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Eosinophils as a novel cell source of prostaglandin D2: autocrine role in allergic inflammation

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Abstract

Prostaglandin (PG)D_2 is a key mediator of allergic inflammatory diseases that is mainly synthesized by mast cells, which constitutively express high levels of the terminal enzyme involved in PGD_2 synthesis, the hematopoietic PGD synthase (H-PGDS). Here, we investigated whether eosinophils are also able to synthesize, and therefore, supply biologically active PGD_2. PGD_2 synthesis was evaluated within human blood eosinophils, *in vitro*-differentiated mouse eosinophils, and eosinophils infiltrating inflammatory site of mouse allergic reaction. Biological function of eosinophil-derived PGD_2 was studied by employing inhibitors of synthesis and activity.

Constitutive expression of H-PGDS was found within non-stimulated human circulating eosinophils. Acute stimulation of human eosinophils with A23187 (0.1 – 5 μM) evoked PGD_2 synthesis, which was located at the nuclear envelope and was inhibited by pre-treatment with HQL-79 (10 μM), a specific H-PGDS inhibitor. Pre-stimulation of human eosinophils with arachidonic acid (AA; 10 μM) or human eotaxin (6 nM) also enhanced HQL-79-sensitive PGD_2 synthesis, which, by acting on membrane-expressed specific receptors (DP_1 and DP_2), displayed an autocrine/paracrine ability to trigger leukotriene (LT)C_4 synthesis and lipid body biogenesis, hallmark events of eosinophil activation. *In vitro*-differentiated mouse eosinophils also synthesized paracrine/autocrine active PGD_2 in response to AA stimulation. *In vivo*, at late time point of the allergic reaction, infiltrating eosinophils found at the inflammatory site appeared as an auxiliary PGD_2-synthesizing cell population.

Our findings reveal that eosinophils are indeed able to synthesize and secrete PGD_2, hence representing during allergic inflammation an extra cell source of PGD_2, which functions as an autocrine signal for eosinophil activation.

Keywords

PGD_2; eosinophils; mast cells; cysteinyl leukotrienes; allergy; lipid droplets; CCL11

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Introduction

Mast cells and eosinophils, two of the principal effector cell types activated at the sites of allergic inflammation, are major participants in the pathogenesis of asthma and other forms of allergic disorders (1,2). Mast cells and eosinophils have the potential to generate and release diverse lipid mediators that are critical to the development and perpetuation of allergic inflammation, including prostaglandins and leukotrienes derived from the oxidative metabolism of arachidonic acid (AA). Both mast cells and eosinophils express the sole leukotriene (LT)C_4-synthesizing enzyme, named LTC_4 synthase (LTC_4S) and are major cell sources of cysteinyl leukotrienes (cysLTs). LTC_4 and its extracellular derivatives, LTD_4 and LTE_4, have many well-recognized functions as mediators of the allergic response, causing bronchoconstriction, mucous hypersecretion, increased microvascular permeability, bronchial hyperresponsiveness, and eosinophil infiltration and activation (3,4). Different from LTC_4-synthesizing capabilities, specific prostanoids produced by mast cells and eosinophils appear to differ according to their differential expression of prostanoid-synthesizing terminal isoenzymes (5,6). Concerning prostanoids with allergy-relevant functions, mast cells are considered the predominant cellular domain of hematopoietic prostaglandin (PG)D_2 synthase (H-PGDS) among resident and recruited cells in allergic inflammatory tissues (7); PGD_2 is also the major prostanoid produced by mast cells (8). Indeed, PGD_2 and its metabolites have been proposed to be selective markers for mast cell activation *in vivo* (9–11). But recently, the dogma that mast cells are the single PGD_2 source in allergic inflammatory conditions has been challenged. New findings unveiled that although PGD_2 synthesis seems to be primarily controlled by allergy-relevant cells, it is not restricted to mast cells. Among these additional PGD_2-synthesizing cellular sources are: (i) both direct and indirect endothelium-mediated generation of PGD_2 (12,13); (ii) Th2 lymphocytes capability of synthesizing small, yet significant, amounts of PGD_2 (14); (iii) skin dendritic cells as supplier of PGD_2 with roles in skin inflammation (15); (iv) host defense-related PGD_2 synthesis by activated macrophages (16,17); and (v) basophil-driven PGD_2 release (18). What about eosinophils? Among prostanoids, it is well accepted that eosinophils are producers of the putative mediators, thromboxane A_2 and PGE_2, rather than mediators of allergic reactions. It is presumed that the lack of PGD_2-synthesizing capability by eosinophils relies on anecdotal evidence of no H-PGDS expression within eosinophils. Nevertheless, while some indications of PGD_2-synthesizing activity may exist (19–21), definitive demonstration that eosinophils can generate PGD_2 is still lacking.

Understanding the mechanisms governing PGD_2 synthesis, including the identification of specific PGD_2 -producing cells, is important as PGD_2 has emerged as a key mediator of the pathogenesis of allergic diseases. PGD_2 recruits and activates eosinophils, as well as basophils and Th2 lymphocytes (22–24). *In vivo*, PGD_2 administration in human volunteers or animals imitates a variety of allergic features (25,26). PGD_2 effects are mediated by the activation of the two known PGD_2 receptors, namely D prostanoid receptor 1 (DP_1) and DP_2 (also known as CRTH2) (22,27–29). Eosinophils co-express both the classic DP_1 receptors coupled to adenylyl cyclase, as well as, pertussis toxin (PTX)-sensitive DP_2 (23). It has been shown that PGD_2 ability to activate eosinophils may be determined by a balance between these DP_1- *versus* DP_2-driven opposing downstream signaling pathways (e.g. PGD_2-induced eosinophil chemotaxis) (23,30,31), but alternatively may well be dependent on an initially unexpected DP_1/DP_2 cooperative effect (e.g. PGD_2-elicited enhanced LTC_4 synthesis by eosinophils) (32). The appeal of PGD_2 as a therapeutic target in allergic diseases, such as asthma, can be promptly attested by the rapid development of selective pharmacological tools to examine the pro-allergic contributions of these two receptors. Of note, since a variety of prostanoid molecules, including PGD_2 metabolites, PGF_2 and 11-dehydro-TBX_2 are capable to activate DP_2 (22,33–38), one can hypothesize physiopathological outcomes of activation of PGD_2 receptors even in the absence of PGD_2 production. However, the

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concentrations of PGD_2 are indeed elevated in a variety of chronic allergic tissues, including in the nasal mucosa of allergic rhinitis (39), the airways of asthmatics (40,41), and the skin of patients with atopic dermatitis (42). Although in these conditions, PGD_2 synthesis is portrayed as a predominantly mast cell-derived product (41), little is known about the alternative and complementary cell sources of PGD_2.

Our study reports that, upon proper stimulation, both human and mouse eosinophils can produce significant amounts of biologically relevant PGD_2. PGD_2 intracellular synthesis within eosinophils was catalyzed by eosinophil-expressed H-PGDS and led to PGD_2 receptor-mediated paracrine/autocrine functions, contributing to eosinophil activation.

