

Chemotactic responses of peripheral blood eosinophils to prostaglandin D₂ in atopic keratoconjunctivitis

Hiroshi Fujishima, MD^{*,†}; Kazumi Fukagawa, MD^{*}; Naoko Okada, MS^{*}; Yoji Takano, MD^{*,†}; Hiroyuki Hirai, PhD[§]; Kinya Nagata, PhD[§]; Ryoichi Hashida, PhD[¶]; Kenji Matsumoto, MD, PhD^{||}; and Hirohisa Saito, MD, PhD^{||}

^{*} Department of Ophthalmology, Keio University School of Medicine, Tokyo, Japan

[†] Department of Ophthalmology, Tsurumi University School of Dental Medicine, Kanagawa, Japan

[§] Department of Ophthalmology, Kitasato Institutional Hospital, Tokyo, Japan

[§] R&D Center, BML, Saitama, Japan

[¶] Patent Group 1, Intellectual Property Department, Eisai Co Ltd, Tsukuba, Japan

^{||} Department of Allergy and Immunology, National Research Institute for Child Health and Development Laboratory, Tokyo, Japan

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ABSTRACT

Background: Eosinophils appear to be key cells in the pathogenesis of conjunctival inflammation in atopic keratoconjunctivitis (AKC). Chemoattractant receptor homologous molecule expressed on T_H2 cells (CRTH2) mediates prostaglandin D₂ (PGD₂)–dependent migration of eosinophils. However, it is unclear whether CRTH2/PGD₂–dependent eosinophil migration is upregulated in allergic diseases.

Objective: To compare the chemotactic responses of peripheral blood eosinophils to prostaglandin D₂ in patients with severe AKC and healthy individuals.

Methods: We used an enzyme immunoassay system to measure PGD₂ levels in tears and blood samples from healthy individuals and patients with AKC. CRTH2 expression on peripheral blood eosinophils was determined using reverse-transcriptase polymerase chain reaction (RT-PCR), flow cytometry, and an oligonucleotide array system. Chemotaxis experiments were performed using a modified Boyden chamber technique and an optical assay system.

Results: The PGD₂ concentrations were higher in tears from patients with severe AKC compared with healthy individuals. RT-PCR (severe and mild cases), flow cytometry (mild cases), and GeneChip analyses revealed a significantly higher expression of CRTH2 on peripheral blood eosinophils in patients with AKC than in healthy individuals. PGD₂ and its stable metabolite 13,14-dihydro-15-keto-PGD₂, a CRTH2 agonist, induced chemotaxis of eosinophils from patients with AKC; chemotaxis was significantly enhanced in eosinophils from patients with severe AKC compared with those from healthy individuals.

Conclusion: CRTH2 is more abundantly expressed on eosinophils from patients with AKC and promoted PGD₂–dependent migration to a greater extent than in healthy individuals.

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Introduction

Eosinophils are implicated in human allergic diseases, such as allergic contact dermatitis and severe ocular allergies.¹ Atopic keratoconjunctivitis (AKC) is a noninfectious inflammatory condition of the chronic ocular surface often experienced by patients with eczema. It may occur during eczema, independent of disease severity.² We previously reported that eosinophils are present in tear fluids of patients with severe AKC, corneal epithelial cells and keratocytes can produce eotaxin, a chemokine specific for

eosinophil recruitment, and that differential regulation of chemokine production from corneal cells induced by interleukin 4 might contribute to selective recruitment of eosinophils to the ocular surface.^{3–5}

Accumulation of eosinophils at the ocular surface involves production of eosinophil chemoattractants at the site of inflammation. Prostaglandin D₂ (PGD₂) is released into tears during episodes of allergic conjunctivitis.^{6–9} PGD₂ is a major metabolite of arachidonic acid produced by mast cells in response to antigen challenge. PGD₂ plays an important role in allergic disorders such as eczema.⁶ Two G protein–coupled receptors for PGD₂, PGD₂ receptor (DP) and chemoattractant receptor homologous molecule expressed on T_H2 (CRTH2),⁷ are expressed on the eosinophil surface. CRTH2 mediates PGD₂–induced eosinophil mobilization.^{8,9} CRTH2 mediates PGD₂–dependent eosinophil migration. The affinity of

Reprints: Hiroshi Fujishima, MD, Department of Ophthalmology, Tsurumi University School of Dental Medicine, 2-1-3, Tsurumi, Tsurumi-ku, Yokohama, Kanagawa 230-8501, Japan; E-mail: fujishima117@gmail.com.

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13,14-dihydro-15-keto-PGD₂ (DK-PGD₂), a PGD₂ metabolite, to CRTH2 is as high as that of PGD₂, whereas the affinity of DK-PGD₂ to DP is at least 600-fold lower than that of PGD₂.⁹ It is unclear whether CRTH2/PGD₂-dependent eosinophil migration is upregulated in allergic diseases.

In this study, we investigated the hypothesis that peripheral blood eosinophils from patients with AKC may express higher levels of CRTH2 than those from healthy individuals and that PGD₂-dependent eosinophil chemotaxis is increased in patients with AKC compared with healthy individuals.

Methods

Study Participants

The study participants were patients with AKC who visited the Department of Ophthalmology at Keio University School of Medicine and had received no prior treatment for AKC at the initial visit. This study was approved with conditions by the Ethics Committee of the Keio University School of Medicine (Tokyo, Japan). The institutional review board (IRB) was concerned about the large blood sample volume required to determine eosinophil characteristics because the number of eosinophils in blood is limited. The IRB also wanted our informed consent forms to address this matter, especially informed consent forms for young children, and to limit the amount of blood drawn to 200 mL or less per study participant and the number of blood drawings to one. Written informed consent was obtained from all study participants or their guardians before participation. We diagnosed AKC as chronic keratoconjunctivitis associated with eczema according to the following criteria: (1) always associated with other atopic eczema; (2) occurred at any time during the associated atopic disease, independent of its severity; and (3) evidence existed for corneal involvement during the disease course. Severe AKC was defined as AKC with severe corneal lesions. Mild AKC was defined as having mild or no corneal complications. The diagnosis of eczema was based on previously reported criteria.¹⁰ The severity of giant papillae formation (GPF) was classified as follows: 3+ and 2+ (giant papillae observed in at least half or less than half the area of the supratarsal conjunctiva, respectively) and 1+ (only short giant papillae present). Affected body surface area (BSA) was quantified. None of the study participants had other systemic diseases. No patients had received systemic corticosteroid therapy before the study, and the use of systemic corticosteroids was prohibited during the study. After completion of the study procedures, participants were treated for AKC with eye drops containing 0.1% dexamethasone, 0.1% fluorometholone, 0.05% cyclosporine A, or cromolyn sodium. Following the instructions of the IRB, one sample of 200 mL or less of blood was drawn per individual; therefore, not all study participants were evaluated for all of the study end points because the amount of eosinophils obtained from each participant was limited.

