

# The murine CCR3 receptor regulates both eosinophilia and hyperresponsiveness in IgE-mediated allergic conjunctivitis

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The present data have not been presented previously.

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## ABSTRACT

**Background/aims** Allergic conjunctivitis is characterised by early-phase clinical symptoms and late-phase inflammation in the conjunctiva. The role of different chemokine receptors in allergic conjunctivitis, especially during the early-phase reaction, is still unclear. We investigated the importance of CC chemokine receptor (CCR) 3 in a murine model of IgE-mediated allergic conjunctivitis using CCR3-deficient (CCR3<sup>-/-</sup>) mice.

**Methods** Allergic conjunctivitis was initiated in wild-type (WT) and CCR3<sup>-/-</sup> mice by passive transfer of ragweed (RW)-specific IgE, followed by topical challenge with RW in eye drops. Early-phase reactions including clinical symptoms and vascular leakage, as well as late-phase eosinophil infiltration of the conjunctiva were evaluated. The expression of mRNAs in the conjunctiva was quantified by real-time PCR analysis.

**Results** The number of infiltrated eosinophils in the conjunctiva following RW challenge, was significantly higher in RW-IgE-sensitised WT mice compared with those sensitised with phosphate-buffered saline for WT, but this was not observed in similarly treated CCR3<sup>-/-</sup> mice. The early-phase clinical symptoms and vascular leakage were also suppressed in CCR3<sup>-/-</sup> mice. The number of conjunctival mast cells were not different between CCR3<sup>-/-</sup> mice and WT mice, and the mRNAs for FcεRIα and the connective tissue-type mast cell proteases were detected in the conjunctiva of both WT and CCR3<sup>-/-</sup> mice. RW-IgE-sensitised CCR3<sup>-/-</sup> mice displayed significantly reduced expression of CCL2, CCL3, and IL-6 compared with WT mice.

**Conclusions** These results demonstrate a direct contribution of CCR3 to both the early-phase reaction and late-phase inflammation during ocular allergy.

The chemokine receptor, CCR3, is an eotaxin receptor expressed on eosinophils and a variety of other cell types, including basophils, T cells, smooth muscle cells, macrophages and mast cells.<sup>2–3</sup> In humans, tryptase-chymase double-positive mast cells, or connective tissue-type mast cells (CTMC), reportedly express CCR3 in the gut and skin.<sup>4</sup> Both human and murine conjunctival mast cells are known to be CTMC.<sup>1–5</sup> Although the expression of CCR3 on human conjunctival mast cells has not yet been demonstrated, it has been observed on mouse conjunctival mast cells.<sup>6</sup> While chemokines are well known to play a critical role in leucocyte recruitment, their involvement in early-phase reactions, driven primarily by mast cell degranulation is poorly defined.

In this paper, we have investigated the direct role of CCR3 in both early-phase hyperresponsiveness and late-phase inflammation in an IgE-mediated type I allergic reaction using CCR3-deficient (CCR3<sup>-/-</sup>) mice.

## METHODS

### Mice

CCR3<sup>-/-</sup> mice and the wild type (WT) counterparts were from Jackson Laboratory (Bar Harbor, ME). The present study conformed to the principles for laboratory animal research outlined by the Animal Welfare Act, the Department of Health, Education, and Welfare (NIH) guidelines, and the Association for Research in Vision and Ophthalmology (ARVO) statement for the Use of Animals in Ophthalmic and Vision Research. The study protocol was approved by the Emory University Committee on Animal Welfare.

### Reagents

Short ragweed pollen (RW) was from Greer, Inc. (Lenoir, North Carolina, USA). Evans blue (EB) was from ICN Biomedicals (Aurora, Ohio, USA).

