

Role of prostaglandin D₂ and CRTH2 blockade in early- and late-phase nasal responses

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Summary

Background Prostaglandin D₂ (PGD₂) plays an important role in allergic inflammation. The PGD₂ receptor, CRTH2, is expressed on basophils, eosinophils, and Th2 lymphocytes and mediates chemotactic activity.

Objective To define the role of CRTH2 in allergen-induced nasal responses in a mouse model of allergic rhinitis (AR), a potent, selective CRTH2 receptor antagonist, ARRY-063 was administered in a model of allergic rhinitis in mice.

Methods ARRY-063 was administered orally to ovalbumin (OVA) sensitized and challenged mice. To assess nasal obstruction, respiratory frequency (RF) was monitored by whole-body plethysmography immediately after the 4th challenge (early-phase response, EPR) and 24 h after the 6th challenge (late-phase response, LPR). Nasal resistance (R_{NA}) was also measured in the LPR. PGD₂ was administered with or without OVA to determine the effect of PGD₂ on nasal responsiveness. Cytokine levels and histopathological changes in nasal tissue were analysed.

Results Instillation of PGD₂ in the nose of sensitized mice together with a low concentration of OVA induced both an EPR and LPR. Treatment with the CRTH2 receptor antagonist prevented the decreases in RF seen immediately following the 4th challenge of sensitized mice (EPR). In the LPR, decreases in RF and increases in R_{NA} were also prevented by antagonist treatment associated with reduced cytokine levels and inflammation in nasal tissues.

Conclusions These data identify PGD₂ as a mediator of both the EPR and LPR in this model of AR and suggest that antagonism of CRTH2 prevents the development of both the EPR and LPR as well as nasal inflammation.

Keywords allergic rhinitis, CRTH2, prostaglandin D₂

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Introduction

Allergic rhinitis (AR) is the most prevalent allergic disease, affecting approximately 600 million people worldwide [1]. AR affects between 10–30% of all adults and as many as 40% of children in the United States [2]. The typical symptoms of AR in humans are sneezing, nasal congestion, itching, and rhinorrhea [3].

Prostaglandin D₂ (PGD₂) is synthesized from arachidonic acid by cyclooxygenases and is released from activated mast cells and eosinophils [4–6]. PGD₂ is recognized as an important lipid mediator in the pathogenesis

of allergic inflammation such as observed in AR or asthma. Binding of PGD₂ to cells occurs through one of three specific G-protein-coupled receptors, DP/DP1 (D prostanoid) receptor [7, 8], DP2/CRTH2 (chemoattractant receptor homologous molecule expressed on Th2 cells) [9, 10], and TP (T prostanoid; thromboxane A₂) receptor [8, 11]. DP1 receptor is expressed on various hematopoietic and non-hematopoietic cells. CRTH2 is also expressed in various organs and particularly on hematopoietic inflammatory cells especially Th2 cells, eosinophils, and basophils. Activation through CRTH2 upregulated Th2-type cytokines in human cells [5, 12–14]. In addition to cells associated with Th2-type inflammation, CRTH2 was also found on neutrophils and Th1 cells [15]. Notably, DP1 and CRTH2 provide reciprocal

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intracellular signals through increases or decreases in the levels of cyclic AMP by up- or down-regulation of adenylate cyclase [4, 16]. Activation of CRTH2 mediates cell migration [9, 17], whereas DP1 mostly functions in an anti-inflammatory signalling pathway [18–20].

Agonist ligation of CRTH2 but not DP1 enhanced airway hyperresponsiveness (AHR) and eosinophilia [19, 21]. In contrast, antagonist blockade of CRTH2 attenuated the development of AHR and airway inflammation in an allergen-induced asthma model in mice [22–24]. In recent clinical studies, a CRTH2 antagonist showed some effects in AR as well as in asthma [25–28]. Based on these findings, we hypothesized that allergen-induced nasal responses would be PGD₂ dependent and that a selective CRTH2 antagonist would prevent the development of both phases of the response to intranasal allergen challenge.

Methods

Animals

Six-week-old female BALB/c mice were obtained from Harlan Laboratories (Indianapolis, IN). The mice were housed under specific pathogen-free conditions and maintained on an ovalbumin (OVA)-free diet at National Jewish Health. All experimental animals used in this study were under a protocol approved by the Institutional Animal Care and Use Committee of National Jewish Health.