Serum IgE Determination

The blood was drawn on the day of the first visit, before the commencement of treatment, but many patients with AKC visit the hospital during the rainy season (June and July) in Japan when their AKC tends to exacerbate. The measurement of serum IgE and identification of the allergens were conducted by SRL Inc (Tokyo, Japan) with their verified method. The collected serum samples were kept at 4°C and transported to SRL on the day of sampling and analyzed within 24 hours. Total serum IgE levels were measured using a fluoroenzyme immunoassay (Thermo Fisher Scientific K.K., Yokohama, Japan), and specific IgE antibodies to 26 allergens were measured using the MAST 26 allergen kit.^{11,12} The MAST 26 test contained *Dermatophagoides farinae*; house dust; cat epithelium;

dog epithelium; a mixture of timothy grass, vernal grass, and ragweed pollens; mugwort pollen; Japan cedar pollen; *Penicillium*; *Cladosporium*; *Candida albicans*; *Alternaria*; *Aspergillus*; wheat; soybean; rice; tuna; salmon; shrimp; crab; cheese; milk; beef; chicken; and egg white allergens. In Japan, many patients test positive for mite antigens (No. 26 of the MAST 26), and therefore this antigen is important. The reference level was MAST 0 and the cutoff level was 1.39 luminescent count or less.

Reagents

Eotaxin was from R&D Systems Inc (Minneapolis, Minnesota), and DK-PGD₂ was from Cayman Chemical Company (Ann Arbor, Michigan). The CRTH2-specific rat monoclonal antibody (mAb) BM7 was obtained as previously described.^{7,8}

Eosinophil Isolation

Eosinophils were isolated from stored whole peripheral blood from patients and healthy individuals as follows: (1) mononuclear cells and granulocytes were separated from whole blood by density gradient centrifugation using metrizoate-based medium (Dainippon Sumitomo Pharma Co Ltd, Osaka, Japan)⁸; (2) Percoll-Conray density gradient centrifugation (490g) at room temperature was applied to separate granulocytes from mononuclear cells; (3) noneosinophil granulocytes were collected and destroyed by negative selection using immunomagnetic beads (Miltenyi Biotec GmbH, Cologne, Germany) coated with anti-CD16 mAb¹³; and (4) eosinophils were finally collected by positive selection using magnetic beads. Diff-Quick stain was used to confirm that eosinophil purity was 98% or greater.

Tear Fluid Collection and Detection of PGD₂

Tear fluid ($\geq 30 \mu\text{L}$) from 11 patients with severe AKC, 7 patients with mild AKC, and 6 healthy individuals was collected from both eyes, pooled in a tube, and centrifuged immediately at 4°C; samples were stored at -80°C until assayed. The PGD₂ concentration was measured using PGD₂-MOX EIA (Cayman Chemical Company).

Detection of CRTH2 Expression by RT-PCR

We determined the eosinophil CRTH2 expression using reverse-transcriptase polymerase chain reaction (RT-PCR), flow cytometry, and GeneChip analysis. Total RNA was obtained from isolated eosinophils ($>95\%$ purity) (RNeasy kit; QIAGEN Sciences, Germantown, Maryland) and treated with DNase (Gibco-BRL; Gel Company, San Francisco, California) before undergoing reverse transcription (GeneAmp kit; PerkinElmer Inc, Waltham, Massachusetts). CRTH2 was amplified by PCR (Advantage PCR GC kit; Clontech Laboratories Inc, Mountain View, California) using the primers 5'-CCTCTGTCCCAGACCCCCACGATGTCCGC and 5'-CACGCCAAGAAGTAGGT GAAGAAG. The PCR reaction solution was denatured (94°C for 1 minute) followed by 25 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 68°C for 1 minute. The PCR reactions were analyzed by electrophoresis. CRTH2 messenger RNA (mRNA) transcripts were quantified by RT-PCR in 3 patients with severe AKC (patients 1–3), 3 with mild AKC (patients 12–14), and 3 healthy individuals (participants 19–21).

CRTH2 Detection by Flow Cytometry

All materials were obtained from Becton Dickinson. The stained cells were analyzed on a FACScan flow cytometer. Suspensions of peripheral blood eosinophils (1×10^5 cells) were preincubated with human IgG (ICN Biomedicals Inc, Santa Ana, California) and incubated with biotinylated CRTH2-specific mAb BM7 or with conjugated biotinylated rat IgG as a control (Fab fractions; BioSource International Inc, Somerset, New Jersey). The samples were then

Table 1
Characteristics of the study participants^a

Patient/participant No.	Sex	Age, y	% of BSA affected	GPF intensity	Corneal complication	Total IgE, U/mL	Specific IgE, No. of allergens
Patients with severe AKC							
1	M	9	70	+++	Ulcer	4,400	21
2	M	38	60	++	Ulcer	6,600	11
3	M	18	60	+++	Ulcer	14,000	9
4	M	34	40	+	Ulcer	3,000	10
5	M	7	40	+++	Ulcer	10,000	7
6	M	24	40	+++	Ulcer	3,300	7
7	F	11	40	++	Ulcer	990	9
8	F	7	20	++	Ulcer	2,200	7
9	M	9	70	+++	Ulcer	14,400	11
10	M	18	60	++	Ulcer	5,600	11
11	M	8	40	+++	Ulcer	12,400	9
Mean ± SD (median)		16.6 ± 11.0	49.0 ± 15.7			12,815 ± 4,895 (6,100)	10.1 ± 3.9
Patients with mild AKC							
12	F	31	60	—	SPK	4,100	7
13	F	28	40	—	SPK	640	4
14	F	24	10	—	SPK	250	3
15	M	33	10	+	—	92	4
16	F	21	60	—	SPK	4,100	7
17	M	8	20	—	—	25	0
18	M	12	30	—	SPK	4500	7
Mean ± SD		22.4 ± 9.4	32.8 ± 21.3			1,958 ± 2,148 (640)	4.5 ± 2.6
Healthy individuals							
19	M	38	0	—	—	25	0
20	M	32	0	—	—	45	0
21	M	22	0	—	—	25	0
22	M	27	0	—	—	0	0
23	F	16	0	—	—	43	0
24	M	18	0	—	—	25	0
Mean ± SD		25.5 ± 8.4				27.1 ± 16.2	

Abbreviations: BSA, body surface area; GPF, giant papillae formation; SPK, superficial punctate keratitis.

