

Activation of the Prostaglandin E₂ receptor EP₂ prevents house dust mite-induced airway hyperresponsiveness and inflammation by restraining mast cells' activity

M. Serra-Pages^{1,*}, R. Torres^{1,2,3,*}, J. Plaza^{1,2}, A. Herrerias¹, C. Costa-Farré⁴, A. Marco⁴, M. Jiménez^{3,5}, M. Maurer⁶, C. Picado^{2,7} and F. de Mora¹

¹Department of Pharmacology, Therapeutics and Toxicology, Universitat Autònoma de Barcelona, Barcelona, Spain, ²CIBER (Centro de Investigación Biomédica en Red) de Enfermedades Respiratorias, Barcelona, Spain, ³Department of Cellular Biology, Physiology and Immunology, Universitat Autònoma de Barcelona, Barcelona, Spain, ⁴Department of Surgery and Animals Medicine, Universitat Autònoma de Barcelona, Barcelona, Spain, ⁵CIBER (Centro de Investigación Biomédica en Red) de Enfermedades Hepáticas y Digestivas, ⁶Department of Dermatology, Charité –Universitätsmedizin Berlin, Berlin, Germany and ⁷Department of Pneumology and Respiratory Allergy, Hospital Clínic, IDIBAPS (Institut d'Investigacions Biomèdiques August Pi i Sunyer), Universitat de Barcelona, Barcelona, Spain

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Summary

Background Prostaglandin E₂ (PGE₂) has been proposed to exert antiasthmatic effects in patients, to prevent antigen-induced airway pathology in murine models, and to inhibit mast cells (MC) activity *in vitro*.

Objective To assess in a murine model whether the protective effect of PGE₂ may be a consequence of its ability to activate the E-prostanoid (EP)₂ receptor on airway MC.

Methods Either BALB/c or C57BL/6 mice were exposed intranasally (i.n.) to house dust mite (HDM) aeroallergens. Both strains were given PGE₂ locally (0.3 mg/kg), but only BALB/c mice were administered butaprost (EP₂ agonist: 0.3 mg/kg), or AH6809 (EP₂ antagonist; 2.5 mg/kg) combined with the MC stabilizer sodium cromoglycate (SCG: 25 mg/kg). Airway hyperresponsiveness (AHR) and inflammation, along with lung MC activity, were evaluated. In addition, butaprost's effect was assessed in MC-mediated passive cutaneous anaphylaxis (PCA) in mice challenged with 2,4-dinitrophenol (DNP).

Results Selective EP₂ agonism attenuated aeroallergen-caused AHR and inflammation in HDM-exposed BALB/c mice, and this correlated with a reduced lung MC activity. Accordingly, the blockade of endogenous PGE₂ by means of AH6809 worsened airway responsiveness in sensitive BALB/c mice, and such worsening was reversed by SCG. The relevance of MC to PGE₂-EP₂ driven protection was further highlighted in MC-dependent PCA, where butaprost fully prevented MC-induced ear swelling. Unlike in BALB/c mice, PGE₂ did not protect the airways of HDM-sensitized C57BL/6 animals, a strain in which we showed MC to be irrelevant to aeroallergen-driven AHR and inflammation.

Conclusions & Clinical Relevance The beneficial effect of both exogenous and endogenous PGE₂ in aeroallergen-sensitized mice may be attributable to the activation of the EP₂ receptor, which in turn acts as a restrainer of airway MC activity. This opens a path towards the identification of therapeutic targets against asthma along the 'EP₂ -MC-airway' axis.

Keywords allergy, asthma, EP₂ receptor, house dust mite, mast cells, PGE₂

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Correspondence:

Fernando de Mora, Department of Pharmacology, Therapeutics and Toxicology Edifici V, Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain.

E-mail: fernando.demora@uab.cat

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Introduction

Although significant efforts are being made to uncover novel antiasthmatic strategies, there is still no cure for asthma, and none of the available treatments has been

proven to alter the natural progression of the disease [1]. To identify new pharmacological targets, creative hypotheses that challenge our current understanding, need to be explored. One such assumptions is the role of prostaglandins in asthma. Whereas these cyclooxygenase (COX) products have been generally viewed as pathogenic in inflammatory diseases, there is clinical

*These two authors have equally contributed to this paper.

and experimental evidence to support a protective function for PGE₂ [2, 3]. We know that locally administered PGE₂ inhibits airway hyperresponsiveness (AHR) and inflammation in experimental human allergic, and non-allergic, asthma [4–6]. Consistently, a diminished PGE₂ production has been demonstrated in airway cells isolated from asthmatic patients [7–10]. However, data regarding the mechanisms underlying this beneficial effect of PGE₂ are scarce, and preclinical studies are needed before entering into clinical research.

Peebles et al. [11, 12] showed that a COX inhibitor worsened ovalbumin (OVA)-induced airway inflammation in mice. Other studies [13–15], as well as ours [16], have demonstrated that the inhibition of COX in OVA-sensitized animals aggravates allergic inflammation and/or AHR. When exogenous PGE₂ was administered, we observed a clear-cut protection against HDM-induced AHR and inflammation [17]. Some authors have reported modulatory properties for PGE₂ at immunologic and inflammatory levels [18], such as the regulation of T-cell differentiation and eosinophil trafficking [19, 20], and the production of pro-inflammatory cytokines [21–23]. Interestingly, we very recently published that PGE₂ might be regulating early immunological events *in vivo* through the modulation of dendritic cell activity [24]. Some of these multiple effects may, to some extent, be additive and potentiate those exerted by glucocorticoids, whose efficacy in asthma is likely attributable to their broad spectrum of action. However, a basic understanding of the PGE₂ receptor, and the molecular and cellular targets involved in the PGE₂-driven beneficial outcome is lacking. One such cellular target may be the airway mast cell (MC) population. The expression of four PGE₂ receptors (EP₁–EP₄) with a broad range of functions (reviewed by Chung et al. [25]) in such a key cell type in allergic diseases [26] suggests a role for MC in the PGE₂ protective action. The identity of the specific receptor that is primarily involved in the PGE₂-driven improvement is a subject of debate. Contrary to others' [27], our data suggest that an EP₃ agonist neither has an effect on HDM-induced pathology in mice, nor does it diminish allergen-induced COX2 and PGE₂ overexpression [28]. EP₂ appears to be a more likely candidate given that there is wide consensus on its ability to attenuate human MC degranulation [29–32]. We have consistently observed in aeroallergen-sensitized mice that PGE₂ exerts an inhibitory effect on lung MC activity *in vivo*, and we hypothesized that EP₂ is responsible for it [17, 28].