OVA sensitization and nasal challenge and PGD₂ administration

Mice were sensitized by intraperitoneal injection of 20 µg of OVA (Fisher Scientific, Pittsburgh, PA, USA)

emulsified in 2.25 mg of alum (Imject[®] Alum; Thermo Scientific Pierce Protein Research Products, Rockford, IL, USA) in a total volume of 100 µL on days 0 and 14. From days 28–33, nasal challenges with 10% (w/v) OVA in saline (15 µL for each nostril) or saline alone as control were administered daily without anaesthesia. Some groups of mice received PGD₂ (0, 1, or 10 nmol/mouse; Cayman Chemical Company, Ann Arbor, MI, USA) intranasally together with 1% (w/v) OVA solution in saline on these days. All of the experiments were carried with 9 mice in each group.

Drug treatments

The CRTH2 antagonist, ARRY-063, was provided by Array BioPharma Inc. (Boulder, CO, USA). Vehicle control (0.5% (w/v) methylcellulose–water) or 0.1, 1, 10 mg/kg of the antagonist was administered orally 1 hr prior to each OVA or OVA with PGD₂ challenge (days 28–33). The properties of ARRY-063 showing specificity for CRTH2 are shown in Table 1. This small molecule demonstrates high binding affinity for CRTH2. ARRY-063 also possesses selectivity versus a panel of other prostanoid receptors. *In vitro* functional activity was demonstrated by antagonism of PGD₂-induced chemotaxis of freshly isolated human basophils and by the inhibition of calcium mobilization. This compound also had high plasma concentrations following oral dosing in mice.

Nasal responsiveness

Nasal responses to allergen were measured as described in previous studies [27–29]. Briefly, in mice sensitized

Table 1. Profile of ARRY-063

Parameter	ARRY-063
Human CRTH2 Binding IC ₅₀ (4% HSA)	107 nM
Human CRTH2 Binding IC ₅₀ (4% HSA)*	66 nM
Human CRTH2 Binding IC ₅₀ (0.1% BSA)*	3 nM
Human Basophil Chemotaxis IC ₅₀ *	1 nM
PGD ₂ -Induced Calcium Mobilization via FLIPR: CRTH2 IC ₅₀ *	4 nM
Ligand-Induced Calcium Mobilization via FLIPR: DP1	<10% Agonist; <10% Antagonist @500 nM
Ligand-Induced Calcium Mobilization via FLIPR: EP1	<10% Agonist; <10% Antagonist @500 nM
Ligand-Induced Calcium Mobilization via FLIPR: EP2	<10% Agonist; <10% Antagonist @500 nM
Ligand-Induced Calcium Mobilization via FLIPR: EP3	<10% Agonist; <10% Antagonist @500 nM
Ligand-Induced Calcium Mobilization via FLIPR: EP4	<10% Agonist; <10% Antagonist @500 nM
Ligand-Induced Calcium Mobilization via FLIPR: FP	<10% Agonist; <10% Antagonist @500 nM
Ligand-Induced Calcium Mobilization via FLIPR: IP	<10% Agonist; <10% Antagonist @500 nM
Ligand-Induced Calcium Mobilization via FLIPR: TP	<10% Agonist; <10% Antagonist @500 nM
Mouse Pharmacokinetics (10 mg/kg po)	C _{max} = 25 µg/mL AUC = 160 µg h/mL F = 50%

FLIPR, Fluorometric Imaging Plate Reader (Molecular Devices, Sunnyvale, CA, USA).

*Assay performed on single active enantiomer of ARRY-063.

to OVA, respiratory frequency (RF) values were assessed using single-chamber whole-body plethysmography (Buxco, Troy, NY, USA). Baseline RF values were determined before the first challenge. RF was determined just prior to and at 0, 4–7, 9–12, 14–17, 19–22, 24–27, and 29–32 min after the 4th OVA challenge. As these responses were detected immediately following allergen challenge, we labelled these as early-phase responses (EPR). In addition, RF values and nasal cavity resistance (R_{NA}) were measured, as described [27–29], 24 h after the 6th OVA challenge and compared with baseline values. As these responses were determined 24 h after the last allergen challenge, we labelled these as late-phase responses (LPR).

