

Original article

CRTH2 mediates the activation of human Th2 cells in response to PGD₂ released from IgE/anti-IgE treated nasal polyp tissue

Background: Mast cells release mediators upon stimulation that contribute to the pathogenesis of chronic airway disease, including the recruitment and activation of Th2 lymphocytes. The objective was to determine the involvement of prostaglandin D₂ (PGD₂) and its receptors in the chemotaxis of Th2 cells, using nasal polyp tissue.

Methods: Tissue explants from ten patients with nasal polyposis were incubated with RPMI alone or RPMI containing IgE/anti-IgE for 30 min. Some samples were treated with diclofenac to inhibit the production of PGD₂. Supernatants were assayed for PGD₂ content and for their ability to promote human Th2 cell chemotaxis in the presence and absence of a CRTH2 antagonist. Transcript levels of D prostanoid receptor type 1 (DP₁), chemoattractant receptor-homologous receptor expressed on Th2 cells (CRTH2) and PGD₂ synthase were analysed by real time PCR.

Results: Increased release of PGD₂ by nasal polyp tissue treated with IgE/anti-IgE was significantly inhibited by preincubation of the tissue with diclofenac. Transcript levels of PGD₂ synthase, DP₁ and CRTH2 receptors increased after stimulation with IgE/anti-IgE. Supernatants from IgE/anti-IgE-stimulated nasal polyp tissue caused significantly increased chemotaxis of Th2 cells. The levels of PGD₂ produced and the degree of Th2 cell chemotaxis were highly correlated. Diclofenac inhibited the production of Th2 cell chemotactic activity, and the chemotactic effect of the supernatant on Th2 cells was inhibited by the CRTH2 antagonist ramatroban.

Conclusion: These data suggest that in immunologically activated nasal polyp tissue, PGD₂ produced by mast cells promotes the migration of Th2 cells through a CRTH2 dependent mechanism.

C. A. Pérez-Novo¹, G. Holtappels¹,
S. L. Vinall², L. Xue², N. Zhang¹,
C. Bachert¹, R. Pettipher²

¹Upper Airways Research Laboratory, Department of Otorhinolaryngology, Ghent University Hospital, Ghent, Belgium; ²Oxagen Ltd, Abingdon, Oxon, UK

Key words: anti-IgE; chemotaxis; CRTH2; Prostaglandin D₂; Th2 cells; upper airways.

Dr R. Pettipher
Oxagen Ltd
91 Milton Park
Abingdon
Oxon
OX14 4RY
UK

Accepted for publication 2 September 2009

Accumulating evidence places mast cells in a position of importance in the pathogenesis of airway disease including chronic rhinosinusitis with nasal polyps (1–3). Mast cells contribute to inflammation directly through the release of inflammatory mediators, cytokines and growth factors and indirectly through the activation of structural cells (3–6). They also play a key role in the induction and/or maintenance of eosinophilic inflammation by a variety of mechanisms, including IgE-dependent and IgE-independent processes (7–9).

Prostaglandin D₂ (PGD₂) is the major mast cell-derived prostanoid, being released in response to IgE-mediated activation of these cells (10). Inhalation

challenge of allergic asthmatic subjects has been shown to increase the levels of this eicosanoid in the bronchoalveolar lavage fluid (11) and in the nasal lavages of patients with allergic rhinitis (12). Prostaglandin D₂ mediates its biological effects through high affinity interactions with the seven transmembrane G-protein coupled receptors D prostanoid receptor type 1 (DP₁) and chemoattractant receptor-like molecule expressed on Th2 cells (CRTH2, also known as DP₂). D prostanoid receptor type 1 is expressed by epithelial goblet cells in nasal and colonic mucosa, serous glands, vascular endothelium, dendritic cells and eosinophils (13). CRTH2 is expressed by CD4⁺ Th2 cells, eosinophils and basophils and mediates chemotaxis of these cell-types in response to PGD₂ (14, 15). Prostaglandin D₂ also stimulates Th2 cells to elaborate cytokines by an action on CRTH2 (16).