To further investigate the protective mechanisms of PGE₂ in allergic asthma, we studied the impact of the selective modulation of the EP₂ receptor on the development of AHR and inflammation in aeroallergen-sensitized mice. We also assessed, by means of various

experimental approaches, the relevance of airway MC to such protection. A detailed understanding of the 'EP₂-MC-airway' connection will likely uncover new pharmacological targets.

Material and methods

Material

PGE₂ and the EP₂ agonist (butaprost) were from Cayman (Europe, Tallinn, Estonia). EP₂ antagonist (AH6809), sodium cromoglycate (SCG), phosphate-buffered saline (PBS), dimethyl sulfoxide, 2,4-dinitrophenol (DNP), Evans Blue, formamide and methacholine were purchased from Sigma-Aldrich (Barcelona, Spain).

Experimental animals

Eight-week-old female BALB/c mice were purchased from Harlan (Sant Feliu de Codines, Barcelona, Spain), and 8-week-old female C57BL/6 wild-type (WT) and C57BL/6 *Kit*^{W-sh/W-sh} (mast cell-deficient) mice were bred at the Department of Dermatology and Allergy (Charité – Universitätsmedizin Berlin, Germany). Finally, 8-week-old female EP₂-knockout (KO) and litter-mate WT (C57BL/6 background strain) mice were bred at the Animal Facility of the Universitat Autònoma de Barcelona from two breeding pairs purchased from Jackson (ref. B6.129-PTGER2 tmBrey/J) (Bar Harbor, ME, USA). All animals were housed under a 12-hour light–dark cycle, and all animal procedures were approved by the Ethics Committee for Animal Research of the Universitat Autònoma de Barcelona (Spain).

Sensitization to house dust mite aeroallergens

Sensitization to HDM was performed according to Cates et al. [33]. Briefly, BALB/c, C57BL/6 and C57BL/6 *Kit*^{W-sh/W-sh} (mast cell-deficient) mice were exposed to a purified HDM extract (Alk-Abelló, Madrid, Spain) with a low lipopolysaccharide content (< 0.5 EU/dose). The aeroallergens were administered intranasally (i.n.) under light isoflurane anaesthesia at a dose of 25 µg/mouse for 10 consecutive days. Non-sensitized (NS, control) animals received i.n. saline solution (Fig. 1a).

Treatment with EP agonists and antagonist and sodium cromoglycate

Sensitized mice were treated with either PGE₂, the EP₂ agonist butaprost (Fig. 1b), or the EP₂ antagonist AH6809 (Fig. 1c). The HDM-sensitized untreated mice received PBS in 0.1% dimethyl sulfoxide (as controls for PGE₂ and butaprost-treated), and the HDM-sensitized untreated mice used as controls for AH6809

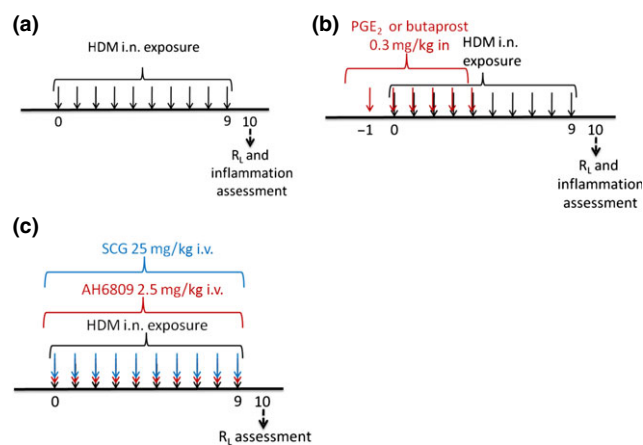


Fig. 1. EP agonist and antagonist treatment protocols. (a) Sensitization to HDM aeroallergens was conducted by means of nasal exposure to an HDM extract (25 μ g/mouse) for 10 consecutive days (days 0 to 9); (b) the treatment with the EP receptor agonist PGE₂ (non-selective), and butaprost (EP₂ selective), was performed through the i.n. administration of 0.3 mg/kg of either agonist one hour before the exposure to the HDM extract, starting 1 day before initiating sensitization (day -1) up to day 4; and (c) the EP₂ antagonist AH6809 was administered intravenously (2.5 mg/kg) one hour before each exposure to HDM for 10 consecutive days (from days 0 to 9), and the MC stabilizer SCG was administered i.p. daily (25 mg/kg) one hour prior to AH6809 treatment.

received physiological serum with 20% ethanol. PGE₂ and butaprost (Fig. 1b) were administered i.n. (0.3 mg/kg) 1 hour before exposure to the HDM extract, starting from one day before initiating sensitization (day -1) up to day 4. The EP₂ antagonist treatment (Fig. 1c) was administered to the corresponding groups intravenously (2.5 mg/kg) one hour before the HDM sensitization for 10 days (from day 0 to day 9). As shown in Fig. 1c, the group of mice receiving the EP₂ antagonist AH6809 was also given the MC inhibitor SCG. SCG was administered daily at a dose of 25 mg/kg in physiological serum by intraperitoneal injection 1 hour prior to AH6809 treatment.

Assessment of airway hyperresponsiveness

Twenty-four hours after exposure to HDM, airway reactivity to increasing doses of methacholine was assessed. Lung resistance (R_L) was measured with the Buxco FinePointe plethysmograph system (Buxco, Troy, NY, USA) in mice anesthetized with ketamine and xylazine. The trachea was cannulated with an 18-gauge needle, and mice were ventilated with a pump ventilator (tidal volume, 12.5 mL/kg; frequency, 120 breaths/min; positive end-respiratory pressure, 2.5–3.0 cm H₂O). Changes from baseline airway reactivity, R_L , were assessed after 1 min of intratracheal nebulization of methacholine. A non-invasive whole-body plethysmography procedure

(WBP Buxco Europe Ltd, Winchester, UK) was also used to screen respiratory function under different experimental conditions [34, 35]. The response to methacholine was averaged and expressed as Penh (Enhanced Pause), a bronchoconstriction index that correlates with lung mechanics [36].

