

Accepted Manuscript

The C-C Chemokine receptor 6 (CCR6) is crucial for Th2-driven allergic conjunctivitis

So-Hyang Chung, Sun Young Chang, Hyun Jung Lee, Seong Hyun Choi

PII: S1521-6616(15)30021-8
DOI: doi: [10.1016/j.clim.2015.08.004](https://doi.org/10.1016/j.clim.2015.08.004)
Reference: YCLIM 7528

To appear in: *Clinical Immunology*

Received date: 15 May 2015
Revised date: 13 August 2015
Accepted date: 16 August 2015



Please cite this article as: So-Hyang Chung, Sun Young Chang, Hyun Jung Lee, Seong Hyun Choi, The C-C Chemokine receptor 6 (CCR6) is crucial for Th2-driven allergic conjunctivitis, *Clinical Immunology* (2015), doi: [10.1016/j.clim.2015.08.004](https://doi.org/10.1016/j.clim.2015.08.004)

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

The C-C Chemokine receptor 6 (CCR6) is crucial for Th2-driven allergic conjunctivitis

So-Hyang Chung^{*†}, Sun Young Chang[‡], Hyun Jung Lee^{*}, Seong Hyun Choi[†]

^{*}Department of Ophthalmology and Visual Science, Seoul St. Mary's Hospital, College of Medicine, The Catholic University of Korea, Seoul, Korea,

[†]Catholic Institute of Visual Science, College of Medicine, The Catholic University of Korea, Seoul, Korea.

[‡]Laboratory of Microbiology, College of Pharmacy, Ajou University, Suwon, Kyeonggi-do, Korea

Running title: CCR6 regulates allergic conjunctivitis

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 (2014R1A1A3052852).

Corresponding author: So-Hyang Chung, MD, PhD

Department of Ophthalmology and Visual Science, Seoul St. Mary's hospital, College of Medicine, The Catholic University of Korea, 505 Banpo-Dong, Seocho-Gu, Seoul, 137-040, Korea. Tel: 82-2-2258-1188, Fax: 82-2-590-2044,

E-mail: chungsh@catholic.ac.kr

Abstract

Allergic conjunctivitis from an allergen-driven Th2 response is characterized by conjunctival eosinophilic infiltration. Although CCL20-CCR6 axis has been reported to play a proinflammatory role in several murine models of autoimmune diseases including allergic diseases, their underlying mechanism need to be investigated. We here examined whether CCL20-CCR6 axis could play a role in the development of allergic conjunctival inflammation using murine experimental allergic conjunctivitis (EAC) model induced by ovalbumin (OVA) allergen. Mice were challenged with consecutive 10 days of OVA via conjunctival sac after systemic challenge with OVA and cholera toxin in alum. Several indicators for allergy were comparatively evaluated in wild-type and CCR6 KO EAC mice. Wild-type mice challenged with OVA via conjunctival sac following systemic challenge with OVA in alum had severe allergic conjunctivitis. The absence of CCR6 suppressed IgE secretion and allergic conjunctival inflammation. Reduced allergic inflammation was ascribable to reduced cytokine responses from Th-2 type in draining lymph node although Th17, regulatory T cells and dendritic cell subsets are not affected by the absence of CCR6. In addition, neutralization of CCR6 ligand, CCL20 could repress allergic conjunctival inflammation. Our findings suggested that CCR6 might be crucial for optimal development of Th2 immune responses and further allergic conjunctival inflammation in EAC model.

Key words: Allergic conjunctivitis, CCR6, Eosinophil, Th2-type immune response

1. Introduction

Allergic conjunctivitis (AC) is one of the most common ocular surface diseases and the incidence of allergic conjunctivitis has increased steadily in the past 30 years. The disease ranges in severity from mild forms, such as seasonal and perennial AC to severe cases, such as vernal and atopic keratoconjunctivitis which may be complicated by corneal damage and may have the potential to cause permanent visual loss.[1]

The immunopathogenic mechanisms in these allergic disorders involve a combination of immunoglobulin E (IgE)-mediated and Th2 cell-mediated responses.[2, 3] The IgE-mediated conjunctival allergic reaction can be reproduced easily by specific conjunctival provocation. It induces an early reaction and degranulation of ocular mast cells leading to release of inflammatory mediators followed by infiltration of inflammatory cells.[4, 5] Eosinophils are the hallmark of allergic disease, particularly in severe chronic ocular allergy where they are easily found in quantity in tears and tissues.[6, 7]

CCL20 (MIP-3 α) is a chemokine that regulates the homeostatic and inflammatory trafficking of leukocytes to mucosal tissue including conjunctiva[8] and chemokine receptor 6 (CCR6) is unique that binds only one chemokine CCL20.[9] CCR6 was found to be expressed on memory T and B, dendritic cells, IL-22 producing NK cells, IL-17 producing $\gamma\delta$ T cell, and Treg cells.[9] The CCL20-CCR6 axis has been reported to play a proinflammatory role in several murine models of allergic diseases, such as allergic diarrhea,[10] and allergic dermatitis.[11] CCR6 KO mice have impaired mucosal but not systemic humoral responses to immunization and rotavirus infection.[12] The CCL20 mRNA expression in the conjunctiva increased in EAC[13] and severe chronic allergic conjunctivitis patients.[14]

In the current study, we hypothesized that this ubiquitous mucosal chemokine CCL20 would play a role in the development of allergic conjunctival inflammation and tested this hypothesis via comparative analysis between wild-type and CCR6 KO mice.

2. Materials and Methods

2.1. Animals

Throughout the study, we followed the protocol of the “ARVO Statement for the Use of Animals in Ophthalmic and Vision Research”. C57BL/6 female mice (8- to 12-wk-old females) were purchased from Charles River Laboratories (Orient Co., Sungnam, Korea). CCR6 gene deficient (CCR6 KO) mice on a C57BL/6 background were purchased from Jackson Laboratories (Orient Co., Sungnam, Korea). All mice were maintained under pathogen-free conditions at the animal facilities of the Catholic University of Korea (Seoul, Korea), where they received sterilized food and water *ad libitum*. All procedures were approved by the Institutional Animal Care and Use Committee.

2.2. Experimental allergic conjunctivitis (EAC)

To generate experimental allergic conjunctivitis (EAC), mice were sensitized *i.p.* with 10 μ g of OVA (Grade V; Sigma-Aldrich), 300 ng of cholera toxin (Sigma-Aldrich) and 200 μ l of 1.5% aluminum hydroxide (ALUM; Pierce), on days 0 and 7, and then challenged daily from day 9 to 18 for 10 days in the conjunctival sac with 250 μ g of OVA according to models from literatures[15, 16] with modifications (Fig. 1A). Control mice were given same doses of OVA, ALUM, and cholera toxin in sensitization stages and PBS in place of OVA in challenge stages. Twenty-four hours after the final challenge with OVA, mice were analyzed to evaluate EAC.

2.3. RNA isolation and Real-Time PCR

Total RNA was isolated using the TRIzol reagent (Gibco-Invitrogen, Grand Island, N.Y., USA) from conjunctiva and CLN. The first strand of complementary DNA (cDNA) was synthesized with random hexamers using SuperScript III RTM reverse transcriptase (Invitrogen). A SYBR Green I real-time PCR method was used and the average threshold

cycle (CT) values for GAPDH were used for internal calibration, to correct for differences in the integrity and amount of total RNA among reactions. We used the $2^{-\Delta\Delta C_t}$ method for relative quantification.[17, 18]

2.4. Histologic analysis of conjunctiva

The eyes including eyelids and the conjunctivas were exenterated. They were harvested and fixed in 10% buffered formalin, cut into horizontal 4 μ m-thick sections and stained with toluidine blue stain for mast cells, and acid giemsa stain for detection of eosinophils. In each section, infiltrating cells in the lamina propria mucosae of the tarsal and bulbar conjunctivas were counted by two masked observers.[17] The sections counted were those of the central portion of the eye, which included the pupil and optic nerve head.

Four- μ m sections were dewaxed by immersion in xylene (twice for 5 min each time) and hydrated by serial immersion in 100%, 90%, 80%, and 70% ethanol and PBS. Antigen retrieval was performed by microwaving sections for 20 min in Target Retrieval Solution (DAKO, Carpinteria, CA). Sections were washed with PBS (twice for 10 min each time), and blocking buffer (10% BSA in PBS) was added for 1 h. Sections were incubated with rat anti-mouse CD4-FITC, CD11c-PE, or F4/80-PE from BD Pharmingen in blocking buffer overnight at 4 °C. The sections were washed with 0.01 M PBS three times and mounted in Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA). The sections were viewed with Zeiss LSM 510 confocal microscopy (Carl Zeiss, Germany).

