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ORIGINAL ARTICLE

TRPV1 Antagonist Suppresses Allergic Conjunctivitis in a Murine Model

Ji Young Kwon, BS¹, Hyun Soo Lee, MD, PhD^{1,2}, and Choun-Ki Joo, MD, PhD^{1,2}

¹Catholic Institute for Visual Science, Seoul St. Mary's Hospital, Seoul, Republic of Korea and ²Department of Ophthalmology, Seoul St. Mary's Hospital, College of Medicine, The Catholic University of Korea, Seoul, Republic of Korea

ABSTRACT

Purpose: To determine the immunologic functions of TRPA1 or TRPV1 in allergic conjunctivitis (AC).

Methods: Mice were sensitized with ovalbumin (OVA), after which TRPA1 antagonist or TRPV1 antagonist was administered before topical OVA challenge. Expression of TRPV1 or TRPA1 in AC was examined by western blotting and multicolor immunofluorescence. Clinical signs, OVA-specific IgE, infiltration of inflammatory cells into conjunctivae (CJs), and Th2 cytokine in draining lymph nodes (LNs) were evaluated by microscopy, flow cytometry, and ELISA.

Results: TRPV1 expression was increased in CJs and LNs from AC mice, but TRPA1 expression was only increased in LNs. TRPV1 antagonist but not TRPA1 antagonist attenuated the clinical signs of AC and OVA-specific IgE in sera. TRPV1 antagonist furthermore inhibited the infiltration of inflammatory cells into CJ and the production of Th2 cytokines in LNs.

Conclusion: TRPV1 antagonist but not TRPA1 antagonist may ameliorate AC by suppressing the Th2 response in LNs.

Keywords: Allergic conjunctivitis, inflammatory cells, Th2 cytokines, TRPA1, TRPV1

The clinical signs of allergic conjunctivitis (AC) include lid swelling, conjunctival chemosis, conjunctival redness, and tearing.¹ A compound IgE-mediated and type 2 immune-mediated response associated with inflammatory conditions are responsible for these symptoms,^{2–4} and this IgE-mediated AC response is produced by a specific conjunctiva (CJ) stimulus,⁵ which results in a response marked by the infiltration of inflammatory cells, such as eosinophils and mast cells.^{6,7} These type 2 immune reactions are dominated by the activation of CD4⁺ Th2 cells and the production of type 2 immune reaction-related cytokines, such as IL-4 and IL-13.⁸

In mammals, the transient receptor potential (TRP) family of ion channels located on the plasma membrane act as receptors for stimuli. The TRP family consists of six subfamilies: TRPC (canonical: TRPC1~TRPC7); TRPV (vanilloid: TRPV1~TRPV6); TRPM (melastatin: TRPM1~TRPM8); TRPP (polycystin: TRPP2, TRPP3, TRPP5); TRPML (mucolipin: TRPML1~TRPML3); and

TRPA (ankyrin: TRPA1).⁹ Among the TRP family, the immunologic functions of TRPA1 or TRPV1 have been researched in terms of their immunologic functions in the context of allergic disease. In allergic asthma, TRPA1 or TRPV1 yielded increased Th2 cytokine levels and induced the infiltration of eosinophils into the lungs.^{10,11}

In an allergic dermatitis model, TRPA1 or TRPV1 induced the infiltration of inflammatory cells into the skin, increased Th2 cytokine levels, and provoked itching.^{12–14} In allergic rhinitis, TRPA1 or TRPV1 increased Th2 cytokine levels and promoted histamine-mediated itching. The increased levels of Th2 cytokines also led to the infiltration of inflammatory cells.^{15,16}

The immunologic functions of TRPA1 or TRPV1 and the mechanisms of their actions in AC, however, remain unknown. In this study, the immunologic functions of TRPA1 or TRPV1 in an ovalbumin (OVA)-sensitized AC murine model were investigated using TRPA1 antagonist or TRPV1 antagonist.

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Correspondence: Choun-Ki Joo, Department of Ophthalmology & Visual Science, Seoul St. Mary's Hospital, 222 Banpo-daero, Seocho-gu, Seoul 137–70, Republic of Korea. E-mail: ckjoo@catholic.ac.kr

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MATERIALS AND METHODS

Animals

Seven-week-old BALB/c male mice were purchased from Orient Bio Inc. (Sungnam, Korea). Mice were cared for in a specific pathogen-free environment at the animal facility, in accordance with the Catholic Institutional Animal Care and Use Committee (IACUC) and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. A mixture of ketamine/xylazine suspensions (120 and 20 mg/kg administered intraperitoneally, respectively) was used for all surgical procedures.

AC Model

The murine model of AC was established as previously described.¹⁷ Briefly, BALB/c mice were immunized by a single intraperitoneal injection with 100 µg OVA in 100 µL PBS that included 1 mg aluminum hydroxide (Alum) and 300 ng pertussis toxin (all purchased from Sigma Aldrich, St. Louis, MO). The mice were sensitized for 2 weeks and then challenged by topical OVA eye drops once daily for at least 13 days.