Material and Methods

Animals

Swiss and BALB/c mice of 16–20 g from both sexes were used. The animals were obtained from the Oswaldo Cruz Foundation breeding unit (Rio de Janeiro, Brazil). The protocols were approved by the Oswaldo Cruz Foundation Animal Welfare Committee.

Allergic pleurisy in sensitized mice

As previously described (43), mice were sensitized with a subcutaneous injection (0.2 mL) of ovalbumin (OVA; 50 μg) and $Al(OH)_3$ (5 mg) in a 0.9% NaCl solution (saline) at days 1 and 7. Allergic challenge was performed at day 14 by means of an intrapleural (i.pl.) injection of OVA (12 μg/cavity; 0.1 mL). Control animals received vehicle (saline; 0.1 mL). The mice were euthanized by CO_2 inhalation 48 h after challenge. The pleural cavities were rinsed with 500 μL of Ca^{2+}/Mg^{2+}free HBSS; pH 7.4 ($HBSS^{-/-}$).

Pleural eosinophil counts

Total leukocyte counts were performed using a Neubauer chamber under an optical microscope, after dilution with Turk fluid (2% acetic acid). Differential counts of mononuclear cells, neutrophils, and eosinophils were performed under an oil immersion objective using cytopins (Cytospin 3; Shandon Inc., Pittsburgh, PA) stained by the May-Grunwald-Giemsa method. Counts are reported as eosinophils *per* cavity.

Isolation of human blood eosinophils

Peripheral blood was obtained with informed consent from normal donors. Briefly, following dextran sedimentation and Ficoll gradient steps, eosinophils were isolated from contaminating neutrophils by negative immunomagnetic selection using the EasySep™ system (StemCell Technologies Inc.), which includes antibodies against human CD2, CD3, CD14, CD16, CD19, CD20, CD36, CD56, CD123 and glycophorin A coupled to magnetic particles (cell purity ~ 99%; cell viability ~ 95%) (32). The protocol was approved by ethical review boards of both the Federal University of Rio de Janeiro and the Oswaldo Cruz Foundation (Rio de Janeiro, Brazil).

In vitro stimulation of human eosinophils

Purified human eosinophils at 2×10^6 cells/mL in Ca^{2+}/Mg^{2+} HBSS ($HBSS^{+/+}$; pH 7.4) were incubated with A23187 (0.1 – 5 μM; Sigma) for 15 min in a water bath (37 °C). Alternatively, eosinophils were stimulated with AA (10 μM; Cayman), human recombinant eotaxin (also known as CCL11 or eotaxin-1; 6 nM; R&D), macrophage migration inhibitory factor (MIF; 50 ng/mL; R&D), PAF (1 μM) or PGD_2 (25 nM) in $HBSS^{-/-}$ for 1 h. To enable detection of released PGD_2 or LTC_4 by EIA, AA or eotaxin-stimulated eosinophils were also challenged with 0.1 μM A23187 (Sigma) for an additional 15 min in $HBSS^{+/+}$.

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Each condition was repeated at least three times with eosinophils purified from different donors.

In vitro eosinophil differentiation from mouse bone marrow cells

With slight modifications, eosinophils were differentiated *in vitro* from mouse bone marrow cells as previously described (44). Briefly, bone marrow cells were collected from femurs and tibiae of wild-type BALB/c mice with RPMI 1640 (Sigma) containing 20% FBS. After RBC lysis, cells were cultured at 10^6 cells/mL in RPMI 1640 containing 20% FBS (VitroCell), 100 IU/ml penicillin, 10 μg/ml streptomycin, 2 mM glutamine (Sigma) and 1 mM sodium pyruvate (Sigma), 100 ng/mL stem cell factor (SCF; PeproTech) and 100 ng/mL FLT3 ligand (PeproTech) from days 0 to 4. On day 4, the SCF and FLT3-L were replaced with IL-5(10 ng/mL; Peprotech). On day 14, eosinophils were enumerated (purity ≥ 75%), resuspended in $HBSS^{-/-}$ (2×10^6 cells/mL) and stimulated with AA (10 μM; Cayman), rm eotaxin (6 nM), PAF (1 μM), or PGD_2 (25 nM).

Treatments

For *in vitro* studies, human or mouse eosinophils in $HBSS^{-/-}$ were pre-treated for 30 min with the H-PGDS inhibitor HQL-79 (10 μM), DP1 receptor antagonist BWA868c (200 nM), or CRTH2 receptor antagonists Bay-u3405 (200 nM) and Cay 10471 (200 nM), or concomitantly with antibodies against PGD_2 (10 μL) (all from Cayman Chemicals) or its isotype control. Notably, pre-treatments did not modify the eosinophil basal lipid body content, nor did they affect eosinophil viability (~90%) (data not shown). For *in vivo* assays using the pleurisy model, animals were pre-treated with i.p. injections of HQL-79 (1 mg/Kg) 30 min before allergic challenge.

Stock solutions of stimuli and inhibitors were prepared in $HBSS^{-/-}$ containing 0.1% endotoxin-free ovalbumin, aliquoted, and stored at −20 °C. Specifically, concentration of HQL-79 stock solution was 5 mM in DMSO (DMSO final concentration during cell incubations was 0.02% and had no effect on eosinophils). A23187, BWA868c Bay-u3405 and Cay 10471 were also diluted in DMSO, while AA, PAF and PGD_2 were diluted in ethanol. The final vehicle concentration was < 0.01% and had no effect on eosinophils.

H-PGDS immunolocalization

Human eosinophils (2×10^5 cells) were cytocentrifuged (500 rpm, 5 min) onto glass slides, and fixed in 2% paraformaldehyde for 10 min. After washing (3 × 10 min) with 0.05% saponin (Sigma) containing 1% BSA (Sigma) in $HBSS^{-/-}$, the slides were incubated for 30 min with rabbit polyclonal antiserum anti-H-PGDS (Cayman) or with normal rabbit serum, washed with saponin/BSA, incubated for 30 min with Alexa Fluor 488-labeled goat anti-rabbit IgG antibody (Molecular Probes), washed with saponin, washed with $HBSS^{-/-}$, and then an aqueous medium containing DAPI (Vector Laboratories). The images were obtained using an Olympus BX51 fluorescence microscope equipped with a Plan Apo 100× 1.4 Ph3 objective and an Olympus 72 digital camera (Olympus Optical CO., Japan) in conjunction with Cell^F Imaging Software for Life Science Microscopy (Olympus Life Science Europe GMBH). The images were edited using Adobe Photoshop 5.5 software (Adobe Systems).

Quantification of eicosanoids

PGD_2, PGE_2 or cysLTs found in cell-free pleural fluid and eosinophil supernatants were measured by commercial EIA kits, according to the manufacturer's instructions (Cayman).