2.5. ELISA for OVA-specific IgE and IgG antibodies 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 using an OVA-specific mouse IgE ELISA kit (AbD Serotec, Raleigh, NC, USA) performed according to the manufacturer's instructions. For OVA-specific IgG1, IgG2a, or IgG detection, the immunoplates (Nalge Nunc International, Naperville, IL) were coated with OVA (1 mg/ml) overnight at 4 °C. After blocking with 1% BSA in PBS for 1h at room temperature, serial dilutions of serum samples were added and incubated for 4 h at room temperature. The plates were then washed with PBS plus 0.05% Tween (PBS/T) and incubated for 2 h at room temperature with horseradish peroxidase (HRP)-conjugated rat anti-mouse IgG1, IgG2a, or IgG antibodies (Southern Biotech, Birmingham, AL). After a wash with PBS/T, color reaction was developed with 3, 3', 5, 5'- tetramethyl-benzidine (Moss Inc., Pasadena, CA) and stopped with 0.1N HCl.

2.6. Lymphoid cell culture and cytokine analysis

Cervical lymph nodes (CLN) were harvested, and a single cell suspension was 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 1×10^6 cells/ml with 1 mg/ml OVA for 96 h in 96-well plates (Nunc, Rochester, NY, USA). To analyze cytokine secretion from helper T cells and regulatory T cells, CD4⁺ T cells were isolated from the CLN and purified using CD4⁺ MicroBeads (Miltenyi Biotec) as per the manufacturer instructions. Isolated CD4⁺ T cells were stimulated with plate-bound CD3 antibody (10 µg/ml) and soluble CD28 antibody (1 µg/ml) for 3 days. Cytokine levels (IFN-γ, IL-4, IL-5, IL-13, and IL-10) of culture supernatants were assayed using BDTM Cytometric Bead Array (CBA) Mouse Th1/Th2 cytokine kit (BD Biosciences, San Diego, CA, USA) for IFN-γ, IL-4, and IL-5, and commercial ELISA kit (BD Biosciences) for IL-13 and IL-10.

2.7. CCL20 Neutralization

WT mice were sensitized *i.p.* with 10 µg of OVA, 300 ng of cholera toxin and 200 µl of 1.5% aluminum hydroxide on days 0 and 7, and then challenged daily from day 9 to 18 in the conjunctival sac with 250 µg of OVA. Mice were randomly assigned to receive either anti-CCL20 neutralizing antibody (1% concentration, R&D systems, Minneapolis, MN) or Rat IgG isotype control (1% concentration, R&D systems). LPS concentration of anti-CCL20 neutralizing antibody or rat IgG isotype control was below 0.01 EU according to the manufacturer. On days 9, 14, and 18 mice were anesthetized and 10 µl of anti-CCL20 neutralizing antibody or Rat IgG was administered via subconjunctival injection (Fig. 7A). Twenty-four hours after the final challenge with OVA, mice were analyzed to evaluate EAC.

2.8. Statistical analysis

Statistical significance between groups was examined by a nonparametric, two-tailed Mann-Whitney t-test using SPSS 14.0 version. We regarded $p < 0.05$ as significant, $p < 0.01$ highly significant, and $p < 0.001$ extremely highly significant. Data are representative of three independent experiments with six mice in each group.

3. Results

3.1. CCL20 expression at the conjunctiva and cervical lymph node (CLN) is upregulated in experimental allergic conjunctivitis (EAC) regardless of CCR6.

Immune cells migrate via the process of chemotaxis, in which cells expressing chemokine receptors are attracted to chemokine ligands in the environment.[19] We investigated the expressions of the corresponding CCR6 ligand, CCL20, in the conjunctiva and draining CLN of normal and allergic conjunctivitis mice. Using real-time PCR, we found that mRNA levels encoding CCL20 in the conjunctiva and CLN of allergic conjunctivitis mice are upregulated approximately 4.61 and 1.87 fold compared with that of PBS control mice, respectively. (Fig.1). In CCR6 KO mice, mRNA levels encoding CCL20 in the conjunctiva and CLN of allergic conjunctivitis mice are also upregulated approximately 4.32 and 1.95 fold compared with that of PBS control mice, respectively (Fig.1). CCL20 expressions were increased in allergic conjunctivitis regardless of CCR6.

3.2. The absence of CCR6 attenuates development of OVA-induced allergic conjunctival inflammation.

To investigate whether CCR6 might be involved in adaptive immune responses to conjunctival allergens, wild type (WT) and CCR6 KO mice were sensitized intraperitoneally with OVA in combination with alum and cholera toxin at days 0, and 7, and then challenged via conjunctival sac with OVA for 10 days. Interestingly, CCR6 KO EAC mice exhibited reductions in macrophage and dendritic cells in conjunctiva (Supplementary Fig. 1). As expected, histologic finding demonstrated significant infiltration of mast cells and eosinophils in the conjunctiva of OVA-challenged mice after systemic priming in WT and CCR6 KO EAC mice (Fig. 2A and 2C). There was a $35.9 \pm 13.7\%$ reduction in mast cell counts in CCR6 KO EAC mice compared to those in WT EAC mice (104.5 ± 20.5 vs. 163.1 ± 16.8) ($p < 0.05$)

(Fig. 2B). Eosinophil counts in the conjunctiva after allergen challenge was also reduced by $50.6 \pm 12.2\%$ in CCR6 KO EAC mice compared to those in WT EAC mice (160.2 ± 15.2 vs. 320.1 ± 25.8) ($p < 0.01$) (Fig. 2D). The infiltration of $CD4^+$ T cells in the conjunctiva after allergen challenge was also decreased in CCR6 KO EAC mice compared to those in WT EAC mice (Fig. 2E). These results indicate that the OVA-induced mast cell, eosinophil, and $CD4^+$ T cell infiltration in the conjunctiva is dependent on CCR6-CCL20 axis.

3.3 Selective reduction of OVA-specific IgE Ab and OVA-specific IgG1/G2a in serum of CCR6 KO EAC mice.

In order to further clarify the role of CCR6 on severity of EAC, we assessed levels of OVA-specific IgE Abs in sera. A combination of systemic priming and local boosting with OVA resulted in significantly higher levels OVA-specific IgE Ab secretion in WT mice ($p < 0.001$) (Fig. 3A). However, OVA-specific IgE Ab concentrations in serum after allergen challenge was reduced by $75.3 \pm 15.1\%$ in CCR6 KO EAC mice compared to those in WT EAC mice ($p < 0.001$). To address overall immunosuppression in CCR6 KO EAC mice, we also assessed levels of OVA-specific IgG antibodies in sera. OVA-specific IgG antibody levels in sera did not decrease in CCR6 KO EAC mice (Fig. 3B). However, OVA-specific IgG1/IgG2a in sera decreased in CCR6 KO EAC mice compared to WT EAC mice (Fig. 3C).

3.4. Th-2 type cytokine responses are inhibited in CLN of CCR6 KO EAC mice.

To investigate the mechanism for specific reduction of IgE response in absence of CCR6, cytokine profiles from T cells were analyzed. Mononuclear cells isolated from CLN of WT EAC mice elicited significantly higher levels of Th1-type cytokine (i.e., IFN- γ), Th2-type cytokine (i.e., IL-4, IL-5, and IL-13), and Treg type cytokine (i.e., IL-10) after re-stimulation with OVA *in vitro* than those from PBS control group (Fig. 4). Th2 type cytokines (i.e., IL-4,

IL-5, and IL-13) were reduced by $50.6 \pm 7.2\%$, $78.3 \pm 14.1\%$ and $77.4 \pm 11.3\%$ respectively in mononuclear cells isolated from CLN from CCR6 KO compared to those from WT EAC mice (Fig. 4). Th1 type cytokine (i.e., IFN- γ) was reduced by $90.4 \pm 15.2\%$ in CCR6 KO mice. Treg type cytokine (i.e., IL-10) was also reduced by $71.2 \pm 10.2\%$ when compared with those from WT EAC mice. However, IL-6 and IL-1 β expressions in the conjunctiva and CLN did not differ between WT and CCR6 KO EAC mice (Fig. 5).

To clarify cytokine responses of helper T cells, the cytokine levels of CD4⁺ T cells isolated from CLN were analyzed following T cell specific stimulation. CD4⁺ T cells from CLN from CCR6 KO EAC mice produced significantly less Th1, Th2, and Treg type cytokine responses compared with those from WT EAC mice (Fig. 6). Especially, OVA-induced Th2 cytokine responses, IL-4, IL-5 and IL-13 were reduced by $55.6 \pm 9.4\%$, $60.4 \pm 12.1\%$ and $80.4 \pm 11.4\%$ respectively in CCR6 KO EAC mice. Taken together, these data demonstrate that the CCR6 during development of EAC is committed to the generation of preferential Th2 cells. These findings indicate that CCR6 is required for optimal development of Th2 immune responses in EAC model. These results indicate that CCR6 deficiency could inhibit Th-2 mediated immune responses resulting in decrease of IgE production without impairment of overall systemic immune responses in EAC model.