In nasal polyp tissue, expression of PGD₂ synthase has been observed in mast cells and inflammatory cells infiltrating the inflamed tissue (13). Localization of DP₁ was observed in vascular endothelial cells and it has been

Abbreviations: COX-1, cyclo-oxygenase-1; COX-2, cyclo-oxygenase-2; CRTH2, chemoattractant receptor-homologous molecule expressed on Th2 cells; HDM, house dust mite; IgE, immunoglobulin E; NP, nasal polyps; IL, interleukin; DP₁, D prostanoid receptor type 1; TP, thromboxane receptor.

proposed to contribute to nasal congestion and the concomitant increase in nasal airway resistance by dilating blood vessels in the nasal mucosa (13). Additionally, this receptor was also expressed on mucus-producing epithelial goblet cells and serous glands suggesting its role in mucus production, which also contributes to nasal airway resistance (13). Furthermore, CRTH2 was also detected in eosinophils, and T-cells invading the nasal mucosa, providing evidence for the facilitation of local entry of eosinophils and Th2 cells into the tissue and the promotion of inflammation (13). Instillation of PGD₂ into the airways promotes eosinophil accumulation, an action that is mimicked by selective CRTH₂ agonists, but not by DP₁ agonists (17), and inhibited by CRTH2 antagonists (18). In addition to promoting increased migration of leucocytes to sites of allergic inflammation, release of PGD₂ in response to allergen exposure can also lead to production of Th2 cytokines including IL-4, IL-5 and IL-13 (19). Studies in CRTH2 knockout mice have implicated CRTH2 in mediating nasal eosinophilia, increased production of Th2 cytokines and antigen-specific IgE/IgG1 in a model of cypress Cry j1-induced pollinosis (20), effects mimicked by small molecule CRTH2 antagonists. While the above studies clearly implicate CRTH2 in aspects of allergic disease, the functional studies conducted to date have been with isolated human cells or in animal models. We therefore investigated whether the PGD₂/CRTH2 pathway is operational in human diseased airway tissue using a nasal polyp explant system. Incubation of nasal polyp tissue with IgE/anti-IgE lead to increased production of PGD₂ and enhanced expression of its receptors. Functional studies highlighted the importance of the PGD₂/CRTH2 pathway in mediating chemotactic activation of human Th2 cells by supernatants from IgE/anti-IgE-treated nasal polyp tissue.

Methods

Patients and sample collection

Study subjects were selected on the basis of a documented medical history for chronic rhinosinusitis with nasal polyps, a pathological

nasal endoscopy and CT-Scan of the sinuses. Clinical diagnosis was performed following the guidelines proposed by the European Academy of Allergy and Clinical Immunology (21). Samples from the ethmoidal sinuses were collected from patients during functional endoscopic sinus surgery (FESS) procedures, indicated independently from this study, at the Department of Otorhinolaryngology at the Ghent University Hospital, following EP3OS guidelines. All patients gave informed consent before their participation and the study was approved by the ethical committee of the Ghent University Hospital. Clinical data of the patients are summarized in Table 1.

The presence of asthma and allergy co-morbidity is reported in Table 1. The use of any oral medication with possible impact on measurements of enzymes or mediators, including systemic glucocorticoids and anti-leukotrienes, or topical glucocorticoids was stopped in all subjects at least 4 weeks before surgery.

Preparation and stimulation of nasal polyp tissue explants

Preparation of nasal tissue explants was performed as described before (22). Briefly, the human nasal mucosa and submucosa were cut and homogenized in culture medium RPMI-1640 (Sigma-Aldrich, Bornem, Belgium) containing 2 mM L-glutamine (Invitrogen, Merelbeke, Belgium), 50 IU/ml penicillin, 50 µg/ml streptomycin; (Invitrogen) and 0.1% BSA (Sigma-Aldrich). Tissue fragments were then weighed, resuspended as 0.02 g tissue/1 ml culture medium and preincubated for 1 h at 37°C 5% CO₂ with 1 µg/ml human myeloma IgE (Calbiochem; VWR International, Leuven, Belgium). As a following step, polyp tissue fragments were stimulated either with RPMI-1640 or 0.5 µg/ml of anti-IgE (Sigma-Aldrich) for 30 min. Some samples were incubated in the presence of various concentrations of diclofenac (Sigma-Aldrich). Then, tissue fragments and supernatants were collected by centrifugation and immediately snap frozen or stored at -20°C until analysis. Samples from 10 donors were used to measure the production of PGD₂ and Th2 cell chemotactic activity. The effect of ramatroban and SQ29548 on the chemotaxis of Th2 cells was studied on four supernatants that showed a significant increase in the production of Th2 cell chemotactic activity (NP tissue from patients 2, 7, 9 and 10).