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EicosaCell for intracellular PGD_2 immuno-detection

By using EicosaCell (45) to detect intracellular PGD_2 at its sites of synthesis, *in vitro*-stimulated eosinophils (in $HBSS^{+/+}$) or pleural cells were mixed with an equal volume of EDAC solution (0.2% in $HBSS^{-/-}$ containing 1% BSA). After a 15 min incubation at 37°C with EDAC, eosinophils were washed with $HBSS^{-/-}$, cytospun onto glass slides, incubated with $HBSS^{-/-}$ with 1% BSA for 30 min, and then incubated with rabbit anti-PGD_2 antibodies (Assay Designs) overnight. Notably, PGD_2 immuno-detection was also achieved when another anti-PGD_2 antibody (Cayman) was employed (data not shown). The anti-ADRP (Adipose differentiation-related protein) antibody was also added overnight to distinguish cytoplasmic lipid bodies within eosinophils. The cells were washed with $HBSS^{-/-}$ containing 1% BSA (3x 10 min) and then incubated with DyLight488 anti-mouse IgG and DyLight549 anti-guinea pig secondary antibodies (Jackson) for 1 h. EicosaCell images were obtained using a LEICA TCS – SP5 confocal fluorescence microscope, equipped with a 63X objective in conjunction with LAS-AF 2.2.0 Software.

Alternatively, to detect PGD_2-synthesizing eosinophils by flow cytometry, after incubation with EDAC, pleural fluid cells were washed with $HBSS^{-/-}$, incubated overnight with rabbit anti-PGD_2 antibodies, washed with $HBSS^{-/-}$, and then incubated with DyLight488 anti-mouse IgG and antibody anti-SiglecF-PE (or isotype-PE) (from eBioscience) for 30 min. After washings, cells were analyzed by flow cytometry in a FACScalibur (BD) flow cytometer.

RT-PCR

mRNA was extracted from 10^6 non-stimulated or AA-stimulated human eosinophils according to the manufacturer's protocol (RNeasy Kit, Qiagen, Germantown, MD). cDNA synthesis and RT-PCR conditions followed standard protocols. Primer sequences for human H-PGDS were the same as previously published (46) and for human β-actin were, 5′-GACAGGATGCAGAAGGAGAT-3′ and 5′-TGTGTGGACTTGGGAGAGGACT-3′ (based on GenBank sequence X00351).

Lipid body staining and enumeration

Cytospun cells, while still moist, were fixed in 3.7% formaldehyde (diluted in $HBSS^{-/-}$), rinsed in 0.1 M cacodylate buffer (pH 7.4), stained with 1.5% OsO_4 for 30 min, rinsed in distilled H_2O, immersed in 1.0% thiocarbohydrazide for 5 min, rinsed in 0.1 M cacodylate buffer, restained with 1.5% OsO_4 for 3 min, rinsed in distilled water, and then dried and mounted. The cell morphology was observed, and the lipid bodies were enumerated by light microscopy. Fifty consecutively scanned eosinophils were evaluated in a blinded fashion by more than one individual, and the results were expressed as the number of lipid bodies *per* eosinophil.

Statistical analysis

The results were expressed as mean ± SEM and were analyzed statistically by means of ANOVA followed by Student-Newman-Keuls test, with the level of significance set at $P < 0.05$.

Results

Human circulating eosinophils are enzymatically competent cells for prompt synthesis of PGD_2

Analyses by fluorescence microscopy demonstrated that H-PGDS is constitutively expressed by human circulating eosinophils freshly isolated from healthy donors. As shown in Figure

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1A, H-PGDS-labeling within non-stimulated human blood eosinophils displayed a cytoplasmic staining pattern. No immunoreactivity was detected within eosinophils incubated with control normal rabbit serum (Figure 1A). Thus, in addition to H-PGDS expression within activated human eosinophils, such as those recruited to nasal mucosa of patients with allergic rhinitis (21) or polyps of chronic rhinosinusitis patients (20), resting eosinophils also contained detectable amounts of cytoplasmic H-PGDS.

It is now well recognized that merely the expression of eicosanoid-forming enzymes does not determine successful eicosanoid synthesis (for a review see (47)). To investigate whether eosinophil H-PGDS could couple to upstream prostanoid-synthesizing enzymes and mount an active PGD_2-synthesizing machinery, human blood eosinophils were stimulated with calcium ionophore A23187. As shown in Figure 1B, A23187 dose-dependently (0.1 – 5μM) elicited acute (within 15 min) secretion of PGD_2 from eosinophils. The pre-treatment with a selective inhibitor of H-PGDS, HQL-79 (10 μM), inhibited PGD_2 synthesis/release induced by A23187 (5 μM) (Figure 1C), but failed to attenuate concomitant LTC_4 secretion (not shown), therefore validating both PGD_2 detection by EIA and the specificity of HQL-79 treatment, while evidencing that eosinophils can rapidly organize an effective H-PGDS-dependent PGD_2 synthesis.

EicosaCell, an immuno-assay that immobilizes the newly formed eicosanoid at its intracellular synthesizing compartments (45), confirmed eosinophil's ability to synthesize PGD_2. As illustrated in Figure 1D, non-stimulated eosinophils exhibited no immunofluorescent staining for PGD_2. In contrast, virtually all eosinophils (more than 90%) activated for 15 min with 0.1 μM A23187 yielded intense and localized immunofluorescent staining for PGD_2 with a perinuclear localization, a well-established eicosanoid-forming site (Figure 1D). The specificity of EicosaCell staining for PGD_2 was ascertained, since (i) there was no immunostaining when an isotype control antibody was used (not shown), and mainly (ii) HQL-79, which blocks H-PGDS activity, completely abolished PGD_2 staining in A23187-stimulated eosinophils (Figure 1D). Although A23187-driven activation easily elicits the enzymatic pathways for eicosanoid production, it is supraphysiologic and may not depict physiopathologic mechanisms of PGD_2 synthesis. Therefore, the unsaturated fatty acid AA, which functions as both substrate and physiologic stimulus of eicosanoid synthesis (48,49), was employed.

As illustrated in Figure 1E, AA (10 μM) very effectively primed eosinophils for increased PGD_2 release in response to a submaximal, 0.1 μM concentration of A23187 (Figure 1E). AA-stimulated eosinophils released about 200 fold as much PGD_2 as eosinophils challenged with 0.1 μM A23187 alone. Moreover, HQL-79 inhibited AA-primed PGD_2 production by eosinophils, while failing to affect concurrent synthesis of PGE_2 (Figure 1E). This inhibition confirmed the specificity of HQL-79 and reinforced H-PGDS role in AA-driven PGD_2 synthesis by eosinophils. Moreover, it is not surprising that while the amounts of secreted PGD_2 are not that different from those picograms of PGE_2 found in the supernatant of AA-stimulated A23187-challenged eosinophils (Figure 1E), under same conditions eosinophils synthesize larger amounts (nanograms) of LTC_4.