3.5. Lack of CCR6 did not influence dendritic cell subsets, Th17 and Treg in EAC mice

CCR6 expression could be found on Treg and Th17 and modulate their function. Especially, Th17 was well-known to be crucial for inflammatory disease and CCR6 expression is representative marker for Th17 in the gut.[20, 21] However, Th17 cells were not changed in the CCR6 KO EAC mice (Supplementary Fig. 2A and 2B). The population of Foxp3⁺ Treg cells were not changed in the CCR6 KO EAC mice although IL-10 cytokines were reduced in

the CCR6 KO mice (Supplementary Fig. 2C and 2D).

Several mucosal dendritic cell (DC) subsets could utilize CCR6 receptor for the migration between draining lymph node and mucosal periphery.[22] $CD8\alpha^-CD11b^+CD11c^+$ DCs might be migratory subsets from mucosal peripheral tissue to CLN to ferry antigen. $CD8\alpha^+CD11c^+$ DCs were regarded as lymph node (LN) resident DCs. When DC subsets were analyzed in CLN of EAC mice, $CD8\alpha^+CD11c^+$ DCs in WT mice was 17.6% and decreased to 12.7% in EAC mice whereas $CD11b^+CD11c^+$ DCs in WT was 70.8% and slightly increased to 75.7% in EAC mice. However, the DC subsets were not changed in absence of CCR6 (data not shown).

3.6. Anti-CCL20 treatment inhibits the development of EAC

To evaluate whether treatment with anti-CCL20 antibody had any effect on the development of EAC, we treated mice with anti-CCL20 antibody or isotype control via subconjunctival injection. Treatment with anti-CCL20 antibody could significantly reduce OVA-specific IgE by 63.5 ± 10.2 . Th1 type cytokine (i.e., IFN- γ) secretion was reduced by $80.6\pm19.4\%$ and Th2 type cytokines (i.e., IL-4, IL-5, and IL-13) were reduced by $36.2\pm6.2\%$, $50.2\pm11.6\%$ and $38.2\pm13.4\%$ respectively in the EAC mice after treatment with anti-CCL20 antibody (Fig. 7). Conjunctiva of mice treated with anti-CCL20 antibody showed significantly reduced numbers of mast cells and eosinophils by $63.9\pm12.2\%$ and $51.2\pm8.3\%$ respectively (Fig. 8). These results suggested that the allergic inflammation in the conjunctiva is dependent on CCR6-CCL20 axis. Further blockade of CCR6-CCL20 such as neutralization of CCL20 could be an additive strategy to alleviate allergic conjunctivitis.

4. Discussion

Our findings provide strong evidence for a role of CCR6 in the development of allergic conjunctivitis in OVA-induced EAC model. The present work provides data indicating that CCR6 was necessary for Th2-driven allergic conjunctival inflammation. CCL20, CCR6 ligand is expressed at high levels in the conjunctiva of EAC model. CCR6 KO mice were significantly protected from allergic conjunctival inflammation as reflected in the cytokine profile and production of antigen-specific IgE Ab. Blockade of CCR6 signaling resulted in an abrogation of significant decrease of allergic conjunctival inflammation via reduced Th2-type immune responses.

The main pathophysiological changes in conjunctival allergic reactions involve a combination of IgE-mediated and Th2 cell-mediated responses resulting in chronic inflammatory cells infiltration into the conjunctiva.[1-3] The IgE-mediated conjunctival allergic reaction induces an early reaction and degranulation of ocular mast cells leading to release of inflammatory mediators in allergic conjunctivitis.[1, 4] In allergic conjunctivitis patients and mice, upregulation of ICAM-1 expression was reported.[23, 24] Mast cells are potent regulator of ICAM-1 and previous study demonstrated that antibody against ICAM-1 significantly inhibited development of allergic conjunctivitis and infiltration of mast cell and eosinophils in EAC.[25] Chronic pathologic processes of allergic conjunctivitis are thought to be mediated by Th2-type cells, which preferentially produce IgE-enhancing cytokines such as IL-4, IL-5, and IL-13.[2, 3, 26] We hypothesized that recruitment of T cells to the conjunctiva through chemokine-driven pathways would be necessary to the development of allergic conjunctival inflammation. We examined the role of mucosal chemokine CCL20 and its receptor CCR6 in the development of EAC using CCR6 KO mice and neutralization of CCL20.

Our study demonstrated for the first time that the absence of CCR6 attenuates Th2

responses in EAC model. When CCR6 KO mice sensitized with OVA on to alum and cholera toxin, OVA-induced Th2-mediated allergic responses, mast cells and eosinophilic infiltration into conjunctiva, Th2 type cytokine secretion, and serum levels of OVA-specific IgE antibodies were impaired in these CCR6 KO EAC model. Previous report in Th2 dependent allergic diarrhea model indicated a mast-cell- and IgE-independent role of CCR6-bearing T cells.[10] CCR6 KO mice were protected from OVA-induced diarrhea but surprisingly were not impaired in mastocytosis or allergen-specific IgE, and showed normal Th2 cytokine expression in draining lymph node.[10] However, in our EAC model, allergen-induced Th1 and Th2 cytokine expression in the draining CLN is dependent on CCR6. A reduction of Th2 responses in CCR6 KO EAC mice was supported by the substantially lower circulating level of OVA-specific IgE antibodies. When mononuclear cells and CD4⁺ T cells from CLN were cultured in the presence of OVA *in vitro*, cells from CCR6 KO EAC mice produced significantly lower amounts of IL-4, IL-5, and IL-13 compared with wild-type EAC mice. Of interest, Th1 type cytokine secretion from CD4⁺ T cells in CLN was not increased in reaction to decreased Th2 type immune responses. These results suggest that allergen-induced Th2 responses were compromised in the absence of signaling via CCR6 in EAC model. Taken altogether, OVA-induced allergic conjunctivitis and immune responses to OVA are dependent on CCR6 signaling.

It has been shown that Treg cells can affect the severity of colitis[27] and CCR6 expression on Treg cells was required for full function of Treg cell-mediated suppression in a model of colitis.[28] Therefore, we evaluated the Treg frequency and IL-10 expression in CLN of WT and CCR6 KO EAC mice. Mononuclear cells and CD4⁺ T cells from CLN of WT and CCR6 KO EAC mice produced more IL-10 compared with those from PBS control mice. Cells from CLN of CCR6 KO EAC mice produced significantly less IL-10 compared with those from WT EAC mice. However, Treg cell differentiation in CLN is not altered after allergen

challenge in both WT and CCR6 KO mice. These results indicate that lack of CCR6 did not change Treg population in EAC model.

Recently, circulating follicular helper T (Tfh) cells ($CD3^+CD4^+CXCR5^+$) are classified into type 1 Tfh (Tfh1), type 2 (Tfh2), and IL-17 producing Tfh (Tfh17) cells based on expression profiles of the chemokine receptors CCR6 and CXCR3, which are shared by Th1 cells and Th17 cells, respectively.[29, 30] In recent study regarding on allergic rhinitis (AR), polarization of Tfh2 ($CD3^+CD4^+CXCR5^+CXCR3^-CCR6^-$) cells and preserved Tfh17 ($CD3^+CD4^+CXCR5^+CXCR3^-CCR6^+$) cells were observed.[31] In our CCR6 KO EAC model, imbalance of circulating Tfh cells might contribute to the direct pathogenesis of allergic inflammation mediated by IgE, which needs to be evaluated in the future.

In a model of allergic airway inflammation induced by cockroach allergen in CCR6 KO and WT mice, the authors demonstrated that high levels of CCL20 are released within hours after allergen challenge and directly regulates the recruitment of conventional DCs into lung and subsequent T cell activation in a Th2 dependent manner.[32] CCR6 expressing DCs could induce more IL-5 producing $CD4^+$ T cells in the pulmonary allergic inflammation model. CLN comprises both LN-resident and migratory DCs from mucosal tissue.[32] When CLN DC subset was analyzed, CCR6 had no influence of the ratio of $CD11b^+$ DCs, migratory subsets from mucosal peripheral tissue to CLN to ferry antigen and $CD8\alpha^+$ DCs, LN-resident subsets.