The clinical evaluation of AC, which involved checking for immediate hypersensitivity responses 10 min post-topical OVA challenge, was conducted in a masked fashion by two independent observers. Clinical scoring con-

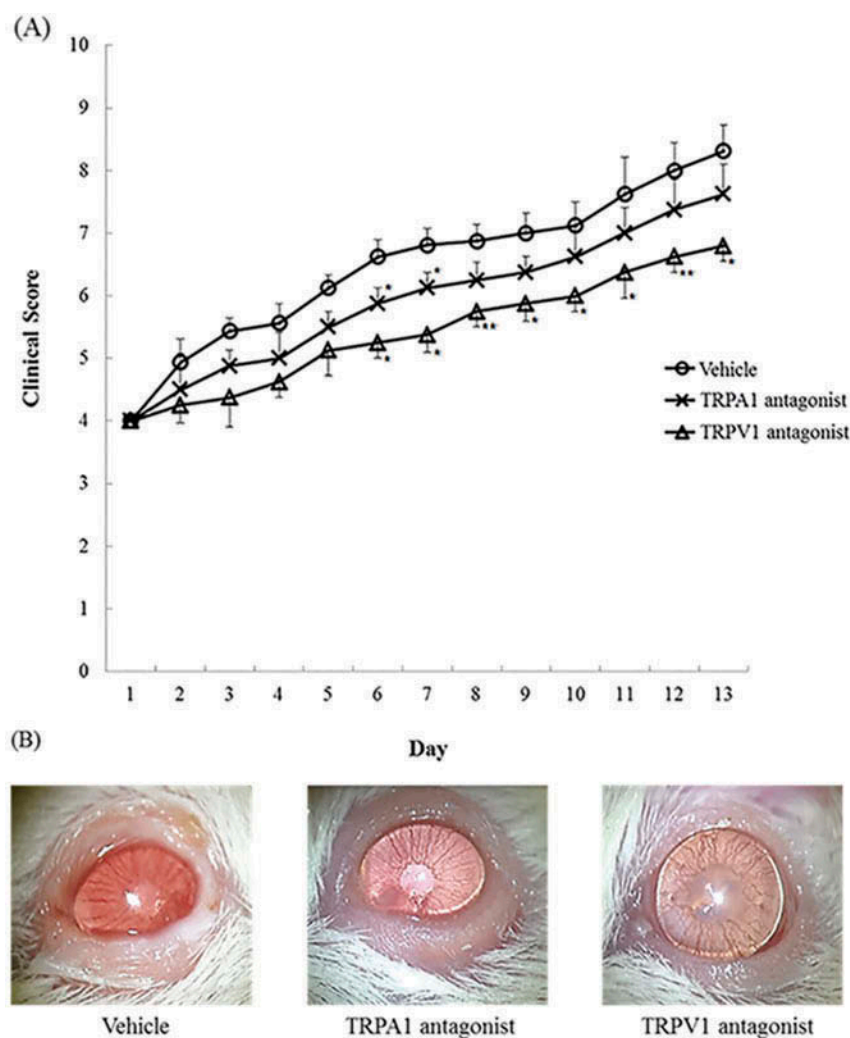


FIGURE 1. (A) Mice were injected intraperitoneally with OVA/Alum, and naive mice served as control animals. After 2 weeks, TRPA1 antagonist, TRPV1 antagonist, or vehicle eye drops were administered 20 min post-topical OVA challenge in the right eye of each mouse once a day for 13 days. The clinical signs were evaluated in a masked fashion 20 min post-topical OVA challenge. The data for the three groups represent three independent experiments and are reported as mean $\pm$ SD (TRPA1 antagonist vs vehicle, TRPV1 antagonist vs vehicle; * p < 0.05, ** p < 0.01). (B) Representative clinical images from TRPA1 antagonist-, TRPV1 antagonist-, or vehicle-treated animals on day 13 of topical challenge.

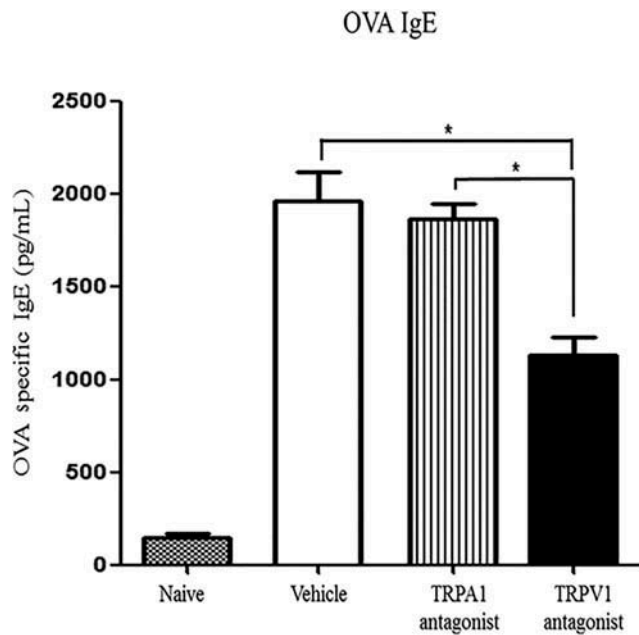


FIGURE 2. The OVA-specific IgE levels in TRPA1 antagonist-, TRPV1 antagonist-, or vehicle-treated animals were compared. Sera were collected from mice ($n = 3/\text{group}$), and OVA-specific IgE levels were measured by ELISA. Data were collected over three independent experiments and are presented as means $\pm$ SEM (* $p < 0.01$).