Although minute quantities of PGD_2 produced by eosinophils stimulated only with AA was not sufficient to be detectable in supernatants by EIA (data not shown), it was intracellularly detected by EicosaCell. As shown in Figure 1F, AA (10 μM) was able to trigger within human eosinophils PGD_2 synthesis which was immuno-detected within 30 min of stimulation. A detailed analysis revealed that part of newly synthesized PGD_2 (green labeling) was in a punctate cytoplasmic pattern proximate to, but separate from, the nucleus and fully consistent in size and form with eosinophil lipid bodies. The specific compartmentalization of newly formed PGD_2 within eosinophil lipid bodies was ascertained

by the co-localization with ADRP, a protein marker of lipid bodies (Figure 1F, red labeling). Virtually no PGD_2 immunolabeling was observed within non-stimulated eosinophils (Figure 1F), whereas about 91% of eosinophils stimulated with AA exhibited lipid body-localized staining for immunoreactive PGD_2, which was fully inhibitable by HQL-79 (Figure 1F). Collectively these data evidences the role of H-PGDS in AA-induced PGD_2 synthesis by eosinophils and ascertains the specificity of the PGD_2 immuno-labeling. Therefore, newly formed lipid bodies of AA-stimulated eosinophils are inducible and enzymatically skilled organelles for effective PGD_2 synthesis. Concurrently, RT-PCR analysis showed that non-stimulated human circulating eosinophils express H-PGDS mRNA, which may represent a potential target for an AA-driven priming effect on PGD_2 production by eosinophils since, in parallel, AA stimulation increased levels of H-PGDS message (Figure 1G).

Eotaxin elicits the synthesis of biologically active PGD_2

Eotaxin – a key mediator in the development of allergic eosinophilia that is known by its potent eosinophilotactic activity – has recently emerged as a potent mediator of eosinophil activation, with the particular ability to enhance LTC_4 synthesis by eosinophils (50). Here, we showed that, aside from activating LO pathways, eotaxin is also able to control COX-driven prostanoid synthesis within eosinophils. As shown in Figure 2A, the pre-stimulation with eotaxin (6 nM) effectively primed eosinophils for HQL-79-sensitive enhanced PGD_2 release in response to suboptimal concentration of A23187. Eotaxin-pre-stimulated eosinophils released about 13-fold as much PGD_2 as did eosinophils challenged with A23187 alone (0.1 μM). Although eosinophils stimulated with eotaxin alone released levels of PGD_2 that are not detectable by EIA (not shown), our findings demonstrate eotaxin ability to initiate H-PGDS-driven PGD_2-synthesizing machinery within eosinophils. To definitively establish eotaxin ability to trigger H-PGDS-driven PGD_2-synthesizing machinery within eosinophils, more sensitive EicosaCell assay was employed with eotaxin stimulation of either human or mouse eosinophils. As shown in Figures 2D and 2F, eotaxin triggered HQL-sensitive lipid body-compartmentalized PGD_2 synthesis within human and mouse eosinophils. In human cells, immuno-fluorescence PGD_2 was detected, as acute as, 4 min of stimulation with eotaxin in about ~ 40% of eosinophils (Figure 2D), whereas about 90% of eotaxin-stimulated mouse eosinophils displayed PGD_2 immuno-labeling (Figure F). Corroborating such cell primed state of *in vitro* differentiated mouse eosinophils, eotaxin stimulation *per se* was able to elicit PGD_2 production that was detected even by EIA in cell supernatants (Figure 2E). It is also noteworthy that any speculation, that other PGD_2-synthesizing cells contaminating eosinophil preparations (e.g. basophils) were the actual cell type responsible for PGD_2 synthesis, was ruled out by the high percentage of cells in eosinophil preparations synthesizing PGD_2. Either in AA-stimulated human eosinophils (Figure 1E) or eotaxin-stimulated mouse eosinophils (Figure 2F), by EicosaCell about 90% of cells were PGD_2 immunoreactive.

To characterize whether eotaxin-induced eosinophil-derived PGD_2 was a bioactive mediator, by employing HQL-79 as a pharmacological strategy, we evaluated the role of endogenous PGD_2 on two parameters of eosinophil functions triggered by eotaxin (6 nM): enhanced LTC_4-synthesizing ability and induction of lipid body biogenesis. As shown in Figure 2B and C, eotaxin-induced enhancement of cysLTs production and lipid body biogenesis were blocked by HQL-79. The inhibition of eotaxin-induced eosinophil functions by HQL-79, besides reinforcing that eosinophils do in fact synthesize PGD_2, shows that eosinophil-derived PGD_2 displays biological activity, which can be very acute (even within 4 min), with allergy-relevant impacts on eosinophil activation.

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Induction of synthesis of bioactive PGD_2 is stimulus-specific

As a functional approach to further screen potential physiological stimuli of PGD_2 synthesis by eosinophils, lipid body biogenesis – a complex cellular outcome (47) that can be triggered by PGD_2 (43) – was induced by a variety of stimuli undergoing HQL-79 treatment. As shown in Figure 3A and B, either AA- and MIF-induced biogenesis of cytoplasmic lipid bodies within eosinophils was significantly reduced by HQL-79, indicating that, at least in part, lipid body assembly triggered by these stimuli is dependent on endogenously produced PGD_2. Among eosinophil-relevant agonists with a recognized capacity to trigger lipid body biogenesis, PAF and PGD_2 itself, also trigger the rapid, receptor-mediated assembly of lipid bodies through signaling mechanisms distinct from those of AA and eotaxin (47). Here, we highlighted such signaling diversity and characterized the eosinophil PGD_2-synthesizing ability as a stimulus-specific process, because HQL-79 treatment failed to modify either PAF- (Figure 3C) or PGD_2-elicited lipid body biogenesis (Figure 3D). Of note, corroborating PAF lack of ability to induce PGD_2 synthesis within eosinophils, distinct from eotaxin (Figure 2D), PAF-stimulated eosinophils fail to mount PGD_2-synthesizing machinery (within 4 min or 1 h), since EicosaCell preparations of PAF-stimulated human eosinophils did not displayed PGD_2 immuno-fluorescence (data not shown).

Mouse eosinophils-derived PGD_2 is also an endogenous bioactive molecule

Similar to human eosinophils, mouse eosinophils-derived PGD_2 also displayed endogenous stimulatory effects. After establishing that exogenous PGD_2 is capable of triggering lipid body biogenesis within mouse eosinophils (Figure 3E), we demonstrated that endogenous PGD_2 released from AA-stimulated (Figure 3F), but not from PAF-stimulated, mouse eosinophils (Figure 3G) also participates in subsequent lipid body assembly. Taken together, these findings discard potential unspecific effects of HQL-79 on eosinophils, confirm that eosinophil-derived PGD_2 contributes to lipid body assembly in a stimulus-specific fashion, and demonstrate the PGD_2-synthesizing properties of mouse eosinophils. Therefore, rather than a species-specific phenomenon, the eosinophil PGD_2-synthesizing ability appears to be a broad function displayed by the eosinophil cell type.

Eosinophil-derived PGD_2 triggers eosinophil activation via interaction with specific receptors

We hypothesized that eosinophil-derived PGD_2 may regulate AA- and eotaxin-induced eosinophil activation by acting on its specific receptors DP_1 and DP_2. Pre-treatments with HQL-79 or Bay-u3405 (antagonist of the PGD_2 receptor DP_2) significantly inhibited eosinophil cysLTs production triggered by AA, thus showing that newly synthesized PGD_2 functions as an agonist of eosinophil-expressed DP_2 receptors under specific stimulation (Figure 4A). Similarly, lipid body assembly triggered by either AA (Figure 4B) or eotaxin (Figure 4C) was significantly reduced when the DP_1 receptor was blocked with the selective DP_1 antagonist BWA868c, indicating that AA- and eotaxin-stimulated eosinophils release biologically active PGD_2, which binds to DP_1 receptors expressed on eosinophils to initiate lipid body biogenesis-eliciting signaling. Figure 4 also shows that pre-treatments with antagonists of the PGD_2 receptor DP_2, Bay-u3405 or Cay10471, as expected, failed to affect AA- (Figure 4B) or eotaxin-induced lipid body biogenesis (Figure 4C), confirming that the lipid body biogenic process within eosinophils triggered by exogenous or endogenous PGD_2 is controlled selectively by DP_1 activation (32).