The limitation of this study is that CCR6 KO may change tissue organization. Several studies demonstrated that loss of CCR6/CCL20 signals can have a profound impact on innate immune cells in mucosal tissues with significant modulation in other lymphoid populations.[33] CCR6 identifies lymphoid tissue inducer cells within cryptopatches[34] and CCR6 KO mice inhibits Treg and M-cell formation inside Peyer's patch.[35] CCR6 KO mice have impaired antibody responses to both oral immunizations and mucosal infections.[19] In

our study, CCR6 KO EAC mice exhibited reductions in both macrophage and dendritic cells in conjunctiva compared to WT EAC mice. We speculate that reduction of innate immune cells in the conjunctiva of CCR6 KO mice may contribute to impaired adaptive immunity in response to OVA challenge. Our previous studies have demonstrated that activation of TLR2 signal by *Staphylococcus Aureus* induced Th2-type immune responses and accelerated EAC.[17]

To evaluate the functional relevance of the CCR6/CCL20 axis in the development of allergic conjunctivitis, we disrupted CCR6 and CCL20 interactions using a CCL20 neutralizing antibody. After treatment with anti-CCL20 antibodies via subconjunctival injection in EAC model, the severity of allergic conjunctival inflammation, OVA-specific IgE secretion, and degree of Th1 and Th2-type immune response was partially decreased. Interestingly, reduction of mast cells was higher in WT EAC mice with anti-CCL20 antibodies compared to that in CCR6 KO EAC mice. In vitro study showed that mast cell-conjunctiva epithelial cell interaction induces CCL2 expression in mast cells and increased CCL2 expressions in conjunctiva of EAC and human chronic allergic conjunctivitis tissue.[13, 14] Therefore, neutralization of CCL2 partially prevents allergic conjunctival inflammation in EAC model.

In summary, we show that CCR6 plays a role in the development of allergic conjunctival inflammation through Th2 cytokine expression in CLN. Our results demonstrate that CCR6 KO mice attenuate mast cell and eosinophil infiltration into the conjunctiva and allergen-specific IgE antibody secretion in serum by decreasing Th2 immune response in CLN. Therefore, it seems reasonable to consider that CCR6 signaling is required for optimal development of polarized Th2 cell-mediated conjunctival inflammation in humans.

Abbreviations

AC Allergic conjunctivitis

AR Allergic rhinitis

ALUM Aluminum hydroxide

CLN Cervical lymph node

DC Dendritic cell

EAC Experimental allergic conjunctivitis

LN Lymph node

OVA Ovalbumin

WT Wild type

References

- [1] S.D. Trocme, K.K. Sra, Spectrum of ocular allergy, *Current opinion in allergy and clinical immunology*, 2 (2002) 423-427.
- [2] D.P. Metz, M. Hingorani, V.L. Calder, R.J. Buckley, S.L. Lightman, T-cell cytokines in chronic allergic eye disease, *The Journal of allergy and clinical immunology*, 100 (1997) 817-824.
- [3] V.L. Calder, G. Jolly, M. Hingorani, P. Adamson, A. Leonardi, A.G. Secchi, R.J. Buckley, S. Lightman, Cytokine production and mRNA expression by conjunctival T-cell lines in chronic allergic eye disease, *Clin Exp Allergy*, 29 (1999) 1214-1222.
- [4] S. Bonini, S. Bonini, A. Vecchione, D.M. Naim, M.R. Allansmith, F. Balsano, Inflammatory changes in conjunctival scrapings after allergen provocation in humans, *The Journal of allergy and clinical immunology*, 82 (1988) 462-469.
- [5] M. Hingorani, V. Calder, G. Jolly, R.J. Buckley, S.L. Lightman, Eosinophil surface antigen expression and cytokine production vary in different ocular allergic diseases, *The Journal of allergy and clinical immunology*, 102 (1998) 821-830.
- [6] A. Leonardi, Tear and mucus eotaxin-1 and eotaxin-2 in allergic keratoconjunctivitis, *Ophthalmology*, 110 (2003) 487-492.
- [7] T.H. Wakamatsu, Y. Satake, A. Igarashi, M. Dogru, O.M.A. Ibrahim, N. Okada, K. Fukagawa, J. Shimazaki, H. Fujishima, IgE and eosinophil cationic protein (ECP) as markers of severity in the diagnosis of atopic keratoconjunctivitis, *British Journal of Ophthalmology*, 96 (2012) 581-586.
- [8] T.H. Dohlman, S.K. Chauhan, S. Kodati, J. Hua, Y. Chen, M. Omoto, Z. Sadrai, R. Dana, The CCR6/CCL20 axis mediates Th17 cell migration to the ocular surface in dry eye disease, *Invest Ophthalmol Vis Sci*, 54 (2013) 4081-4091.
- [9] E. Schutyser, S. Struyf, J. Van Damme, The CC chemokine CCL20 and its receptor CCR6,

Cytokine & growth factor reviews, 14 (2003) 409-426.

[10] A.B. Blazquez, A.K. Knight, H. Getachew, J.S. Bromberg, S.A. Lira, L. Mayer, M.C. Berin, A functional role for CCR6 on proallergic T cells in the gastrointestinal tract, *Gastroenterology*, 138 (2010) 275-284 e271-274.

[11] T.J. Paradis, S.H. Cole, R.T. Nelson, R.P. Gladue, Essential role of CCR6 in directing activated T cells to the skin during contact hypersensitivity, *The Journal of investigative dermatology*, 128 (2008) 628-633.

[12] D.N. Cook, D.M. Prosser, R. Forster, J. Zhang, N.A. Kuklin, S.J. Abbondanzo, X.D. Niu, S.C. Chen, D.J. Manfra, M.T. Wiekowski, L.M. Sullivan, S.R. Smith, H.B. Greenberg, S.K. Narula, M. Lipp, S.A. Lira, CCR6 mediates dendritic cell localization, lymphocyte homeostasis, and immune responses in mucosal tissue, *Immunity*, 12 (2000) 495-503.

[13] S. Iwamoto, Y. Asada, N. Ebihara, K. Hori, Y. Okayama, J. Kashiwakura, Y. Watanabe, S. Kawasaki, N. Yokoi, T. Inatomi, K. Shinomiya, A. Murakami, A. Matsuda, Interaction between conjunctival epithelial cells and mast cells induces CCL2 expression and piecemeal degranulation in mast cells, *Invest Ophthalmol Vis Sci*, 54 (2013) 2465-2473.

[14] N. Inada, A. Ishimori, J. Shoji, CCL20/MIP-3 alpha mRNA expression in the conjunctival epithelium of normal individuals and patients with vernal keratoconjunctivitis, *Graefe's archive for clinical and experimental ophthalmology = Albrecht von Graefes Archiv fur klinische und experimentelle Ophthalmologie*, 252 (2014) 1977-1984.

[15] H.S. Lee, S. Schlereth, P. Khandelwal, D.R. Saban, Ocular allergy modulation to hi-dose antigen sensitization is a Treg-dependent process, *PloS one*, 8 (2013) e75769.

[16] H.S. Lee, D. Hos, T. Blanco, F. Bock, N.J. Reyes, R. Mathew, C. Cursiefen, R. Dana, D.R. Saban, Involvement of corneal lymphangiogenesis in a mouse model of allergic eye disease, *Invest Ophthalmol Vis Sci*, 56 (2015) 3140-3148.

[17] S.-H. Chung, K.-H. Nam, M.-N. Kweon, *Staphylococcus aureus* accelerates an

experimental allergic conjunctivitis by Toll-like receptor 2-dependent manner, *Clinical immunology*, 131 (2009) 170-177.

[18] S.H. Chung, S.H. Choi, K.J. Cho, C.K. Joo, Toll-like receptor 4 signalling attenuates experimental allergic conjunctivitis, *Clinical & Experimental Immunology*, 164 (2011) 275-281.

[19] S.K. Bromley, T.R. Mempel, A.D. Luster, Orchestrating the orchestrators: chemokines in control of T cell traffic, *Nat Immunol*, 9 (2008) 970-980.

[20] S. Brand, Crohn's disease: Th1, Th17 or both? The change of a paradigm: new immunological and genetic insights implicate Th17 cells in the pathogenesis of Crohn's disease, *Gut*, 58 (2009) 1152-1167.

[21] C. Wang, S.G. Kang, J. Lee, Z. Sun, C.H. Kim, The roles of CCR6 in migration of Th17 cells and regulation of effector T-cell balance in the gut, *Mucosal immunology*, 2 (2009) 173-183.