Assessment of cytokine levels in nasal tissue homogenates

After measurement of the LPR, mice were euthanized by exsanguination from the inferior vena cava. The skulls were skinned, and the nasal portion was excised from the head along a line in front of the zygomatic arch. The tip of the nose area containing the nasal vestibule and incisor teeth were also excised. The tissues were weighed and completely homogenized on ice in 1 mL of PBS (pH 7.4) containing 0.1% (v/v) Triton X-100 and proteinase inhibitor (Complete®; Roche Diagnostics, Indianapolis, IN, USA) using a tissue homogenizer (Tissue Tearor; BioSpec, Bartlesville, OK, USA) and then centrifuged at $1000 \times g$ for 5 min at 4°C. Supernatants were collected and frozen at –80°C until further analysis. Cytokine levels were assessed by ELISA (eBioscience, San Diego, CA, USA), according to the manufacturer's directions and corrected for the tissue weight of the sample.

Histological studies

Nasal tissue, prepared as described above without homogenization, was fixed in 10% (v/v) formalin and decalcified with decalcifying solution (Richard-Allan Scientific, Kalamazoo, MI, USA). Eosinophils were identified by immunohistochemical staining as previously described with rabbit anti-mouse major basic protein (kindly provided by Dr. J.J. Lee, Mayo Clinic, Scottsdale, AZ, USA) [30].

Statistical analyses

All values were expressed as mean $\pm$ SEM. The RF values were analysed with repeated-measures two-way ANOVA, followed by Bonferroni correction as a post hoc test. All other values were analysed with one-way ANOVA, followed by Bonferroni correction. Statistical analyses were performed using GRAPHPAD PRISM 5.0d (GraphPad

Software Inc., La Jolla, CA, USA). A P value < 0.05 was considered statistically significant.

Results

Intranasal PGD₂ induces an EPR and LPR in sensitized and challenged mice

To directly identify the role of PGD₂ in allergen-induced nasal responsiveness, we determined the impact of intranasal administration of PGD₂ on nasal responsiveness in BALB/c mice (Fig. 1a). Mice, which received 10 nmol/mouse PGD₂ alone, did not show any alterations in RF, similar to mice which received saline (Figs. 1b, c). We then examined the effects of PGD₂ in mice that were first sensitized to OVA and challenged with OVA. Following OVA sensitization, when mice were challenged with 1% (w/v) OVA together with 10 nmol PGD₂, they developed an EPR (Fig. 1b) and LPR as indicated by changes in RF (Fig. 1c) and R_{NA} (Fig. 1d); 1 nmol PGD₂ together with 1% OVA failed to alter nasal responsiveness. These data indicated that in sensitized and 1% OVA-challenged mice, intranasal PGD₂ triggered the development of both an EPR and LPR.

CRTH2 antagonist treatment prevents the development of an EPR and LPR

To determine the effects of CRTH2 antagonist treatment on allergen-induced nasal responses, ARRY-063 or vehicle was administered prior to each of the allergen challenges. In these experiments, 10% OVA was administered to induce an EPR and LPR (Fig. 2a).

In the EPR, which was monitored immediately after the 4th OVA challenge, sensitized mice treated with vehicle demonstrated significant reductions in RF values (Fig. 2b). Treatment of sensitized mice with the CRTH2 antagonist on the days of challenge prevented the development of the EPR, manifested as a change in RF, in a dose-dependent manner. In a similar fashion, antagonist treatment also prevented the development of the LPR dose-dependently 24 h after the 6th challenge (Fig. 2c). In parallel to changes in RF in the LPR, R_{NA} was also measured. R_{NA} values in mice sensitized and challenged to OVA and treated with vehicle increased to 1.41 ± 0.05 cmH₂O·s/mL from a baseline R_{NA} of 0.70 ± 0.02 cmH₂O·s/mL in OVA-sensitized but saline-challenged and vehicle-treated mice. Following treatment with 10 mg/kg of the antagonist, R_{NA} values were significantly reduced (Fig. 2d).

Cytokine levels in nasal tissue homogenates were measured after assessment of the LPR. Levels of IL-4, IL-5, and IL-13 were increased following OVA challenge of sensitized mice. Th1 cytokine levels such as IL-12