### IgE-mediated allergic conjunctivitis in mice

IgE-mediated allergic conjunctivitis was induced in mice by passive immunisation with RW-specific IgE, followed by challenge with RW applied to the conjunctiva using eye drops.<sup>1</sup> Mice were intravenously injected with RW-specific IgE<sup>7</sup> or phosphate-buffered saline (PBS). Twenty-four hours later, the mice were challenged with RW in PBS (2 mg in 10 µl) administered as eye drops. Fifteen minutes later, clinical symptoms were evaluated based on criteria previously described<sup>8</sup> in a double-blind fashion. Chemosis, conjunctival redness, lid swelling and tearing were each graded

## INTRODUCTION

Ocular allergic diseases are induced by a type I allergic reaction in the conjunctiva. This reaction consists of the early-phase reaction and late-phase inflammation. Early-phase responses are induced by degranulation of mast cells resulting in many of the acute anaphylactic symptoms, such as tearing, redness, conjunctival oedema and itching. In the late-phase reaction, inflammatory cells, mainly eosinophils, infiltrate into the conjunctiva several hours after allergen exposure. Using a passive sensitisation model with transfer of antigen-specific IgE into wild-type (WT), mast cell-deficient *Kit*<sup>W<sup>sh</sup>/W<sup>sh</sup></sup> mice, and mast cell-reconstituted *Kit*<sup>W<sup>sh</sup>/W<sup>sh</sup></sup> mice, we have recently shown that conjunctival mast cells play critical roles not only in the early-phase reaction, but also in late-phase inflammation in IgE-mediated allergic conjunctivitis.<sup>1</sup>

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on a 0–3 scale. The total score consisted of the sum of scores in each of these four categories. The highest score of 12 was given to eyes which had severe oedema in conjunctival and lid tissue, tearing and redness due to vasodilation; a score of eight was given to eyes with moderate chemosis and lid oedema; and a score of four was given to eyes with mild conjunctival oedema and erythema. Two hours later, the eyes were harvested for RNA extraction. Twenty-four hours after antigen challenge, the conjunctivas were harvested for eosinophil counting.

### Measurement of vascular permeability

Vascular permeability was assessed by the EB dye extravasation method performed during the immediate hypersensitivity period. Twenty-four hours after passive immunisation by IgE transfer, EB (1 mg/ml, 15 µl/g body weight) was injected intravenously. Then, the left eye was challenged with RW challenge introduced as eye drops, while the right eye was challenged with PBS. Thirty minutes later, the conjunctivas were harvested and weighed, and EB was extracted from the conjunctivas and measured according to previously described methods.<sup>9</sup> Data are presented as the % of control (the EB levels (absorbance)/tissue weight in the PBS-treated eye).

### Quantitative RT-PCR

RNA was extracted from conjunctival tissues using the RNeasy Mini kit (Qiagen, Valencia, California, USA), and cDNA was synthesised with the QuantiTect Reverse Transcription Kit (Qiagen). Relative expression levels of RNAs were determined using real-time PCR with the QuantiTect SYBR Green RT-PCR kit (Qiagen) and an ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, California, USA), as described

previously.<sup>1</sup> Primers corresponding to mouse mast cell proteases (mMCPs) and glyceraldehyde-3-phosphate dehydrogenase are described in the Supplement. Other primers were from Qiagen. Relative RNA expression was determined by using the comparative method of relative quantification ( $2^{-DDCt}$ ) and normalised to glyceraldehyde-3-phosphate dehydrogenase levels.

### Histological analysis

The harvested conjunctivas were fixed in 4% paraformaldehyde. Vertical 5-µm-thick sections were cut and stained with Giemsa. Infiltrating eosinophils in the lamina propria mucosae of the tarsal and bulbar conjunctivas throughout each section were counted by blinded observers.<sup>9</sup> Sections counted were from the central portion of the eye, including the pupil and optic nerve head. The data are presented as the mean (SEM) for all mice examined.