Assessment of airway inflammation

HDM-induced airway inflammation in treated and untreated mice was assessed 24 hours after exposure to aeroallergens through differential cell count from bronchoalveolar lavage (BAL), and lung histology study. BAL was performed by slowly infusing 0.3 mL PBS twice and recovering it by gentle aspiration. BAL cells were stained with Turk solution (0.01% crystal violet in 1% acetic acid). Total airway cellularity and differential cell counts (Diff-Quick, Sigma-Aldrich, Barcelona, Spain) were performed in BAL cytopspins. Relative numbers of eosinophils, lymphocytes, macrophages, and neutrophils were then determined. For the subsequent histology study, the left lung was perfused with formaldehyde, immersed in the fixative, and embedded in paraffin. Paraffin-embedded lung sections (5 μ m) were stained with haematoxylin and eosin for the histopathological evaluation.

mMCP-1 determination

For the evaluation of airway MC activity, right lung lobes were collected for protein extraction using a lysis buffer containing protease inhibitors (Mini Complete Tablet, Roche Diagnostics, Barcelona, Spain). Lysis buffer (250 μ L) was added to the tissue samples, and the resulting solutions were homogenized. The samples were centrifuged, and the supernatants were collected to measure mouse mast cell protease-1 (mMCP-1) levels using a sandwich enzyme-linked immunosorbent assay (ELISA) (Moredun Scientific Ltd., Scotland, UK).

Passive cutaneous anaphylaxis

A group of EP₂-knockout (KO) and litter-mate WT (C57BL/6 background strain) mice was passively sensitized with an intradermal injection of 75 ng of DNP-specific mouse IgE kindly provided by Dr. Juan Rivera from the Immunogenetics laboratory at the National Institute of Arthritis and Musculoskeletal and Skin Diseases, National Institutes of Health (Bethesda, MD, USA) [37], in a 20 μ L volume into the right ear, while the contralateral ear was injected with 20 μ L PBS as a negative control. After 24 h, mice were challenged intravenously with 200 μ g antigen (Ag, DNP-human serum albumin) in PBS containing 1% Evans blue

(100 µL). Mice undergoing PCA were treated with an intravenous injection of 0.3 mg/kg of butaprost in 100 µL PBS or vehicle (100 µL PBS with 0.1% dimethyl sulfoxide) 30 minutes before the Ag injection. Mice were then euthanized with CO₂. The right and left ears were then cut, minced, and the Evans blue dye was extracted with 700 µL formamide at 55°C for 2 h. The absorbance of Evans blue in the extract was determined at 620 nm. The net absorbance was determined by subtracting the baseline absorbance₆₂₀ in the ear receiving PBS from the absorbance₆₂₀ in the Ag-injected ear.

Statistical analysis

Data are represented as means ± standard error of the mean (SEM). Statistical significance was assessed using a two-tailed Student's *t*-test for mean-to-mean comparisons (inflammation, mMCP-1 and PCA). For a comparative assessment of the R_L and Penh curve shapes, differences among the treatments were evaluated by a two-way analysis of variance with a Bonferroni post hoc test. A *P*-value of < 0.05 was considered significant.

Results

Selective effect of EP₂ on the airways reaction to HDM, and on airway MC activity

To specifically study the relevance of the EP₂ receptor in PGE₂-driven protection, HDM-sensitized BALB/c mice were exposed i.n. to the EP₂-selective agonist butaprost, or to control vehicle. AHR, inflammation, and lung MC activity were assessed (See treatment protocol in Fig. 1b).

The local administration of butaprost caused a significant decrease in aeroallergen-induced AHR as assessed by whole-body plethysmography (Fig. 2a). The use of an invasive method to measure AHR showed a very similar outcome, that is a 50% decrease in airway resistance (R_L) in butaprost-treated versus vehicle-treated mice (Fig. 2b). A significant butaprost effect was seen with methacholine concentrations as low as 2.5 mg/mL under invasive conditions, and 12.5 mg/mL using WBP. Such EP₂-mediated attenuated smooth muscle reactivity was equivalent to that exerted by the non-selective EP agonist PGE₂ in HDM-sensitized BALB/c mice [24]. A differential inflammatory cell count in the airways was also performed in mice exposed to butaprost (Fig. 2c). A strong eosinophilic recruitment was induced in HDM-sensitized mice. Such eosinophilia was significantly prevented by the selective activation of the local EP₂ receptor, similarly to what we had shown using a

non-selective agonist [24]. Butaprost reduced eosinophilic, but not lymphocytic or neutrophilic, infiltration by up to 84% in mice exposed to HDM aeroallergens. Finally, lung MC activity was evaluated *in vivo* by measuring the concentration of the mMCP-1 protein in the airways (Fig. 2d). mMCP-1 was strongly overexpressed locally by a factor of 20 in HDM-exposed versus non-exposed mice, and the EP₂ agonist butaprost administered i.n. at a dose of 0.3 mg/kg decreased MC activity *in vivo* by 40%.

Effect of the blockade of the endogenous PGE₂-EP₂ pathway and/or MC activity in HDM-induced allergic airway disease

To uncover the relevance of MC in mediating PGE₂-driven protection, and to elucidate the role of the EP₂ receptor, the EP₂ antagonist AH6809 was administered to both HDM-sensitized and non-sensitized BALB/c mice in the presence of the MC stabiliser SCG (see treatment protocol in Fig. 1c).

As shown in Fig. 3a, exposure to HDM aeroallergens induced a methacholine concentration-dependent AHR, which was further increased in the presence of the EP₂ antagonist. The worsening of AHR under AH6809 was fully reversed by SCG. Although no statistical significance was detected, a similar trend was observed in airway inflammation (Fig. 3b), where the EP₂ antagonist aggravated the eosinophilic infiltrate, and SCG prevented such worsening.

Effect of EP₂ agonism in a fully MC-dependent reaction in vivo

PCA, a fully MC-dependent *in vivo* model of allergy, was used to further highlight the relevance of MC to PGE₂-EP₂-mediated protection (Fig. 4).