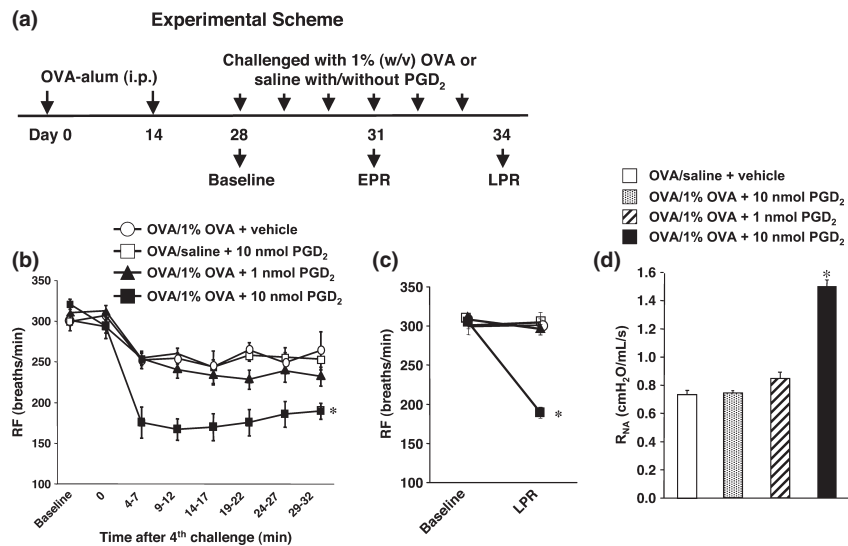


Fig. 1. PGD₂-induced nasal obstruction in the EPR and LPR. (a) Experimental protocol for OVA sensitization and OVA with PGD₂ challenge. Mice were sensitized with OVA-alum on days 0 and 14 and then challenged with 1% OVA with/without PGD₂ on days 28–33. (b) PGD₂-induced RF values in the EPR. BALB/c mice were sensitized with OVA-alum on days 0 and 14 and challenged with 0, 1, 10 nmol/mouse PGD₂ with/without 1% (w/v) OVA or saline. Baseline RF values were obtained at prior to the 1st challenge on day 0, and RF values were obtained on days 4 (before 4th challenge), 4–7, 9–12, 14–17, 19–22, 24–27, and 29–32 min after the 4th challenge. (c) RF values in the LPR. In the LPR, RF values were measured 24 h after the 6th challenge. (d) R_{Na} values in LPR. Shown are the means $\pm$ SEM, $n = 9$ in each group. * $P < 0.05$ compared with the OVA/1% (w/v) OVA + vehicle group.

p70 and IFN- γ were also increased following OVA sensitization and challenge. All of these increases in cytokine levels were significantly inhibited following treatment with 1 and 10 mg/kg of the CRTH2 antagonist (Fig. 2e).

Histological analysis of nasal tissue stained with rabbit anti-mouse major basic protein demonstrated that sensitization and intranasal allergen challenge elicited a marked eosinophilic inflammatory response. CRTH2 antagonist (10 mg/kg) treatment markedly decreased the numbers of eosinophils in the nasal tissue (Fig. 2f).

Discussion

Despite the availability of antihistamines, leukotriene modifiers, and intranasal corticosteroids to target the clinical symptoms of AR, there is a well-recognized and widespread failure in many patients to achieve control, especially in nasal obstruction [31]. In the present study, we determined whether treatment with a potent and selective CRTH2 receptor antagonist could prevent the development of allergen-induced nasal responses in an experimental model in mice. In this model, what have been labelled as EPR and LPR have been well-characterized with the latter response showing features of nasal obstruction as detected by changes in RF, R_{Na}, and cellular inflammation [27–29]. As PGD₂ has been implicated in the development of AR [32] as well as enhancing allergen-induced AHR and airway eosinophilia in a mouse model of asthma [21], we examined

the role of PGD₂ in the development of allergen-induced nasal responses. Intranasal administration of PGD₂ enhanced allergen-induced nasal responsiveness in both the EPR and LPR. Of interest, the activity of PGD₂ could only be demonstrated in mice that were sensitized to allergen and challenged with low concentrations of allergen that were unable to elicit responses on their own. It is unclear at present why the response to PGD₂ alone was insufficient to elicit a response. The responses elicited when combined with challenge with limiting amounts of allergen may suggest that in addition to PGD₂, other mediators released in response to allergen contributed to the development of the EPR and LPR. When larger amounts of allergen were used in the challenge phase, the role of PGD₂ in triggering both the EPR and LPR was clearly shown in experiments administering the CRTH2 antagonist. In both the PGD₂-induced responses and in the face of higher concentrations of allergen in the challenge phase, CRTH2 antagonist treatment significantly prevented the development of both the EPR and LPR.