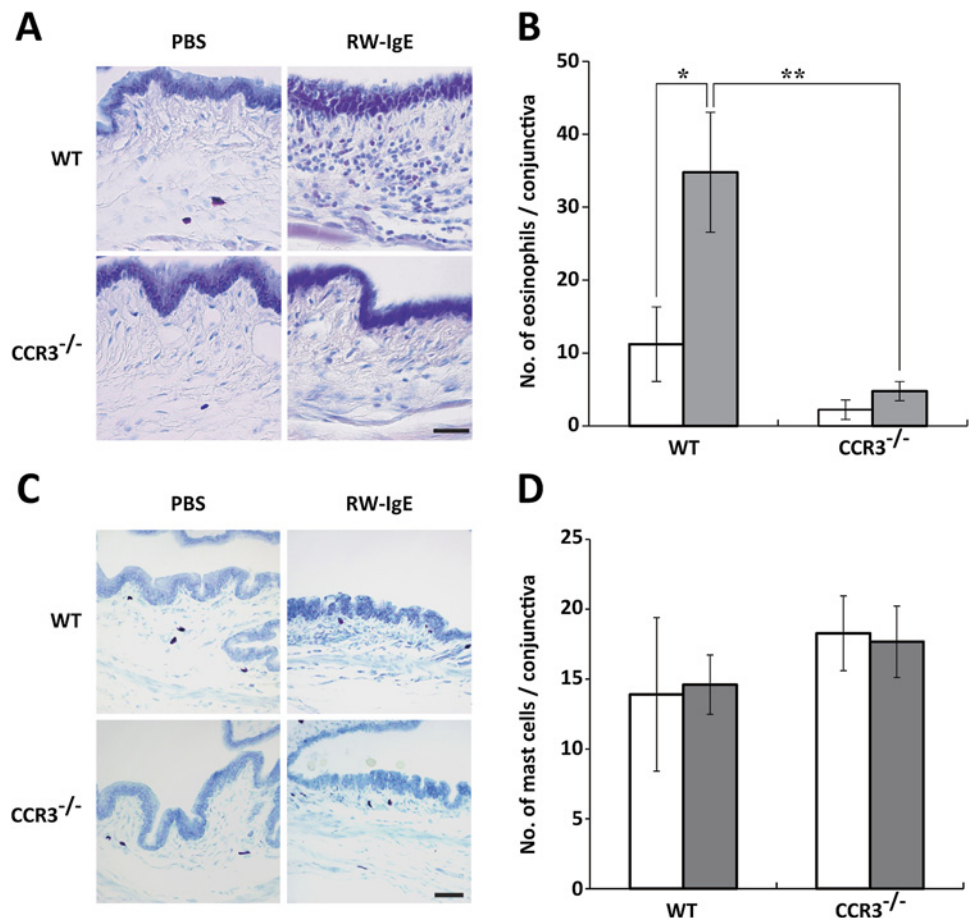
### Statistical analysis

Data are presented as means±SEM. Results were compared between two groups with the unpaired Student t test, and among four groups by Tukey–Kramer multiple comparison test. The Bonferroni and Šidák corrections were applied, and they confirmed the statistical significance of our comparisons. A p value of <0.05 was considered statistically significant.

### RESULTS

IgE-mediated allergic conjunctivitis was induced in mice by passive immunisation with RW-specific IgE, followed by RW challenge topically onto the conjunctiva as previously described.<sup>1</sup> First, we examined the role of CCR3 in late-phase conjunctival inflammation at 24 h after antigen challenge in IgE-

**Figure 1** IgE-mediated late-phase inflammation in WT and CCR3<sup>-/-</sup> mice. Mice were passively sensitised by means of transfer of phosphate-buffered saline (open bars) or ragweed-specific IgE (closed bars), followed by RW challenge through eye drops. Twenty-four hours after antigen challenge, conjunctivas were harvested for histologic analyses. Representative microphotographs of conjunctiva in each group are shown. (A) Giemsa staining; (C) toluidine blue staining, scale bar, 20 µm). Infiltrating eosinophils (B) and mast cells (D) were counted in the lamina propriae. The averages±SEMs of four mice per group are shown. \*p<0.05, \*\*p<0.01 (Tukey–Kramer test) versus indicated values.



mediated allergic conjunctivitis. As shown in figure 1, the number of infiltrating eosinophils following RW challenge was significantly increased in RW-IgE-sensitised WT mice compared with those sensitised with PBS. By contrast, no increase in eosinophil infiltration into conjunctiva was observed in RW-IgE-sensitised and RW-challenged CCR3<sup>-/-</sup> mice. Conjunctival mast cell numbers did not significantly differ between WT and CCR3<sup>-/-</sup> mice (figure 1C,D).