When wild-type mice were locally sensitized with an intradermal injection of DNP-specific IgE in the right ear, and intravenously challenged with DNP, they exhibited a significant extravasation of Evans Blue compared with the non-sensitized left ear. As also shown in Fig. 4, PCA was decreased by 45% when the EP₂ agonist butaprost was pre-administered (expressed as absorbance₆₂₀Ag ear-absorbance₆₂₀PBS ear). To support the fact that butaprost is selectively acting through the PGE₂-EP₂ pathway in mice, the same experimental approach in the PCA model was performed using EP₂ KO mice. Untreated EP₂ receptor-deficient mice developed a significant PCA response upon IgE/DNP intradermal sensitization and challenge. When the EP₂ KO animals were exposed to the EP₂ agonist, neither a reduction nor an increase in the IgE/DNP-induced reaction was observed.

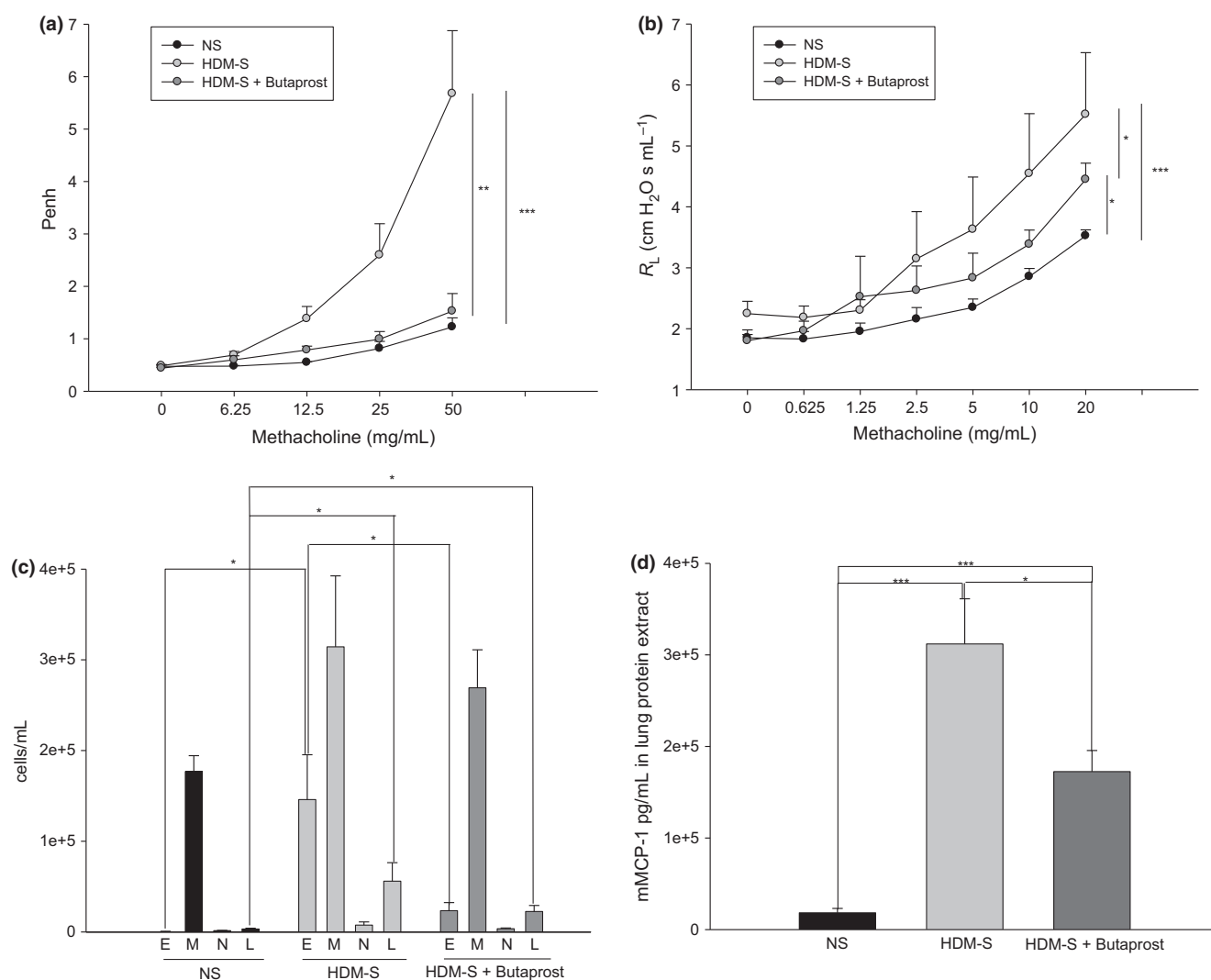


Fig. 2. Airway response to HDM exposure of BALB/c mice treated with the selective EP₂ agonist butaprost (N = 8–13). Butaprost, an EP₂ agonist, given i.n. (protocol Fig. 1a), prevented the development of airway hyperresponsiveness assessed through both (a) a non-invasive and (b) an invasive system. (c) Differential inflammatory cell count per mL determined from counting at least 300 cells (E, eosinophils; M, macrophages; L, lymphocytes; N, neutrophils) and (d) airway MC activity assessed in sensitized, treated, and untreated mice by measuring the local production of mouse mast cell protease-1 (m-MCP1). (**P*-value < 0.05, ***P*-value < 0.01, and ****P*-value < 0.001).

PGE₂ protects against MC-dependent (BALB/c), but not MC-independent (C57BL/6) HDM-induced airway response

As C57BL/6 mice have been proposed to develop airway sensitivity to Ag through MC-independent mechanisms, this mouse strain was used to further elucidate the relevance of MC to PGE₂-driven protection.

We first demonstrated that MC-deficient C57BL/6 mice (Kit^{W-sh/W-sh}) developed a similar airway reaction to the WT C57BL/6 control – non-MC-deficient – animals (see sensitization protocol in Fig. 1a). Total airway inflammatory cell count (Fig. 5a) and eosinophilic inflammation (Fig. 5b and c) were equivalent in both C57 MC-deficient and wild-type animals sensitized to

HDM aeroallergens. In addition, a qualitative lung pathology study further revealed that the nature of the airway inflammatory process was analogous in both MC-deficient and control mice (Fig. 5d). Furthermore, baseline airway functional reactivity to methacholine was lower in mice lacking MC than in the corresponding WT controls. Following exposure to HDM, the MC-deficient mice developed an AHR statistically comparable to that of WT animals (Fig. S1). Lung mMCP-1 protein was measured and, as expected, was shown to be significantly upregulated upon aeroallergen challenge in the WT mice, but not in the MC-deficient animals (data not shown).