^aThe extent (severity) of GPF is represented by a plus sign. Negative areas or absence of complications is represented by a minus sign. Median values are shown in parentheses.

incubated with phycoerythrin-labeled streptavidin. CRTH2 eosinophil expression was quantified by the mean fluorescence intensity of the positive cell population. Flow cytometry was performed in 4 patients with severe AKC (patients 2–4 and 6), 4 with mild AKC (patients 12–15), and 3 healthy individuals (participants 19–21).

CRTH2 Detection by GeneChip Expression Analysis

Human genome-wide gene expression was examined using the U95A probe array (GeneChip; Affymetrix Inc, Santa Clara, California).¹⁴ Total RNA (3–10 µg) was extracted from approximately 10⁷ cells from 4 patients with severe AKC (patients 8–11), 2 patients with mild AKC (patients 17 and 18), and 2 healthy individuals (participants 23 and 24).

Chemotaxis Assay

The chemotactic response of eosinophils was determined in multiwell chambers fitted with 3-µm-pore polycarbonate filters (Chemotacell; Kurabo Industries Ltd, Osaka, Japan).¹⁵ The eosinophil suspension (60 µL; 1 × 10⁶ cells/mL) was placed in the upper chamber; 0.1 mL of culture medium with or without PGD₂ was added to the lower chamber. The chemotaxis chambers were incubated at 37°C for 30 minutes under 5% carbon dioxide. The fluid was then aspirated from the lower compartment and the chamber disassembled.

The migrated eosinophils on the lower surface were counted by determining eosinophil peroxidase activity.¹⁶ The remaining cell suspension was incubated at 37°C and the cells counted to construct a standard curve. For confirmation, another chemotaxis assay was conducted at room temperature using an assembled KK-chamber (TAXIScan; GE Healthcare K.K., Tokyo, Japan) containing 2 sets of 6 channels. Medium was added to the common space at the upper end of each channel, and 1 µL of culture medium containing

eosinophils was injected into 1 of the 2 compartments. The cells began to flow after drawing 5.3 µL of medium from the common space to the vicinity of the microchannel. The drawing rate of the medium was determined by the flow rate of the cells, which was consistently less than 40 µm/s. Then, 1 µL of culture medium containing specific concentrations of PGD₂ was injected into the compartment opposite the compartment containing the cells. Cell migration in the channel was monitored using a charge-coupled device video camera. Images of the cells in 6 channels per experiment were recorded at timed intervals using a charge-coupled device camera with coaxial episcopic illumination (Fit; Suwa, Nagano, Japan).¹⁷ The PGD₂ and eotaxin potencies for inducing eosinophil chemotactic responses were compared. The results were expressed as the number of cells that migrated to the opposite side of the channel. The chemotaxis assay was performed using eosinophils from 5 patients with severe AKC (patients 2–4, 6, and 9) and 5 healthy individuals (participants 20–24).

Statistical Analysis

In this study, the sample size was not the mean the number of blood samples per patient but rather the number of patients (eg, n = 4 means blood samples from 4 patients not 4 samples per patient). Chemotaxis in each patient was examined in triplicate, and the value of the second determination was used for analysis. Data are expressed as absolute values, means ± SD, and medians. Data were analyzed using analysis of variance, the Welch *t* test, or the unpaired 2-tailed *t* test. *P* < .05 was considered significant.

The antigen specific IgE was analyzed by counting the number of positive antigens and calculating the mean number of positive antigens for the severe AKC, mild AKC, and healthy groups (indicated by means ± SD). These means were compared by the *t* test.

Results

We were limited by the IRB to collecting one blood sample of 200 mL or less from each participant. The amount of eosinophils in 200 mL or less of whole blood was not enough to evaluate all the study end points, and therefore the number of individuals for each analysis or evaluation varied. Owing to this limitation, sample size does not mean the number of measurements per sample but rather the number of different samples or study participants, except in reference to eosinophil chemotaxis.

Participant Characteristics

The participant characteristics are listed in Table 1. Eleven patients with severe AKC, 7 patients with mild AKC, and 6 healthy participants were enrolled. No significant differences in age were found among the 3 groups. The difference in BSA between patients with severe ($49.0\% \pm 15.7\%$) and mild ($32.8\% \pm 21.3\%$) AKC was not significant ($P = .08$). All patients with severe AKC had greater GPF severity than patients with mild AKC and healthy participants. The patients with AKC had elevated total serum IgE and allergen specific IgE antibodies against multiple allergens. The mean total IgE level was significantly greater in patients with severe AKC ($6,990 \pm 4,895$ U/mL; $n = 11$) than in patients with mild AKC ($1,958 \pm 2,148$ U/mL; $n = 7$; $P = .009$). The mean number of positive specific IgEs was significantly greater in patients with severe AKC (10.1 ± 3.9 antigens; $n = 11$) than in patients with mild AKC (4.5 ± 2.6 antigens; $n = 11$; $P = .004$). Healthy participants had no history of AKC diseases; their total serum IgE level (27.1 ± 16.2 U/mL; $n = 6$) and number of antigen specific IgEs (all zero) were within normal ranges.

PGD₂ in Tear Fluids

The participants' tear PGD₂ concentrations are shown in Figure 1. The tear PGD₂ concentration was significantly higher in patients with severe AKC (753.6 ± 729.0 pg/mL, $P = .03$, $n = 11$) than in healthy individuals (27.9 ± 5.0 pg/mL, $n = 6$).

CRTH2 Expression on Blood Eosinophils

Only a small number of patients gave their informed consent for blood sampling; therefore, a fewer number of blood samples were collected to determine CRTH2 expression. RT-PCR revealed significantly higher eosinophil expression of CRTH2 in patients with AKC compared with those from healthy individuals. Densitometric analysis of bands normalized for β -actin expression revealed that CRTH2 mRNA expression was comparable in patients with mild and severe AKC, and CRTH2/ β -actin expression in both groups with AKC (mild: $P = .03$, $n = 3$, patients 12–14; severe: $P = .008$, $n = 3$, patients 1–3) was significantly greater than in healthy individuals ($n = 3$) (eFig 1). Fluorescence-activated cell sorter (FACS) analysis revealed higher numbers of eosinophils expressing CRTH2 in patients with severe AKC ($n = 3$, patients 1–3) than in healthy individuals ($n = 3$, participants 19–21). Representative FACS histograms are shown in eFigure 2. On the basis of mean fluorescence intensity, the surface expression of CRTH2 relative to IgG was significantly greater in patients with mild AKC (3.17 ± 0.537 , $P = .005$, $n = 4$) compared with healthy individuals (1.60 ± 0.185 , $P = .08$, $n = 3$), whereas no difference was found between patients with severe AKC (3.16 ± 1.232 , $n = 4$, patients 2–4 and 6) and healthy individuals (eFig 3). Gene expression was adjusted for expression of housekeeping genes by referring to internal standards. CRTH2 mRNA expression was examined using the GeneChip system and was more than 3-fold higher in patients with AKC than in healthy individuals (eFig 4).

