was presented as the mean $\pm$ standard deviation (SD) for each group.

2.4. Analysis of conjunctiva histology

The conjunctiva and the eyelids of the mice were harvested and fixed in 10% buffered formalin, then embedded and cut into horizontal 4 μ m-thick sections. Acid Giemsa staining was used for the detection of eosinophils. The sections used were those that included the pupil and the optic nerve head in the central portion of the eye. The cells infiltrating into the lamina propria mucosae of the bulbar and tarsal conjunctivas were counted by two masked observers [18].

For immunofluorescence, 4 μ m cut sections underwent a de-waxing process by immersion in xylene for 40 min. The slides were subsequently hydrated by serial immersion in 100%, 95%, 90%, 80%, and 70% ethanol and PBS. Antigen retrieval was achieved by microwaving section slides for 15 min in Target Retrieval Solution (DAKO, Carpinteria, CA). Sections were then washed with PBS plus 0.05% Tween (PBST) three times for 2 min each, and incubated in blocking buffer (10% normal goat serum in PBST) for 1 h. The sections were incubated with anti-CD11c, CD4 or ATG5 Abs in PBST overnight at 4 °C. They were washed with PBST and incubated with Alexa Fluor 488 or 546-conjugated anti-mouse and rabbit IgG Ab, as appropriate. After PBST washing three times, the sections were mounted in Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA). The sections were viewed with Zeiss LSM 800 with Airyscan confocal microscopy (Carl Zeiss,

Oberkochen, Germany) and the data shown was in mean $\pm$ SD for each group.

2.5. ELISA for total and OVA-specific IgE and IgG1/IgG2a ratio in serum

Twenty-four hours after OVA challenge of immunized mice, blood was collected and kept at room temperature to allow coagulation. Serum was separated via centrifugation and then collected and stored at -70°C until analyzed. For OVA-specific IgE detection, samples were analyzed in triplicate using a 96-well format with an OVA-specific mouse IgE ELISA kit (Cayman Chemical, Ann Arbor, MI) performed according to the manufacturer's instructions. For OVA-specific IgG1 or IgG2a detection, the immunoplates (Nalge Nunc International, Naperville, IL) were coated with OVA (1 mg/ml), and for total IgE Ab detection, coated with purified rat anti-mouse IgE mAb (2 $\mu\text{g}/\text{mL}$, R35-72, BD Pharmingen, San Diego, CA) overnight at 4°C . After blocking with 1% bovine serum albumin (BSA) in PBS for 1 h at room temperature, serial dilutions of serum samples were added and incubated for 4 h at room temperature. The plates were then washed with PBST and incubated for 2 h at room temperature with horseradish peroxidase (HRP)-conjugated rat anti-mouse IgE or IgG antibodies (Southern Biotech, Birmingham, AL). After a wash with PBST, color reactions were developed with 3, 3', 5, 5'- tetramethyl-benzidine (Moss Inc., Pasadena, CA) and stopped with 0.1N HCl. The ratio of IgG1/IgG2a was determined by dividing the concentrations of IgG1 by IgG2a from the same serial dilution point.

2.6. Lymphoid Cell Culture and Cytokine Analysis

Cervical lymph nodes (CLN) were harvested, and single-cell suspensions were cultured in RPMI 1640 medium supplemented with 2 mM L-glutamine, 50 mM 2-mercaptoethanol, and 10% heat-inactivated fetal calf serum (all from Invitrogen Life Technologies, Carlsbad, CA, USA). Cells were cultured at 1×10^6 cells/ml and treated with a final concentration of 1

mg/ml OVA in each well for 96 h in 96-well plates (Nunc, Rochester, NY, USA). Cytokine levels (IL-4, IL-5, IL-13, IFN- γ , IL-10, and TGF- β) of culture supernatants were assayed using R&D Systems (Minneapolis, MN, USA).

2.7. Flow Cytometry

Cervical lymph nodes from mice were harvested into RPMI, and isolated under a dissecting microscope. They were filtered through a 70- μ m mesh cell strainer (Corning, NY, USA) before proceeding with flow cytometry staining. The single-cell suspensions were labeled with the following antibodies for analysis: FITC Rat Anti-Mouse CD4, PE Rat Anti-Mouse CD25, PE Rat Anti-mouse CD103 all purchased from BD biosciences (San Jose, CA) and FITC Rat Anti-mouse CD11b and APC Rat Anti-mouse FOXP3 (eBioscience, San Diego, CA). The samples were analyzed using a FACS Calibur and FACS Canto II system (BD Biosciences).

2.8. RNA isolation and real-time PCR.

Conjunctivas were extracted and total RNA isolated by TRIzol reagent (Gibco-Invitrogen, Grand Island, N.Y., USA). SuperScript III reverse transcriptase (Invitrogen) was used for the synthesis of complementary DNA (cDNA). The real-time PCR method of SYBR Green I was utilized and GAPDH as the internal calibration of the average threshold cycle values. For relative quantitation, the $2^{-\Delta\Delta C_t}$ method was applied [18]. The primer sequences used in this study are listed in Table 1.

2.9. Statistical Analysis

Statistical significance between groups was examined by a nonparametric, two-tailed Mann-Whitney t-test using SPSS 17.0 version. We regarded $p < 0.05$ as significant, $p < 0.01$ as

highly significant, and $p < 0.001$ as extremely highly significant. Data are representative of three independent experiments with six mice in each group.

3. Results

3.1. Administration of rapamycin significantly reduces the clinical signs of allergic conjunctivitis in EAC mice.

To investigate the role of rapamycin on the development of EAC, rapamycin was administered IP 30 mins before OVA challenge (Fig. 1A). A total of four clinical signs were evaluated by masked observers on the last day after 20 mins of OVA challenge. The clinical signs were rated on a scale of 0 to 3, the higher number indicating increasing severity. As shown in Fig. 1B, mice challenged with OVA demonstrated moderate to severe signs of all four clinical parameters compared to the control PBS group. Interestingly, rapamycin-treated mice showed reduced clinical signs compared to the non-treated OVA group with a clinical score of 1.6 ± 0.5 vs. 8.6 ± 0.9 ($p < 0.001$). These results demonstrate rapamycin treatment is effective in reducing the clinical signs of allergic conjunctivitis in EAC.

3.2. Rapamycin drastically decreases conjunctival inflammation in EAC mice.

The attenuating effects of rapamycin on clinical scores in EAC led us to further investigate the histology of the conjunctiva. Immunologically, eosinophils play a significant role in the pathogenesis of chronic allergic inflammations. As can be observed from Fig. 2A, Acid-Giemsa staining showed that the number of eosinophil infiltration in the conjunctiva was prominently lower in the rapamycin-treated group compared to the non-treated OVA group. The rapamycin-treated group showed an average three-fold decrease in eosinophil cell counts compared to the non-treated OVA group (271.0 ± 10.6 vs. 675.0 ± 8.3 , $p < 0.001$). Immunohistochemistry of conjunctiva shown in Fig. 2B illustrated a decrease in the number of CD4⁺ T cells and CD11c⁺ dendritic cells (DCs) in the rapamycin-treated group compared to the non-treated OVA group, with a difference of 272.5 ± 26.13 ($p < 0.05$) and 195.8 ± 15.6 ($p < 0.05$), respectively. When DC subsets were analyzed in CLN of EAC mice, though the

infiltration of CD11c⁺ DCs decreased in the rapamycin-treated group, the percentage of CD103⁺ CD11c⁺ DCs and CD11b⁺ CD11c⁺ DCs demonstrated no significant differences between the non-treated OVA groups and the rapamycin-treated groups (Supplementary Fig. 1). The pronounced decrease in the infiltration of eosinophils, CD11c⁺ DCs and CD4⁺ T cells strengthens the immunomodulating role of rapamycin in EAC.

3.3. Total IgE, OVA-specific IgE and IgG1/IgG2a in serum decreased in rapamycin-treated EAC mice.

For further examination of the effects of rapamycin on EAC, total and OVA-specific IgE levels and IgG1/IgG2 were examined in the sera of mice. As can be seen from the results of Fig. 3A-B, a significant increase of total and OVA-specific IgE levels in the non-treated OVA group were observed but were shown to be greatly reduced in the rapamycin-treated group (3326.3 ± 12.1 vs. 1350.9 ± 179.9 , $p < 0.01$ for total IgE and 1377.2 ± 221.1 vs. 559.8 ± 160.2 , $p < 0.001$ for OVA-specific IgE). Similarly, in conjunction with IgE, OVA-specific IgG1/IgG2a was increased in the non-treated OVA group, but demonstrated drastic attenuations in the rapamycin-treated group (7.0 ± 0.7 vs. 3.2 ± 0.1 , $p < 0.01$) (Fig. 3C).

