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Systemic overexpression of IFN- γ and IL-5 exacerbates early phase reaction and conjunctival eosinophilia, respectively, in experimental allergic conjunctivitis

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ABSTRACT

Aims/background: To investigate how systemic overexpression of IL-4, IL-5 and IFN- γ affects the severity of experimental conjunctivitis (EC) in mice.

Methods: The tibialis anterior muscle of naive BALB/c mice was electroporated with IL-4, IL-5, IFN- γ or a control gene, and then the mice blood and conjunctivas were harvested to measure the eosinophil content in these tissues. To evaluate the effects of cytokine gene electroporation on the early-phase reaction (EPR), cytokine gene-electroporated ragweed (RW)-immunised mice were intravenously injected with Evans Blue (EB) and then challenged with RW in eye-drops. Thirty minutes later, their conjunctivas were harvested to extract EB and evaluate the EPR. Additionally, 24 h after RW challenge, conjunctivas were harvested from cytokine gene-electroporated RW-immunised mice, which had not received intravenous injection of EB, to measure conjunctival eosinophilia.

Results: Significantly more eosinophils were detected in the blood and conjunctivas of IL-5-electroporated mice in which EC was not induced. The intensity of the EPR was significantly greater in IFN- γ -electroporated mice. Significantly greater eosinophil infiltration was seen in the conjunctivas of IL-5-electroporated EC-developing mice.

Conclusions: It appears that systemic IL-5 and IFN- γ play different roles in the development of EC.

produce IL-4. Thus, in EC, it is believed that IL-4 plays an important role in the IgE-mediated EPR, which can be regulated by IFN- γ , whereas IL-5 is involved in conjunctival eosinophilia. In this study, we have investigated how IL-4, IL-5 and IFN- γ participate in the development of EC by introducing cytokine genes into mice by electroporation.

MATERIALS AND METHODS

Mice

Inbred wild-type (WT) BALB/c mice were purchased from Japan SLC, Hamamatsu, Shizuoka, Japan. The age- and gender-matched mice were used when they were 6–12 weeks old. The mice were maintained under specific pathogen-free conditions at the Kochi Medical School animal facility. All procedures were carried out in accordance with the Association for Research in Vision and Ophthalmology's Statement for the Use of Animals in Ophthalmic and Vision Research.

Reagents

Short ragweed (RW) pollen was purchased from Polysciences, Warrington, Pennsylvania. RW extract was obtained from LSL (Tokyo). Aluminium hydroxide (alum) was purchased from Sigma (St Louis, Missouri). Concanavalin A (Con A) was purchased from Wako Pure Chemical Industries (Osaka, Japan). Biotinylated goat polyclonal antirabbit immunoglobulin was purchased from BD Biosciences Pharmingen (San Diego, California), and an Avidin-Biotin-Complex kit was obtained from Vector Laboratories (Burlingame, California).

Electroporation of cytokine genes

Mice were electroporated as previously described.¹¹ Briefly, the tibialis anterior muscles of mice were injected with a DNA plasmid expressing IL-4, IL-5 or IFN- γ , as the vector, after which a pair of electrode needles were inserted into the DNA injection site to deliver electric pulses. As a control, the same DNA plasmid with a non-cytokine gene was injected. It was reported that in vivo electroporation increased both the number of muscle fibres taking up plasmid DNA and the copy number of plasmids introduced into the cells, and so it is accepted that the electroporated muscle cells produce the cytokine.¹¹

The term allergic conjunctivitis (AC) encompasses a variety of diseases ranging from mild forms such as seasonal AC, to severe forms including vernal keratoconjunctivitis (VKC).¹ The major signs and symptoms of mild AC are conjunctival hyperaemia and itching that are mediated by the early-phase reaction (EPR) of the type I allergic response.² The typical findings of severe AC are giant papillary formations in the tarsal conjunctivas and corneal ulcers³ that are predominantly mediated by eosinophils.⁴ We have shown using an animal model that Ag-specific T cells, rather than Ag-specific IgE, play an important role in conjunctival eosinophil infiltration during the development of AC (experimental conjunctivitis, EC).⁵