Given the role of PGD₂ in allergic inflammation, examination of the function and targeting of the PGD₂ receptors, DP1 and CRTH2, have been the focus of attention. To a large extent, signalling through CRTH2 has been viewed as pro-allergic, whereas signalling through DP1 may be anti-inflammatory [19, 21]. It is known that intracellular events following activation of DP1 and CRTH2 may antagonize each other; activation through DP1 increases intracellular cAMP, whereas signalling

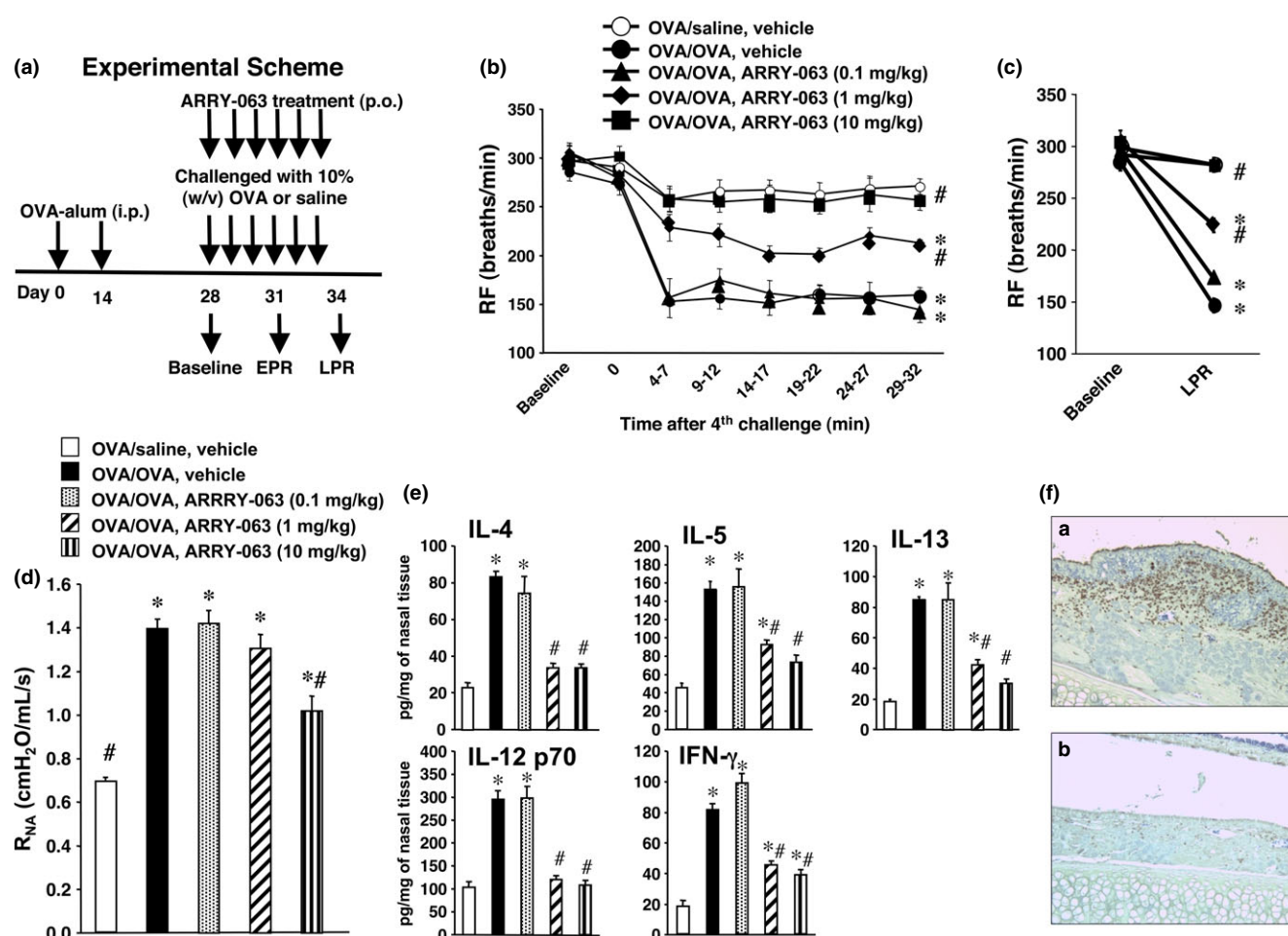


Fig. 2. Effect of CRTH2 antagonist on nasal obstruction in EPR and LPR. (a) Experimental protocol for CRTH2 antagonist treatment in AR model. Mice were sensitized with OVA-alum on days 0 and 14 and then challenged with 1% OVA with/without PGD₂ on days 28–33. CRTH2 antagonist was administered 1 h prior to each challenge. (b) RF values in EPR. (c) RF values in LPR. (d) R_{NA} values in LPR. (e) Cytokine levels in nasal tissue homogenates. Shown are the means $\pm$ SEM, $n = 9$ in each group. * $P < 0.05$ compared with the OVA/saline, vehicle group. # $P < 0.05$ compared with the OVA/10% (w/v) OVA, vehicle group. (f) Immunohistochemical staining to detect eosinophils (brown staining) in nasal tissue using an anti-mouse major basic protein antibody. (a) OVA/OVA, vehicle, (b) OVA/OVA, ARRY-063 (10 mg/kg).

through CRTH2 decreases intracellular cAMP levels through inhibition of adenylate cyclase [22, 33]. As a consequence, in a PGD₂ abundant environment, blockade of CRTH2 may result in increased activation through DP1 and enhance anti-inflammatory effects on receptor-expressing cells. A DP1 agonist has been shown to suppress allergen-induced asthma in mice [19], but in patients with asthma or allergic rhinitis, a DP1 antagonist did not show any beneficial effects [20].

In animal models of allergic disease, including allergen-induced nasal responses, the role of CRTH2 has been determined using receptor antagonists. Ramatroban, which is a dual CRTH2/TP antagonist, was shown to decrease the frequency of sneezing, nasal rubbing, and nasal tissue inflammation in a model of AR [34, 35]. Treatment with a more selective CRTH2 antagonist, as shown here, reduced symptoms of allergen-induced