sisted of four parameters: lid swelling, conjunctival chemosis, conjunctival redness, and tearing. Each limitation was scored on a scale from 0 (absence of signs) to 3 (maximal), and the sum of the scores for the four parameters yielded the total score for each animal.^{18,19}

Topical Administration of TRPA1 Antagonist or TRPV1 Antagonist

The TRPA1 antagonist, HC-030031 (Abcam, Cambridge, MA), was diluted to 10 mM in 1% dimethyl sulfoxide (DMSO) and PBS. The TRPV1 antagonist, Capsazepine (R&D Systems, Minneapolis, MN), was diluted to 10 mM in 1% DMSO and PBS. The vehicle control consisted of 1% DMSO in PBS. TRPA1 antagonist, TRPV1 antagonist, or vehicle eye drops were administered 20 min before topical OVA eye drops (500 $\mu\text{g}/\text{mL}$) once daily to prevent the dilution of TRPA1 antagonist, TRPV1 antagonist, or vehicle and OVA.

ELISA for OVA-specific IgE in Serum

Following topical challenges for 13 days, blood was collected from mice euthanized by cardiac puncture. Sera were isolated via coagulation and centrifugation before being analyzed using an ELISA kit for OVA-specific IgE according to the manufacturer's instructions (AbD Serotec, Raleigh, NC).

Western Blot Analysis

Following topical challenges for 13 days, CJs and ipsilateral cervical lymph nodes (CLNs) were collected from naive (untreated) mice or AC mice. Proteins were extracted from each tissue using RIPA buffer and were quantified using a bovine serum albumin (BSA) standard curve, after which concentrations were adjusted to allow for equal loading. Together with appropriate markers, the proteins were subjected to 10% SDS PAGE and were then transferred onto PVDF membrane. The membranes were blocked for 1 h with 3% BSA (in PBST; PBS containing 0.5% Tween 20) before being incubated overnight at 4°C with anti-TRPA1 or anti-TRPV1 (1:500) antibody diluted in the 3% BSA solution. After being washed with PBST, the membranes were incubated with anti-rabbit IgG-HRP (1:1000 dilution in 3% BSA) at room temperature for 1 h. Finally, specific protein bands were visualized using chemiluminescent HRP substrate solution.

Immunofluorescence

Following topical challenges for 13 days, ipsilateral whole eyes including CJs and CLNs were collected from naive (untreated) mice or AC mice. Cryosections (7 μm) were air dried for 30 min and washed with PBS five times (3 min/wash). The CJs and CLNs were blocked with 1% BSA in PBS at room temperature for 1 h. The CJs were incubated in 1% BSA in PBS with mixtures of anti-TRPA1 antibody (or anti-TRPV1 antibody) and Alexa Fluor 488-conjugated anti-mouse CD4 antibody (BioLegend, San Diego, CA) overnight at 4°C. The CLNs were incubated in 1% BSA in PBS with mixtures of anti-TRPA1 antibody (or anti-TRPV1 antibody), Alexa Fluor 488-conjugated anti-mouse CD4 antibody and Pacific Blue-conjugated anti-mouse/human CD45R/B220 antibody (BioLegend) overnight at 4°C. The CJs and CLNs were incubated in BSA in PBS with mixtures of secondary antibodies (Dylight 594-conjugated goat anti-rabbit polyclonal antibody) for 1 h at room temperature.

Quantitation of Conjunctival Mast Cells and Eosinophils

As described previously,^{17,19} mice were euthanized 20 min after the challenge and CJs were collected. The conjunctival tissues were digested with 2 mg/mL collagenase (Roche, Mannheim, Germany) and 0.05 mg/mL DNase I (Roche). The suspensions were filtered through a 70- μm cell strainer and washed carefully. The cells were resuspended in 0.5% FBS buffer and treated with anti-FcR (CD16/CD32, BioLegend) antibodies to block Fc-mediated reactions, as per the manufacturer's instructions. The cells were subsequently stained with an Alexa Fluor 488-conjugated anti-