[22] J.H. Song, J.I. Kim, H.J. Kwon, D.H. Shim, N. Parajuli, N. Cuburu, C. Czerkinsky, M.N. Kweon, CCR7-CCL19/CCL21-regulated dendritic cells are responsible for effectiveness of sublingual vaccination, *Journal of immunology*, 182 (2009) 6851-6860.

[23] M.T. Magone, C.C. Chan, L.V. Rizzo, A.T. Kozhich, S.M. Whitcup, A novel murine model of allergic conjunctivitis, *Clinical immunology and immunopathology*, 87 (1998) 75-84.

[24] G. Ciprandi, S. Buscaglia, G. Pesce, B. Villaggio, M. Bagnasco, G.W. Canonica, Allergic subjects express intercellular adhesion molecule--1 (ICAM-1 or CD54) on epithelial cells of conjunctiva after allergen challenge, *The Journal of allergy and clinical immunology*, 91 (1993) 783-792.

[25] S.M. Whitcup, C.C. Chan, A.T. Kozhich, M.T. Magone, Blocking ICAM-1 (CD54) and LFA-1 (CD11a) inhibits experimental allergic conjunctivitis, *Clinical immunology*, 93 (1999)

107-113.

- [26] A. Ozaki, Y. Seki, A. Fukushima, M. Kubo, The control of allergic conjunctivitis by suppressor of cytokine signaling (SOCS)3 and SOCS5 in a murine model, *Journal of immunology*, 175 (2005) 5489-5497.
- [27] D. Haribhai, W. Lin, B. Edwards, J. Ziegelbauer, N.H. Salzman, M.R. Carlson, S.H. Li, P.M. Simpson, T.A. Chatila, C.B. Williams, A central role for induced regulatory T cells in tolerance induction in experimental colitis, *Journal of immunology*, 182 (2009) 3461-3468.
- [28] K. Kitamura, J.M. Farber, B.L. Kelsall, CCR6 marks regulatory T cells as a colon-tropic, IL-10-producing phenotype, *Journal of immunology*, 185 (2010) 3295-3304.
- [29] R. Morita, N. Schmitt, S.E. Bentebibel, R. Ranganathan, L. Bourdery, G. Zurawski, E. Foucat, M. Dullaers, S. Oh, N. Sabzghabaei, E.M. Lavecchio, M. Punaro, V. Pascual, J. Banchereau, H. Ueno, Human blood CXCR5(+)CD4(+) T cells are counterparts of T follicular cells and contain specific subsets that differentially support antibody secretion, *Immunity*, 34 (2011) 108-121.
- [30] H. Ueno, J. Banchereau, C.G. Vinuesa, Pathophysiology of T follicular helper cells in humans and mice, *Nat Immunol*, 16 (2015) 142-152.
- [31] R. Kamekura, K. Shigehara, S. Miyajima, S. Jitsukawa, K. Kawata, K. Yamashita, T. Nagaya, A. Kumagai, A. Sato, H. Matsumiya, N. Ogasawara, N. Seki, K. Takano, Y. Kokai, H. Takahashi, T. Himi, S. Ichimiya, Alteration of circulating type 2 follicular helper T cells and regulatory B cells underlies the comorbid association of allergic rhinitis with bronchial asthma, *Clinical immunology*, 158 (2015) 204-211.
- [32] S.K. Lundy, S.A. Lira, J.J. Smit, D.N. Cook, A.A. Berlin, N.W. Lukacs, Attenuation of allergen-induced responses in CCR6^{-/-} mice is dependent upon altered pulmonary T lymphocyte activation, *Journal of immunology*, 174 (2005) 2054-2060.
- [33] T. Ito, W.F.t. Carson, K.A. Cavassani, J.M. Connett, S.L. Kunkel, CCR6 as a mediator of

immunity in the lung and gut, *Experimental cell research*, 317 (2011) 613-619.

[34] A. Lugering, M. Ross, M. Sieker, J. Heidemann, I.R. Williams, W. Domschke, T. Kucharzik, CCR6 identifies lymphoid tissue inducer cells within cryptopatches, *Clinical and experimental immunology*, 160 (2010) 440-449.

[35] A. Lugering, M. Floer, S. Westphal, C. Maaser, T.W. Spahn, M.A. Schmidt, W. Domschke, I.R. Williams, T. Kucharzik, Absence of CCR6 inhibits CD4⁺ regulatory T-cell development and M-cell formation inside Peyer's patches, *Am J Pathol*, 166 (2005) 1647-1654.

Figure 1. Time course for development of experimental allergic conjunctivitis (A). CCL20 expression at the conjunctiva (B) and cervical lymph node (CLN; C) is upregulated in wild type and CCR6 KO experimental allergic conjunctivitis mice. WT and CCR6 KO mice were sensitized to OVA and challenged with OVA or PBS (control). ALUM; aluminum hydroxide, CT; cholera toxin, *, $p < 0.05$, **, $p < 0.01$ ($n = 3$ in each group, three independent experiments)

Figure 2. The absence of CCR6 attenuates development of OVA-induced allergic conjunctival inflammation. Infiltration of the mast cells, eosinophils, and $CD4^+$ T cells into the conjunctiva decreased in CCR6 KO experimental allergic conjunctivitis (EAC) mice than WT EAC mice. **A, C, and E.** Representative image of infiltration of mast cells (A), eosinophils (C), and $CD4^+$ T cells (E). **B and D,** Number of mast cells and eosinophils. Bars = 20 μm . *, $p < 0.05$, **, $p < 0.01$ ($n = 6$ in each group, three independent experiments)

Figure 3. Selective reduction of IgE Ab secretion in serum of CCR6 KO experimental allergic conjunctivitis (EAC) mice. **A**, The absence of CCR6 led to a profound decrease of Ag-specific IgE Ab secretion in serum. **B**, OVA-specific IgG antibody levels in sera did not decrease in CCR6 KO EAC mice. **C**, OVA-specific IgG1/G2a in sera decreased in CCR6 KO EAC mice compared to WT EAC mice. *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$ (n=6 in each group, three independent experiments)

Figure 4. OVA-induced Th2 cytokine responses in the cervical lymph nodes (CLN) are impaired in CCR6 KO experimental allergic conjunctivitis (EAC) mice. CLN cells were restimulated with OVA, and IFN- γ , IL-4, IL-5, IL-13, and IL-10 were measured in culture supernatants. CLN cells from CCR6 KO EAC mice produced significantly less IFN- γ (A), IL-4 (B), IL-5 (C), IL-13 (D), and IL-10 (E) compared with those from WT EAC mice. *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$ (n=6 in each group, three independent experiments)

Figure 5. IL-6 and IL-1 β expressions in the cervical lymph nodes (CLN) and conjunctiva does not differ between WT and CCR6 KO experimental allergic conjunctivitis (EAC) mice.

Figure 6. OVA-induced Th2 cytokine responses from CD4⁺ T cells in the cervical lymph nodes (CLN) are impaired in CCR6 KO experimental allergic conjunctivitis (EAC) mice. CD4⁺ T cells from CLN from CCR6 KO EAC mice produced significantly less IFN- γ (A), IL-4 (B), IL-5 (C), IL-13 (D), and IL-10 (E) compared with those from WT EAC mice. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ (n=6 in each group, three independent experiments)

Figure 7. The treatment of anti-CCL20 antibody via subconjunctival injection inhibited experimental allergic conjunctivitis. (A) OVA-specific IgE secretion in serum and (B-E) OVA-induced cytokine responses in the cervical lymph nodes (CLN). *, $p < 0.05$, **, $p < 0.01$, (n=6 in each group, three independent experiments)

Figure 8. The treatment of anti-CCL20 antibody via subconjunctival injection attenuates development of OVA-induced allergic conjunctival inflammation. Infiltration of the mast cells and eosinophils into the conjunctiva decreased after treatment with anti-CCL20 antibody. **A** and **C**, Representative image of infiltration of mast cells (A) and eosinophils (C). **B** and **D**, Number of mast cells and eosinophils. Bars = 20 μm . **, $p < 0.01$ (n=6 in each group, three independent experiments)