nasal responses pointing to a critical role for CRTH2 in these responses and the importance of the PGD₂-CRTH2 axis. In support of this notion, we demonstrated the efficacy of the CRTH2 antagonist in preventing allergen-induced nasal obstruction in both the EPR and LPR. The antagonist also prevented the increases in levels of both Th2 and Th1 cytokines in the nasal tissues during the LPR. Previously, in this model we showed that mast cells play an essential role in the development of the EPR but not the LPR [27], whereas IL-13 was essential in the LPR [28]. Both IL-13-producing Th2 cells and type 2 innate lymphoid cells express CRTH2 [5, 12, 36, 37], and our data suggest that CRTH2 antagonism reduces levels of IL-13, accounting for the inhibition of the LPR. As CRTH2 is also expressed on IFN-γ- and/or IL-12-producing cells such as dendritic cells or macrophages, and Tc2 and Th1 cells in mice

[5, 12, 13, 15, 38], preventing migration of these cells through blocking CRTH2 likely resulted in the decreased levels of IFN- γ and IL-12p70.

CRTH2 has also been shown to play a critical role in the recruitment of eosinophils. Intravenous injection of a CRTH2-specific agonist such as Δ^{12} -prostaglandin J₂ or 13,14-dihydro-15-keto-prostaglandin D₂ induced eosinophil recruitment from the bone marrow into the bloodstream [39], and PGD₂ induced the mobilization and activation of inflammatory cells such as eosinophils *in vitro* [10, 40]. Levels of vasoactive intestinal peptide shown to upregulate CRTH2 on eosinophils and promote their recruitment were increased in the nasal tissue of patients with AR [41]. In accord with these findings, we showed that CRTH2 antagonist treatment markedly reduced nasal tissue eosinophilia induced by allergen sensitization and intranasal challenge.

The mechanisms underlying the development of the EPR is the result of cross-linking allergen-specific IgE bound to Fc ϵ RI [27, 29, 42]. Both mast cells and basophils express Fc ϵ RI and CRTH2 [10, 43]. Activation of Fc ϵ RI triggers degranulation of mast cells and the release of PGD₂ [5, 44, 45]. The activation of mast cells and release of histamine in the nasal tissue may be

essential for the recruitment of basophils to the nasal tissue and their subsequent activation and release of IL-13 [46]. These events induce recruitment of eosinophils, which may be further enhanced by PGD₂ as they also express CRTH2. Interference with CRTH2 may therefore be critical at different stages of the response, interfering with the development of both the EPR and LPR through independent mechanisms. These results on allergen-induced nasal responses suggest that targeting CRTH2 with a specific antagonist potentially may have benefits in allergic rhinitis, in addition to those achieved with antihistamines or corticosteroids.

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Conflict of interests

The authors declare they have no conflicts of interest.

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