Endogenous PGD_2 displays an autocrine/paracrine, rather than an intracrine, effect on eosinophils

Acting extracellularly, PGD_2 has emerged as key paracrine mediator pertinent to asthma and other allergic diseases. However, it is increasingly accepted that eicosanoids including PGD_2

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may also display intracrine roles in regulating cell functions. Indeed, signaling evoked by intracellular eicosanoid receptors has been shown in eosinophils and other cells (51–53). To verify whether autocrine/paracrine *versus* intracrine activity of eosinophil-synthesized PGD_2 control eosinophil activation, intact viable eosinophils were pre-treated with anti-PGD_2 antibodies whose neutralizing activity is excluded from intracellular compartment. Pertinent to PGD_2 functions as a paracrine/autocrine mediator of eosinophil activation (Figure 4), the neutralization of endogenous PGD_2 by the anti-PGD_2, but not by an isotype control (data not shown), inhibited lipid body biogenesis induced by either AA (Figure 4D) or eotaxin (Figure 4E) within human eosinophils. Supporting antibody specificity, anti-PGD_2 treatment failed to affect PAF-induced lipid body formation within eosinophils (Figure 4F). Thus, rather than functioning as an intracrine signal-transducing molecule, eosinophil-derived PGD_2 is secreted from eosinophils and then, by acting extracellularly on PGD_2 specific receptors, mediates eosinophil activation initiated by AA or eotaxin.

Eosinophils recruited to sites of allergic inflammation function as a late cellular source of PGD_2

As activation of infiltrating eosinophils is a critical feature in the pathogenesis of allergic diseases, we hypothesized that activated eosinophils found at sites of allergic inflammation may synthesize and release PGD_2. As previously described (43,54,55), allergic challenge in actively sensitized mice induces a marked eosinophil recruitment to the pleural cavity in a mouse model of allergic inflammation. Infiltrating eosinophils are not detectable within 1 h, peak at 24 h, are detectable within the pleural space up to at least 96 h after allergic challenge, and are concurrent with a resident population of mast cells and a discrete but significant accumulation of other mononuclear cells (macrophages and lymphocytes), but no neutrophil is found (54). It is noteworthy that at acute time points (within 1 h) in absence of allergic eosinophilia, pleural inflammatory fluid presents a significant increase in PGD_2 amounts (from 13 ± 10 to 61 ± 12 ng/cavity of PGD_2 in saline- *versus* OVA-challenged mice, respectively; $p \leq 0.05$; $n = 3$); such synthesis can be attributed to resident mast cells. Notably, 48 h-related pleural eosinophilia (Figure 5A), which is insensitive to HQL-79, parallels an increased pleural level of PGD_2 (Figure 5B). Such delayed allergen-elicited PGD_2 production appears to depend on H-PGDS activity, since HQL-79 impairs it (Figure 5B). To identify the cell source of delayed pleural PGD_2, EicosaCell preparations of pleural leukocytes recovered from allergic inflammatory sites were immuno-labeled with anti-PGD_2. As shown in Figure 5C (left panel), virtually all eosinophils infiltrating the pleural space yielded focal immunofluorescent staining for PGD_2. Of note, in EicosaCell preparations, the eosinophil population was readily identified by visual inspection of nucleus morphology, since as defined by direct counting of eosin-stained cells, the eosinophils (~ 35 % of total pleural cells) were the single polymorphonucelar cell type found infiltrating allergic site of inflammation. The specificity of PGD_2 immunostaining within recruited eosinophils was validated because (i) leukocytes from sensitized, non-challenged mice exhibited no PGD_2 staining; (ii) pre-treatment with HQL-79 completely abolished PGD_2 staining within infiltrating eosinophils (Figure 5C, right panel);and (iii) mononuclear cells (e.g. macrophages), also found in the pleural space after an allergic challenge in sensitized mice, did not show any staining for intracellular PGD_2 (Figure 5C, left panel) at this time point. Even though mast cells are the professional PGD_2-synthesizing cells, at late time points mast cell contribution to PGD_2 generation appears to be very limited (Figure 5C, left panel – arrows). The ability of eosinophils recruited to site allergic inflammation to produce PGD_2 was further confirmed by analyzing EicosaCell preparations by flow cytometry (Figure 5 D). To study eosinophils, we employed double-immunolabeling by anti-SiglecF, an effective approach at detecting eosinophils in mixed cell populations (56). Indeed, as expected the SiglecF staining yielded results that matched visual inspection of eosin-stained cytospin preparations by specifically labeling eosinophilic polymorphonuclear cells (Figure

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5D, left panel), immunodetecting 32.1 ± 6.2 % of cells infiltrating allergic inflammatory space as SiglecF$^+$ eosinophils (n = 5). Specifically analyzing these SiglecF$^+$ cells in Eicosacell preparations of allergic reaction by flow cytometry (Figure 5D, right panel), we extended our microscopic findings by demonstrating that all recruited SiglecF$^+$ eosinophils synthesized PGD_2, as evidenced by an HQL-79-sensitive uniformly positive, unimodal pattern of intracellular PGD_2 immunoreactivity (Figure 5D). Collectively, our data implicates infiltrating eosinophils as an additional cell population responsible for maintenance of PGD_2 production during allergic inflammatory reactions.

Discussion

PGD_2 is a key lipid mediator of allergic airway inflammation that is released following allergen exposure in patients with asthma (40,41). Because PGD_2 modulates key aspects of this prevalent pathology, PGD_2 has emerged as a major mediator of allergic inflammatory disorders and, therefore, is an interesting target for anti-allergic treatments. Among PGD_2-driven asthma-relevant actions are the synthesis of cysLTs, as well as both recruitment and subsequent activation of eosinophils, which is one of the principal cell types recruited to and activated at sites of allergic inflammation.