Next, the severity of clinical symptoms in the early-phase reaction, including conjunctival oedema, conjunctival redness, tearing and lid swelling, was scored 15 min after the adminis-

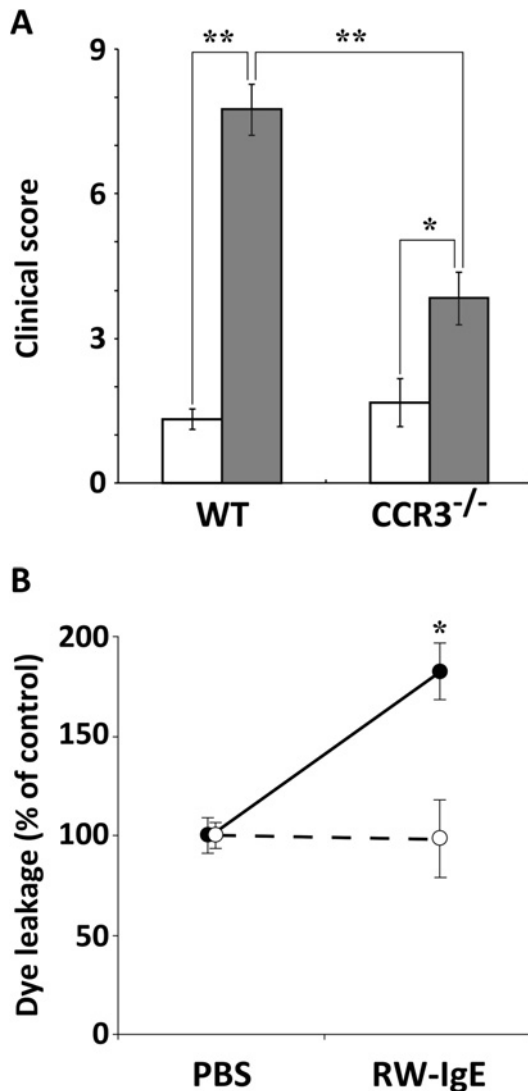


**Figure 2** IgE-mediated early-phase reaction in wild-type (WT) mice and CCR3<sup>-/-</sup> mice. Mice were passively sensitised by means of transfer of phosphate-buffered saline (PBS) (open bars) or ragweed (RW)-specific IgE (closed bars) followed by RW challenge through eye drops. (A) Fifteen minutes after antigen challenge, clinical symptoms were observed. The averages  $\pm$  SEM of six mice per group are shown. \* $p < 0.05$ , \*\* $p < 0.01$  (Tukey-Kramer test) versus indicated values. (B) For vascular leakage, Evans blue was injected intravenously before antigen challenge. Thirty minutes later, the conjunctivas in WT (closed circles) and CCR3<sup>-/-</sup> mice (open circles) were harvested and weighed, and Evans blue was extracted and then measured. Data are presented as the % of PBS-injected control mice for dye content (absorbance)/tissue weight (g). \* $p < 0.05$  (unpaired Student t test) versus values for PBS-injected mice.

tration of RW eye drops. As shown in figure 2, WT mice sensitised with RW-specific IgE followed by RW challenge displayed significantly more severe symptoms than did mice sensitised with PBS, suggesting that these symptoms were due to an antigen-specific allergic reaction. The clinical scores for RW-IgE-sensitised CCR3<sup>-/-</sup> mice were significantly lower following allergen challenge compared with those for RW-IgE-sensitised WT mice. In addition, vascular permeability, as assessed by EB dye leakage, was induced in RW-IgE-sensitised WT mice, but not in RW-IgE-sensitised CCR3<sup>-/-</sup> mice following RW challenge. These results suggest that CCR3 is essential not only for late-phase eosinophilic inflammation, but also for the early-phase anaphylactic reaction in IgE-mediated allergic conjunctivitis.