Cytokines are key molecules that dictate whether an immune response will be dominated by a type I allergic response or T cells.⁶ IL-4 is a crucial molecule for the IgE class switch.⁷ IL-5 is important for the maturation and activation of eosinophils.⁸ IFN- γ is involved in the macrophage-mediated, delayed-type hypersensitivity⁹ and suppresses Th2 cells,¹⁰ which

Effects of electroporation on conjunctival inflammation

Effects of electroporation on mice not sensitised with RW

Seventeen days after the electroporation procedure ($n = 3$ per group), the mice were challenged with RW in phosphate-buffered saline (PBS) (2 mg in 10 μ l per eye) in eye-drops, and 24 h later, the spleens, conjunctivas and blood were collected for flow-cytometric analysis, histological analysis to determine conjunctival eosinophil numbers and haematological analysis to determine the percentage of peripheral blood eosinophils and measurement of serum cytokine levels, respectively. In a separate experiment, 18 days after electroporation of the IL-5 gene or control gene ($n = 3$ per group), the conjunctivas were harvested from mice that had not received a RW challenge. The conjunctivas were then subjected to histological analysis to determine the conjunctival eosinophil numbers.

Induction of EC by active immunisation in mice that received electroporation of cytokine genes: effects on the EPR

BALB/c mice were immunised with RW in alum as previously described.⁵ Eleven days later, the mice were electroporated with IL-4 ($n = 11$), IL-5 ($n = 12$), IFN- γ ($n = 11$) or the control gene ($n = 11$). Three days later, the mice were injected intraperitoneally with RW in alum. Fourteen days later, after collecting an aliquot of blood to measure the levels of total serum IgE, Evans Blue (EB, 1 mg/ml, 15 μ l/g body weight) was injected intravenously. Then, an RW challenge was introduced into the left eye with eye-drops, while the right eye was challenged with PBS. Thirty minutes later, the conjunctivas were harvested and weighted, and EB was extracted from the conjunctivas and then measured according to previously described method.¹² Data are presented as the differences in the EB levels (μ g)/tissue weight (g) between the left and right eyes.

Induction of EC by active immunisation in mice that received electroporation of cytokine genes: effects on conjunctival eosinophilia

Seven days after the electroporation procedure ($n = 6$ per group), RW adsorbed on alum was injected into the left hind footpad and the tail base. Fifty microlitres of emulsion (50 μ g of RW and 675 μ g of alum) was injected into each site. The eyes of the immunised mice were challenged with RW on day 10. Twenty-four hours later, conjunctivas, blood and spleens were harvested for histological analysis, haematological analysis to determine the percentage of peripheral blood eosinophils and measurement of RW-specific Ig levels and cytokine assays.

Flow-cytometric analysis

A single cell suspension was prepared from harvested spleens. The splenocytes were then subjected to flow-cytometric analysis to evaluate their forward and side scatters. As previously shown,^{13, 14} cells with high levels of side-scatter are considered to be eosinophils. Splenocytes were stained with FITC-labelled anti-CD3 (145-2C11), FITC-labelled anti-CD45R/B220 (RA3-6B2) or PE-labelled anti-F4/80 (A3-1) (Caltag Laboratories, Burlingame, California). The cells were sorted using a FACScan (Becton Dickinson, Mountain View, California), and data were analysed using CellQuest software. Data are expressed as a percentage of positively stained with each Ab out of whole splenocytes.