PGD_2 is a major COX pathway product of mast cells, which are acknowledged as a key cell population providing PGD_2 within inflammatory sites of allergic reactions. This view is mostly based on a rather restricted expression of the limiting PGD_2-forming enzyme H-PGDS within mast cells. Extending the findings of an earlier study that evaluated human tissue eosinophils found infiltrating mucosa of patients with allergic rhinitis (21) or polyps of chronic rhinosinusitis patients (20), H-PGDS expression (both at mRNA and protein levels) was detected within human circulating eosinophils freshly isolated from healthy donors, thus indicating that, in addition to mast cells, eosinophils could also contribute to PGD_2 synthesis in allergic inflammatory sites. However, it is now well established that the successful production of PGD_2, or any other eicosanoid, is not merely determined by the proper expression of restrictive enzymes. In addition, it requires AA availability, the presence of all other relevant protein/enzymes, coordinated phosphorylation of some enzymes, correct spatial assembly of enzymatic complexes, and regulated intracellular compartmentalization of these complexes (for a review, see (47)). By simply finding H-PGDS within eosinophils, it cannot be ascertained whether these cells are capable of mounting a successful PGD_2 production and, consequently, contribute to allergy-related elevated PGD_2 generation. Therefore, our main aim here was to prove that, as in mast cells, eosinophil H-PGDS could couple to an active prostanoid-synthesizing machinery at specific, intracellular compartments within eosinophils, which would generate and release bioactive PGD_2. By employing several experimental techniques, we attest that both human and mouse eosinophils are indeed able to synthesize PGD_2 because (i) PGD_2 was detected within supernatants of purified human eosinophils stimulated *in vitro* with A23187; (ii) AA and eotaxin were able to up-regulate PGD_2 production/release by eosinophils; (iii) AA- and eotaxin-primed, as well as A23187-induced, PGD_2 production/release were inhibited by HQL-79; (iv) A23187- and AA-induced, HQL-79-sensitive PGD_2 synthesis was differentially compartmentalized within the perinuclear membrane and lipid bodies, respectively; and (v) functional assays showing that AA- and eotaxin-induced eosinophil activation were inhibited by pre-treatments with HQL-79, antibody anti-PGD_2 or receptor antagonists of DP_1 and DP_2. More specifically, these data show that such PGD_2 synthesis by eosinophils is an H-PGDS-dependent event that culminates in an extracellular release of a biologically active PGD_2, which displays autocrine/paracrine activities on eosinophils via the activation of its specific receptors, DP_1 and DP_2.

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What about eosinophil contribution to allergic airway inflammation as a PGD_2 source following allergen exposure? To definitively demonstrate that eosinophils also play such an additional role in allergic inflammatory reactions by providing PGD_2, we employed a direct strategy for the *in situ* immunolocalization of intracellular PGD_2 to identify the cell population responsible for PGD_2 production in a mouse model of allergic inflammation that displays increased levels of PGD_2 at later time points. Eosinophils recruited to the inflammatory site, which also had concurrent macrophage and mast cell populations, was the predominant cell type generating PGD_2, thus challenging the prevailing notion of mast cells as the single PGD_2 cell source during allergic reactions and placing eosinophils as responsible for continued production of PGD_2.

Eosinophil-derived, biologically active PGD_2 may modulate eosinophil activation in sites of allergic inflammation. PGD_2-driven eosinophil activation during allergic airway inflammation is known to elicit LTC_4 production by eosinophil themselves (43). Therefore, even at minute amounts and in part due to its autocrine feature, such eosinophil-derived PGD_2 activity emerges as a key up-regulatory mechanism for the local generation of pro-allergic mediators. Considering both our findings and the disappointing clinical trial results of DP_1 antagonist laropiprant in asthmatics and allergic rhinitis patients (57), it appears that therapies targeting PGD_2 synthesis, rather than receptor antagonism, may display superior beneficial outcomes. Therefore, our data, in addition to reinforcing the notion of eosinophils as major effector cells of allergic disorders, identify the PGD_2-synthesizing property of eosinophils as a novel alternative target for anti-allergic therapies.

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Abbreviations

5-LO	5-lipoxygenase
COX	cyclooxygenase
AA	arachidonic acid
ADRP	adipose differentiation-related protein
CRTH2	chemoattractant receptor-homologous molecule expressed on T helper type 2 cell (Th2) cells
cysLTs	cysteinyl leukotrienes
DP_1	D prostanoid receptor 1
DP_2	D prostanoid receptor 2
EDAC	1-ethyl-3-(3-dimethylamino-propyl) carbodiimide
LTC_4	leukotriene C_4
PGD_2	prostaglandin D_2
PAF	platelet-activating factor

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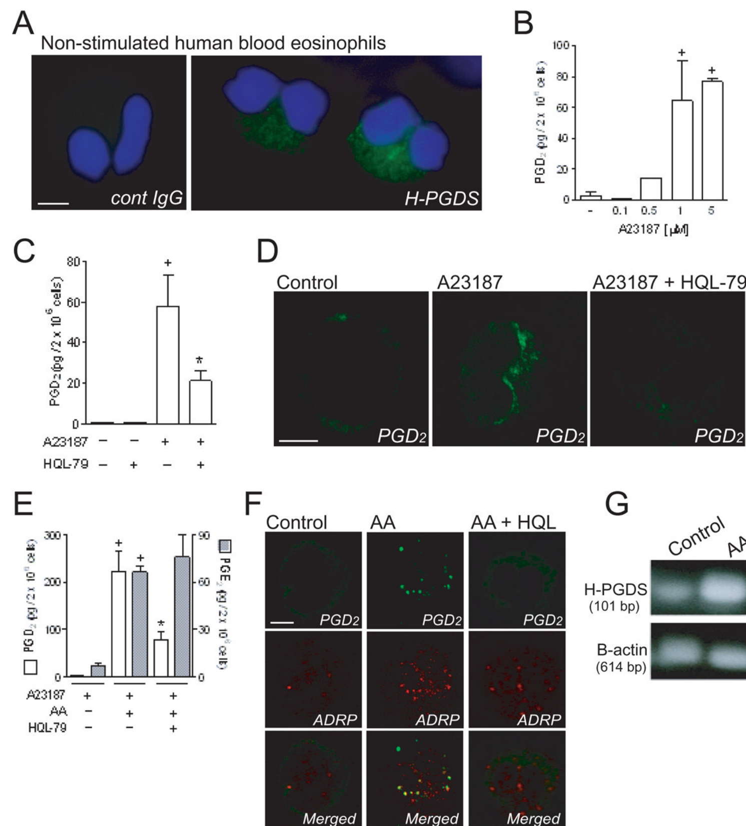

Figure 1. Human eosinophils are able to synthesize PGD_2 in an H-PGDS-dependent manner

Panel **A** presents epifluorescence images of cytoplasmic immuno-detection of H-PGDS (green) in non-stimulated human eosinophils. Blue fluorescence shows eosinophil nuclei stained with DAPI. Eosinophils incubated with isotype irrelevant IgG are shown. Panel **B** shows PGD_2 amounts secreted by human eosinophils stimulated for 15 min with A23187 (0.1 to 5 μM). In **C**, eosinophils were pre-treated for 30 min with HQL-79 and then stimulated with 5 μM of A23187. Panel **D** displays confocal images of intracellular immuno-fluorescence for PGD_2 in non-stimulated, A23187 (0.1 μM)-stimulated, and HQL-79-treated A23187-stimulated human eosinophils (as indicated). Arrows indicate PGD_2 immuno-staining at the nuclear envelope of A23187-stimulated human eosinophils. Panel **E** shows production of PGD_2 and PGE_2 by human eosinophils stimulated for 1 h with AA (10 μM) and then challenged with A23187 (0.1 μM). Eosinophils were pre-treated for 30 min with HQL-79. In **F**, confocal images of EicosaCell preparations display intracellular immuno-detection of newly formed PGD_2 (green) and of ADRP (red) in human eosinophils stimulated with AA (10 μM). Eosinophils were pre-treated with HQL-79 for 30 min. Overlay images of identical fields are shown in the larger images. In **G**, constitutive levels of H-PGDS mRNA and its up-regulation in human eosinophils that were stimulated for 1 h with AA (10 μM) were assessed by RT-PCR. Values are expressed as means ± SEM of at least three distinct donors. $^{+}$ $P \leq 0.05$ compared with control. * $P \leq 0.05$ compared with A23187- or AA-stimulated eosinophils. All the images are representative of three independent experiments with distinct donors. Bar, 5 μm.