Levels of PGD₂ after IgE stimulation

Concentration of PGD₂ was measured by Enzyme Linked Immunoassay (ELISA) purchased from Cayman Chemicals (Ann Arbor, MI, USA). Sample extraction procedures for protein removal and eicosanoid stabilization were performed according to the provider's instructions. The assay has a detection range between 250 and 2 pg/ml, and intra- and inter-assay coefficient of variation was <10%.

Table 1. Patient's clinical data

Subject	Gender	Age	Diagnosis	Asthma	Aspirin Intolerance	Skin prick test	Seasonal or perennial allergy
1	M	68	NP	No	No	Positive	HDM
2	M	62	NP	Yes	No	Negative	
3	M	68	NP	No	No	Positive	HDM
4	M	45	NP	Yes	No	Negative	
5	F	45	NP	No	No	Negative	
6	F	44	NP	Yes	No	Positive	HDM, grass
7	M	50	NP	No	No	Positive	HDM
8	M	53	NP	No	No	Negative	
9	F	45	NP	No	No	Positive	HDM
10	F	72	NP	No	No	Negative	

Human CRTH2 + CD4 + Th2 cell culture

Human CRTH2 + CD4 + Th2 cells were prepared using a modified method described previously (16). Briefly, peripheral blood mononuclear cells were isolated from buffy coats from blood donors of unknown atopic status (National Blood Service Bristol, UK) by Ficoll Hypaque (Amersham Biosciences, Buckinghamshire, UK) density gradient centrifugation, followed by CD4⁺ cell purification using MACS CD4⁺ T-cell isolation kit II (Miltenyi Biotec, Bisley, Surrey, UK). After 7 day culture in X-VIVO 15 medium containing 10% human serum, 50 U/ml IL-2 and 100 ng/ml IL-4, CRTH2 positive cells were isolated from the CD4⁺ cultures by positive selection using an anti-human CRTH2 MicroBead Kit (Miltenyi Biotec). The harvested CD4 + CRTH2+ cells were treated as Th2 cells and were further amplified by stimulation with the T-cell Activation/Expansion Kit (Miltenyi Biotec) and grown in X-VIVO 15 medium containing 10% human serum and 50 U/ml IL-2 before use.

Chemotaxis assays

For measurement of chemotaxis, Th2 cells were resuspended in X-VIVO media at 2×10^6 cells/ml; 25 μ l of cell suspension and test samples (29 μ l) prepared in X-VIVO media were applied to the upper and lower chambers of a 5 μ m-pore sized 96-well ChemoTx plate (Neuroprobe, Gaithersburg, MD, USA). After incubation at 37°C for 60 min, any cells remaining on top of the filter were wiped off and plates were centrifuged at 300 g for 2 min to collect any cells on the underside of the filters. The upper membrane was carefully removed and cell migration was quantified by fluorescence activated cell sorter analysis. Background cell migration was determined by measuring the response to media alone.

To define the role of CRTH2 in the chemotactic response of Th2 cells, some chemotaxis assays were conducted in the presence of the dual CRTH2/TP antagonist ramatroban or the selective TP antagonist SQ29548 (both from Cayman Chemicals, Ann Arbor).