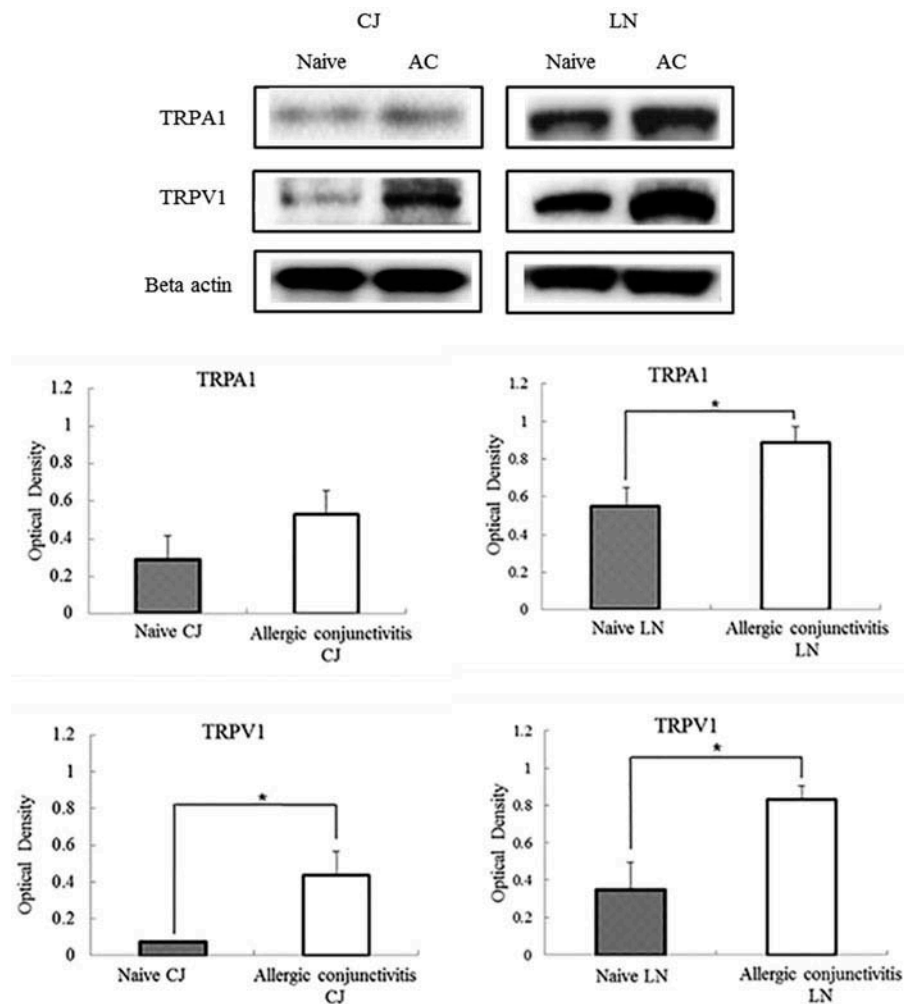


FIGURE 3. TRPA1 or TRPV1 expression in the CJs and CLNs of naive or AC mice was assessed by western blot ($n = 4/\text{group}$). Data were collected over three independent experiments and are reported as mean $\pm$ SEM ($*p < 0.05$).

CD45 antibody (BioLegend), a PE-Cy7-conjugated anti-c-kit (BioLegend) antibody, and a PE-conjugated sialic acid-binding immunoglobulin-type lectin-F (Siglec-F; BD Biosciences, East Rutherford, NJ) antibody. The isotype control was stained with appropriate matched antibodies (BioLegend). Additionally, all samples were analyzed using an LSRFortessa flow cytometer (BD Biosciences).

Measurement of T-cell Responses to Recall Allergen Stimulation

Single cells from CLNs collected from euthanized mice^{17,19} were plated on 96 wells, as indicated above. Following 72 h of OVA stimulation, the cultures were restimulated with phorbol myristate acetate/ionomycin (Sigma Aldrich, St. Louis, MO) for up to 6 h. The IL-4 and IL-13 cytokines in the collected supernatant were quantified by ELISA as per the manufacturer's instructions (Ready-set-go ELISA kit, eBioscience, San Diego, CA).

Statistical Analyses

Data are expressed as mean $\pm$ standard deviation (SD) or mean $\pm$ standard error of the mean (SEM). Differences between groups were analyzed by analysis of variance (ANOVA) or two-tailed Student's *t*-test as indicated. Differences with p values < 0.05 were considered statistically significant.

RESULTS

TRPV1 Antagonist Reduced Clinical Signs of Allergic Reaction

First, we aimed to clarify whether topical application of the TRPA1 antagonist or TRPV1 antagonist modulates immune reactions in AC. In a previous study, we demonstrated AC reactions to topical OVA eye drops in mice that were sensitized by intraperitoneal injection of OVA.^{17,19} In this study, topical TRPA1