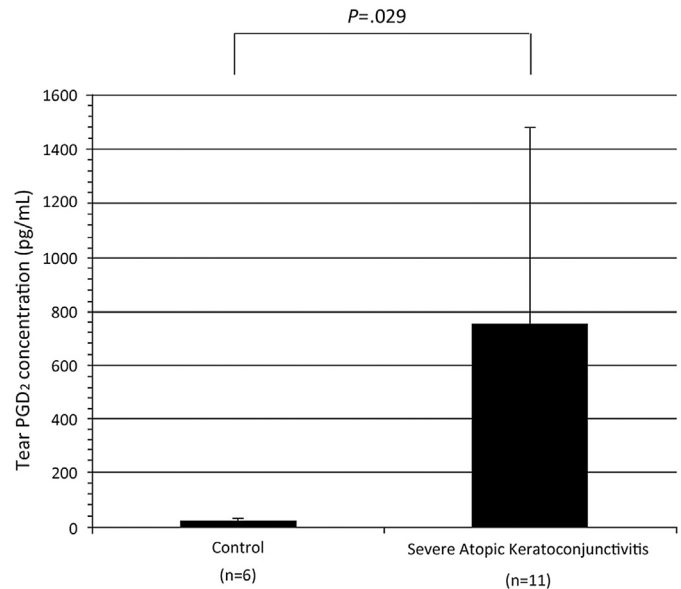


Figure 1. Detection of tear prostaglandin D₂ (PGD₂) concentrations in healthy individuals and patients with severe atopic keratoconjunctivitis (AKC). Tear PGD₂ levels were significantly higher in patients with severe AKC (median, 556 pg/mL; $P = .03$; $n = 11$) than in healthy individuals (median, 28.7 pg/mL; $n = 6$).

Eosinophil Counts and Chemotactic Responses to PGD₂

The effect of PGD₂ on the chemotaxis of peripheral blood eosinophils was compared between patients with severe AKC ($n = 2$) and healthy individuals ($n = 4$). Chemotaxis of unstimulated eosinophils from patients with severe AKC was not much different than that of chemoattractant-treated eosinophils from healthy individuals, whereas eosinophils from patients revealed only a weak chemotactic response to PGD₂, suggesting that the eosinophils from patients had already been stimulated in vivo. Treating eosinophils with 1,000 ng/mL of PGD₂ had comparable effects to treatment with 10 ng/mL of eotaxin (Fig 2). The chemotactic index of PGD₂-stimulated eosinophils to unstimulated controls was determined for patients with severe AKC and healthy individuals. An index of 1.0 indicates no net cell migration in response to the stimulus. After treatment with 100 ng/mL of PGD₂, the chemotactic index was significantly greater in patients with severe AKC ($7.08\% \pm 2.29\%$, $P = .04$) than in healthy individuals ($2.96\% \pm 1.56\%$).

The chemoattractant effect of DK-PGD₂, a metabolic product of PGD₂ that acts as a selective CRTH2 agonist, was almost equivalent to that of PGD₂ in eosinophils from patients with severe AKC (eFig 2). The effects of 100 and 1,000 ng/mL of DK-PGD₂ ($n = 4$ and $n = 5$, respectively) were not significantly different ($P = .07$).

Optical Assay System

Medium containing PGD₂ (0, 30, 100, 300, 1,000, or 3,000 ng/mL) was injected into the opposite compartment, and the eosinophils that migrated toward the middle of the channel were counted. The number of migrated eosinophils is shown in eFigure 5, and eFigure 7 shows the means and SD for 4 patients. Eosinophils from patients with AKC had greater migration in response to 10, 100, and 1,000 ng/mL of PGD₂ compared with eosinophils from healthy individuals (Fig 2). The effects of 100 or 1,000 ng/mL DK-PGD₂ were not significantly different ($P = .07$; eFig 5). The number of eosinophils that reached the middle of opposite side of the channel was scored as follows: 0 for 0 to 5 cells, 0.5 for 6 to 10 cells, 1.0 for 11 to 20 cells, 2.0 for 21 to 30 cells, and 3.0 for 31 cells or more. The scores for patients with AKC ($n = 7$ per dose) increased significantly as PGD₂ increased from 30 to 1,000 ng/mL, when compared with scores for

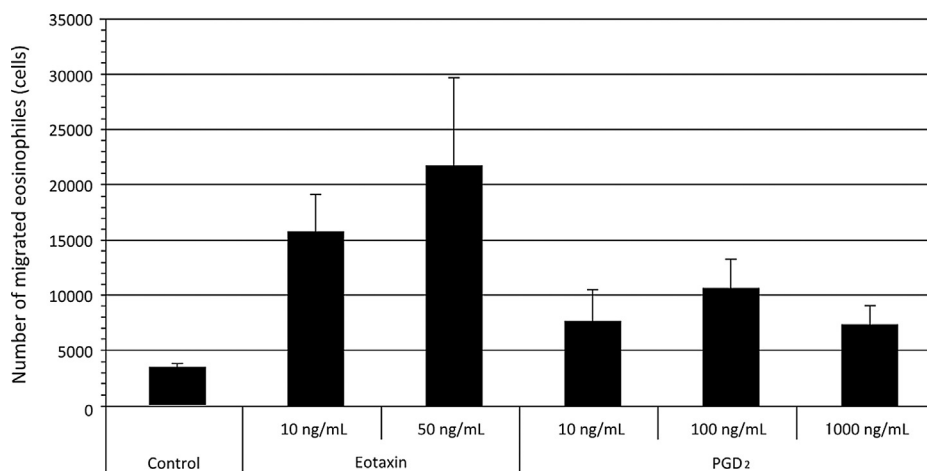


Figure 2. Induction of blood eosinophil chemotaxis by eotaxin and prostaglandin D₂ (PGD₂). Numbers of blood eosinophils that migrated to the middle of the opposite side of the channel are shown. The number of blood eosinophils from patients with atopic keratoconjunctivitis (number of study participants = 5 per dose) that migrated to the middle of the opposite side of the channel in response to 10 to 1,000 ng/mL of PGD₂ was significantly increased, compared with the number of blood eosinophils from healthy participants (number of study participants = 5 per dose; $P < .001$; analysis of variance).

healthy individuals ($n = 6$ per dose; $P < .001$, analysis of variance) and independent groups ($P < .001$). However, the scores at 3,000 ng/mL of PGD₂ were not significantly different among the groups ($P = .38$; Fig 2). Eosinophil chemotaxis was recorded with an EZ-TAXIScan at 30-second intervals for 30 to 60 minutes (eFig 6). The images showed different locomotion patterns of eosinophil chemotaxis in response to PGD₂. For example, panels 2 and 3 show migration of multiple cells toward PGD₂ using eosinophils from patients with severe AKC, indicating marked variation in cell sensitivity to the chemoattractant.