Quantitative real time PCR

Quantitative real time PCR was used to quantify mRNA levels of PGD₂ synthase and DP₁ and CRTH2 receptors. Nasal tissue fragments were collected and immediately snap frozen after stimulation. Then, total RNA was extracted using the Aurum mini kit from BioRad-Laboratories (Hercules, CA, USA). Concentration and RNA quality were determined using the Nanodrop and Agilent 2100 Bioanalyzer systems respectively, both from Agilent Technologies (Palo Alto, CA, USA). Subsequently, 2 μ g of RNA were reverse transcribed with the iScript cDNA synthesis kit (BioRad-Laboratories). Real time PCR reactions contained 30 ng of cDNA (total RNA equivalent) in 1 $\times$ SYBR Green I PCR Mastermix (BioRad-Laboratories) and 250 nM of Fwd-DP₁: 5'-TCTGCGCG-CTACCTT-CATG-3' and Rev-DP₁: 5'-TCCTCGTGGACCATCTGGATA-3' (Genbank accession number NM_000953 and amplicon size 85 bp). For amplification of CRTH₂ and PGD₂ synthase genes, we use TaqMan technology and PCR reactions contained 30 ng cDNA, 100 nM of TaqMan probe and 200 nM of forward and reverse primers and 1 $\times$ iQ Supermix (BioRad-Laboratories). Primers sequences were: Fw-CRTH₂: 5'-GCGCTGTC-CGACCTGTTG-3', Rev-CRTH₂: 5'-GATGGAGGAGTGCAGTTTGCA-3', Probe-CRTH₂: 6-FAM- 5'-TCACCTACTTCTTGGCCGTGGGCC-3'-TAMRA (Genbank accession number AB008535 and amplicon size 110 bp); Fw-PGD₂S: 5'-GGGCAGAGAAAAAGCAA-GATGT-3', Rev-PGD₂S: 5'-CCCCCTAAATATGTGTCCAAG-3', Probe-PGD₂S: 6-FAM-5'-CAATGAGCTGCTACGTATAATGCGCC-

(BHQ-1)-3' (Genbank accession number NM_014485 and amplicon size 102 bp). Amplifications were performed in an iQ5 Real Time PCR System (BioRad-Laboratories) and the thermal cycling conditions were: for SYBr-Green applications: 95°C for 10 min followed by 40 cycles (95°C for 30 s, 60°C during 1 min). For TaqMan amplifications, the protocol consisted of 1 cycle at 95°C for 1:30 min followed by 50 cycles (95°C for 15 s then 60°C for 1 min and 72°C for 1 min). All results were normalized against the amplification data of three reference genes ACTB (Actin Beta), HMBS (Hydroxymethylbilane synthase) and EF-1 (Elongation factor 1). Primer sequences and qPCR conditions are reported previously (23, 24). Relative quantities were calculated from the obtained C_T values using the QBASE software (25).

Statistical data analysis

The data generated in this study were analysed using the MEDCALC software version 6.0 (Mariakerke, Belgium). Data comparison within different patient groups was performed using the Kruskal-Wallis test. The *Mann-Whitney U-test* was applied to evaluate the statistical differences between two patient groups. *P*-values ≤ 0.05 was regarded as significant.

The Th2 cell chemotaxis data were analysed using one-way analysis of variance (ANOVA) followed by the Newman-Keuls test. The correlation was conducted using GRAPHPAD prism software (La Jolla, CA, USA).

Results

PGD₂ levels and CRTH₂, DP₁ and PGD₂ synthase mRNA expression

Levels of PGD₂ were negligible in the supernatants from tissue explants cultured in media alone. However, the concentrations of the eicosanoid increased substantially following treatment of nasal polyp tissue with IgE and anti-IgE antibodies for 30 min (Fig. 1). Production of PGD₂ in response to IgE/anti-IgE was significantly inhibited by preincubation of the tissue with diclofenac (10^{-4} , 10^{-5} and 10^{-6} M).

Transcript levels of the PGD₂ receptors DP₁ and CRTH₂ and PGD₂ synthase were significantly increased 30 min after stimulation with IgE/anti-IgE compared with the levels observed in nonstimulated tissue (Fig. 2).