Figure 6 shows airway responses to HDM exposure in PGE₂-treated wild-type C57BL/6 mice (see treatment

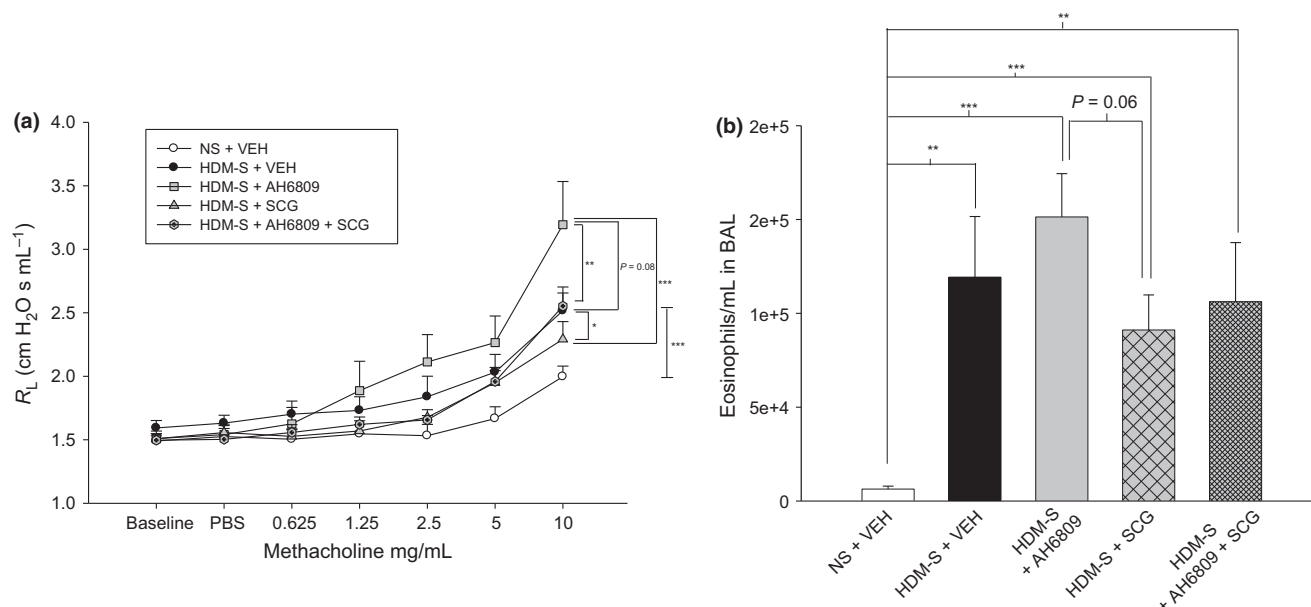


Fig. 3. HDM-induced airway reactivity after blocking endogenous PGE₂ and/or mast cell activity ($N = 7-8$). (a) Airway hyperresponsiveness to methacholine was assessed by measuring lung resistance (R_L) in HDM-sensitized mice treated with the EP₂ antagonist AH6809, and either exposed or not to SCG, the mast cell stabilizer. AH6809 worsened airway reactivity when pre-administered to HDM-sensitized mice. BALBc mice subject to treatment with the AH6809, and treated with SCG, recovered from the deleterious effect of AH6809 (protocol Fig. 1c). (b) Similar trends were observed in lung eosinophilia. (* P -value < 0.05, ** P -value < 0.01, and *** P -value < 0.001).

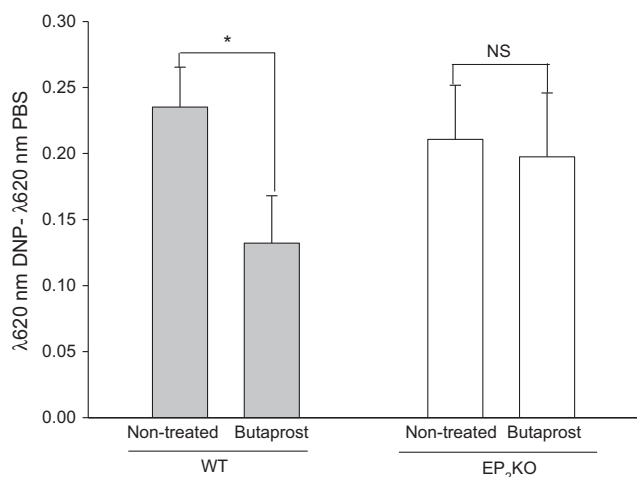


Fig. 4. EP₂ agonism on IgE/DNP-mediated skin reaction (PCA) in wild-type and EP₂-knockout mice ($N = 6-8$). The absorbance of Evans blue dye was measured at 620 nm from sensitized and non-sensitized ears in butaprost (EP₂ agonist)-treated and non-treated C57BL/6 WT or EP₂ KO mice. The net absorbance was calculated by subtracting baseline absorbance. Butaprost significantly reduced Evans blue extravasation in WT mice, but did not reduce the skin reaction in the EP₂ KO mice. (* P -value < 0.05).

protocol in Fig. 1a). Local PGE₂ treatment neither prevented eosinophilia (Fig. 6a) nor AHR (Fig. 6b) in the HDM-sensitized C57BL/6 mice. As per mMCP-1 assessment in the mice's airways, the activity of lung MC in both PGE₂-treated and untreated C57 mice was

statistically equivalent (Fig. 6c). This is in contrast to our previous observation of PGE₂ fully abrogating HDM-induced AHR in BALB/c mice, paralleled by a significant reduction in eosinophil recruitment and by a decrease in lung MC activity [24].