Discussion

We focused on the immunologic responses involved in eosinophil chemotaxis in atopic disease because activated T_H2-like cells are involved in local infiltration and activation of eosinophils.¹⁸ We detected greater PGD₂ concentrations in tear fluid from patients with AKC compared with healthy individuals, suggesting that PGD₂ is involved in atopic diseases such as AKC (Fig 1). CRTH2 mRNA was detected in peripheral blood eosinophils from healthy individuals and patients with AKC. However, eosinophil CRTH2 expression was more intense in patients with mild or severe AKC than in healthy individuals (eFigs 1–4). To our knowledge, this is the first report indicating that CRTH2 mRNA and protein expression in eosinophils is greater in patients with AKC than in healthy individuals. Eosinophil CRTH2 expression was not significantly different between patients with mild or severe AKC, prompting the conclusion that AKC severity is unrelated to the extent of eosinophil CRTH2 expression. CRTH2 might contribute to the pathogenesis of atopic disease, as shown in this study, possibly via enhanced PGD₂-mediated eosinophil chemotaxis or other relevant effectors. Human eosinophils express both DP and CRTH2 receptors at the mRNA level,¹⁹ and PGD₂ shows greater affinity for CRTH2 than for DP.²⁰

CRTH2 expression relative to IgG was significantly greater in the blood eosinophils from patients with mild AKC compared with healthy individuals but not from patients with severe AKC (eFig 3). This apparent paradox could be explained by chemotaxis of blood eosinophils with upregulated expression in patients with severe AKC and then their removal from the circulation, leaving only blood eosinophils without upregulated expression.

From these results, we hypothesize that PGD₂ in conjunction with CRTH2 might control eosinophil function and therefore have an important role in AKC. We found a marked difference in

chemotaxis of eosinophils from patients with AKC and healthy individuals because the former had a significant dose-dependent chemotactic response to PGD₂ in the range of 10 to 1,000 ng/mL. As reported by Nitta et al,²¹ eosinophil chemotaxis was confirmed to be higher in eosinophils from patients with AKC than those from healthy individuals using an optical assay system. Our results suggest that PGD₂ could stimulate chemotaxis (directed migration toward a chemotactic factor) rather than chemokinesis (random locomotion in the presence of chemical agents). DK-PGD₂ is a stable PGD₂ metabolite that also acts as a CRTH2 selective agonist. DK-PGD₂ also induces eosinophil chemotaxis in healthy individuals,⁹ and we found that DK-PGD₂ induced marked eosinophil chemotaxis in patients with severe AKC. Interestingly, the chemotactic activity of 1,000 ng/mL of DK-PGD₂ was not significantly different from that of 100 ng/mL of PGD₂ (eFigs 3 and 4). The CRTH2-specific mAb BM7 but not the control rat IgG suppressed the migratory responses of eosinophils to PGD₂, including spontaneous migration induced by PGD₂.⁹ These results strongly support the hypothesis that the chemotactic effect of PGD₂ is predominantly mediated via CRTH2 signaling.

We previously studied PGD₂/CRTH2 eosinophil chemotaxis using CD16⁺ eosinophils and found striking differences in terms of PGD₂-mediated migration between AKC and normal eosinophils. It is difficult to purify CD16⁺ eosinophils, and even when positive selection of CD9 was performed, the eosinophils were activated.^{22,23} We also detected migratory differences in CD16⁺ eosinophils, but further studies are needed to examine the differences in disease effects and chemotactic responses between CD16⁺ and CD16⁺ eosinophils.

The mechanism underlying increased CRTH2 expression is still unknown; however, studying CRTH2 expression on eosinophils induced by PGD₂ may provide insight into the pathogenesis of atopic disease. Our focus is now on factors that may potentially activate CRTH2, leading to chemotaxis of T_H2 cells, basophils, and eosinophils, which show high CRTH2 expression.⁸ CRTH2 is the most reliable marker for monitoring the T_H1 and T_H2 balance in any tissue and in some pathologic conditions. The T_H1/T_H2 balance is important for controlling the immune balance within the body and for effective immune responses to invading pathogens.

These results suggest that CRTH2 is an important receptor on eosinophils, which accumulate in sites of inflammation in patients with AKC and related diseases. Determination of CRTH2 expression on eosinophils might help with the diagnosis and evaluation of

prognosis of AKC. PGD₂-mediated inflammatory responses involved in eosinophil activation, eosinophil accumulation at sites of allergic reactions through CRTH2 activation, and the effects of PGD₂-induced CRTH2 activation were completely inhibited by Ramatroban, which is currently the only CRTH2 antagonist available in Japan for treating allergic rhinitis. Thus, clinical information on CRTH2 antagonists is currently limited to Ramatroban data.²⁴ Therapeutic anti-CRTH2 agents with a novel mechanism of action that downregulate CRTH2 expression may become a viable treatment alternative for AKC.

The limitation of this study is that no group of patients with eczema without AKC was included for comparison. Such a group would be required to confirm our results more precisely because increased CRTH2 expression has been reported in eczema, asthma, and the nose of allergic rhinitis patients. Although participants in this study were all ophthalmology outpatients, it was difficult to establish a group of patients with eczema only. This comparison will be the focus of future investigation.

In summary, our study found that CRTH2 is more abundantly expressed on peripheral blood eosinophils obtained from patients with AKC compared with healthy individuals. PGD₂ plays important roles in AKC reactions, including eosinophil infiltration at sites of inflammation. CRTH2 antagonists may therefore provide a novel therapeutic strategy to control allergic ocular diseases.

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Supplementary Data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.anai.2013.05.027>