Mast cell expression of neutral proteases typically ascribed to the mucosal mast cell subset (mMCP-1 and mMCP-2) or the CTMC subset (mMCP-4, -5, -6, and carboxypeptidase A (CPA)), was examined by real-time PCR analysis in the WT and CCR3<sup>-/-</sup> mice.<sup>10</sup> We have previously revealed that murine conjunctival mast cells are CTMC.<sup>1</sup> Consistent with this report,<sup>1</sup> mRNAs for Fc $\epsilon$ RI $\alpha$  and the CTMC proteases mMCP-4, -5, -6 and CPA were detected in the eyelid of WT mice (figure 3). In WT mice, the expression of mRNAs for Fc $\epsilon$ RI $\alpha$ , mMCP-5, mMCP-6, mMCP-7 and CPA tended to increase following RW challenge in RW-IgE-sensitised mice. Transcripts for the CTMC proteases and Fc $\epsilon$ RI $\alpha$  were detected in the conjunctiva of untreated CCR3<sup>-/-</sup> mice at higher levels than observed in WT mice. In contrast with the observations in WT mice, the expression of Fc $\epsilon$ RI $\alpha$  and CPA significantly decreased after RW challenge in RW-IgE-sensitised CCR3<sup>-/-</sup> mice. The decreases in expression of mMCP-5, -6, and -7 in response to RW-IgE sensitisation and RW challenge in CCR3<sup>-/-</sup> mice were not statistically significant.

Since CCRs on mast cells play important roles in mast cell activation,<sup>11 12</sup> and mast cells are known to synthesise various chemokines and cytokines, such as IL-6 and IL-13 by cross-linking of Fc $\epsilon$ RI,<sup>13 14</sup> levels of representative CC chemokines and cytokines in the conjunctiva were examined by real-time PCR analysis. In WT mice, the chemokines CCL2, CCL3 and CCL4, and the cytokines IL-6 and IL-13 increased following RW challenge in RW-IgE-sensitised mice (figure 4). However, the expression of mRNAs for these chemokines did not increase in CCR3<sup>-/-</sup> mice following RW challenge; indeed, RW-IgE-sensitised CCR3<sup>-/-</sup> mice exhibited a significant reduction of CCL2, CCL3, and IL-6 expression as compared with WT mice.

## DISCUSSION

In this paper, using a passive sensitisation model with transfer of antigen-specific IgE into WT and CCR3<sup>-/-</sup> mice, we have shown that CCR3 plays critical roles not only in late-phase eosinophilic infiltration but also in the early-phase anaphylactic responses during allergic conjunctivitis in vivo.

Chemokines play important roles in the migration of inflammatory cells into tissues. The research using genetic deletions of either chemokine receptor ligands or the receptor itself in mice have been investigated; for example, genetic modification of the CCR2-MCP1-axis results in profound reduction of inflammation in various disease models.<sup>15</sup> The principal receptor involved in eosinophil infiltration is CCR3.<sup>2</sup> CCR3 is also reportedly expressed by Th2 cells, basophils, macrophages and mast cells.<sup>3</sup> All these cell types contribute to the development of allergic inflammation. In contrast to CCR1, CCR2 and CCR5, CCR3 is only expressed at low levels on monocytes/macrophages. However, specifically, the expression of CCR3 is closely related to allergic diseases. In this study,

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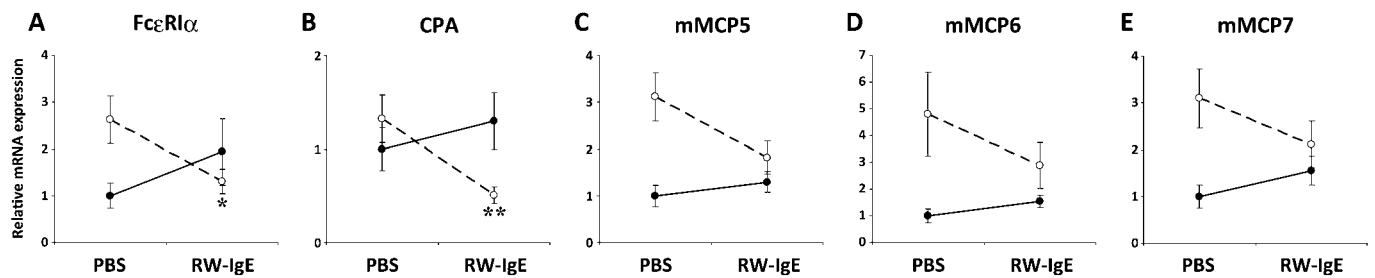