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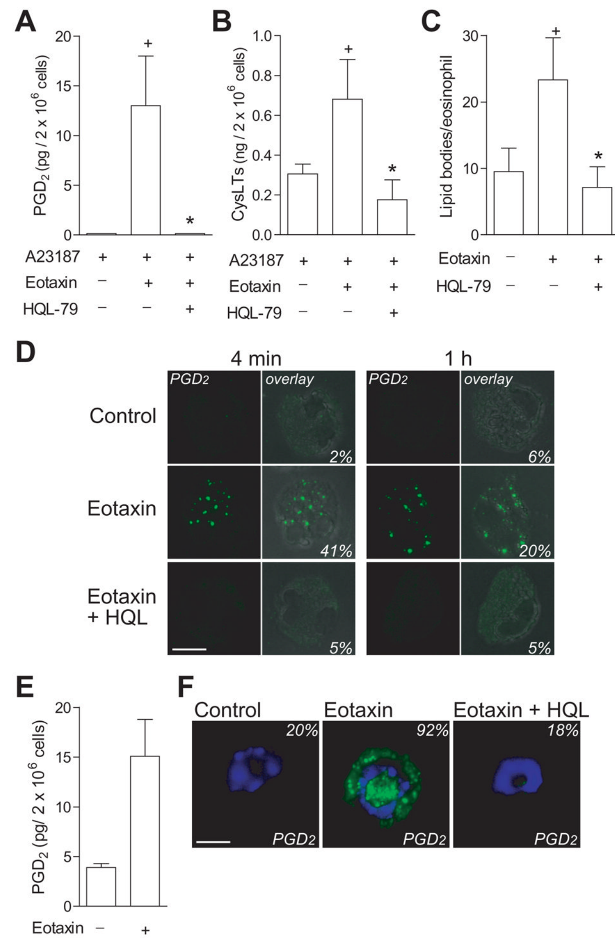

Figure 2. Eotaxin elicits production of biologically active PGD_2

Both human and mouse eosinophils were pre-treated for 30 min with HQL-79 before eotaxin stimulation. In **A** and **B**, for analysis of PGD_2 and cysLTs synthesis, human eosinophils were stimulated for 1 h with eotaxin (6 nM) and then challenged with A23187 (0.1 μM). In **C**, for analysis of lipid body biogenesis, human eosinophils were stimulated for 1 h with eotaxin (6 nM). In **D**, confocal images of EicosaCell preparations display intracellular immuno-detection of newly formed PGD_2 (green) in human eosinophils stimulated with rh eotaxin (6 nM). Overlay images of immuno-fluorescence and light microscopy of identical fields are shown in the larger images. In **E**, for analysis of PGD_2 synthesis, mouse eosinophils were stimulated for 1 h with rm eotaxin (6 nM). Panel **F** shows confocal images of PGD_2 immuno-detection of H-PDS (green) in mouse eosinophils stimulated with rm eotaxin (6 nM). Blue fluorescence shows eosinophil nuclei stained with DAPI. Bar, 5 μm. Results are expressed as means ± SEM for at least three independent experiments with eosinophils from distinct donors. $^{+}P \leq 0.05$ compared with control. $^{*}P \leq 0.05$ compared with eotaxin-stimulated eosinophils.

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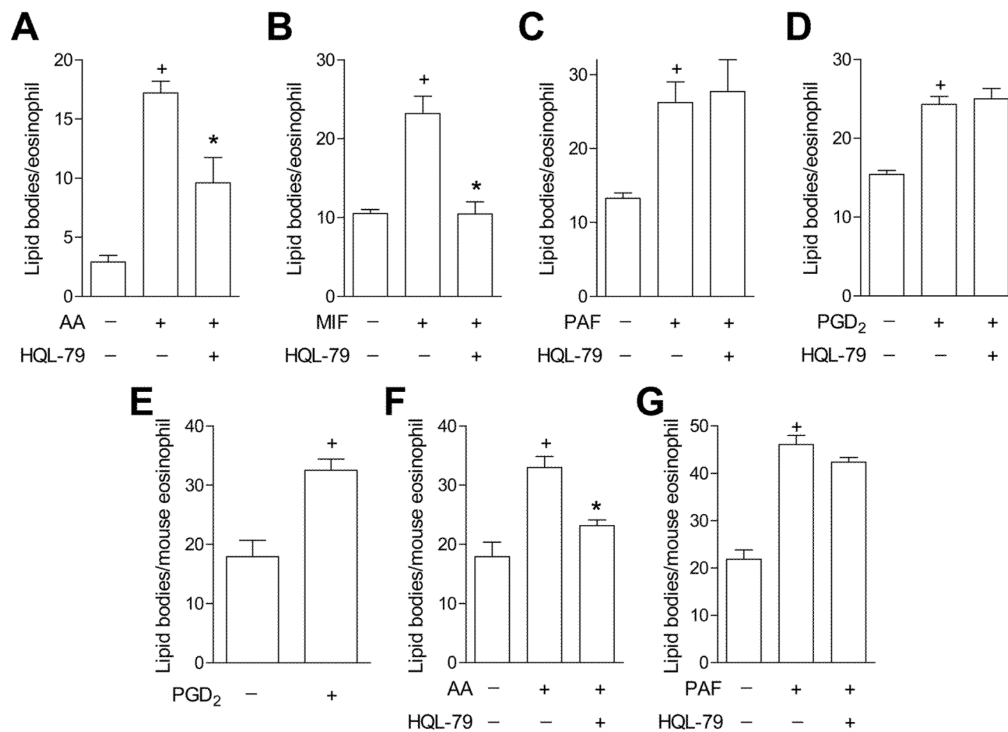

Figure 3. Endogenous eosinophil-derived PGD_2 mediates AA- and eotaxin-, but not PAF or PGD_2-induced lipid body biogenesis

For *in vitro* analysis of lipid body biogenesis, human (**A**, **B**, **C** and **D**) or mouse (**E**, **F** and **G**) eosinophils were pre-treated for 30 min with HQL-79 and then stimulated for 1 h with AA (10 µM; **A** and **F**), MIF (50 ng/mL; **B**), PAF (1 µM; **C** and **G**) or PGD_2 (25 nM; **D** and **E**). *In vitro* results are expressed as means ± SEM for at least three independent experiments with eosinophils from distinct donors. $^+ P \leq 0.05$ compared with control. $^* P \leq 0.05$ compared with stimulated eosinophils.