References

- [1] Takano Y, Fukagawa K, Dogru M, et al. Inflammatory cells in brush cytology samples correlate with the severity of corneal lesions in atopic keratoconjunctivitis. *Br J Ophthalmol*. 2004;88:1504–1505.
- [2] Calonge M, Herreras JM. Clinical grading of atopic keratoconjunctivitis. *Curr Opin Allergy Clin Immunol*. 2007;7:442–445.
- [3] Fukagawa K, Tsubota K, Shimura S, et al. Chemokine production in conjunctival epithelial cells. *Adv Exp Med Biol*. 1998;438:471–478.
- [4] Takano Y, Fukagawa K, Shimura S, et al. IL-4 regulates chemokine production induced by TNF-alpha in keratocytes and corneal epithelial cells. *Br J Ophthalmol*. 1999;83:1074–1076.
- [5] Fujishima H, Fukagawa K, Okada N, et al. Prostaglandin D2 induces chemotaxis in eosinophils via its receptor CRTH2 and eosinophils may cause severe ocular inflammation in patients with allergic conjunctivitis. *Cornea*. 2005;24:S66–S70.
- [6] Matsuoka T, Hirata M, Tanaka H, et al. Prostaglandin D2 as a mediator of allergic asthma. *Science*. 2000;287:2013–2017.
- [7] Nagata K, Tanaka K, Ogawa K, et al. Selective expression of a novel surface molecule by human Th2 cells in vivo. *J Immunol*. 1999;162:1278–1286.
- [8] Nagata K, Hirai H, Tanaka K, et al. CRTH2, an orphan receptor of T-helper-2 cells, is expressed on basophils and eosinophils and responds to mast cell-derived factor(s). *FEBS Lett*. 1999;459:195–199.
- [9] Hirai H, Tanaka K, Yoshie O, et al. Prostaglandin D2 selectively induces chemotaxis in T helper type 2 cells, eosinophils, and basophils via seven-transmembrane receptor CRTH2. *J Exp Med*. 2001;193:255–261.
- [10] Ricci G, Dondi A, Patrizi A. Useful tools for the management of atopic dermatitis. *Am J Clin Dermatol*. 2009;10:287–300.
- [11] Fujishima H, Sahashi N, Shimazaki J, Tsubota K. Allergic conjunctivitis caused by sugi (*Cryptomeria japonica* D. Don) pollen out of season. *Asian Pac J Allergy Immunol*. 1995;13:113–117.
- [12] Dogru M, Matsumoto Y, Okada N, et al. Alterations of the ocular surface epithelial MUC16 and goblet cell MUC5AC patients with atopic keratoconjunctivitis. *Eur J Allergy Clin Immunol*. 2008;63:1324–1334.
- [13] Hansel TT, De Vries IJ, Iff T, et al. An improved immunomagnetic procedure for the isolation of highly purified human blood eosinophils. *J Immunol Methods*. 1991;145:105–110.
- [14] Matsumoto K, Fukuda S, Nakamura Y, et al. Amphiregulin production by human eosinophils. *Int Arch Allergy Immunol*. 2009;149(suppl 1):39–44.
- [15] Kusugami K, Ando T, Ohsuga M, et al. Mucosal chemokine activity in *Helicobacter pylori* infection. *J Clin Gastroenterol*. 1997;25(suppl 1):S203–S210.
- [16] Strath M, Sanderson CJ. Production and functional properties of eosinophils from bone marrow cultures. *J Cell Sci*. 1985;74:207–217.
- [17] Kanegasaki S, Nomura Y, Nitta N, et al. A novel optical assay system for the quantitative measurement of chemotaxis. *J Immunol Methods*. 2003;282:1–11.
- [18] Kaminuma O, Fujimura H, Fushimi K, et al. Humoral immunity is dispensable for the local recruitment of antigen-specific Th2 cells. *Int Arch Allergy Immunol*. 2001;125(suppl 1):17–18.
- [19] Gervais FG, Cruz RP, Chateaufort A, et al. Selective modulation of chemokinesis, degranulation, and apoptosis in eosinophils through the PGD2 receptors CRTH2 and DP. *J Allergy Clin Immunol*. 2001;108:982–988.
- [20] Sugimoto H, Shichijo M, Iino T, et al. An orally bioavailable small molecule antagonist of CRTH2, Ramatroban (BAY u3405), inhibits Prostaglandin D2-induced eosinophil migration in vitro. *J Pharmacol Exp Ther*. 2003;305:347–352.
- [21] Nitta N, Tsuchiya T, Yamauchi A, et al. Quantitative analysis of eosinophil chemotaxis tracked using a novel optical device-TAXIScan. *J Immunol Methods*. 2007;320:155–163.
- [22] Kim JT, Gleich GJ, Kita H. Roles of CD9 molecules in survival and activation of human eosinophils. *J Immunol*. 1997;159:926–933.
- [23] Matsumoto K, Bochner BS, Wakiguchi H, et al. Functional expression of transmembrane 4 superfamily molecules on human eosinophils. *Int Arch Allergy Immunol*. 1999;120(suppl 1):38–44.
- [24] Shirasaki H, Kikuchi M, Kanaizumi E, et al. Accumulation of CRTH2-positive leucocytes in human allergic nasal mucosa. *Ann Allergy Asthma Immunol*. 2009;102:110–115.

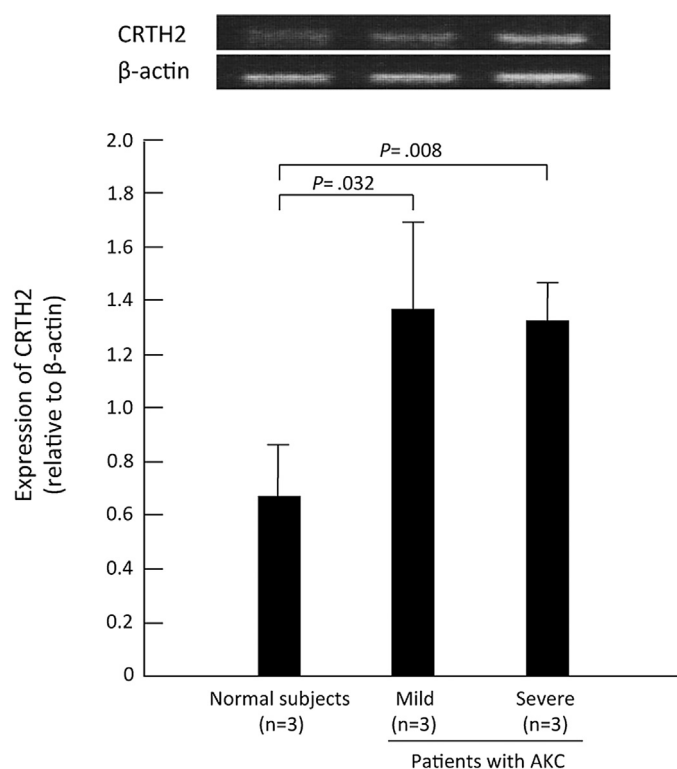


Figure 1. Analysis of expression of chemoattractant receptor homologous molecule expressed on T_H2 cells (CRTH2) on peripheral blood eosinophils by reverse-transcriptase polymerase chain reaction. The expression of CRTH2 relative to β -actin was quantified by densitometry. CRTH2 messenger RNA expression was significantly higher in both groups with atopic keratoconjunctivitis (AKC) (mild: $P = .03$, $n = 3$, patients 12-14; severe: $P = .008$, $n = 3$, patients 1-3) than in healthy individuals ($n = 3$). An unpaired 2-tailed t test was conducted for each comparison.