Effect of nasal polyp supernatants on chemotaxis of human Th2 cells

Supernatants collected from nasal polyp tissue incubated in medium without IgE/anti-IgE did not stimulate increased migration of Th2 cells compared with background responses to media alone (Figs 3 and 4). Some NP samples (but not all) produced Th2 cell chemotactic activity when incubated with IgE/anti-IgE, and there was a significant correlation between the magnitude of Th2 cell chemotactic response and the levels of PGD₂ produced by nasal polyp tissue (Fig. 3). Samples from patients 2, 7, 9 and 10 produced the highest levels of Th2 cell chemotactic activity (twofold to threefold compared with unactivated NP tissue), and these samples were used

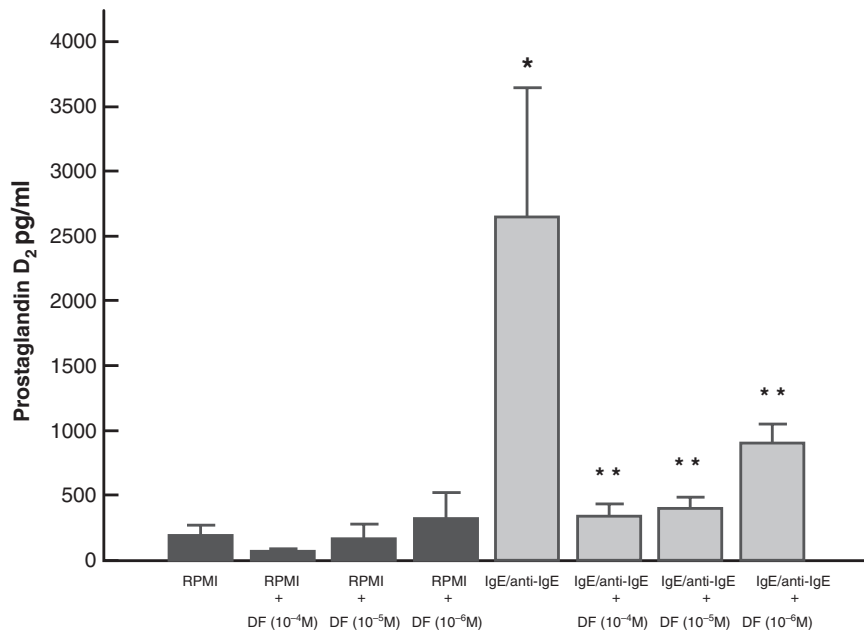


Figure 1. Concentrations of PGD₂ in nasal polyp tissue explants after 30 min incubation with RPMI or IgE/anti-IgE in the presence or absence of diclofenac (10⁻⁴, 10⁻⁵ and 10⁻⁶ M). **P*-value < 0.05 compared with cultures containing RPMI alone; ***P*-value < 0.05 between cultures stimulated with IgE/anti-IgE and cultures containing IgE/anti-IgE preincubated with diclofenac.

to study the effect of diclofenac on the production of the factor and ramatroban and SQ29548 on the responses of the Th2 cells to the factor. Treatment of the nasal polyp tissue with diclofenac inhibited the production of the Th2 cell stimulatory activity (Fig. 4). The chemotactic response of the Th2 cells to the supernatants was inhibited by the dual CRTH2/TP antagonist ramatroban but not by the selective TP antagonist SQ29548.

Discussion

Nasal polyp tissue expressed increased levels of PGD₂ synthase mRNA and increased production of PGD₂ in response to treatment with IgE/anti-IgE. Both mast cells and Th2 cells present within nasal polyps are potential sources of PGD₂, but mast cells are the most likely source given the high levels detected over the short 30 min incubation period and are far richer source of PGD₂ than Th2 cells. The 30 min incubation time was selected based on previous studies, which showed substantial release of histamine, leukotrienes and PGD₂ over this time period (26). There was considerable variability in the production of PGD₂ by nasal polyp, which may reflect differences in the atopic status of the donors and/or cellular heterogeneity of the samples collected. However, there was no obvious association between the production of Th2 cell chemotactic activity and the atopic status of those patients that produced the highest levels (patients 2, 7, 9 and 10), measured by either the presence of co-morbid asthma or by skin prick test positivity. It is evident, however, that activation of nasal polyp tissue with IgE/