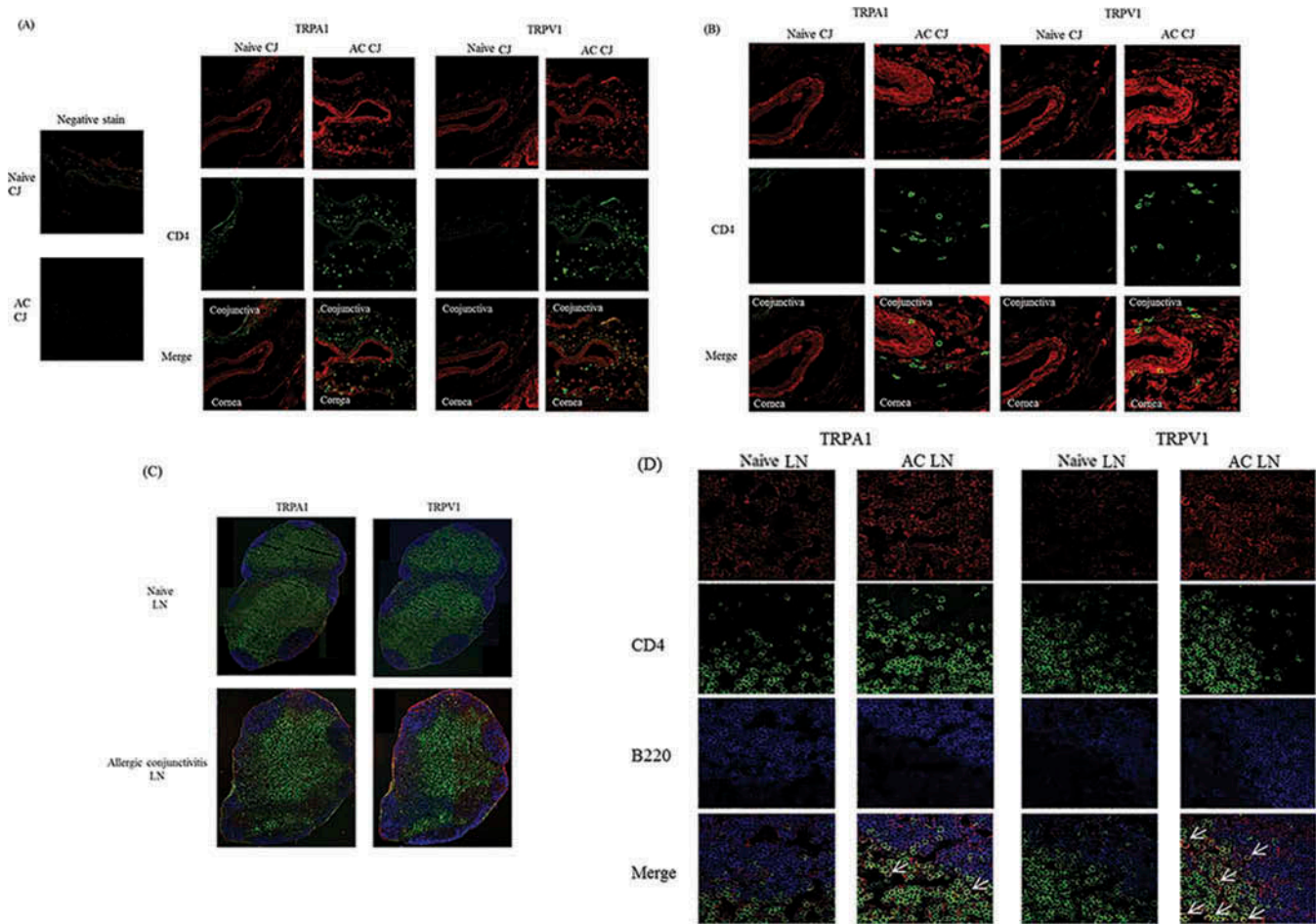


FIGURE 4. The localizations of TRPA1 or TRPV1 in the CJs and CLNs of mice were assessed in naive or AC animals. (A,B) Representative images of immunofluorescence for TRPA1 (red) or TRPV1 (red), and CD4+ T cells (green) in CJs ($n = 2/\text{group}$). Original magnification (A) $\times 200$, (B) $\times 400$. (C,D) Representative images of immunofluorescence for TRPA1 (red) or TRPV1 (red), with B cells (blue) and CD4+ T cells (green) in CLNs ($n = 2/\text{group}$). Original magnification (C) $\times 50$, (D) $\times 400$. The arrow indicates co-localization of TRPA1 or TRPV1 and CD4+ cells in CJ or LN.

antagonist, TRPV1 antagonist, or vehicle eye drops were applied with topical OVA challenge once daily to OVA-sensitized mice for 13 days. To evaluate AC responses, clinical signs of AC were scored about 10 min post-challenge.^{18,19} The results from this experiment demonstrated that the topical TRPA1 antagonist-treated mice did not have improved clinical AC symptoms compared with the vehicle-treated mice, while topical TRPV1 antagonist-treated mice showed an improvement in clinical AC symptoms compared with the vehicle-treated mice (Figure 1). The TRPV1 antagonist but not the TRPA1 agonist was thus shown to suppress the clinical signs of AC.

TRPV1 Antagonist Suppresses Serum OVA-specific IgE Levels in AC

Next, the OVA-specific IgE levels in the sera of mice were assessed (Figure 2) and it was shown that OVA-specific IgE levels in TRPV1 antagonist-treated mice

were significantly lower than those in the vehicle-treated mice ($p = 0.0017$ vs vehicle). The levels of OVA-specific IgE were furthermore shown to be lower in TRPV1 antagonist-treated mice than in TRPA1 antagonist-treated mice ($p = 0.0016$ vs TRPA1 antagonist).