3.4. Th1 and Th2 type cytokine responses are attenuated in the cervical lymph nodes (CLN) of rapamycin-treated EAC mice

To elucidate the total and allergen-specific decline of IgE responses in the rapamycin-treated group, Th2 type cytokines (IL-4, IL-5, and IL-13), Th1 cytokines (INF- γ) and Treg cytokines (IL-10 and TGF- β) along with Treg cell populations were investigated. As demonstrated in Fig. 4A-C, rapamycin-treated mice demonstrated greatly reduced secretions of IL-4, IL-5 and IL-13 from the CLN compared to the non-treated OVA mice, with a difference of 7.6 ± 1.9 ($p < 0.001$), 286.0 ± 31.8 ($p < 0.001$), and 917.3 ± 33.3 ($p < 0.01$),

respectively. This tendency could also be observed in the reduced secretions of Th1 cytokine, INF- γ , in rapamycin-treated mice compared to the non-treated OVA mice shown in Fig. 4D, with a difference of 37.7 ± 18.3 ($p < 0.01$). Interestingly, IL-10 and TGF- β cytokine levels (Fig. 4E, F) and percentage of Treg cells in the CLN (Fig. 5) did not show significant differences between the non-treated OVA and rapamycin-treated group. Thus, it would be speculated from the results that rapamycin endows its effects on EAC with the attenuation of both Th1 and Th2 type cytokines, independent of the T regulatory immune responses.

3.5. Rapamycin attenuates the gene expressions of CC chemokines and adhesion molecules in the conjunctiva of EAC.

CC Chemokines (RANTES, eotaxin) and leukocyte-endothelial adhesion molecules (p-selectin, VCAM-1) are important in the recruitment and migration of leukocytes to the sites of inflammation [10,11]. Expression of these molecules is modulated by cytokines such as IL-4, [20] IL-5, IL-13 [21,22] and to a lesser degree, IFN- γ [23]. In view of the suppressive effect of rapamycin on these cytokine levels, their effects on RANTES, eotaxin, p-selectin and VCAM-1 expressions in the conjunctiva was examined. Rapamycin-treated group demonstrated a decrease in mRNA levels for RANTES and eotaxin compared to the non-treated OVA group, with differences of 8.7 ± 0.6 and 3.1 ± 0.4 ($p < 0.001$), respectively (Fig. 6A, B). Rapamycin-treated group also showed attenuated mRNA levels of p-selectin and VCAM-1 than the non-treated OVA group, with differences of 6.6 ± 1.5 and 2.3 ± 1.2 ($p < 0.01$), respectively (Fig. 6C, D). These results demonstrate that rapamycin attenuates the expression of chemokines and adhesion molecules in conjunctiva resulting in decreased infiltration of eosinophils and immune cells into the conjunctiva of EAC mice.

3.6. Rapamycin-treated EAC mice show decreased gene expression of proteins in the mTOR pathway

mTOR is the master regulator of cell growth and metabolism, predominantly by virtue of controlling the phosphorylation of at least two regulators of protein synthesis: p70 ribosomal S6 kinase (p70S6K) and an inhibitor of translation initiation, eukaryotic initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1) [24]. These two components lead to an active translation of mRNAs in which hypoxia-inducible factor 1- α (HIF-1 α) and its target gene vascular endothelial growth factor A (VEGF-A) expression is involved. Rapamycin is an inhibitor of mTOR, and thus sought to examine if key proteins in the mTOR pathway was inhibited in rapamycin-treated EAC mice. Therefore, in order to discern the allergic conjunctivitis inhibitory effects of rapamycin through the mTOR pathway, the expressions of mTOR and its effector p70S6K were observed in the conjunctiva of EAC mice. Additionally, mRNA expression of HIF-1 α , known to be one of the key factors regulating inflammatory responses and related to the expression of above two molecules, and VEGF-A, modulated by HIF-1 α , were also investigated. As can be observed from Fig. 7, the mRNA levels of proteins in the mTOR pathway, such as mTOR, p70S6K, HIF-1 α , and VEGF-A showed significant decrease in rapamycin-treated group compared to the non-treated OVA group mice with differences of 5.8 ± 1.4 , 2.5 ± 0.6 , 7.1 ± 1.3 , and 8.0 ± 1.0 ($p < 0.001$), respectively. These results indicate the induction of HIF-1 α and its target gene VEGF-A in allergic conjunctivitis which is activated by mTOR/p70S6K and is attenuated by rapamycin in EAC models.

3.7. Topical application of rapamycin attenuates EAC.

With the results of the attenuating effects on EAC by IP injection of rapamycin, it was applied topically as an eye drop for the purpose of future clinical applications. As revealed in Fig. 8A, BALB/c mice were sensitized at days 0 and 7 intraperitoneally with OVA and alum.

From days 15 to 18, the mice were challenged topically on the conjunctival sac with OVA and rapamycin was administered topically 30 mins before OVA challenge. Fig. 8B indicated similar reduced clinical scores in rapamycin IP and eye drop group, with a difference of 5.9 ± 0.3 and 5.7 ± 0.3 ($p < 0.001$), respectively, compared to the non-treated OVA group. Additionally, similar inclinations of reductions could be observed in the inhibition of eosinophils, with a difference of 337.0 ± 6.8 and 374.0 ± 10.6 ($p < 0.001$), and OVA-specific IgE levels with a difference of 1120.1 ± 20.3 ($p < 0.001$) and 1031.7 ± 28.2 ($p < 0.01$), respectively, both compared to the non-treated OVA group. Th2 levels in the CLN were also similarly decreased in both the rapamycin IP injection group and eye drop group compared with the non-treated OVA group, with a difference of 259.9 ± 10.0 ($p < 0.01$) and 182.9 ± 2.9 ($p < 0.05$) for IL-5 levels, and 947.3 ± 19.5 ($p < 0.01$) and $954.9 \pm 69.$ ($p < 0.05$) for IL-13 levels, respectively. These results indicate the potential of rapamycin eye drops in conjunction with IP rapamycin injection, as it demonstrated similar attenuating effects of EAC.

4. Discussion

The present study elucidates the application of rapamycin in attenuating and alleviating allergic conjunctivitis in an OVA-induced EAC model. Allergic conjunctival inflammation is developed by an allergen specific IgE production and Th2 cell-mediated immune responses. Recruitment and infiltration of inflammatory cells, such as T-lymphocytes and eosinophils, into the conjunctiva are the main pathological features mediated by Th2 immune responses in allergic conjunctivitis. In our study, rapamycin treatment attenuated clinical signs and conjunctival infiltration of eosinophils in EAC.

To elucidate the immunologic mechanisms of systemic treatment of rapamycin on attenuating allergic conjunctivitis, we evaluated Th2 cytokine (IL-4, IL-5 and IL-13 production) responses from the CLN in EAC. Rapamycin is known to inhibit the overall proliferation of CD4⁺ T cells with a preferential inhibition of Th2 cell proliferation [25]. Th2 cytokines IL-5 and IL-13 play a central role in the differentiation, activation, and survival of eosinophils [26,27]. In conjunction with these studies, our results have revealed a decrease of Th2 cytokines such as IL-4, IL-5 and IL-13 in the CLN of rapamycin-treated mice, leading to the attenuation of eosinophils and CD4⁺ T cell infiltrations into the conjunctiva. Studies in allergic airway inflammation have also defined the effects of rapamycin on attenuating eosinophil infiltration by reducing IL-13 and IL-5 cytokines [28,29]. Our results have also demonstrated rapamycin to inhibit Th1 cytokine, IFN- γ , in the CLN which is in alignment with previous studies displaying the suppression of Th1 and Th2 cytokines by rapamycin[25,30].