anti-IgE is a necessary requirement for significant PGD₂ synthesis – nasal polyp tissue produced only low levels of PGD₂ spontaneously in the absence of allergic stimulation. Treatment with IgE/anti-IgE also increased the expression of mRNA of the PGD₂ receptors DP₁ and CRTH2, illustrating that after allergic stimulation nasal polyp tissue has both the capacity to produce PGD₂ and the potential to give a heightened response to this mediator. Supernatants from nasal polyp tissue activated with IgE/anti-IgE increased the chemotaxis of Th2 cells, while supernatants from nasal polyp tissue incubated with media alone did not. The degree of Th2 chemotaxis correlated with the levels of PGD₂ produced by nasal polyp tissue, and the production of the chemotactic activity was effectively inhibited by diclofenac at concentrations that blocked the synthesis of PGD₂. Diclofenac is an inhibitor of COX-1 and COX-2 that inhibits the production of PGD₂ by human mast cells but does not inhibit the release of histamine or leukotriene C₄ (27). Furthermore, diclofenac does not impair the response of Th2 cells to PGD₂ (28), indicating that this drug inhibits the production but not the action of PGD₂. Taken together, these data suggest that PGD₂ is the dominant mediator produced upon mast cell activation that mediates chemoattraction of human Th2 cells. The effects of nasal polyp supernatants on Th2 cell chemotaxis are mediated by the activation of the CRTH2 receptor expressed on Th2 cells, as the response was inhibited by the dual CRTH2/TP antagonist ramatroban, but not by the selective TP antagonist SQ29548. The concentration of ramatroban was selected based on previous experiments demonstrating that this drug causes

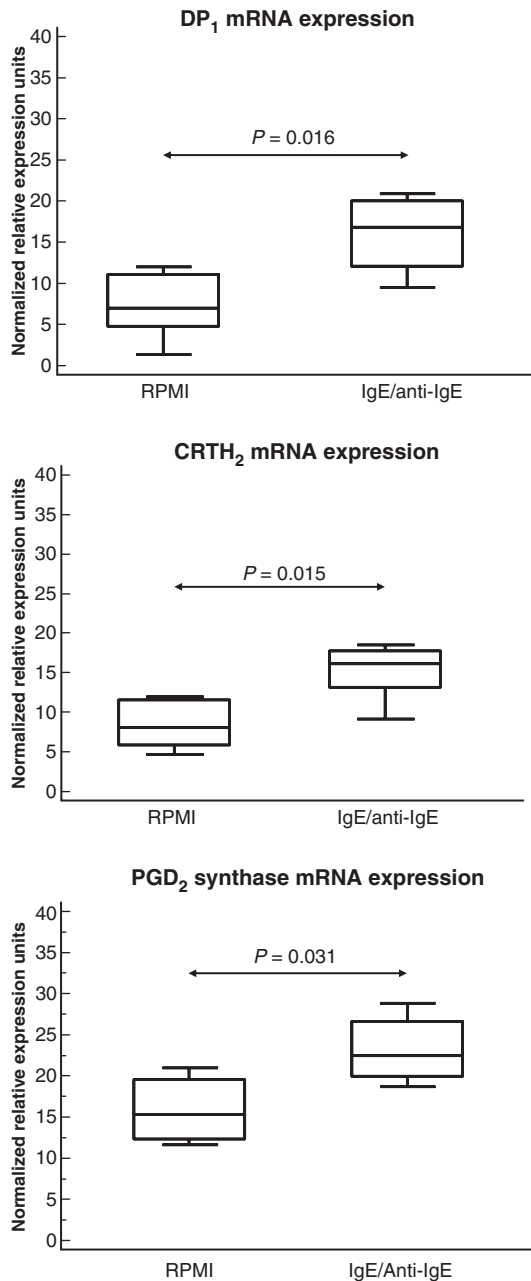


Figure 2. Relative mRNA expression of PGD₂ synthase, DP₁ and CRTH2 receptors in nasal polyp tissue explants before and after exposure to RPMI and/or IgE/anti-IgE for 30 min. *P*: represent the *P*-values after a Nonparametric Mann–Whitney *U*-test.

concentration-dependent inhibition of responses of Th2 cells to PGD₂ with potency consistent with CRTH2 antagonism (28, 29). While previous studies have demonstrated an important role for CRTH2 in mediating interactions between mast cells and Th2 cells (28, 29), this is the first demonstration that the PGD₂/CRTH2 pathway is functionally active in a model system involving intact human diseased airway tissue.