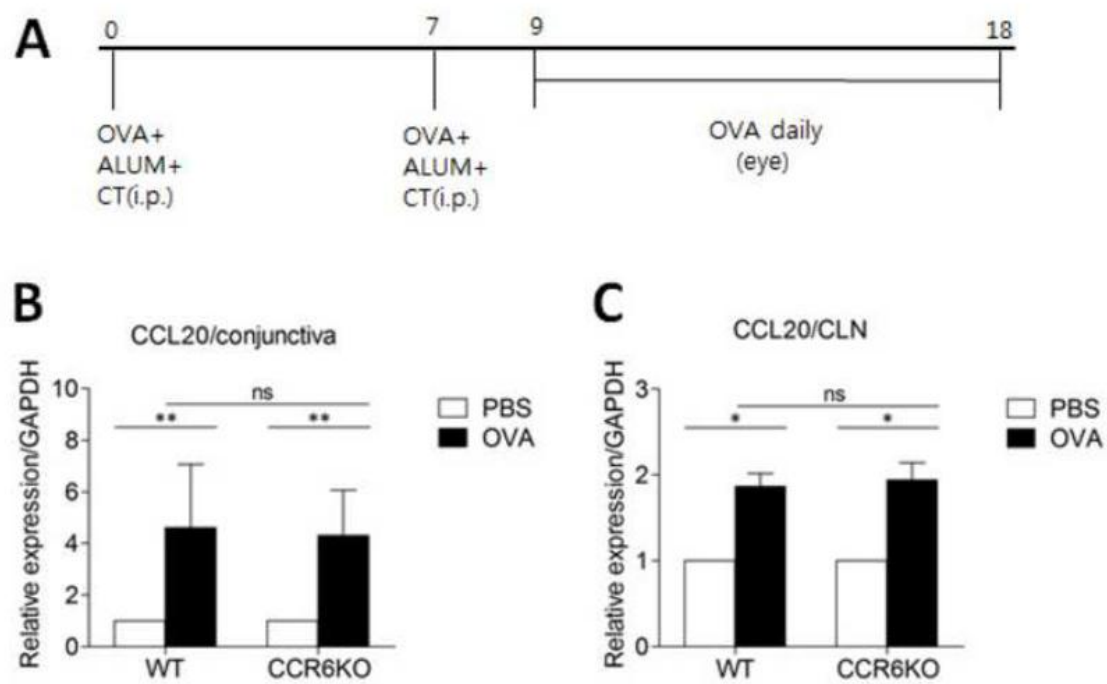


Fig. 1

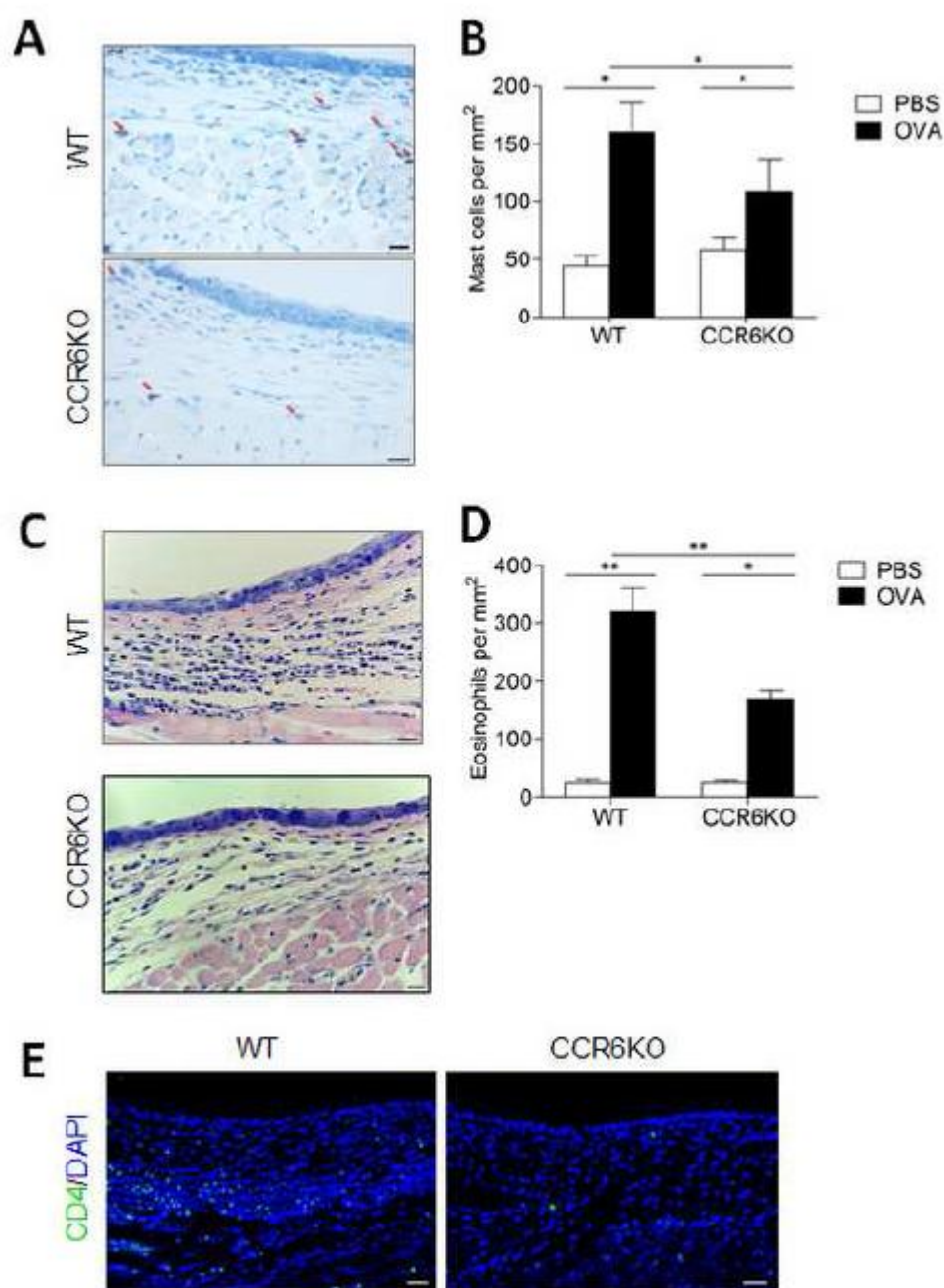


Fig. 2

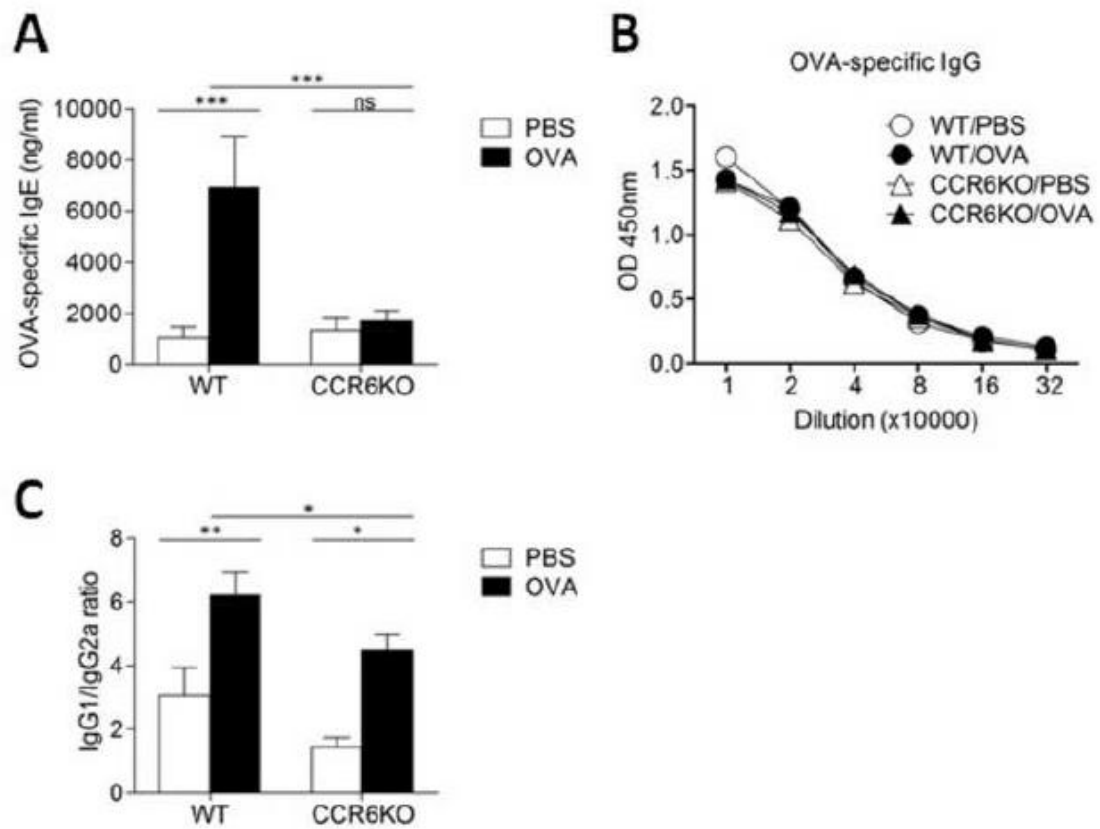


Fig. 3

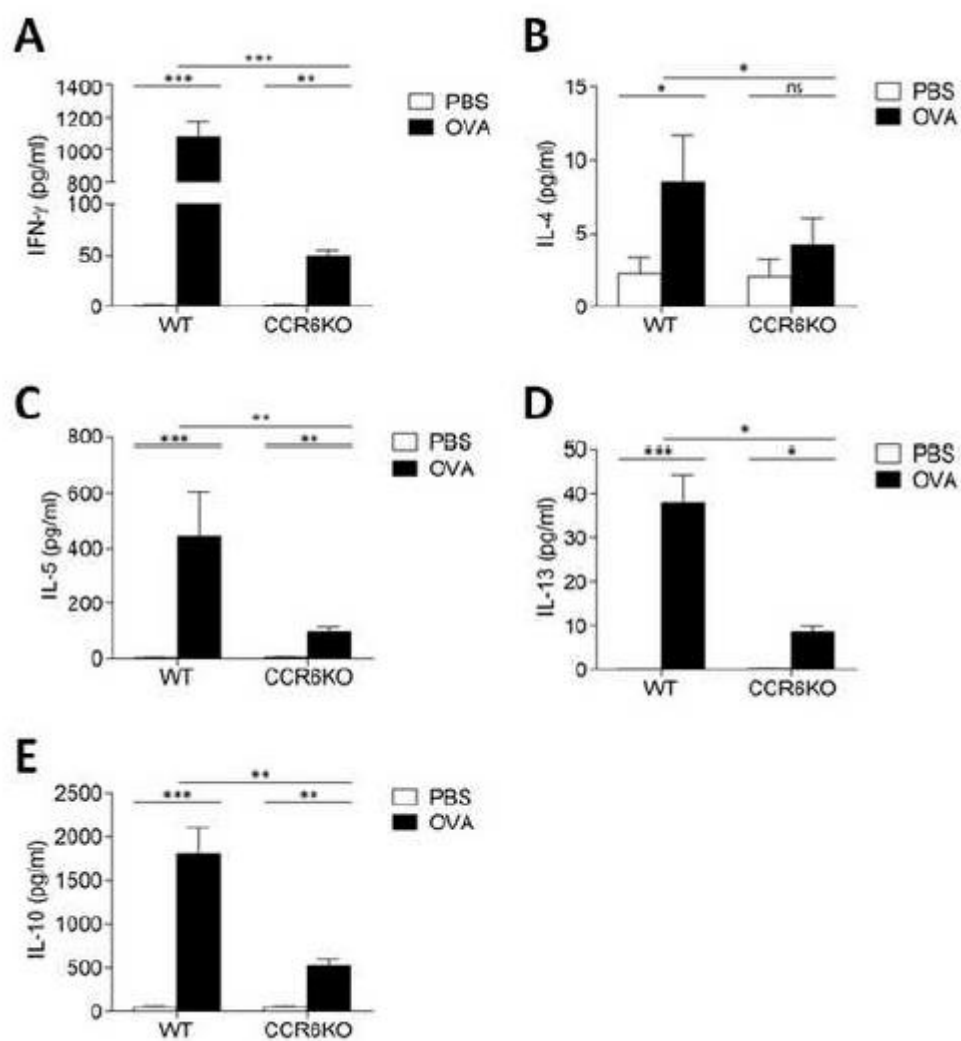


Fig. 4

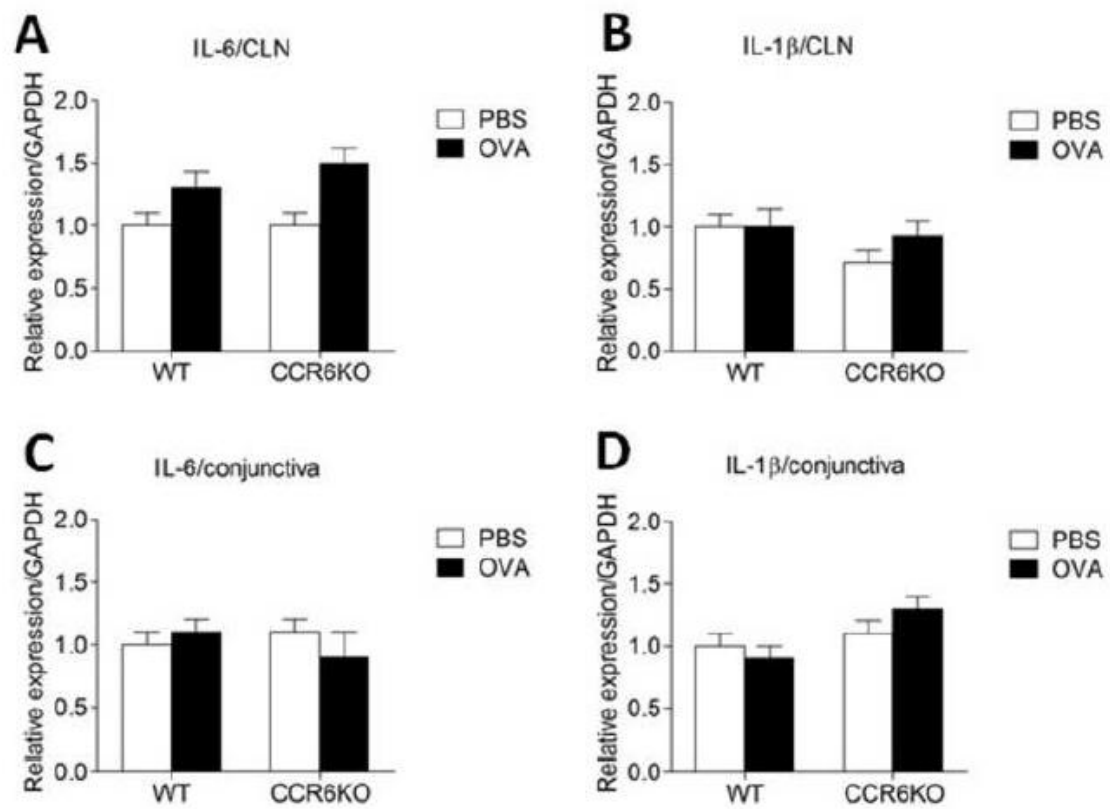


Fig. 5