Intriguingly, mononuclear cells from the CLN of rapamycin-treated EAC mice demonstrated similar TGF- β and IL-10 levels compared to the non-treated EAC mice, which were also evident in Treg cells. Rapamycin inhibits the proliferation of CD4 T cells, including Th1 and Th2 responses through the mTOR pathway [25], without blocking Treg

cells [31,32]. TGF- β orchestrates the development and differentiation of Tregs [33,34]. IL-10 is secreted by Th2 and Treg cells, which is crucial for Treg cell-mediated inhibition of immune responses. It also increases Tregs for the maintenance of the antigen specificity of peripheral T cell tolerance [35,36]. In our study, rapamycin attenuated Th2 cytokines, but did not block Treg cells as well as TGF- β and IL-10 production, indicating that IL-10 might be mainly produced by Treg cells in an OVA-induced EAC model.

To confirm the effects of rapamycin as a mTOR inhibitor, we evaluated mTOR signaling proteins in EAC. mTOR is the master regulator of cell growth and metabolism, predominantly by virtue of controlling the phosphorylation of p70S6K and 4E-BP1 leading to stimulation of HIF-1 α and its target gene VEGF-A expression [37]. Our findings demonstrate that expressions of proteins crucial in the mTOR pathway (mTOR, p70S6K, HIF-1 α , and VEGF-A) were increased in OVA-induced EAC mice and attenuated with rapamycin treatment. The mTOR pathway exerts a stimulatory effect on the development of airway diseases, and can stimulate HIF-1 α , which contributes to the inflammation and airway remodeling in asthma [38]. This finding confirms that rapamycin inhibited mTOR pathway proteins which contribute to the development of allergic conjunctivitis.

Interestingly, our findings demonstrated reduced infiltration of CD11c⁺ DCs into the conjunctiva after rapamycin treatment in EAC. Dendritic cells play a pivotal role in the triggering of allergic immune responses upon provocation [39]. CD11c⁺ DCs encompasses both CD11b⁺ and CD103⁺, with the former residing in the stroma and the latter in the intraepithelial, respectively [40]. However, DC subsets of CLN were not changed in rapamycin-treated EAC mice (Supplementary Figure 1). Exposure of human DC in rapamycin proved to prominently downregulate the phosphorylation of mTOR and p70S6K [41]. Narasimhan et al. demonstrated that rapamycin downregulated the phosphorylation of mTOR and its proteins, such as p70S6K and 4E-BP1, which is crucial for DC protein

synthesis. Several studies indicate that blocking mTOR in vitro by rapamycin resulted in a reduction of lysosome tubulation, an important factor in aiding antigen presentation for maturing dendritic cells [42,43]. It was shown that rapamycin inhibited the IL-4 dependent maturation and T-cell stimulatory activity of bone marrow-derived DCs. In vivo rapamycin administration also impaired not only production of pro-inflammatory cytokines, but also DC costimulatory molecule upregulation [44]. HIF-1 α and its target genes are crucial for the differentiation of immature DCs, and inhibition of mTOR attenuates these responses [45]. Thus, rapamycin might bestow inhibitory effects on the maturation of DCs in the EAC model [46].

As rapamycin was known to induce autophagy in human DCs through mTOR inhibition [41], we investigated autophagy marker ATG5 expression in the conjunctiva of EAC mice (Supplement Fig. 2). Our data showed increased ATG5 expressing CD11C⁺ DC in the conjunctiva of rapamycin-treated mice and ATG5 gene expression levels in the conjunctiva was also enhanced in rapamycin treated mice. Therefore, it was hypothesized that the administration of rapamycin would inhibit DCs metabolism and functions leading to decreased infiltration of CD11C⁺ cells in the conjunctiva of EAC mice. The number of DCs uptaking antigens from the conjunctiva and moving to the CLN might be also decreased as well, leading to an inhibition of antigen specific Th2 cell proliferation in rapamycin-treated EAC mice.

In addition, our findings demonstrate that rapamycin treatment decreased conjunctival expressions of chemokines (RANTES, eotaxin) and leukocyte-endothelial adhesion molecules (VCAM-1, p-selectin), which may reduce the recruitment and migration of eosinophils and CD4⁺ T cells in the conjunctiva of EAC mice. Expression of these molecules is modulated by cytokines such as IL-4,[20] IL-5 and IL-13 [21,22] and to a lesser degree, IFN- γ [23]. Suppressive effects of rapamycin on these cytokine levels may result in decreased

expressions of chemokines and adhesion molecules in the conjunctiva of rapamycin-treated EAC mice.

To investigate the therapeutic potential of rapamycin eye drop in alleviating EAC, we applied rapamycin eye drop as IP injection once a day. Of interest, rapamycin eye drop showed similar reductions of clinical signs and eosinophilic infiltration into the conjunctiva through the inhibition of IgE production and Th2 cytokine responses in EAC.

Conclusively, this current study demonstrates the alleviating effects of rapamycin on allergic conjunctivitis along with the inhibition of mTOR. Considered together with our experiments on attenuated Th2-type cytokine levels, along with chemokine eotaxin that is responsible for eosinophil chemoattraction and activation, the role of rapamycin in allergic conjunctivitis through the inhibition of eosinophil differentiation and function can be clearly observed. Thus, these results impart various implications for the therapeutic potential of rapamycin for the treatment of allergic conjunctivitis in humans.

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Figure Legends

Fig. 1. Rapamycin treatment drastically reduced the clinical sings of experimental allergic conjunctivitis. (A) Experimental protocol time frame. In PBS mice, only aluminum hydroxide (ALUM) was included intraperitoneally on days 0 and 7. Rapamycin was administered intraperitoneally 30 minutes before OVA challenge for 4 days from day 15 to 18. (B) Representative photo and clinical scoring (mean $\pm$ SD) at 20 minutes after OVA challenge. ***, $p < 0.001$ ($n = 6$ in each group, 3 independent experiments).

Fig. 2. Rapamycin treatment attenuates allergic conjunctival inflammation in experimental allergic conjunctivitis (A) Eosinophilic infiltration was attenuated in the rapamycin-treated group, as shown in the representative images for the infiltration and number of eosinophils (mean $\pm$ SD). Red arrows indicate eosinophils. Bars = 30 μm . ***, $p < 0.001$ ($n = 6$ in each group). (B) CD11C⁺ dendritic cells and CD4⁺ T cells infiltration in the conjunctiva of rapamycin-treated group were reduced compared with the non-treated OVA group. Bars = 50 μm . *, $p < 0.05$. (A, B) Data shown are representative of 3 different experiments, ($n = 6$ in each group).

Fig. 3. IgE levels and IgG1/IgG2a in serum decreased in rapamycin-treated experimental allergic conjunctivitis mice. (A, B) Total and OVA-specific IgE Ab levels in sera profoundly decreased in rapamycin-treated group mice compared to the non-treated OVA group. (C) Similar with IgE levels, rapamycin-treated mice also showed a decrease in IgG1/IgG2a. **, $p < 0.01$, ***, $p < 0.001$ ($n = 6$ in each group, 3 independent experiments).

Fig. 4. Rapamycin-treated mice showed a dramatic decrease in T helper type 2 (Th2) immune responses. (A-C) Th2 cytokines IL-4, along with IL-5 and IL-13 that play a role in eosinophil activation and proliferation showed prominent decreases in rapamycin-treated group. (D) Th1 cytokine, IFN- γ , also demonstrated attenuated levels in the rapamycin-treated group compared with the non-treated OVA group. (E, F) IL-10 and TGF- β , which are the main cytokines for T regulatory immune responses, showed minimal changes in the rapamycin-treated group compared to the non-treated OVA group. **, $p < 0.01$, ***, $p < 0.001$ ($n = 6$ in each group, 3 independent experiments).

Fig. 5. Rapamycin does not exert its attenuating effects on T regulatory cell immune responses. (A, B) Flow cytometry results for T regulatory cells showed a similar percentage of cells between non treated OVA group and rapamycin-treated group, which is consistent with the results for IL-10 and TGF- β cytokines. ($n = 6$ in each group, 3 independent experiments).

Fig. 6. The expressions of CC chemokines and adhesion molecules declined in rapamycin-treated experimental allergic conjunctivitis mice. (A, B) In the conjunctiva of rapamycin-treated group, mRNA levels of CC chemokines by real time PCR were significantly decreased compared to the non-treated OVA group. (C, D) mRNA levels of cell adhesion molecules were also attenuated in the conjunctiva of rapamycin-treated group. **, $p < 0.01$, ***, $p < 0.001$ ($n = 6$ in each group, 3 independent experiments).