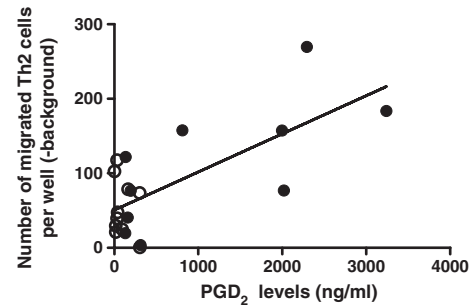


Figure 3. Correlation between the production of PGD₂ by nasal polyp tissue in the presence (●) and absence (○) of IgE/anti-IgE ($r = 0.7$, $P = 0.0005$). Results are expressed as the paired mean values from 10 nasal polyp tissue explants.

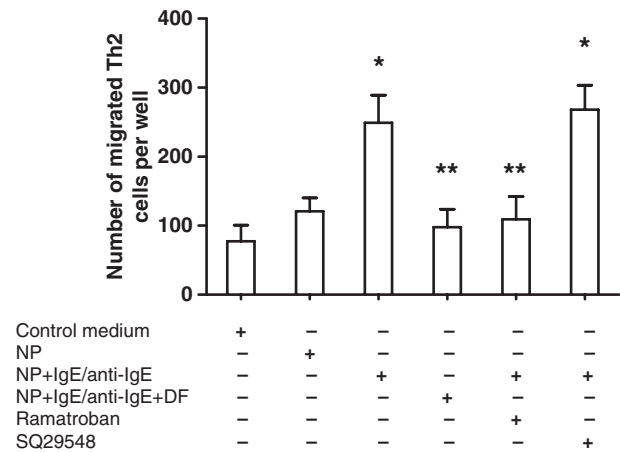


Figure 4. Chemotactic responses of human Th2 cells to supernatants from nasal polyp tissue explants incubated for 30 min with RPMI or anti-IgE in the presence and absence of diclofenac (10^{-5} M). The effects of the supernatants were also tested on Th2 cells incubated with the dual CRTH2/TP antagonist ramatroban or the selective TP antagonist SQ29548. Results are expressed as the mean $\pm$ SEM from 4 nasal polyp tissue explants. * $P < 0.05$ by ANOVA (followed by Newman–Keuls test) for IgE/anti-IgE alone and IgE/anti-IgE + SQ29548 compared with media alone and supernatants from nasal polyps without IgE/anti-IgE and ** $P < 0.05$ by ANOVA for diclofenac and ramatroban treated samples compared with IgE/anti-IgE alone. There was no significant difference ($P > 0.05$) between the effects of media alone, nasal polyps incubated without IgE/anti-IgE and samples treated with ramatroban or diclofenac.

It is of interest that immunologically activated nasal polyp tissue can stimulate Th2 lymphocytes independently of T-cell receptor activation. This finding is consistent with the results of studies in IgE transgenic mice where mast cell activation leads to the accumulation of Th2 cells in the airways independently of antigen-specific T-cell activation (8) and the classical observation that a late phase lymphocytic response can be induced in nonatopic subjects by passive transfer of serum containing IgE (30). In addition to promoting chemotaxis,

activated mast cells can stimulate Th2 cells to produce cytokines including IL-5 (27). This may be particularly relevant to the pathogenesis of nasal polyposis as this condition is associated with high levels of IL-5 with accompanying eosinophilia (31). Production of IL-5 is clearly linked to disease progression as therapy with the anti-IL-5 antibody mepozulimab reduced nasal polyp size and improved symptom scores in those subjects with high nasal concentrations of IL-5 (32).

In summary, mast cells within nasal polyp tissue produce high levels of PGD₂ in response to their activation by IgE/anti-IgE, which promotes migration of Th2 cells through a CRTH2-dependent mechanism. This is the first demonstration of a functional role for CRTH2 in upper airway disease such as nasal polyps, suggesting that CRTH2 antagonists may be useful in treating this and other upper airway diseases involving mast cell degranulation.

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