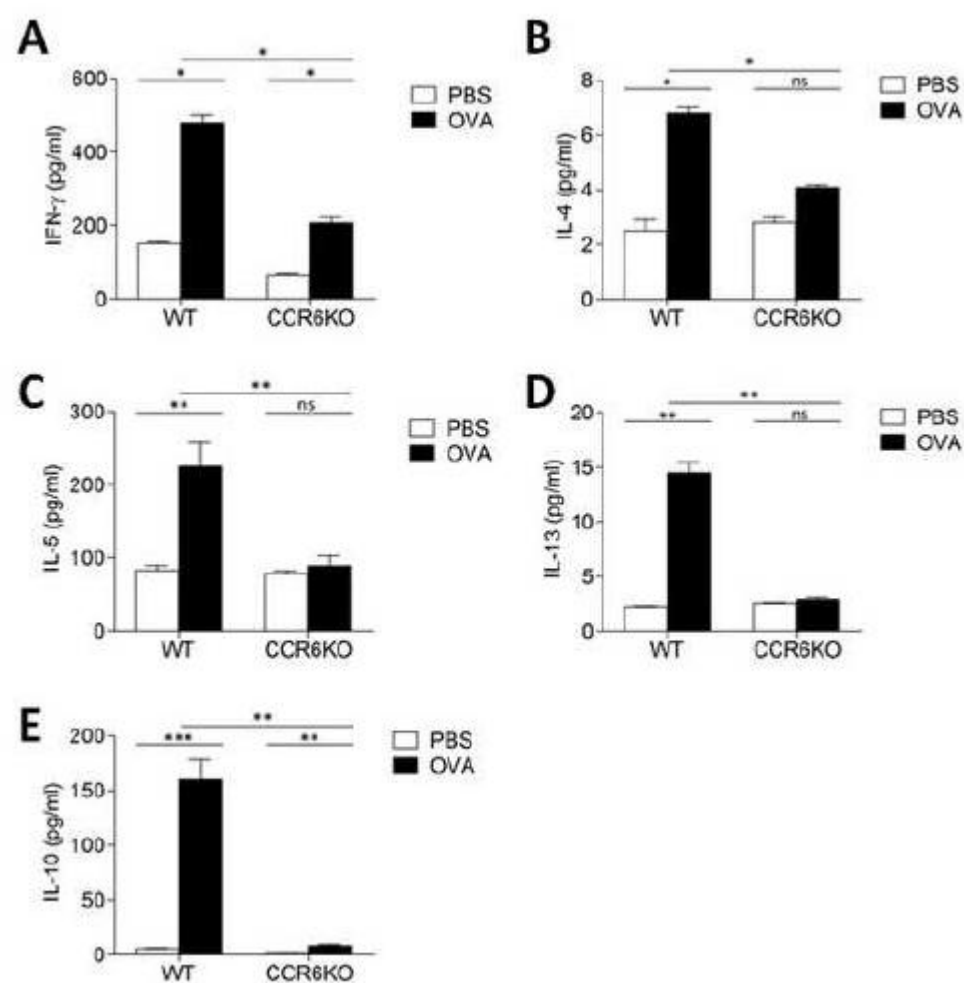


Fig. 6

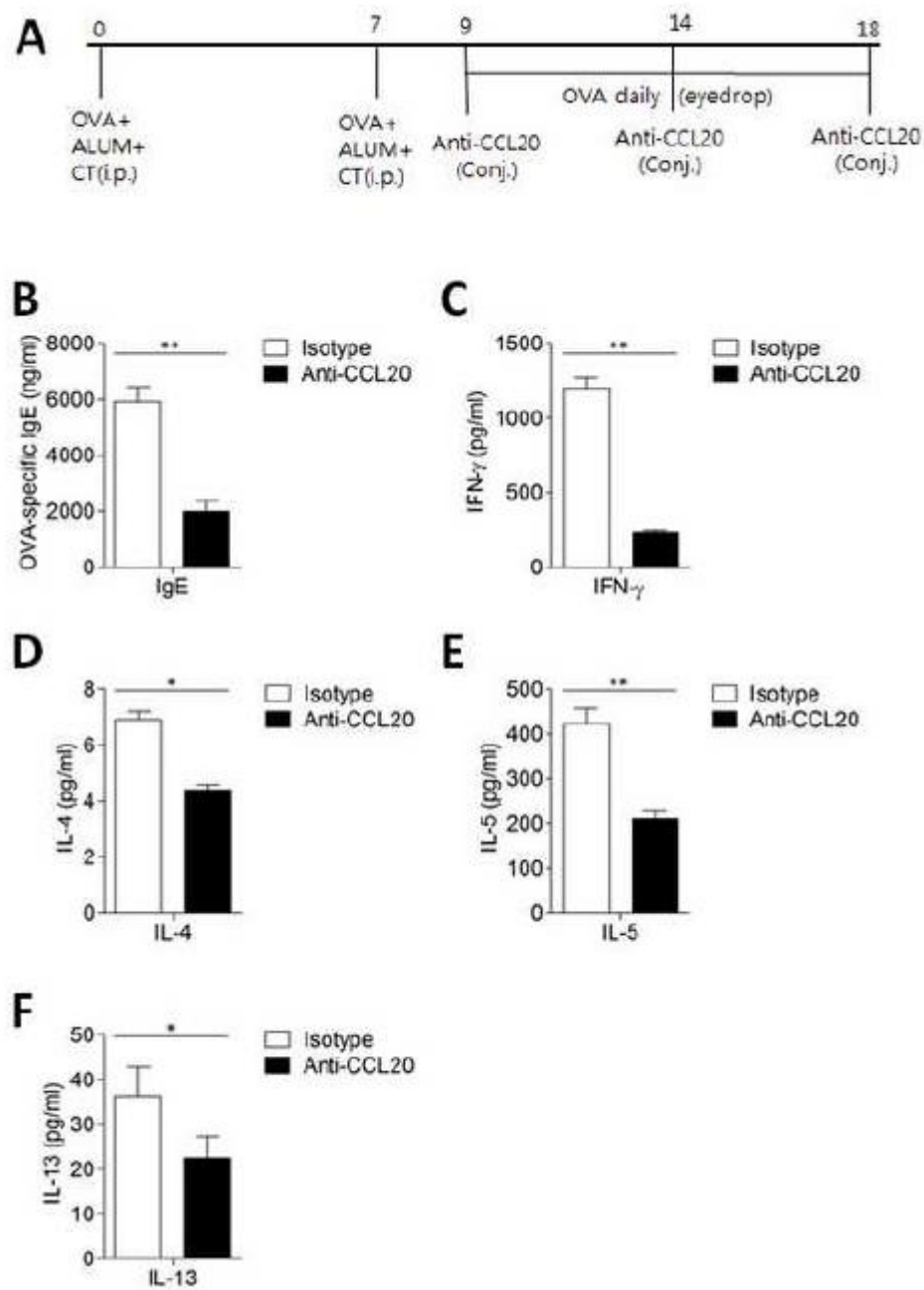


Fig. 7

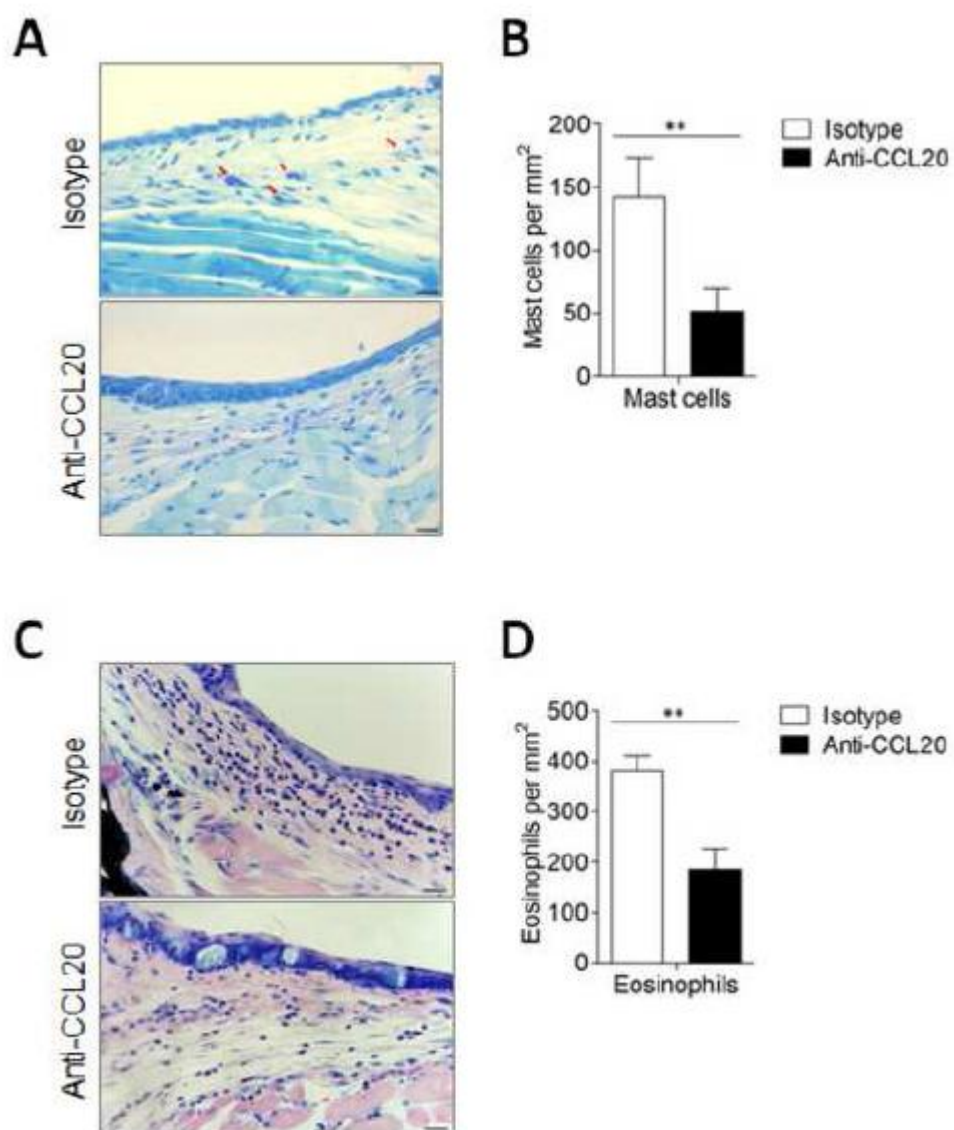


Fig. 8

Highlights

Allergic conjunctivitis from an allergen-driven Th2 response is characterized by conjunctival eosinophilic infiltration.

C-C Chemokine receptor 6 mice were significantly protected from allergic conjunctival inflammation as reflected in the cytokine profile and production of antigen-specific IgE Ab.

Neutralization of CCR6 ligand, CCL20 inhibits the development of experimental allergic conjunctivitis.