Fig. 7. Rapamycin-treated mice showed decreased expression levels of proteins in the mTOR pathway. (A-D) mRNA expression levels in the conjunctiva of mice by real time PCR of key proteins in the mTOR pathway were mitigated with treatment of rapamycin. ***, $p < 0.001$ ($n = 6$ in each group, 3 independent experiments).

Fig. 8. Rapamycin eye drops attenuated experimental allergic conjunctivitis. (A) Experimental protocol time frame. Rapamycin eye drops were administered 30 minutes before OVA challenge via the conjunctival sac for 4 days from day 15 to 18. (B) Clinical scoring at 20 minutes after OVA challenge. (C) Eosinophil counts in the conjunctiva. (D) OVA-specific IgE Ab levels in sera. (E, F) IL-5 and IL-13 cytokine levels in cervical lymph nodes. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ ($n = 6$ in each group, 3 independent experiments).

Table 1. List and sequence of primers used for real time PCR

Gene	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
GAPDH	GACATCAAGAAGGTGGTGAAGCAC	ATACCAGGAAATGAGCTTGACAAA
RANTES	CCCTCACCATCATCCTCACT	CCTTCGAGTGACAAACACGA
Eotaxin	TCCACAGCGCTTCTATTCCT	CTATGGCTTTCA GGGTGCAT
P-selectin	GTCCACGGAGAGTTTGGTGT	AAGTGGTGTTCCGACCAAAG
VCAM-1	ATTTTCTGGGGCAGGAAGTT	ACGTCAGAACCAACCGAATCC
mTOR	CTCAAGCGATCCAGTTGTCA	AGAAGGTGGGGACACTGATG
p70S6K	GCTGTGGATTGGTGGAGTTT	GTGTGAGGTAGGGAGGCAAA
HIF-1 α	GAAATGGCCCA GTGA GAAAA	CTTCCACGTTGCTGACTTGA
VEGF-A	CCAGGAGGACCTTGTGTGAT	GGGAAGGGAAGATGAGGAAG
ATG5	AGATGGACAGCTGCACACAC	GCTGGGGGACAATGCTAATA