Expression of TRPA1 or TRPV1 in CJs and Lymph Nodes (LNs)

In this study, the expression levels of TRPA1 or TRPV1 were assessed in naive (untreated) mice and in AC mice (topical OVA in OVA-sensitized mice). Western blotting showed that the expression of TRPA1 was significantly higher in the LNs of AC mice than in the LNs of naive mice ($p = 0.011$). TRPV1 expression was, moreover, found to be significantly higher in the CJs and LNs of AC mice compared with the CJs ($p = 0.019$) and LNs ($p = 0.007$) of naive mice (Figure 3). To demonstrate the localization of the expression of TRPA1 or TRPV1 and CD4+ T cells in the CJs and

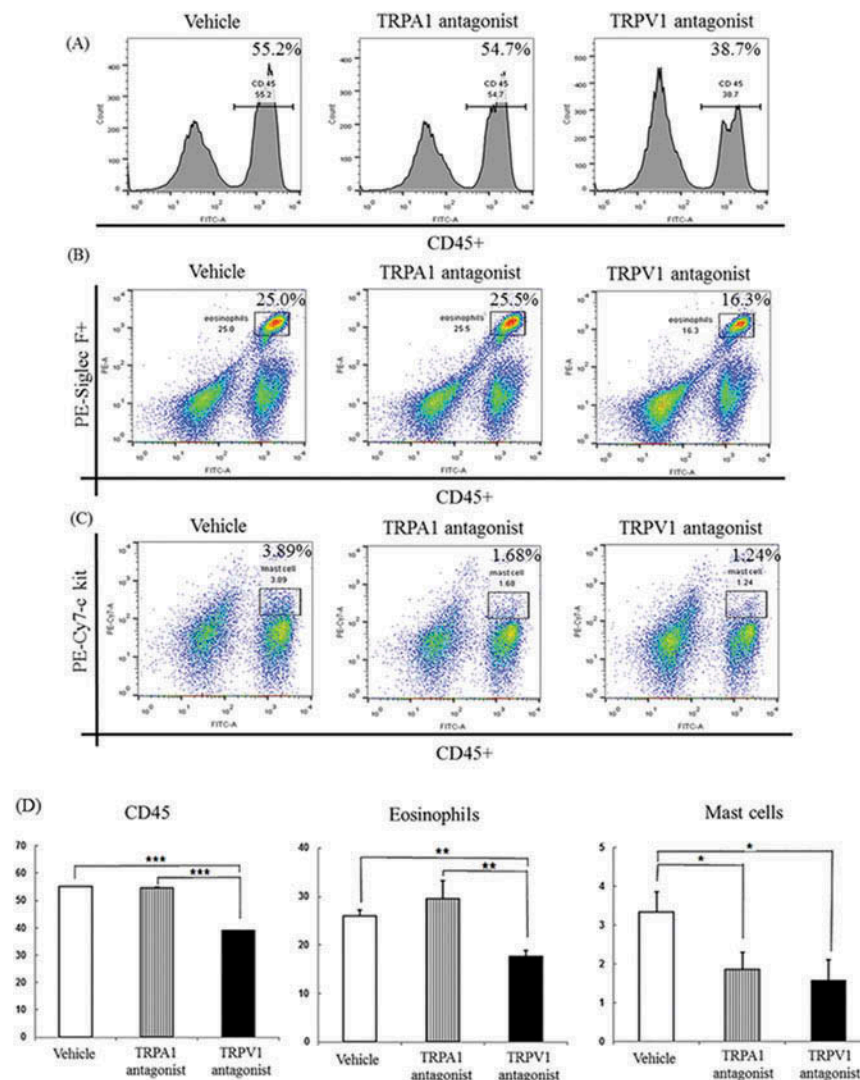


FIGURE 5. Topical TRPA1, TRPV1, or vehicle eye drops applied 20 min before topical OVA challenge were administered in the right eye of OVA-sensitized mice once a day for 13 days. CJs were collected from these and naive control (untreated) mice and were analyzed for CD45+ cells (A), eosinophils (B; CD45+ Siglec-F+), and mast cells (C; CD45+ c-kit+) by flow cytometry ($n = 4$ /group). The figures represent multiple independent experiments. (D) Data from three independent experiments ($n = 4$ mice per group/each experiment) are reported as mean $\pm$ SEM (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$).

LN of AC mice and naive mice, multicolor immunofluorescence staining was performed (Figure 4). The immunofluorescence staining revealed that the expression level of TRPA1 or TRPV1 was increased in the CJs and LNs of AC mice. Interestingly, TRPV1 exhibited co-localization with the CD4+ T cell marker in the CJs and LNs of AC mice.