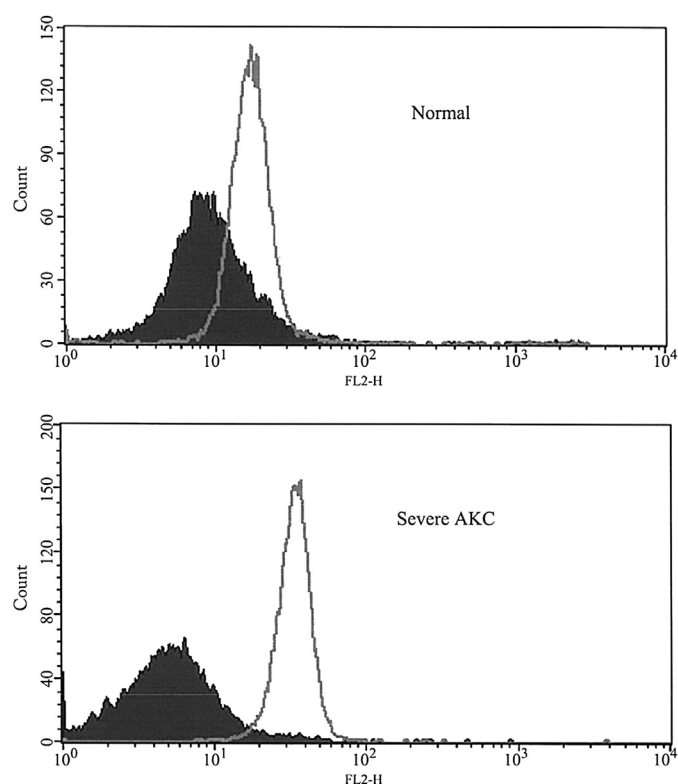


Figure 2. Representative histograms of flow cytometry (fluorescence activated cell sorting [FACS]) of eosinophils expressing chemoattractant receptor homologous molecule expressed on T_H2 cells (CRTH2) in patients with severe atopic keratoconjunctivitis (AKC) and healthy individuals. Data were collected on the FL2-H (green) FACS channel. Analysis revealed higher numbers of eosinophils expressing CRTH2 in patients with severe AKC ($n = 3$, patients 1-3) than in healthy individuals ($n = 3$, participants 19-21).

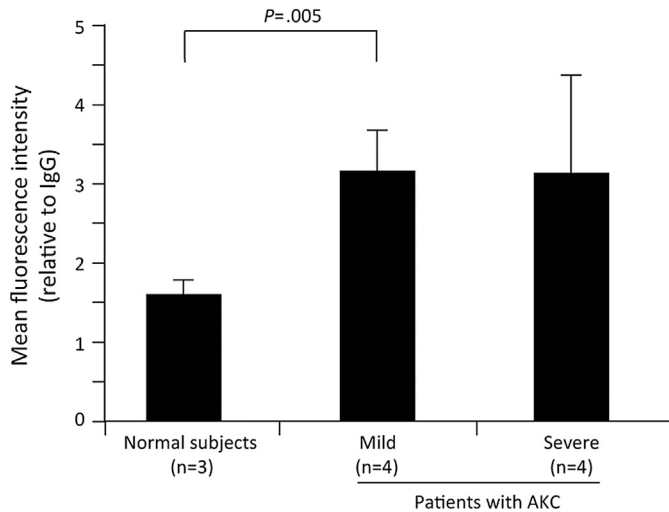


Figure 3. Mean fluorescence intensity in healthy individuals and patients with atopic keratoconjunctivitis (AKC). The surface expression of chemoattractant receptor homologous molecule expressed on T_H2 cells (CRTH2) relative to control IgG was significantly greater in patients with mild AKC (3.17 ± 0.537 , $P = .005$, number of participants = 4) compared with healthy individuals (1.60 ± 0.185 , $P = .08$, number of participants = 3), whereas no significant difference was found between patients with severe AKC (3.16 ± 1.232 , number of participants = 4, patients 2–4 and 6) and healthy individuals. An unpaired 2-tailed t test was conducted for each comparison.

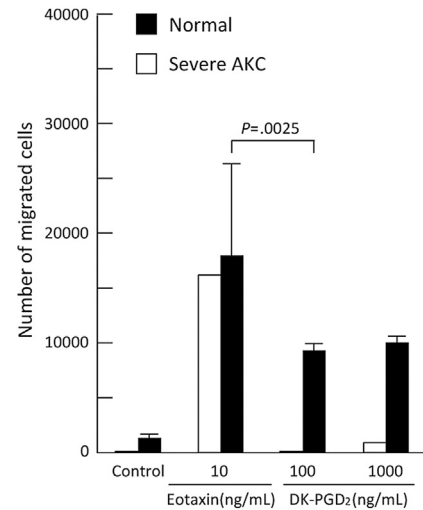


Figure 5. Induction of blood eosinophil chemotaxis by eotaxin or 13,14-dihydro-15-keto-prostaglandin D_2 (DK-PGD₂). The mean number of migrating eosinophils in 4 chemotactic chambers was determined. The effect of DK-PGD₂ at either 100 or 1,000 ng/mL was not statistically different ($n = 5$ each, $P = .07$).