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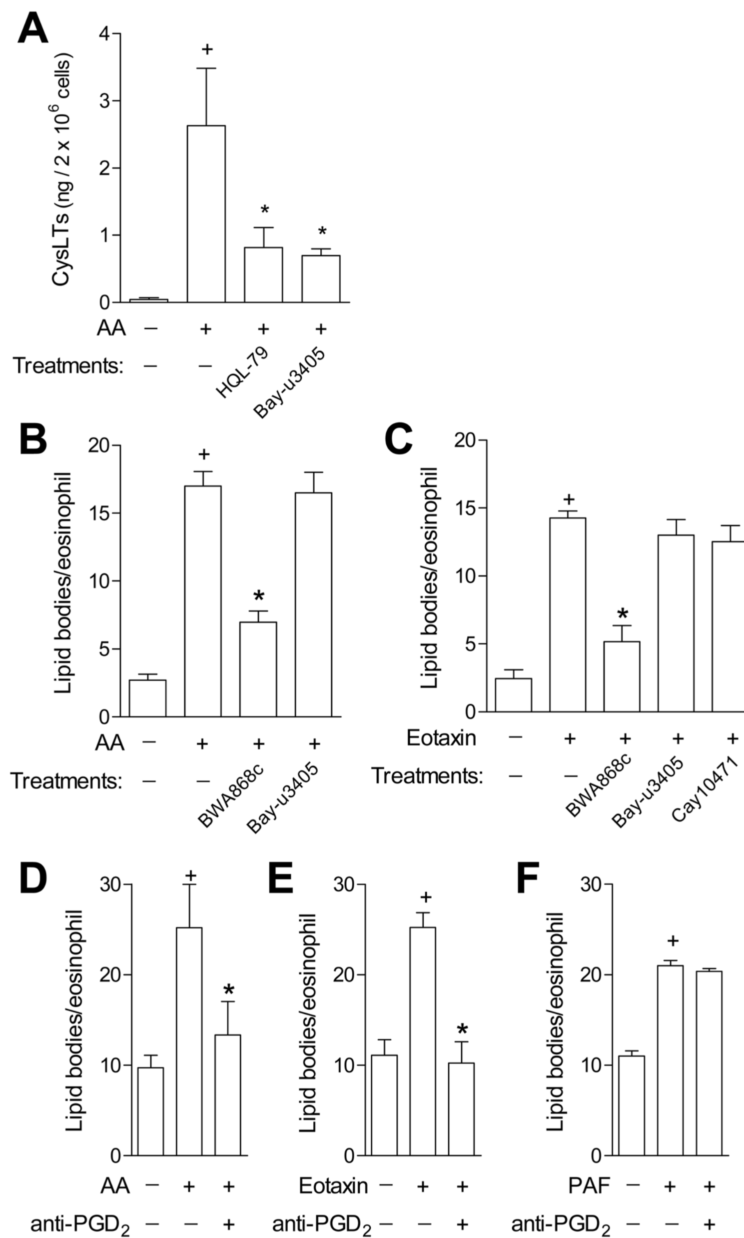

Figure 4. Eosinophil-derived PGD_2 controls eosinophil activation via interaction with specific PGD_2 receptors

In **A**, for analysis of cysLTs production, human eosinophils were pre-treated for 30 min with HQL-79 or Bay-u3405, stimulated for 1 h with AA (10 μM) and then challenged with A23187 (0.1 μM). From **B** to **F**, for *in vitro* analysis of lipid body biogenesis, human eosinophils were pre-treated for 30 min with neutralizing anti-PGD_2 antibody, BWA868c, Bay-u3405 or Cay10471 and then stimulated for 1 h with AA, eotaxin, or PAF, as indicated. The results are expressed as the means ± SEM for at least three independent experiments with eosinophils purified from distinct donors. $^{+}$ $P \leq 0.05$ compared with control. * $P \leq 0.05$ compared with stimulated eosinophils.

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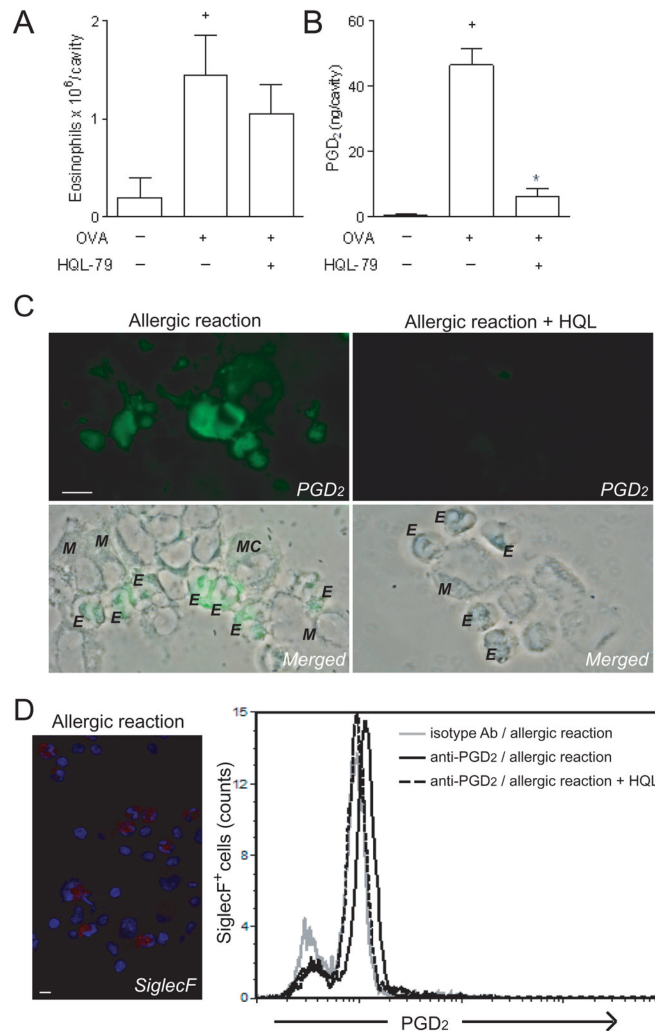

Figure 5. Infiltrating eosinophils are an auxiliary cell source of PGD_2 at sites of allergic inflammation

In **A** and **B**, for *in vivo* analysis of eosinophil influx and PGD_2 production, respectively, sensitized mice were sacrificed 48 h after allergic challenge with ovalbumin. Animals received HQL-79 1 h prior to allergic challenge. *In vivo* results are expressed as the means ± SEM for at least eight animals. $^{+}$ $P \leq 0.05$ compared with control. * $P \leq 0.05$ compared with ovalbumin-challenged animals. In **C**, EicosaCell images show intracellular immuno-fluorescence for PGD_2 pleural leukocytes recovered from non- and HQL-79-treated ovalbumin-challenged mice (as indicated). Overlay images of identical phase contrast fields are shown to facilitate the identification of the immuno-fluorescent cell type. E, M, and MC indicate eosinophils, macrophages, and mast cells, respectively. Arrows show immuno-labeled PGD_2 within a resident mast cell. Bar, 10 μm. In **D**, epifluorescence image (left panel) shows allergic infiltrating eosinohils as SiglecF^{+}PE-labeled (red staining) cells with DAPI-stained polymorphic nuclei (blue staining). Right panel shows EicosaCell analysis of PGD_2 synthesis within SiglecF^{+} pleural eosinophils found in the site of allergic inflammation of HQL-treated and non-treated animals. Histogram is a representative data of 5 animals *per* group.