Infiltration of Inflammatory Cells in CJ

To determine the functions of the TRPA1 antagonist or TRPV1 antagonist in the conjunctival inflammatory reaction in AC, CJs were collected and the CD45+ cells (Figure 5A), eosinophils (CD45+ Siglec-F+; Figure 5B), and mast cells (CD45+ c-kit+; Figure 5C) in the CJs were

counted using flow cytometry. TRPA1 antagonist-treated mice showed significantly reduced infiltration of mast cells ($p = 0.019$ vs vehicle) into CJs relative to the vehicle-treated mice (Figure 5D). TRPV1 antagonist-treated mice exhibited significantly reduced infiltration of CD45+ cells ($p < 0.0001$ vs vehicle), eosinophils ($p = 0.001$ vs vehicle), and mast cells ($p = 0.015$ vs vehicle) into CJs relative to the vehicle-treated mice (Figure 5D). The TRPV1 antagonist-treated mice furthermore displayed significantly reduced infiltration of CD45+ ($p < 0.0001$ vs TRPA1 antagonist) cells and eosinophils ($p = 0.007$ vs TRPA1 antagonist) into CJs relative to the TRPA1 antagonist-treated mice (Figure 5D). These findings indicate that the TRPA1 antagonist did not inhibit the infiltration of eosinophils in the conjunctival inflammatory reaction in AC, but that the TRPV1 antagonist

inhibited the infiltration of inflammatory cells in the conjunctival inflammatory reaction in AC.

Regulating the Level of Th2 Cytokines in LNs

To determine whether the TRPA1 antagonist or TRPV1 antagonist led to allergic response regulation in AC, drained LNs were collected 13 days after daily TRPA1 antagonist or TRPV1 antagonist and OVA administration, and purified T cells were stimulated with OVA *in vitro*. Secretions of Th2 cytokines, such as IL-4 and IL-13 into the supernatants were analyzed by ELISA (Figure 6). The level of IL-4 in TRPA1 antagonist-treated mice was not significantly reduced compared with that in the vehicle-treated mice, while the level of IL-13 was significantly lower in TRPA1 antagonist-treated mice compared with vehicle-treated mice ($p = 0.0095$ vs vehicle). In TRPV1 antagonist-treated mice, the levels of Th2 cytokines such as IL-4 ($p = 0.0003$ vs vehicle) and IL-13 ($p < 0.0001$ vs vehicle) were significantly reduced compared with those in the vehicle-treated mice. The levels of Th2 cytokines such as IL-4 ($p = 0.0004$ vs TRPA1 antagonist) and IL-13 ($p = 0.0083$ vs TRPA1 antagonist) were furthermore significantly lower in TRPV1 antagonist-treated mice than in TRPA1 antagonist-treated mice.

DISCUSSION

Allergic conjunctivitis (AC) responses involve augmented levels of IgE in sera and the infiltration of eosinophils and mast cells into CJ, mediated by Th2 cytokines.^{20,21}

The TRPA1 or TRPV1 has been studied in terms of their immunologic functions in allergic diseases. In allergy, increased calcium ion levels were shown to activate TRPA1 or TRPV1.¹⁶ The activated TRPA1 or TRPV1 have been shown to induce the infiltration of inflammatory cells into tissues and to increase Th2 cytokine levels.^{10,12}

In this study, the immunologic functions of TRPA1 or TRPV1 were assessed using TRPA1 antagonist or TRPV1 antagonist in an OVA-sensitized AC murine model. The topical application of the TRPA1 antagonist did not relieve the clinical symptoms of AC in this murine model and did not decrease the serum levels of OVA-specific IgE. In contrast, the topical application of the TRPV1 antagonist relieved the clinical symptoms of AC in this murine model and decreased the serum levels of OVA-specific IgE. In a recent study on the sensory neural mechanisms of AC, particularly on whether the TRPA1 antagonist or TRPV1 antagonist is associated with the clinical symptoms of AC, TRPA1 antagonist-treated rats showed reduced blinking without changes in tearing, while the TRPV1 antagonist-treated rats showed a reduction in both blinking and tearing.²² Together with the present findings, this indicates that TRPV1 plays a more important role in regulating the clinical symptoms of AC compared with TRPA1.

Expression levels and protein localization in the CJs and CLNs of naive mice or AC mice were assessed and it was found that, compared with the expression levels in naive mice, TRPA1 expression was elevated in the CLNs of AC mice and TRPV1 expression was elevated in the CJs and CLNs of AC mice. It was furthermore revealed that TRPV1 co-localized with the CD4+ T cell in the CJs and LNs of AC mice. In agreement with

