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The C-C chemokine receptor 6 (CCR6) is crucial for Th2-driven allergic conjunctivitis☆,☆☆

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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 needs 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.

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-3α) 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 γδ 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.

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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-week-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 μg of OVA (Grade V; Sigma-Aldrich), 300 ng of cholera toxin (Sigma-Aldrich) and 200 μl 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 μg 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 IIITM 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 $2^{-\Delta\Delta Ct}$ 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 μm-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-μm 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 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 PBS plus 0.05% Tween (PBS/T) and incubated for 2 h at room temperature with

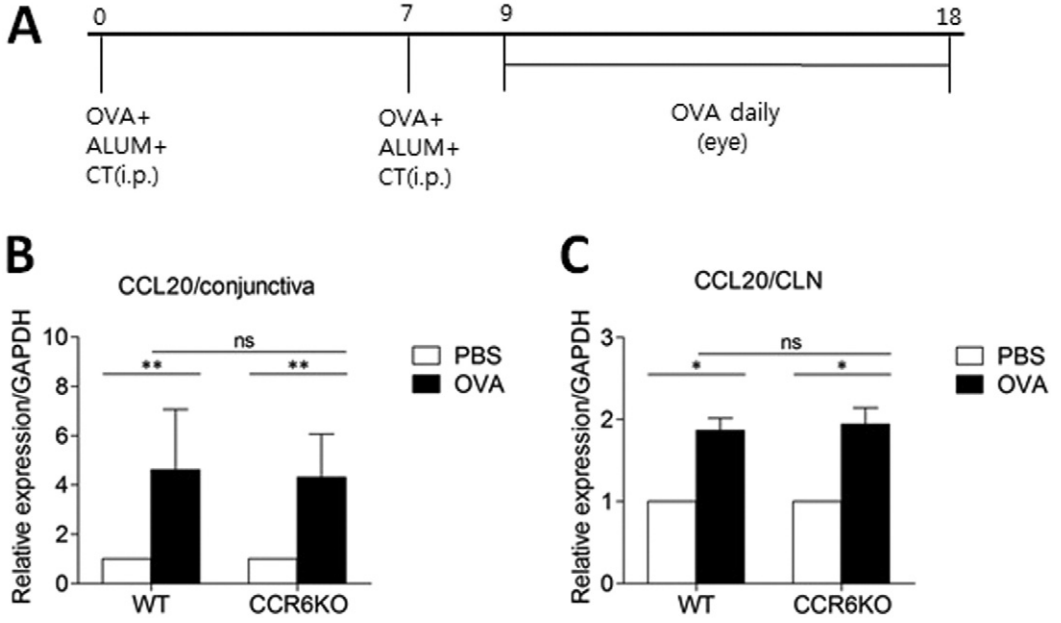

Fig. 1. Time course for development of experimental allergic conjunctivitis (A). CCL20 expression at the conjunctiva (B) and cervical lymph node (CLN; C) is upregulated in wild type and CCR6 KO experimental allergic conjunctivitis mice. WT and CCR6 KO mice were sensitized to OVA and challenged with OVA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