Measurement of cytokines in serum and culture supernatant

Serum was prepared from blood and kept frozen until just prior to testing. For the culture supernatant, red-blood-cell-depleted

splenocytes (10^7 cells/ml) were stimulated in vitro with 25 μ g/ml RW extract or 5 μ g/ml Con A in 96-well flat-bottom plates in a final volume of 0.2 ml RPMI 1640 medium supplemented with 10% FCS and 50 μ M 2-ME. Forty-eight hours later, culture supernatants were collected and kept frozen until just prior to testing. The levels of IL-2, IL-4, IL-5, IL-10, IL-12 and IFN- γ in serum and the culture supernatants were measured using a Luminex100 system (Luminex Corporation, Austin, Texas) and the Bio-Plex Cytokine Assay Panel (Bio-Rad Laboratories, Hercules, California) according to the manufacturers' instructions.

RT-quantitative PCR analysis

Five days after the electroporation procedure, the tibialis anterior muscles were collected. Total RNA was prepared by TRIzol according to the manufacturer's instructions (Invitrogen, Carlsbad, California). RNA was treated with DNase I (Ambion, Austin, Texas) for 20 min at 37°C, and DNase I was inactivated using DNase I Inactive Reagent (Ambion). 2 μ g of DNase I-treated RNA was used to synthesise cDNA using a cDNA synthesis kit (Invitrogen). For RT-quantitative PCR (qPCR), the PCR reaction and analysis of data were performed following the manufacturer's instructions (User Bulletin #2, ABI PRISM 7700 Sequence Detection System, Applied Biosystems, Austin, Texas). All runs were accompanied by hypoxanthine guanine phosphoribosyltransferase (HPRT) gene as an internal control. Samples were normalised to HPRT RNA, giving arbitrary values representing a ratio of experiment to control. Results are expressed as relative mRNA levels. Additionally, PCR products were run on a gel and presented as photo data.

Evaluation of peripheral blood and splenic eosinophils

Smears were prepared by dropping an aliquot of blood onto a glass slide. After the blood had dried, slides were stained with Giemsa. Eosinophils were distinguished from neutrophils, monocytes and lymphocytes based on eosinophilic staining in the cytoplasm. The number of eosinophils per 100 white blood cells was counted for each mouse. To further distinguish eosinophils from other types of granulocytes, the slides (splenocytes smear) were stained with rabbit antimouse major basic protein (MBP, kindly provided by J J Lee, Mayo Clinic) and a secondary Ab, and then Ab binding was revealed using the Avidin-Biotin-Complex kit followed by development with 3,3'-diaminobenzidine tetrahydrochloride.

Histological analysis

The harvested conjunctivas were fixed in 10% buffered formalin. Vertical 2 μ m 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 two blinded observers. 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 the mice examined.

Measurement of total and RW-specific IgG1 and IgE in the serum

Total and RW-specific IgG1 and IgE in serum were measured by ELISA as previously described.¹⁵ Total and RW-specific IgE data are presented as ng/ml, while RW-specific IgG1 concentrations are expressed as units (U)/ml relative to the pooled hyper-immune serum.