Discussion

We have demonstrated, by means of a broad and diverse experimental approach, that MC are mediating the protective effect exerted by PGE₂ *in vivo* in HDM-sensitized mice and that EP₂ is likely to be the critical receptor responsible for this beneficial effect. Indeed, the modulation of MC activity by PGE₂ acting selectively on the EP₂ receptor has a direct impact on antigen-induced AHR and inflammation.

We first analysed the effect exerted by the selective EP₂ agonist butaprost in HDM-induced airway reactivity, and lung MC function, in BALB/c mice. Butaprost did prevent HDM aeroallergens-induced airway response. As for the assessment of airway reactivity to methacholine, both invasive and non-invasive procedures were used to demonstrate the benefit of i.n. butaprost on AHR. To our knowledge, this is the first demonstration of the ability of EP₂ to protect from AHR. Eosinophil recruitment was also significantly prevented with butaprost treatment. Our data support the demonstration by Sturm et al. [20] of an over 50% reduction in eosinophilia due to endogenous PGE₂

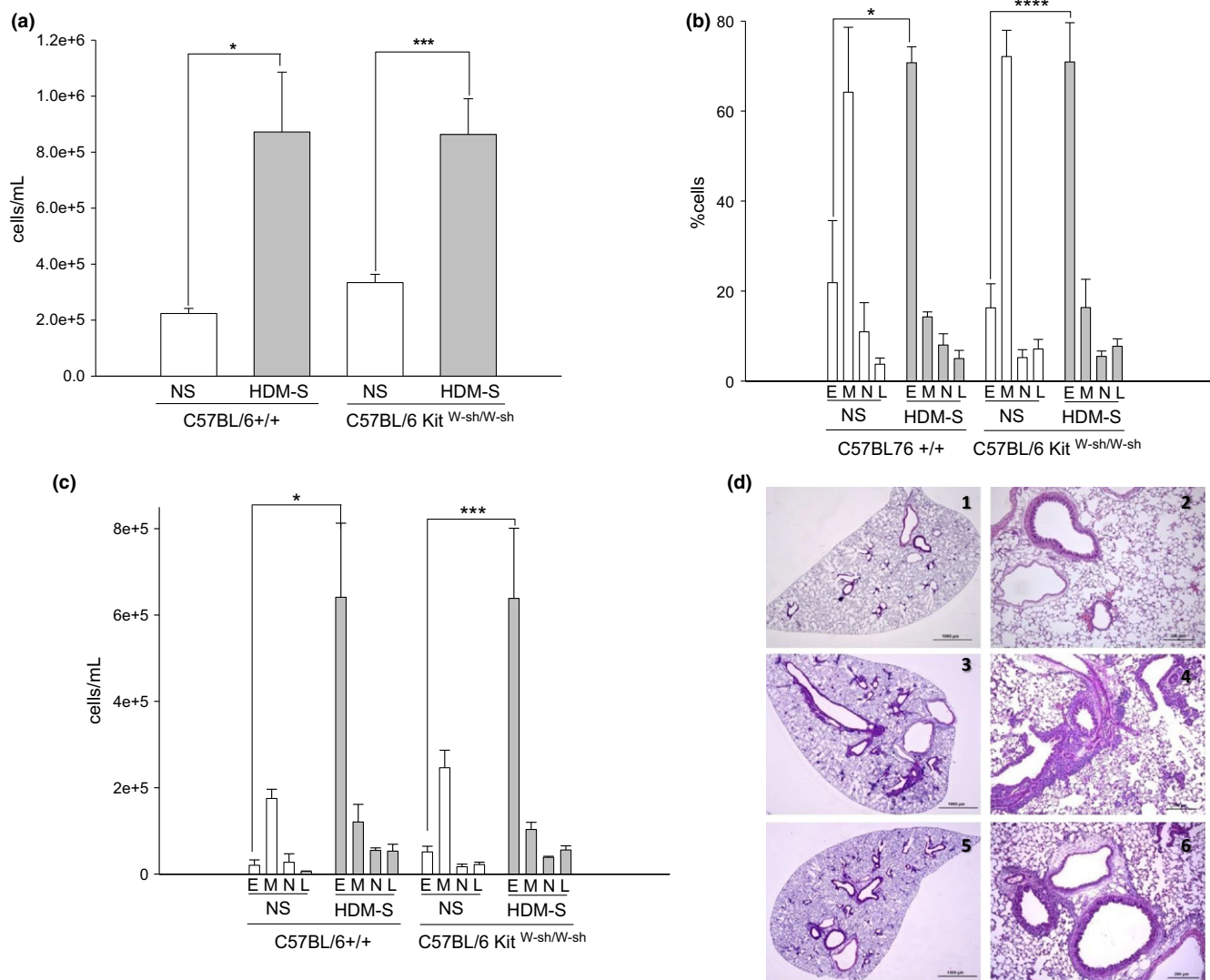


Fig. 5. Airway pathology of HDM-sensitive mast cell-deficient *C57BL/6 Kit^{W-sh/W-sh}* mice. (*N* = 5–6). (a) Total inflammatory cell count in bronchoalveolar lavage (BAL), (b) differential inflammatory cell count, (c) inflammatory cell subtypes per mL determined from combining the bronchoalveolar lavage (BAL) total cell count and the differential count, and (d) microphotographs of haematoxylin and eosin stained lung sections from wild-type (WT) mice (NS: 1,2, HDM-S: 3,4) and MC-deficient mice (HDM-S: 5, 6). Scale bar equivalent to 1000 μ m (E, eosinophils; M, macrophages; L, lymphocytes; N, neutrophils). HDM-induced airway inflammation (under protocol Fig. 1a) developed in the absence of MC in *C57BL/6 Kit^{W-sh/W-sh}* mice. (**P*-value < 0.05, ****P*-value < 0.001 and *****P*-value < 0.0005).

acting on EP₂. In the HDM murine model, we had discarded EP₃ as a beneficial molecule [17]. More recently, others have also discounted EP₃ as the receptor causing the protective effect [38, 39]. Hence, Kunikata et al. [27] suggestion that EP₃ activation may be beneficial, using an OVA model in EP₃ KO mice, is probably explained by their experimental approach, that is the use of a different antigen, through a different route and, possibly, in a mouse model where the EP receptor expression pattern may be modified. It is also to note that Kunikata et al. [27] did not see any benefit from injecting 16,16-dimethyl (dm)-PGE₂, a modified PGE₂ molecule, while in a similar OVA model, De Campo and Henry did [40]. Irrespective of

the reasons for discrepancy, in our view, the HDM-sensitive murine model provides ideal conditions to further elucidate PGE₂-induced anti-inflammatory and immunoregulatory mechanisms [24]. The selective EP₂ agonist butaprost did also reduce, by approximately 50%, the ability of airway MC to release mMCP-1, a MC-specific protease. We anticipated the effect of the EP₂ agonist on lung MC activity *in vivo* from our consistent *in vitro* observation that butaprost was able to inhibit murine MC activity [41]. Furthermore, we know that human airway MC bear EP receptors and that EP₂ activation has indeed been described as 'the' inhibitory receptor, and no inhibitory action was attributed to the MC EP₃ receptor [29–31].