**Figure 3** Expression of mRNAs for various mast cell neutral proteases in the conjunctiva of wild-type (WT) and CCR3<sup>-/-</sup> mice. Mice were passively sensitised by intravenous injection of phosphate-buffered saline (PBS) or ragweed-specific IgE (RW-IgE) and challenged with ragweed eye drops in WT (closed circles) and CCR3<sup>-/-</sup> mice (open circles). The amounts of mRNA (A: FcεR1α, B: CPA, C: mMCP5, D: mMCP6, E: mMCP7) in the conjunctivas (N=6 per group) were determined by reverse transcription and real-time PCR analysis. Levels were normalised to those of GAPDH mRNA and are expressed as mean arbitrary units±SEM. \*p<0.05, \*\*p<0.01 (unpaired Student t test) versus values for PBS-injected mice.

infiltration of eosinophils into the conjunctiva significantly decreased in CCR3<sup>-/-</sup> mice, consistent with previous reports in several allergic disease models.<sup>16 17</sup> Conflicting results for mast cells and hyperresponsiveness in CCR3<sup>-/-</sup> mice were reported in these papers, however. In a mouse model of allergic asthma in which mice were sensitised with antigen in artificial adjuvant by intraperitoneal injection, CCR3<sup>-/-</sup> mice exhibited increased numbers of mast cells in the trachea, resulting in enhanced airway hyperresponsiveness.<sup>16</sup> In contrast, another report showed no differences in airway hyperresponsiveness or tracheal mast cell numbers between WT and CCR3<sup>-/-</sup> mice using a model in which mice were epicutaneously sensitised without adjuvant and challenge in the airways.<sup>17</sup> In our study, comparable numbers of mast cells were observed in the conjunctivas of WT and CCR3<sup>-/-</sup> mice regardless of the sensitisation with IgE and antigen challenge. This result suggests that CCR3 is not important for conjunctival mast cell trafficking and recruitment to conjunctiva, or that redundant pathways induce this response.

The extent to which mast cells can contribute to the hyper-reactivity and inflammation associated with models of allergic conjunctivitis and asthma in mice is known to be highly dependent on the specific experimental model investigated.<sup>18–21</sup> In studies in which mice were sensitised with antigen mixed with an artificial adjuvant, mast cell-deficient mice did not display a significant reduction in allergic responses compared with WT mice.<sup>18 19</sup> An adjuvant, such as aluminium hydroxide, can promote a strong non-specific antibody response, or have other effects that can influence features of the allergic disease model, thereby masking the contribution of mast cells to allergic responses. We have previously demonstrated that conjunctival

mast cells play a critical role in various features of allergic conjunctivitis by means of a passive sensitisation model in which antigen-specific IgE was transferred into genetically mast cell-deficient, congenic normal and mast cell-reconstituted mice.<sup>1</sup> In this study, we employed a passive sensitisation model because we were interested in the role of CCRs on mast cell activation.<sup>11 12</sup>

Collington *et al* have recently compared the bone marrow-derived mast cells from WT and CCR3<sup>-/-</sup> mice, and found that there were no differences in mast cell phenotype or ability to migrate to mast cell chemoattractants.<sup>22</sup> We examined mast cell degranulation using a β-hexosaminidase release assay following IgE sensitisation and antigen stimulation in cultured bone marrow-derived mast cells from WT mice and CCR3<sup>-/-</sup> mice, and found no difference in degranulation between WT and CCR3<sup>-/-</sup> mast cells (data not shown). Although different proteases expressed in mast cells do not necessarily mean a different cell type, conjunctival mast cells in CCR3<sup>-/-</sup> mice display a CTMC phenotype similar to that of WT mice, as determined by the expression pattern of mMCPs. Higher transcript levels for FcεR1α and CPA in untreated CCR3<sup>-/-</sup> mice might imply that the phenotype of mast cells in vivo from CCR3<sup>-/-</sup> mice differs in more than just the CCR3 expression.