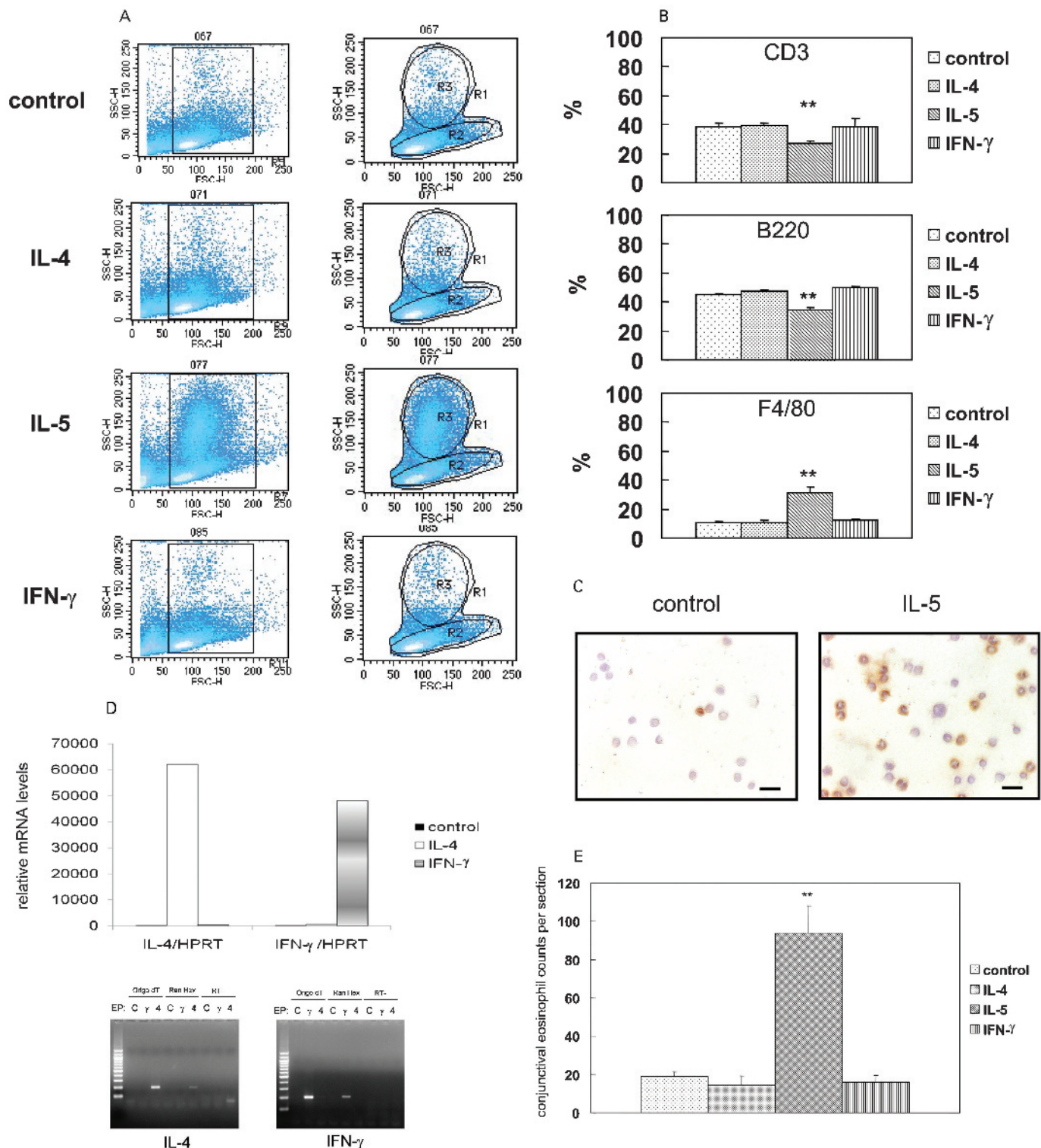


Figure 1 Effects of cytokine gene introduction on systemic and conjunctival eosinophils in mice without experimental conjunctivitis. Naive BALB/c mice were electroporated with the IL-4, IL-5, or IFN- γ gene or a control gene. Seventeen days later, mice were challenged with ragweed, and 24 h later, blood, spleens and conjunctivas were collected. (A, B) Flow-cytometric analysis of splenocytes. R3 indicates cells with high side scatter. R1 = R2+R3. Representative data are shown (A). ** $p < 0.01$ compared with the control group (B). (C) Immunocytochemical analysis of splenocytes. Eosinophils were confirmed by major basic protein expression. Representative data are shown. Bar = 20 μ m. (D) Quantitative PCR analysis of cytokine expression in the tibialis anterior muscles. Results are expressed as relative mRNA levels compared with the control hypoxanthine guanine phosphoribosyltransferase (HPRT) gene. PCR products were run on a gel and shown as a band. "EP: C γ 4" indicates electroporation of the control gene, IFN- γ gene or IL-4 gene. With regard to PCR cycle number, 30 and 35 cycles were performed to detect IFN- γ and IL-4, respectively. Representative data are shown. (E) Counts of eosinophils in the conjunctiva. ** $p < 0.01$ compared with the control group. FSC-H, forward light scatter—height; Ran Hex, random hexamer; RT—, reverse transcription was not conducted; SSC-H, side light scatter—height.