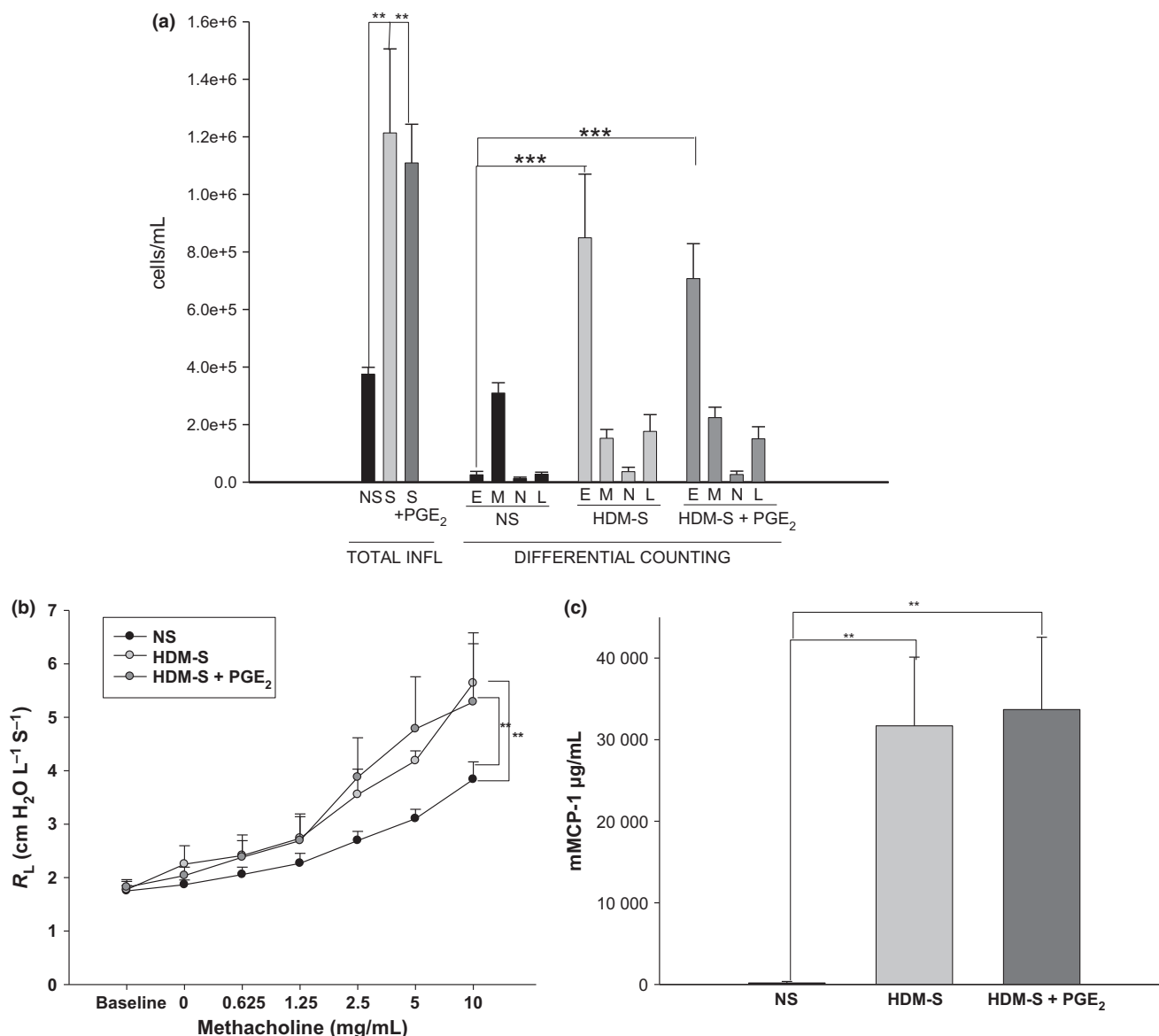


Fig. 6. HDM-induced airway response and mast cell activity following PGE₂ treatment of C57BL/6 mice ($N = 7-8$). (a) Total inflammation assessed on fresh BAL samples and differential inflammatory cell count of BAL cells per mL determined from counting at least 300 cells (E-eosinophils, M-macrophages, L-lymphocytes, N-neutrophils), (b) AHR measured as lung resistance (R_L), and (c) airway mast cell activity assessed by measuring local lung protein production of mouse mast cell protease-1 (m-MCP1). PGE₂ treatment in C57BL/6-sensitized mice (under protocol Fig. 1b) did neither prevent HDM-induced airway response nor MC increased activity. (** P -value < 0.01 and *** P -value < 0.001).

Thus, EP₂ agonism prevents the development of AHR and inflammation, and such reduced response is associated with a diminished lung MC activity. The quantitative effect exerted by butaprost is similar to that of the non-selective EP agonist (PGE₂) as for our previous studies [3]. This highlights the likely primary role of EP₂ in PGE₂-driven protection in asthma [4–6] and supports the hypothesis that PGE₂ acts through a restraining effect on MC. It is worthwhile highlighting that the route of administration and the dose used in our experiments were chosen on the basis of our previous observations [17, 24, 28], where such treatment

conditions exerted optimal responses, very much in agreement with others' studies [39]. The ability of butaprost and PGE₂ to modulate HDM-induced airway pathology through an i.n. pharmacological intervention underlines the potential for the identification of local anti-asthma targets with a favourable safety-to-efficacy ratio.