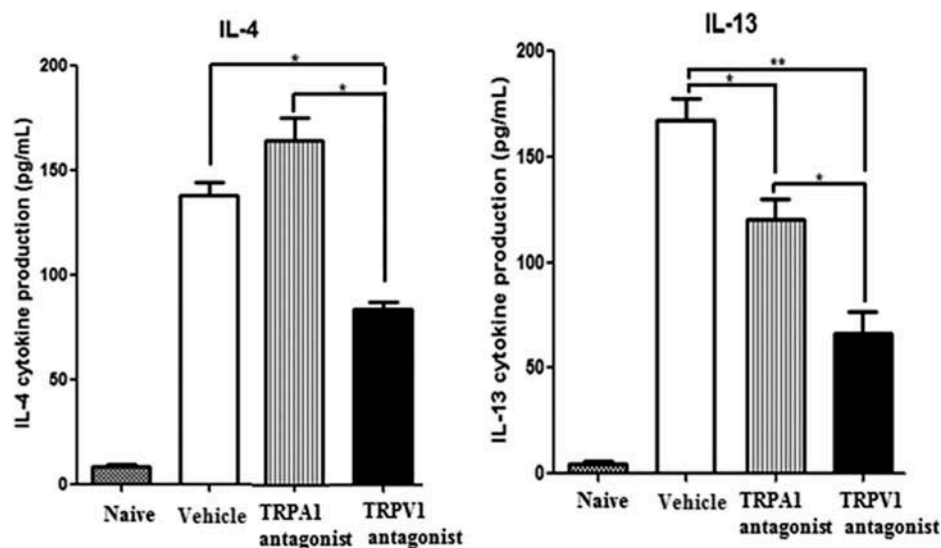


FIGURE 6. Drained LNs were harvested after administration of TRPA1 antagonist, TRPV1 antagonist, or vehicle eye drops ($n = 3$ mice/group). Single-cell suspensions from LNs were stimulated with OVA for 72 h and were then restimulated with phorbol myristate acetate/ionomycin for up to 6 h. IL-4 ($*p < 0.001$) and IL-13 ($*p < 0.01$, $**p < 0.0001$) levels in culture supernatants were quantified by ELISA.

these findings, Bertin et al. showed that TRPV1 is functionally expressed in CD4⁺ T cells and plays a crucial role of in the activation and acquirement of inflammatory character in CD4⁺ T cells.²³

The topical application of TRPA1 antagonist resulted in the inhibited infiltration of mast cells into the CJ, but did not block the infiltration of eosinophils into the CJ. The topical application of TRPV1 antagonist, on the other hand, blocked the infiltration of eosinophils and mast cells into the CJ. In previous studies, the TRPA1 and TRPV1 receptors were shown to be expressed in mast cells. Chen and Hackos showed that the TRPA1 antagonist inhibits the infiltration of mast cells and attenuates itch-scratching behavior,²⁴ and van Diest et al. showed that the TRPV1 antagonist prevents the infiltration of mast cells and pain responses.²⁵ Jian et al. furthermore showed that the H4 receptor was activated by TRPV1, but not by TRPA1, and that the H4 receptor in terms of histamine-dependent itching was expressed mainly in eosinophils.²⁶ Previous studies demonstrated that both the infiltration into tissues and inflammatory functions of mast cells were mediated by IL-13 in allergy model and IL-4 participated in eosinophil infiltration in a murine allergy model.^{27,28} In the present study, the TRPA1 antagonist was shown to decrease IL-13 levels and to suppress the infiltration of mast cells, as previously reported. TRPA1 antagonist, however, did not suppress IL-4 levels in the T cells of LNs and eosinophil infiltration in CJs.

TRPV1 antagonist not only inhibited the infiltration of eosinophils and mast cells, but also attenuated IL-4 and IL-13 in the T cells of CLN in this study. Rehman et al. reported that the siRNA-mediated knockdown of TRPV1 attenuated allergic inflammation in IL-13,²⁹ and Biro et al. showed that the TRPV1 antagonist inhibited the induction of IL-4 production in murine mast cells.³⁰ Ramachandran et al. reported that TRPV1 antagonist-treated mice exhibited decreased allergic inflammation and decreased Th2 cytokine production, demonstrating that TRPV1 inhibition reduced allergic inflammation.³¹

The CJ is supplied by capillaries and lymphatics³² and the administration of drugs for AC may thus be cleared through blood and lymph. The conjunctival blood vessels do not form a tight junction barrier,³³ which means that small molecule drugs can penetrate the blood circulation by pinocytosis and/or convective transport through paracellular pores in the vascular endothelial layer. The conjunctival lymphatics act as an efflux system for efficient elimination from the CJ. In a previous study, at least 10% of a small molecule (sodium fluorescein) compound administered in the subconjunctiva was shown to be eliminated into LNs and into the systemic circulation via the lymphatics within the first hour in rat eyes.³⁴ The topical administration of β -1,3-glucan was furthermore shown to suppress the systemic immune responses

including serum IgE and the production of Th2 cytokines by T cells in LNs in a murine model of AC.¹⁹ Drugs transported by lymphatics in conjunction with the elimination by blood circulation may therefore contribute to systemic exposure via the interstitial fluid returned to the systemic circulation after filtration through LN.

In this study, the immunologic function of TRPV1 was assessed using a TRPV1 antagonist, which was shown to inhibit the infiltration of eosinophils and mast cells by inhibiting Th2 response, specifically by decreasing the levels of IL-4 and IL-13 in CLNs. The TRPV1 antagonist, and not the TRPA1 antagonist, was thus shown to suppress allergic responses in the OVA-sensitized AC murine model. Although further studies are required to understand the mechanisms of TRPA1 or TRPV1 in AC, the findings reported here suggest that topical TRPV1 antagonist eye drops may be a novel therapeutic drug for regulating Th2 immune reactions, including the infiltration of eosinophils and mast cells into the CJ.

DECLARATION OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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