Statistical analysis

Differences in serum Ig and cytokine levels, cytokine production by splenocytes and eosinophil numbers in spleens, peripheral blood and conjunctivas between the cytokine gene-electroporated groups and the control gene-electroporated groups were evaluated by ANOVA and then by the Fisher Protected Least Significant Difference method. *p* Values less than 0.05 were considered significant.

RESULTS

Effects of cytokine gene introduction on systemic and conjunctival eosinophils

Splenic eosinophils, as determined by their high side scatters, were abundant in the IL-5-electroporated group compared with the other three groups (fig 1A), and the difference was statistically significant (the average percentage of IL-5-electroporated group = 27, the average percentage of other three groups <4). Flow-cytometric analysis using Abs revealed that the percentage of CD3-positive and B220-positive cells was significantly lower in the IL-5-electroporated group, while that of F4/80-positive cells was significantly higher (fig 1B). The latter data were similar to those in fig 1A. Additionally, the increased numbers of eosinophils in IL-5-electroporated group were confirmed by an immunocytological technique (fig 1C). Cytokine analysis of serum demonstrated that the levels of IL-5 were significantly higher in the IL-5-electroporated group than in the other three groups (the average concentration was 37, 37, 134 and 38 pg/ml in a control gene-, IL-4-, IL-5- and IFN- γ -electroporated group, respectively). However, IL-4 levels did not differ between the four groups (the average concentration was 42 pg/ml in every group). Levels of IFN- γ in serum were below the threshold of detection. To confirm whether the gene transfer was performed successfully, 5 days after electroporation, the tibialis anterior muscles were collected from mice electroporated with IL-4 or IFN- γ gene to evaluate cytokine mRNA expression. IL-4 mRNA was detected in IL-4- but not IFN- γ -electroporated mice (fig 1D). Similarly, IFN- γ mRNA was detected in IFN- γ - but not IL-4-electroporated mice (fig 1D). The data were also shown as photographs of PCR products on a gel (fig 1D). The peripheral blood eosinophil content was significantly higher in the IL-5-electroporated group (average = 24%) than in the other three groups (average <3%). A greater number of eosinophils was also seen in the conjunctivas of IL-5-electroporated mice, and the number of conjunctival eosinophils was also significantly higher in this group (fig 1E). To examine whether an RW challenge administered in eye-drops affects conjunctival eosinophil infiltration, the IL-5 gene or a control gene was introduced into mice, and 18 days later, their conjunctivas were harvested. Similar to what is shown in fig 1E, the number of conjunctival eosinophils was significantly higher in the IL-5-electroporated group (average = 94) than in the control group (average = 19).

Effects of cytokine gene introduction on the EPR in murine EC development

To examine the effects of cytokine gene electroporation on the EPR in EC, conjunctival vascular permeability was evaluated by measuring leakage of EB into the conjunctivas. The concentration of total IgE in serum did not differ between the four groups (fig 2A). The level of EB in the conjunctivas was significantly higher in the IFN- γ -electroporated group than in the control group (fig 2B).