We then intended to elucidate the consequences of PGE₂-associated MC modulation on aeroallergens-induced AHR and inflammation. We performed two experiments. First, we blocked the endogenous PGE₂ pathway in HDM-sensitized mice by administering an

EP₂ receptor antagonist (AH6809). The choice of EP₂ versus other EP receptors was based on the butaprost *in vivo* data and observations of the inhibitory potential of selective EP₂ agonist in human and murine MC in *in vitro* systems [32, 41]. Despite the fact that AH6809 is thought to also have a weak antagonistic effect on the EP₁ receptor, it has been established that EP₁ is not functionally expressed on MC populations [31, 32, 41]. The inhibition of the endogenous PGE₂-EP₂ pathway in the HDM-sensitive mice did by itself worsen methacholine-induced AHR (a trend towards a similar worsening was observed at the inflammatory level). This novel piece of information suggests that mice exposed to HDM aeroallergens may trigger a spontaneous PGE₂-orchestrated endogenous compensatory mechanism to attenuate the airway pathology induced by aeroallergens [17], hence supporting the hypothesis that deficiencies in the regulation of COX2-PGE₂ may be a contributing factor to the progression of asthma [9]. Interestingly, the harm induced by the blockade of the PGE₂-EP₂ pathway was fully neutralized by the MC stabilizer SCG. This experiment provided evidence to support that locking spontaneous PGE₂ protection does trigger MC hyperreleasability, and such MC over-activity is directly responsible for the increased airway inflammation and AHR induced by HDM aeroallergens. The second experimental approach to further prove that the modulation of MC activity by EP₂ may correlate with the Ag-induced reaction was the testing of the effect of an EP₂ agonist in an *in vivo* MC-dependent model of allergy, PCA [42]. In this model, the injection of specific IgE and DNP lead to MC-driven ear swelling. When butaprost was given to these passively sensitized mice, the MC-triggered ear reaction was substantially prevented. It is to note that, in order to ascertain that EP₂ was the unique EP receptor mediating such effect, we also demonstrated that the magnitude of the PCA response was equivalent in butaprost-treated and non-treated EP₂ KO mice. The lack of protection against IgE-DNP-induced swelling in the ears of EP₂ KO mice does therefore seem to exclude the involvement of other receptors. The fact that baseline swelling is not worse in EP₂ KO mice may be attributable to two phenomena: a maximum swelling response is reached, and/or a compensatory overexpression of other EP receptors has developed in the EP₂ KO mice. Taken together, both experiments suggest that MCs are implicated as effector players in the PGE₂-mediated benefit in antigen or allergen-induced damage. Consistent with the outcome observed as a result of using the selective EP₂ receptor agonist in HDM-sensitized mice, a likely direct connection between EP₂ protection, reduced MC activity, and reduced aeroallergen-caused AHR and inflammation has therefore been established.

As a follow-up of our observations on the protective nature of exogenous PGE₂ in HDM-sensitive BALB/c mice [24], we have undertaken a study aimed at further demonstrating the relevance of MC to the PGE₂-driven outcome. We have first shown that C57BL/6 *Kit*^{W-sh/W-sh} MC-deficient mice exposed to HDM aeroallergens develop both AHR and eosinophilic inflammation to the same extent as the corresponding C57BL/6 wild-type control animals, in spite of exhibiting a reduced lung MC activity. Furthermore, we have uncovered that the inflammatory infiltrate in the mice lacking MC was qualitatively analogous to that of the control animals. This is the first report showing that HDM aeroallergens, which are the primary cause of human asthma, can induce AHR through a non-MC-mediated mechanism, a finding very much along the lines of those reported by other authors using the C57BL/6 strain, but exposing the animals to different antigens [43, 44]. This result suggests that, contrary to BALB/c mice, in a C57BL/6 genetic background, exposure to aeroallergens can trigger airway pathology through a non-IgE-committed response, possibly through mechanisms of innate immunity [45]. This is an interesting finding that may reflect the non-Th2 asthma endotype (MC-independent) in patients [46]. No matter the underlying explanation for the functional irrelevance of airway MC in sensitized C57BL/6 mice, this model provided ideal grounds for the evaluation of the importance of MC to PGE₂ protection. Thus, by comparing the effect of exogenous PGE₂ in HDM-sensitized wild-type C57BL/6 mice with our previous observation on BALB/c animals, we have shown that PGE₂ protection only occurs when MC do play a significant role. It is to note that PGE₂ does not inhibit lung MC activity in C57BL/6 mice *in vivo*, but it does prevent the BALB/c airway MC population from being fully activated [24]. All in all, this experimental approach further supports the hypothesis of MC as central functional players in mediating PGE₂-driven protection in HDM-sensitized mice.

Interestingly, a recent paper by Zaslon et al. [39] suggested the beneficial effect of misoprostol (PGE₁ analogue) in OVA-sensitized C57BL/6 mice, possibly via its interaction with the T cells' EP₂ receptor. Additional studies are needed to conciliate both set of findings, but it is worth being highlighted that their data do not exclude other cell populations from being relevant in mediating the observed protective effect. The use of OVA, instead of natural aeroallergens, may be crucial to the relative importance of T *versus* other cell types. Also, the fact that misoprostol's primary target is the EP₃ receptor rather than EP₂ may translate into the different outcome. Noticeably, the importance of the interaction of PGE₂, or analogues, with other cell populations cannot be excluded in the light of our

results. In fact, PGE₂ effects have been ascribed to its functional interaction with other cell populations such as T cell and eosinophils [19, 20], or even dendritic cells, as we recently published [24].

Our results highlight the likely relevance of the EP₂-MC axis in the beneficial effect exerted by PGE₂ in experimental allergic asthma. The protection, achieved by means of a selective EP₂ activation, may be, at least in part, the result of a direct interaction of PGE₂ with airway MC. Our data reveal that in turn, MC play a role in the regulation of the severity of allergic airway disease as a result of EP₂ receptor modulation. In summary, our observations reveal the presence of a relevant anti-asthma target along the 'EP₂-MC-airway' pathway.

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Conflict of interest

The authors declare no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Airway pathology in HDM-sensitized mast cell-deficient C57BL/6 Kit^{W-sh/W-sh} mice (N = 6).