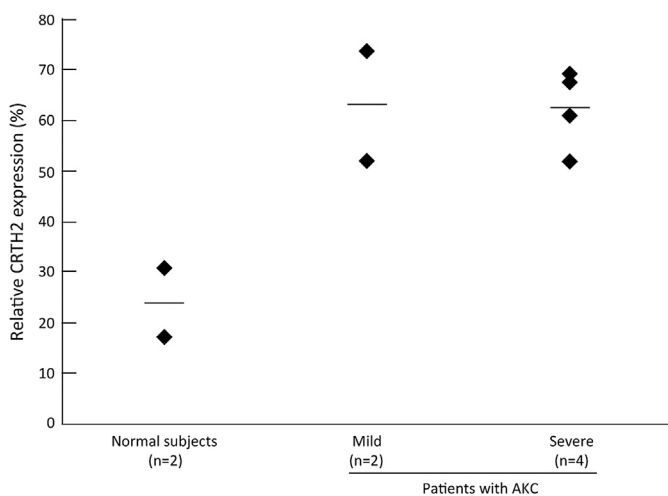
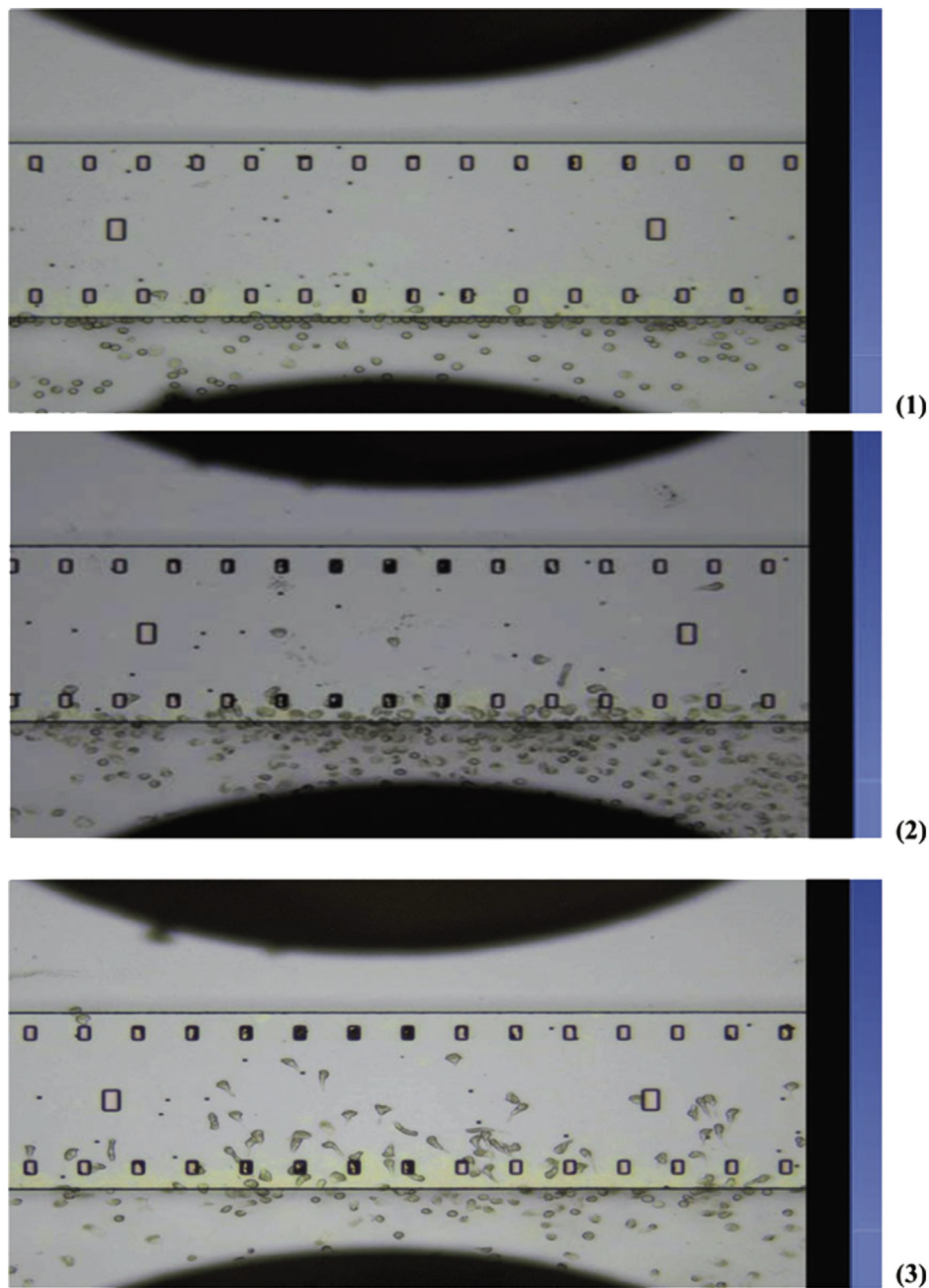


Figure 4. GeneChip analysis of expression of chemoattractant receptor homologous molecule expressed on T_H2 cells (CRTH2) in healthy individuals and patients with atopic keratoconjunctivitis (AKC). The expression was normalized to the expression of the internal housekeeping gene. The messenger RNA expression of CRTH2 was more than 3-fold greater in patients with mild ($n = 2$) or severe ($n = 4$) AKC than in healthy individuals ($n = 2$).



eFigure 6. Representative images obtained with the EZ-TAXIScan to demonstrate the different patterns of eosinophil locomotion in response to prostaglandin D₂ (PGD₂). Panel 1 shows blood eosinophils (1 μ L of 2×10^6 cells/mL) from healthy volunteers in the lower compartment and 1 μ L of culture medium without PGD₂ in the upper compartment. Panels 2 and 3 show examples of blood eosinophils from patients with severe atopic keratoconjunctivitis as they migrate toward the chemoattractant, PGD₂ (100 ng/mL).

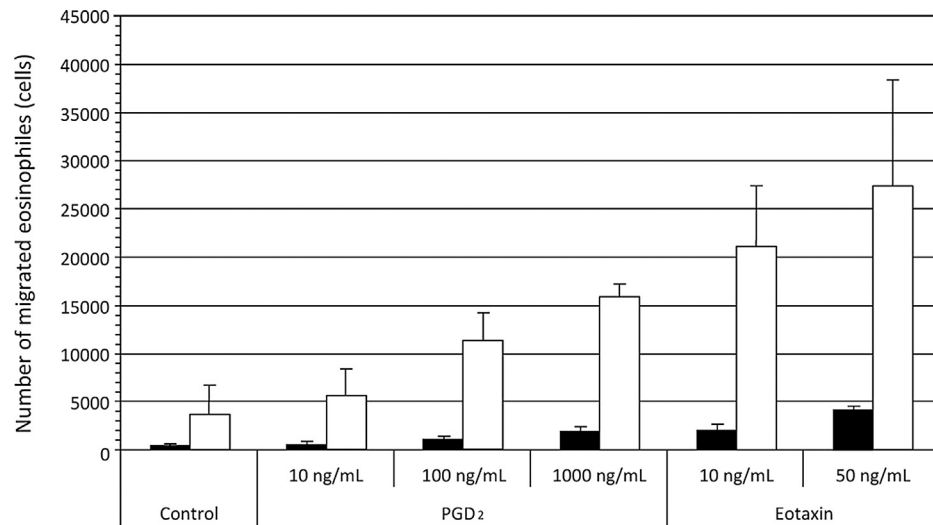


Figure 7. Effect of prostaglandin D₂ (PGD₂) and eotaxin on the chemotaxis of peripheral blood eosinophils from patients with severe atopic keratoconjunctivitis (AKC) and from healthy individuals. The number of migrated eosinophils is shown. No significant difference exists between unstimulated eosinophils from patients with severe AKC (control; n = 5) and chemoattractant-positive eosinophils from healthy individuals (n = 5), whereas eosinophils from patients had only a weak chemotactic response to PGD₂, suggesting that the eosinophils from patients had already been stimulated in vivo. Eosinophils from patients with AKC (compared with eosinophils from healthy individuals) showed significantly greater migration in response to 10, 100, and 1,000 ng/mL of PGD₂. An unpaired 2-tailed *t* test was conducted for each comparison.