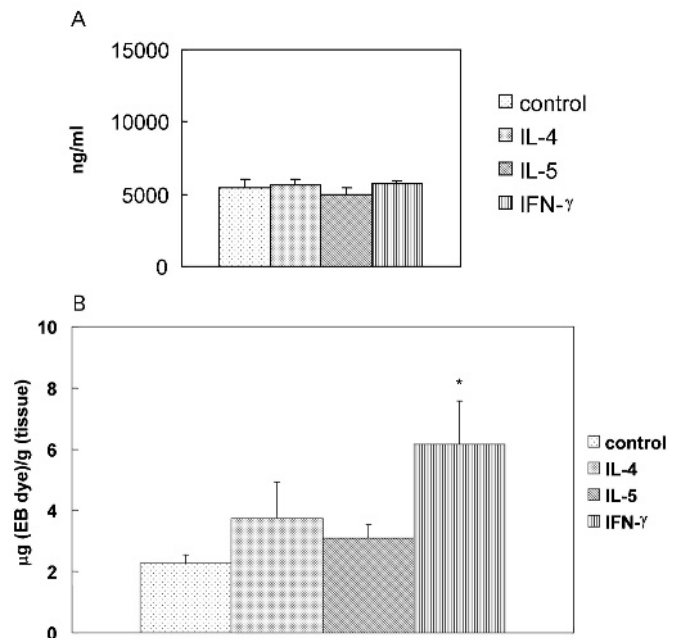


Figure 2 Effects of cytokine gene introduction on the early-phase reaction (EPR) in mice with experimental conjunctivitis. Eleven days after initial immunisation with ragweed (RW) in alum, mice were electroporated with IL-4, IL-5, IFN- γ or the control gene. Three days later, the mice were injected intraperitoneally with RW in alum. Fourteen days later, after collecting an aliquot of blood to measure the levels of total IgE in serum (A), Evans Blue (EB) was injected intravenously. Then, the left and right eyes were challenged with RW and phosphate-buffered saline, respectively. Thirty minutes later, the conjunctivas were harvested and weighed, and EB was extracted and then measured (B). Data are presented as the differences of EB content (μ g)/tissue weight (g) between the left and right eyes. **p* < 0.05.