Early-phase responses induced by mast cell degranulation were suppressed in CCR3<sup>-/-</sup> mice, however, and the expression of chemokines/cytokines was not increased in response to antigen challenge in these mice. It is possible that certain factors from local tissue affect the expression of CCR3, which may in turn regulate cell function in resident CTMC. Impaired early-phase reactions in allergic conjunctivitis were consistent with

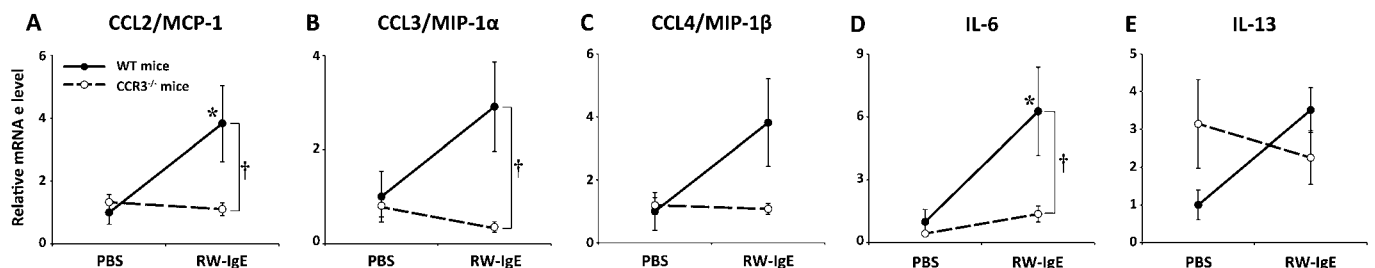


**Figure 4** IgE-mediated local chemokine/cytokine expression in wild-type (WT) mice and CCR3<sup>-/-</sup> mice. Mice were passively sensitised by intravenous injection of phosphate-buffered saline (PBS) or ragweed-specific IgE (RW-IgE) and challenged with ragweed eye drops in WT (closed circles) and CCR3<sup>-/-</sup> (open circles) mice. The levels of chemokine and cytokine mRNA (A: CCL2, B: CCL3, C: CCL4, D: interleukin (IL)-6, E: IL-13) in the conjunctiva were determined by real-time RT-PCR 2 h after ragweed instillation. The averages±SEMs of six mice per group are shown. \*p<0.05, (Tukey-Kramer test) versus values for PBS-injected mice. †p<0.05 (Tukey-Kramer test) versus indicated values.

our previous studies using a CCR3 antagonist and eotaxin-deficient mice.<sup>6 23</sup>

Soluble anti-chemokine/anti-chemokine receptor treatment is an interesting and promising option for other systemic inflammatory/allergic diseases.<sup>24</sup> Systemic blockade of CCR3 by a specific antagonist significantly suppressed allergen-induced clinical signs, mast cell degranulation and eosinophilic inflammation in a murine model of allergic conjunctivitis induced by active immunisation.<sup>23 25</sup> Clustering analysis of the transcriptome during the early phase also revealed that CCL2 was upregulated by antigen challenge and was suppressed by CCR3 blockade.<sup>25</sup> These results using CCR3 antagonists were similar to current results using CCR3<sup>-/-</sup> mice. Given that the conjunctiva is located at the surface of the eye, topical administration with chemokine or chemokine receptor antagonists may be effective for the treatment of ocular allergic diseases. The precise mechanisms of the interaction of CCR3 and mast cell function remain to be elucidated. Taken together, our results suggest that CCR3 does not have a major effect on mouse mast cell phenotype or migration, and is more likely to function on mature mast cells in the conjunctiva. CCR3 plays critical roles in mast cell-mediated early-phase reaction, and may therefore, be a possible target for the treatment of ocular allergy.

**Contributors** KF and SJO designed research; KF, C-HK, and KM performed research; F-TL contributed by providing critical reagents; KF and C-HK analysed data; KF, F-TL, and SJO wrote the paper.

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**Competing interests** None.

**Patient consent** It is an animal experiment.

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