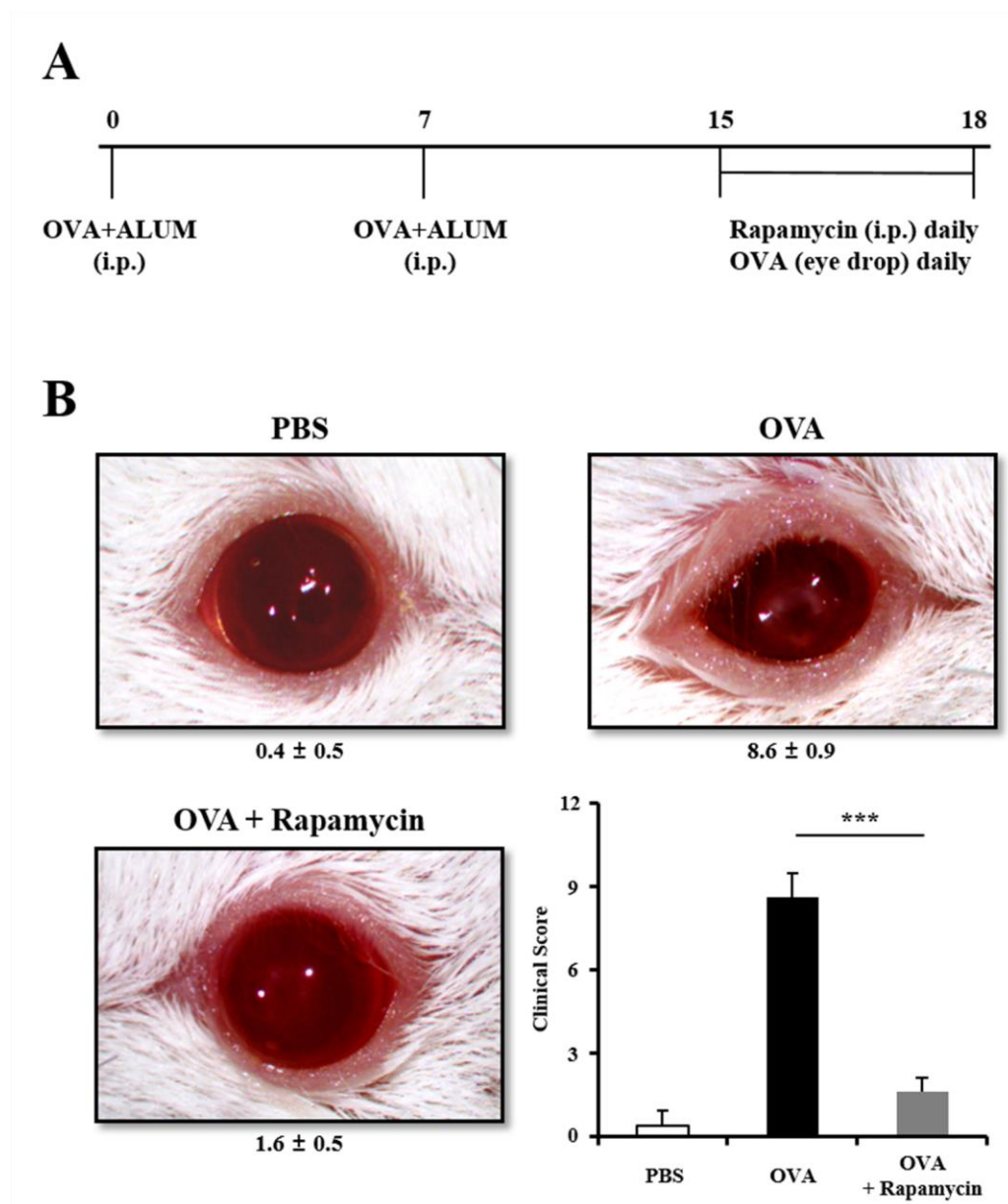


Figure 1

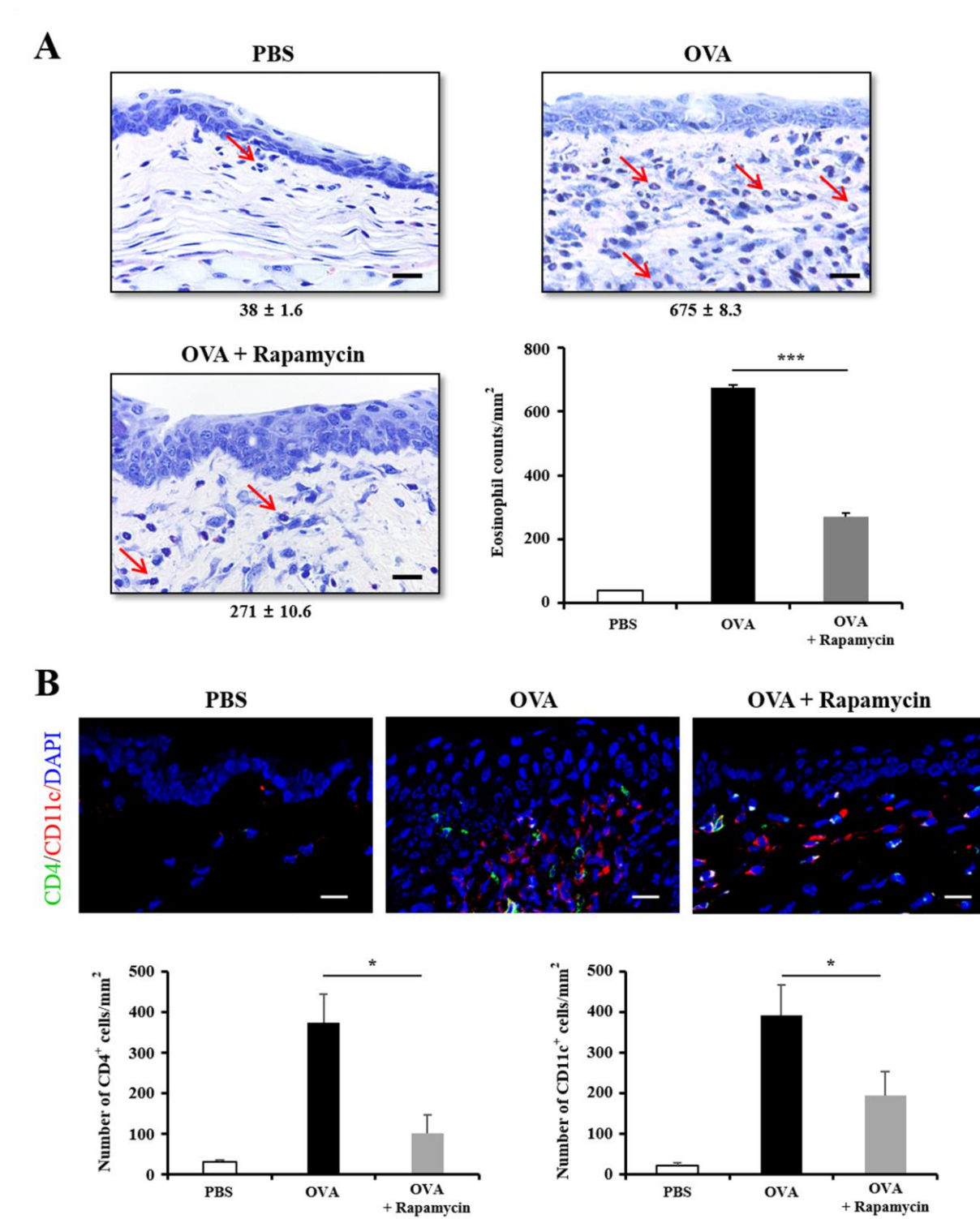


Figure 2

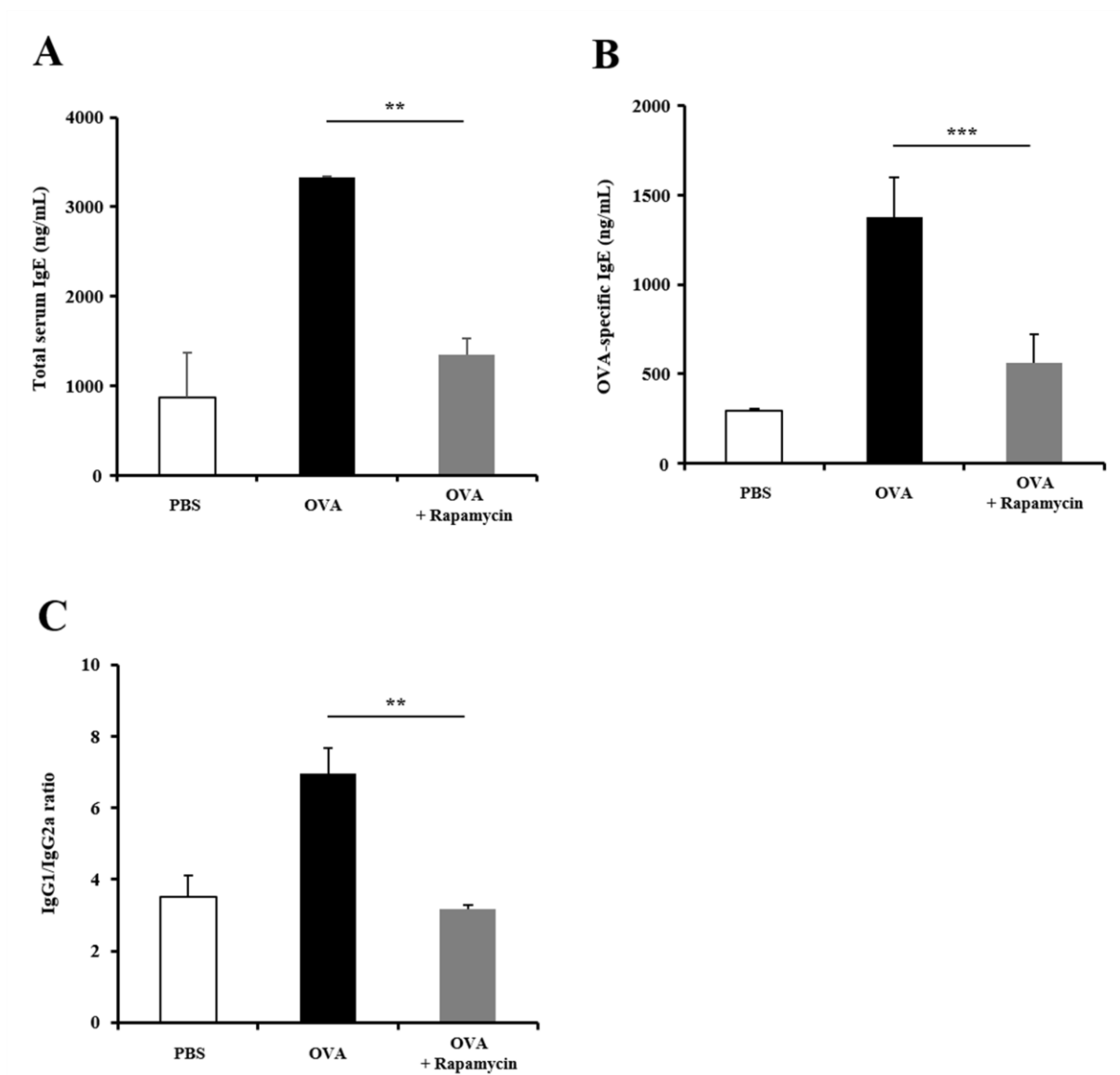


Figure 3