Effects of cytokine gene introduction on conjunctival eosinophilia induce by EC

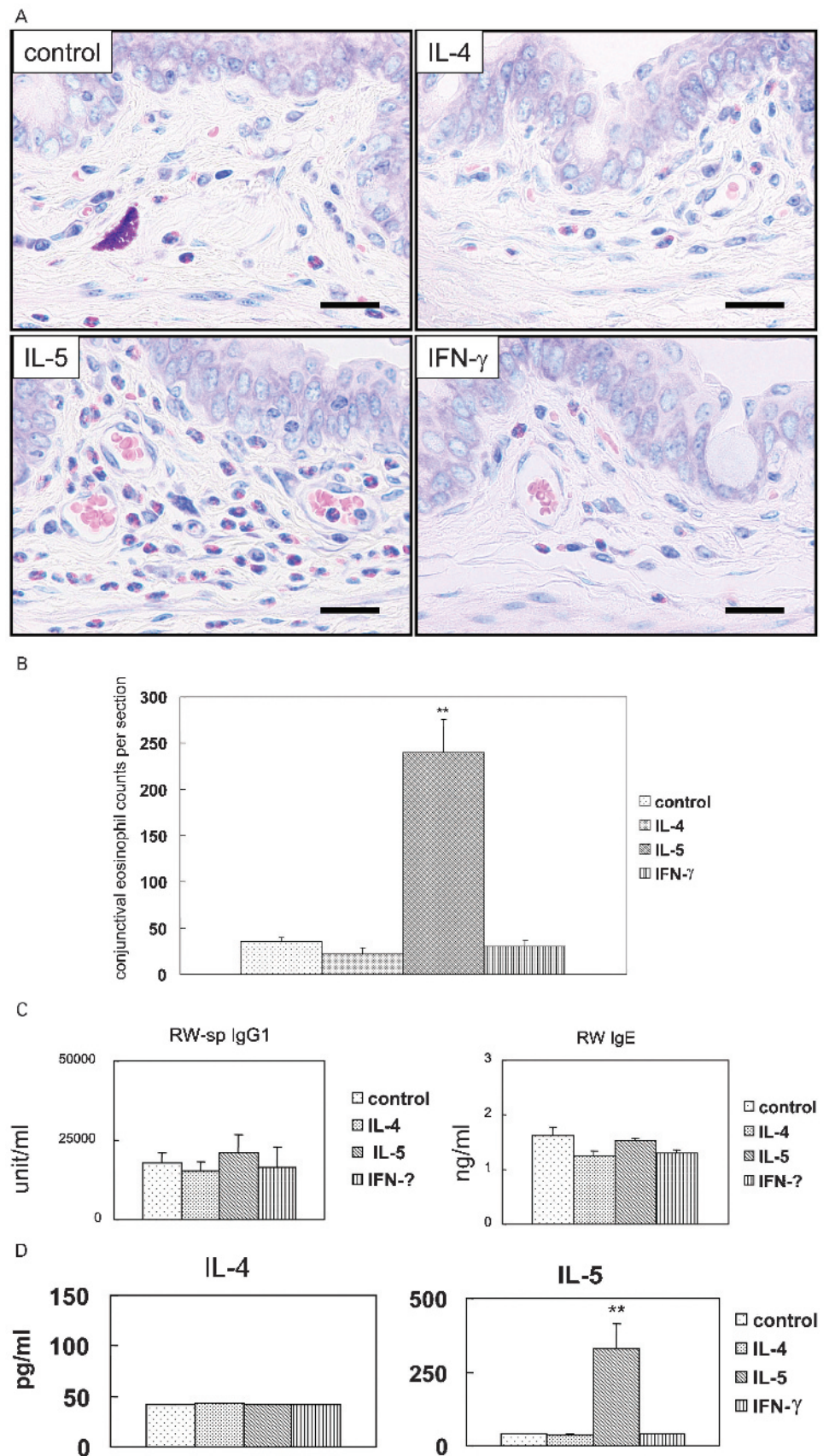
IL-4, IL-5, IFN- γ or a control gene was electroporated into naive BALB/c mice, 7 days before EC induction. Peripheral blood eosinophil content was significantly higher in the IL-5-electroporated group (average = 32%) than in the other three groups (average <3%), as similarly noted in unsensitised mice. In addition, more eosinophils were present in the conjunctivas of IL-5-electroporated mice (fig 3A), and the number of conjunctival eosinophils was also significantly higher in this group (fig 3B), as similarly noted in unsensitised mice (fig 1E). Immunohistochemical analysis revealed that B cell numbers in the conjunctiva were few and were not different between the IL-4-electroporated group and the three other groups (data not shown). RW-specific IgG1 and IgE levels did not differ between the four groups (fig 3C). IL-5 was significantly higher in the serum of the IL-5-electroporated group compared with the other three groups (fig 3D), although there was no significant difference between the groups in the levels of IL-4 (fig 3D) or IFN- γ (below the threshold of detection). Splenocytes from mice in all four groups showed a similar production of IFN- γ , IL-2, IL-4, IL-5, IL-10 and IL-12 in response to RW and Con A (data not shown).

DISCUSSION

Here, we have demonstrated that systemic overexpression of IL-5 increases eosinophils in the peripheral blood and conjunctivas of mice, regardless of EC induction. The data suggest that

Laboratory science

Figure 3 Effects of cytokine gene introduction on conjunctival eosinophilia in mice with EC. Naive BALB/c mice were electroporated with IL-4, IL-5, IFN- γ or the control gene. Seven days later, these mice were immunised with ragweed (RW) in alum, and 10 days later, they were challenged with RW in eye-drops. Twenty-four hours later, the blood, conjunctivas and spleens were collected. (A) Microphotographs of conjunctivas. Bar = 20 μ m. (B) Eosinophil counts in the conjunctivas. ** $p < 0.01$ compared with the control group. (C) RW-specific IgG1 and IgE levels in serum. (D) Cytokine levels in serum. ** $p < 0.01$ compared with the control group.



circulating eosinophils increased by systemic IL-5 electroporation, and thereby, more eosinophils infiltrated into the conjunctiva even when EC was not induced. However, systemic overexpression of IL-4 and IFN- γ did not affect conjunctival

eosinophil infiltration. Thus, it appears that IL-5 specifically promotes conjunctival eosinophil infiltration.