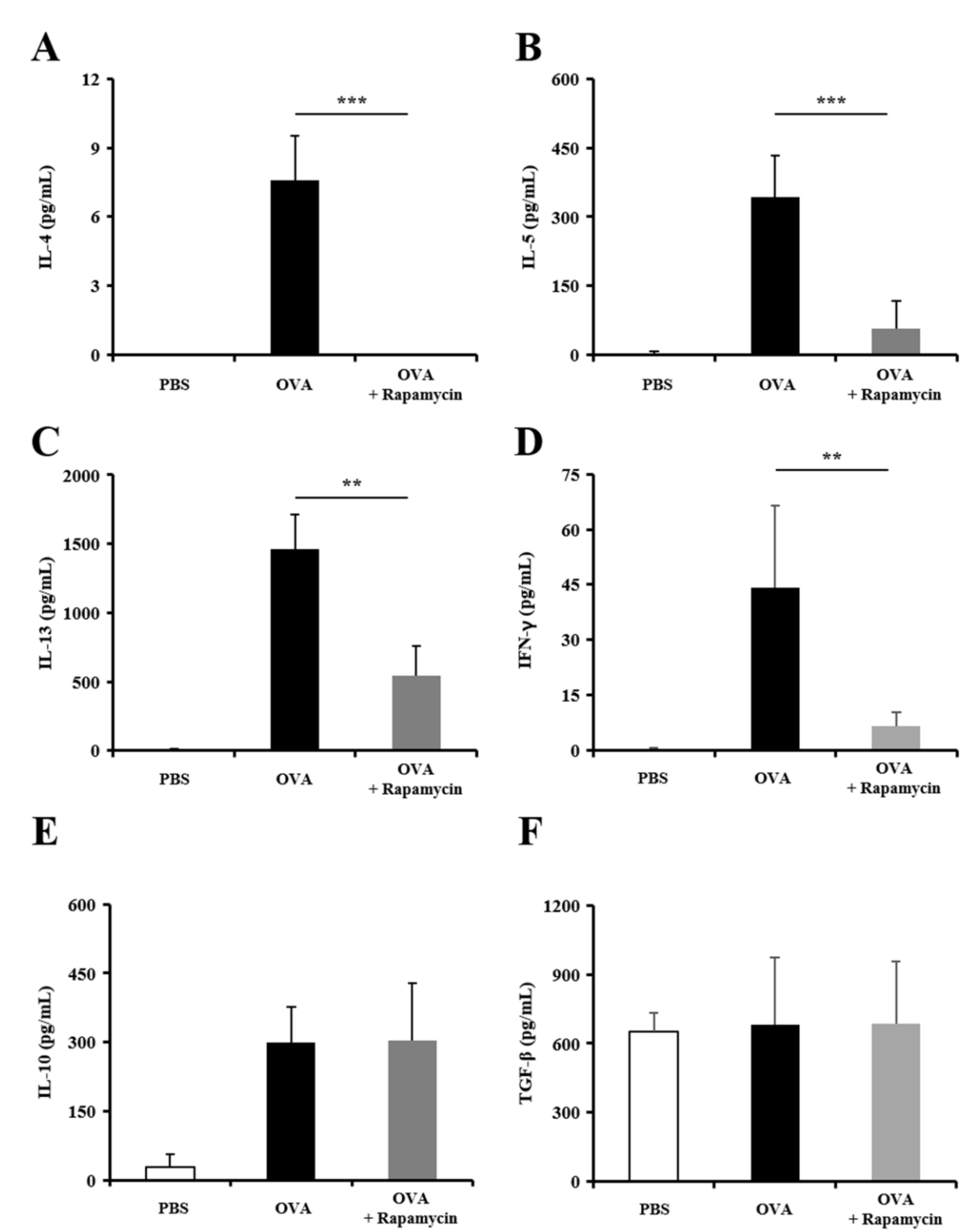


Figure 4

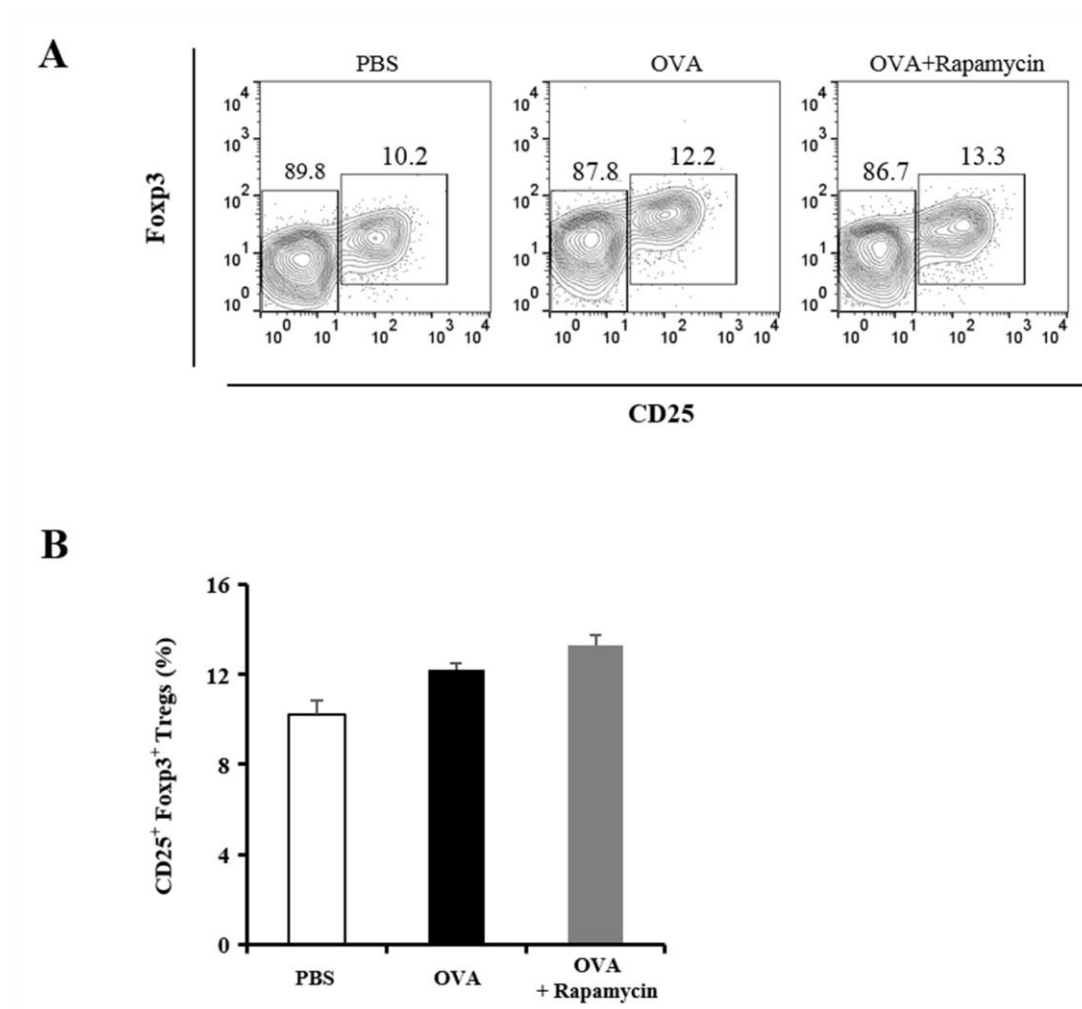


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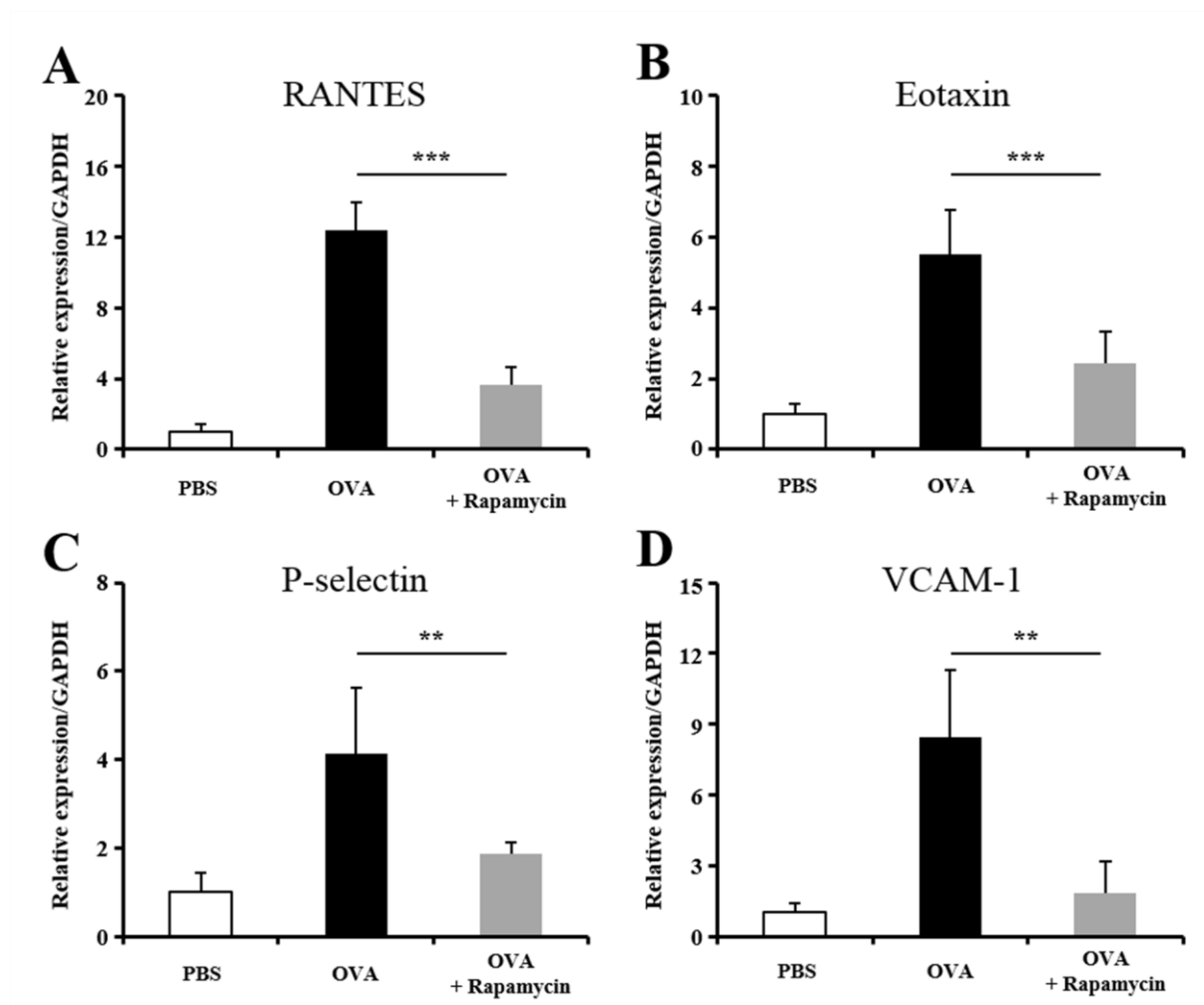


Figure 6

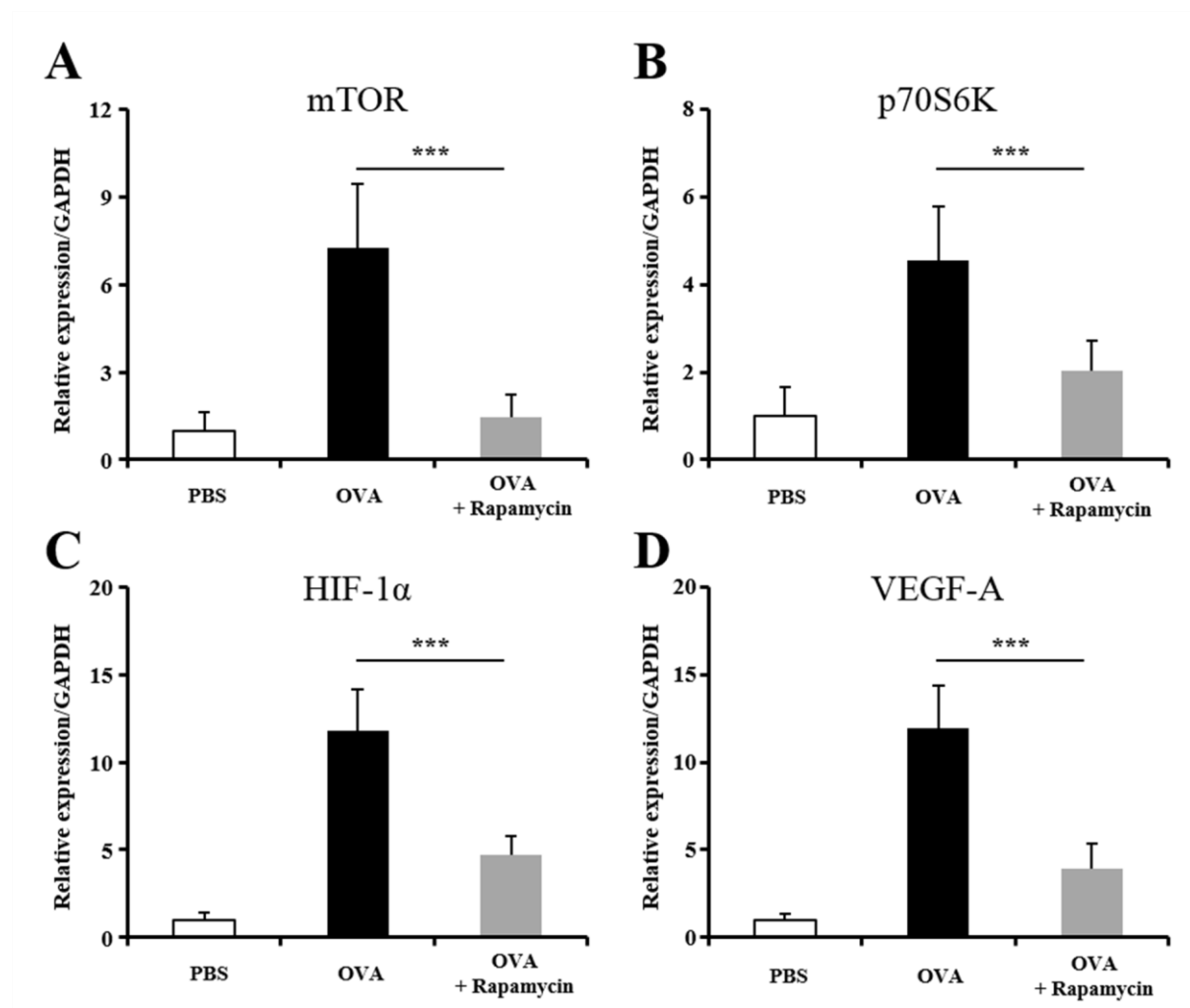


Figure 7

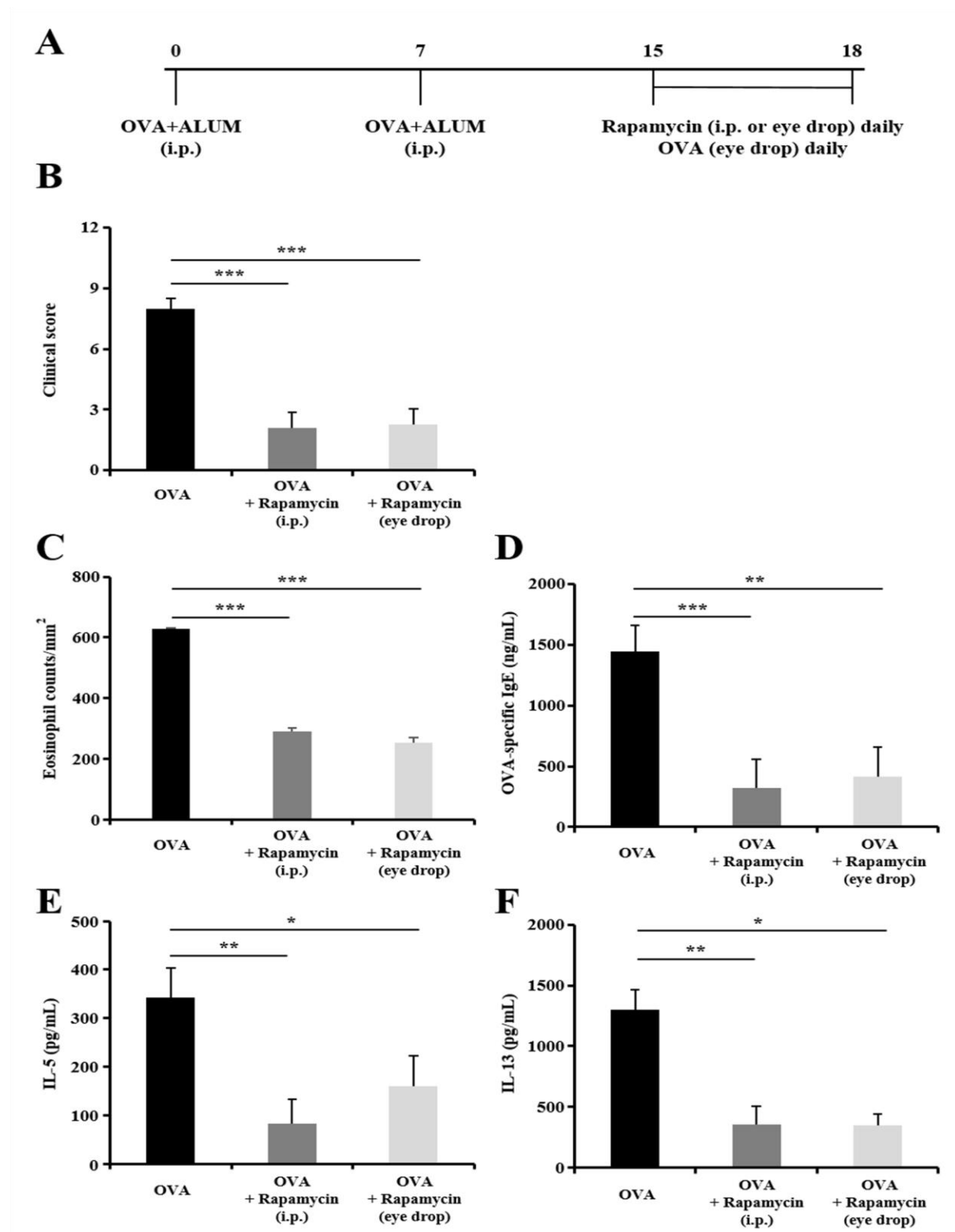


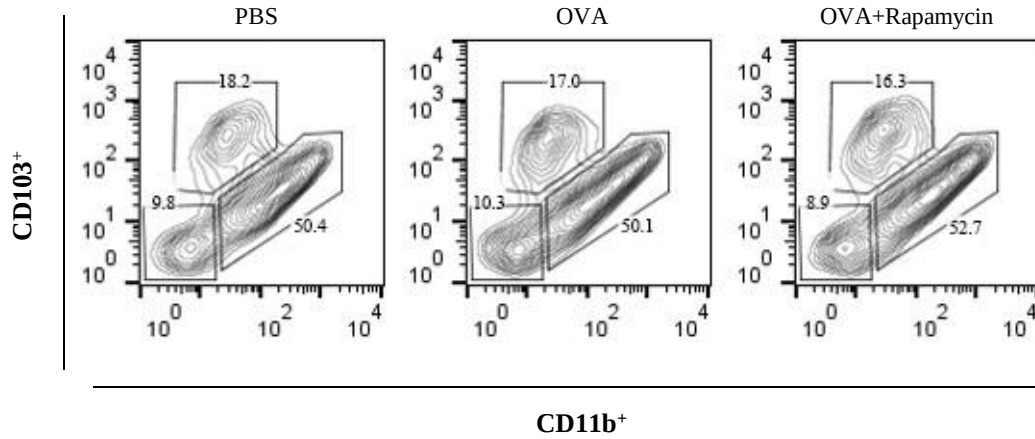
Figure 8

Highlights

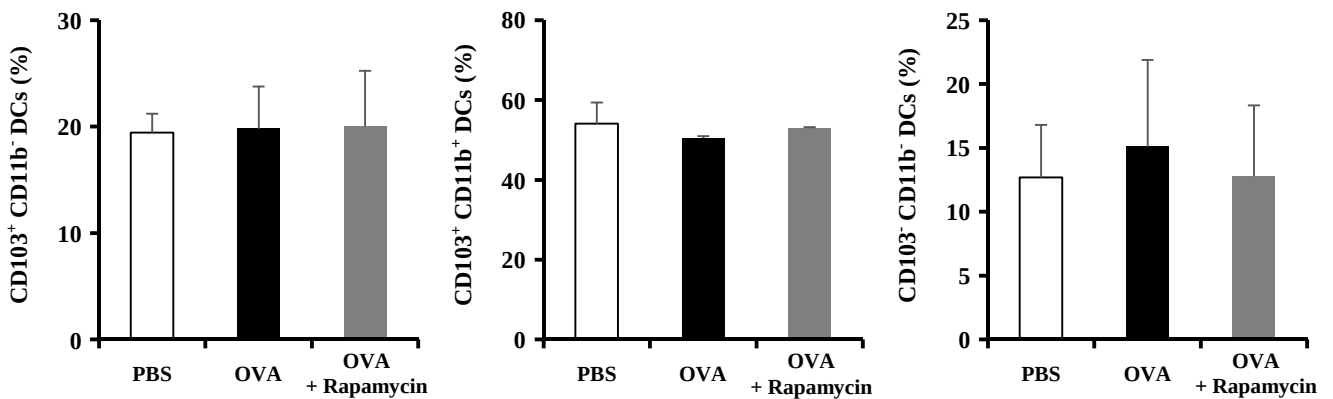
- Rapamycin treatment reduces clinical signs, conjunctival eosinophilic infiltration, IgE in serum and Th2 immune responses in OVA-induced experimental allergic conjunctivitis.
- The expressions of chemokines and adhesion molecules are reduced by rapamycin treatment in experimental allergic conjunctivitis.
- The attenuation of the expression of proteins in mTOR pathways contribute to the alleviating effects of rapamycin on allergic conjunctivitis.

Supplementary 1

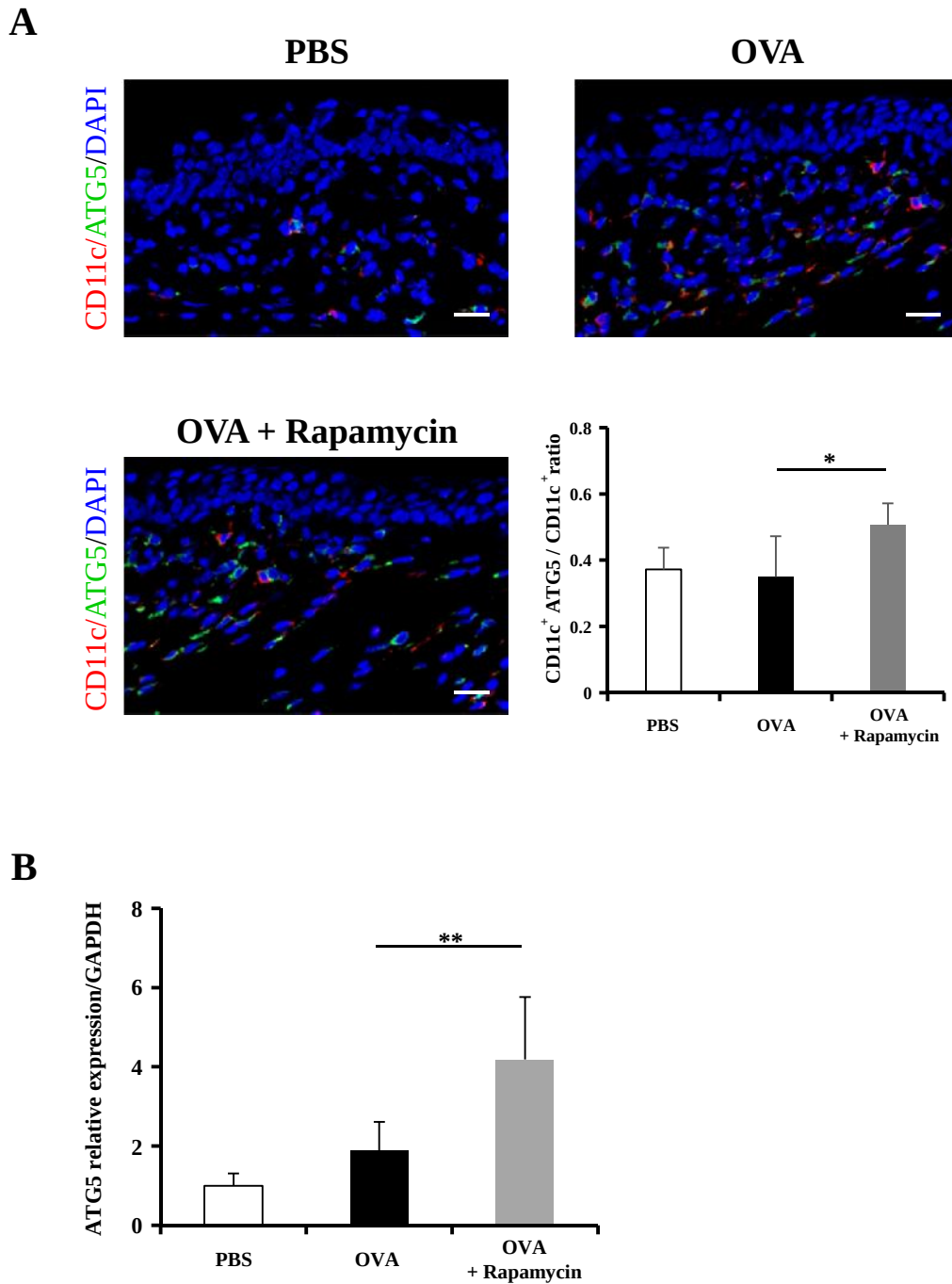
A



B



Supplementary Figure 1. Percentage of CD103⁺ and CD11b⁺ cells in experimental allergic conjunctivitis. Cells from the cervical lymph nodes that were gated positively for CD11c⁺ were further analyzed for CD103⁺ and CD11b⁺ by flow cytometry ($n = 6$ in each group, 3 independent experiments).



Supplementary Figure 2. Ratio of CD11c⁺ and ATG5 co-stained stained cells and ATG5 gene expression levels in experimental allergic conjunctivitis.

(A) Analysis of CD11c⁺ and ATG5 co-stained stained cells out of total CD11c⁺ stained cells in the conjunctiva demonstrated slight increases in rapamycin-treated mice. (B) mRNA expression levels of ATG5 in the conjunctiva were significantly increased in rapamycin-treated mice compared to the non-treated OVA mice. Bars = 50μm. *, $p < 0.05$, **, $p < 0.01$. Data shown are representative of 3 different experiments, $n = 6$ in each group.