In contrast to the case of conjunctival eosinophil infiltration, which is a hallmark of the severe forms of AC,⁴ the EPR was

significantly augmented by IFN- γ but not by IL-4 or IL-5. IL-4 is a crucial molecule for IgE class switching,⁷ and so we predicted that overexpression of IL-4 would augment the EPR. However, the serum IgE levels of mice electroporated with the IL-4 gene and then immunised with RW were similar to those electroporated with IL-5, IFN- γ or a control gene. This may be due to the fact that we used an experimental protocol in which electroporation was conducted between the initial immunisation and the second boosting immunisation. Maximal upregulation of IL-4 in sera using this electroporation method was observed 5 to 12 days after injection, as previously described.^{13 14} These two factors may account for the fact that electroporation of IL-4 did not significantly affect IgE production or the EPR. In contrast, electroporation of IFN- γ resulted in significant upregulation of the EPR. The reason for this is not clear. It is possible that upregulation of IFN- γ may have affected the conjunctival microvasculature, since it was reported that IFN- γ affects vascular permeability.¹⁶ Furthermore, it was reported that IFN- γ appears to contribute to the pathogenesis of murine allergic conjunctivitis at the effector phase, by using murine allergic conjunctivitis induced by repeated topical application of RW.¹⁷ These two reports may account for the upregulation of EPR by electroporation of IFN- γ . It is obviously important to investigate the expression of IFN- γ in the conjunctiva, especially the types of cells in the conjunctiva that do express IFN- γ . Future studies will focus on how systemic electroporation of IFN- γ affects the conjunctival IFN- γ expression, in relation to the EPR.

The upregulation of conjunctival eosinophilia by IL-5 electroporation was also observed in mice that developed EC. Electroporation of the IL-5 gene has a synergistic rather than additive effect, since the average numbers of conjunctival eosinophils were 94, 36 and 240 in IL-5-electroporated naive mice, control-electroporated RW-immunised mice challenged with RW, and IL-5-electroporated RW-immunised mice challenged with RW, respectively. The induction of EC leads to T cell activation in the conjunctiva and then promotes conjunctival cytokine and chemokine production, which attracts inflammatory cells to the conjunctivas.¹⁸ IL-5 electroporation increased serum IL-5 levels but did not affect other systemic immune responses. Thus, we believe that the induction of EC facilitated the infiltration of the increased number of circulating eosinophils in the peripheral blood resulting from electroporation of the IL-5 gene. These data also suggest that an eosinophil-rich state worsens conjunctival allergy. Indeed, it has been reported that eosinophilia during infancy is associated with allergic disease during the first 6 years of life.¹⁹ Thus, the inhibition of eosinophilia may be a therapeutic strategy for conjunctival allergic eosinophil infiltration, as well as for asthma, for which treatment with anti-IL-5 Ab has been shown to be effective in a subset of patients with asthma.²⁰

The examined cytokines (IL-4 and IFN- γ) in this study are known to be the key cytokines affecting Th1/Th2 balance. Generally, Th1 cells regulate Th2 cells,²¹ and so several reports demonstrated that allergic diseases mediated by Th2 cells were suppressed by augmentation of Th1 responses.^{22 23} In contrast, it was also reported that Th1 cells assist Th2-mediated allergic airway inflammation.²⁴ How Th1 cells assist the development of Th2-mediated allergic diseases has not been fully clarified; however, it can be speculated that Th1 cytokines upregulate the expression of adhesion molecules in vessels,^{17 25} and then inflammatory reactions occur in the target organs. As discussed above, this possibility is also the case in our study, since electroporation of IFN- γ upregulated the conjunctival EPR.

In conclusion, we have shown that systemic IL-5 and IFN- γ participate in conjunctival eosinophilia and the EPR, respectively. Although it is apparent that systemic IL-5 upregulates circulating eosinophil levels in the peripheral blood and thereby augments conjunctival eosinophil infiltration, it is not clear how IFN- γ mediates upregulation of the EPR in EC. Further studies are necessary to clarify the role of systemic IFN- γ in the EPR of EC.

Competing interests: None.

Provenance and peer review: Not commissioned; externally peer reviewed.

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