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Prostaglandin D_2 Causes Preferential Induction of Proinflammatory Th2 Cytokine Production through an Action on Chemoattractant Receptor-Like Molecule Expressed on Th2 Cells

Luzheng Xue,1,2 Shân L. Gyles,1 Frank R. Wettey, Lucien Gazi,3 Elizabeth Townsend, Michael G. Hunter, and Roy Pettipher

PGD_2, produced by mast cells, has been detected in high concentrations at sites of allergic inflammation. It can stimulate vascular and other inflammatory responses by interaction with D prostanoid receptor (DP) and chemoattractant receptor-like molecule expressed on Th2 cells (CRTH2) receptors. A significant role for PGD_2 in mediating allergic responses has been suggested based on the observation that enhanced eosinophilic lung inflammation and cytokine production is apparent in the allergen-challenged airways of transgenic mice overexpressing human PGD_2 synthase, and PGD_2 can enhance Th2 cytokine production in vitro from CD3/CD28-costimulated Th2 cells. In the present study, we investigated whether PGD_2 has the ability to stimulate Th2 cytokine production in the absence of costimulation. At concentrations found at sites of allergic inflammation, PGD_2 preferentially elicited the production of IL-4, IL-5, and IL-13 by human Th2 cells in a dose-dependent manner without affecting the level of the anti-inflammatory cytokine IL-10. Gene transcription peaked within 2 h, and protein release peaked ~8 h after stimulation. The effect of PGD_2 was mimicked by the selective CRTH2 agonist 13,14-dihydro-15-keto-PGD_2 but not by the selective DP agonist BW245C, suggesting that the stimulation is mediated by CRTH2 and not DP. Ramatroban, a dual CRTH2/thromboxane-like prostanoid receptor antagonist, markedly inhibited Th2 cytokine production induced by PGD_2, while the selective thromboxane-like prostanoid receptor antagonist SQ29548 was without effect. These data suggest that PGD_2 preferentially up-regulates proinflammatory cytokine production in human Th2 cells through a CRTH2-dependent mechanism in the absence of any other costimulation and highlight the potential utility of CRTH2 antagonists in the treatment of allergic diseases. The Journal of Immunology, 2005, 175: 6531–6536.

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1 L.X. and S.L.G. contributed equally to this work.
2 Address correspondence and reprint requests to Dr. Luzheng Xue, Oxagen Limited, 91 Milton Park, Abingdon, Oxon OX14 4RY, U.K. E-mail address: l.xue@oxagen.co.uk
3 Current address: CareX S. A., Bioparc, Parc d’Innovation, Boulevard Sébastien Brandt, BP 30442, 67412 Illkirch, Cedex, France.

4 Abbreviations used in this paper: DP, D prostanoid receptor; CRTH2, chemoattractant receptor-like molecule expressed on Th2 cell; TP, thromboxane-like prostanoid receptor; DK-PGD_2, 13,14-dihydro-15-keto-PGD_2.
IL-4, IL-5, and IL-13 by Th2 cells without affecting the production of IL-10. Ramatroban (BAY u 3405), originally defined as a thromboxane-like prostanoid receptor (TP) antagonist and recently identified to also be a potent CRTH2 antagonist (20, 21), abolished Th2 cell responses to PGD2. This inhibition was mediated by CRTH2 because SQ29548, a specific TP antagonist, did not mimic the effect of ramatroban. This study illustrates a key mechanism whereby PGD2 may contribute to Th2-mediated diseases through activation of CRTH2.

### Materials and Methods

#### Reagents

Recombinant human IL-2 and IL-4 were purchased from Sigma-Aldrich and R&D Systems Europe, respectively. [3H]PGD2 was from Amershams Biosciences. PGD2, 13,14-dihydro-15-keto-PGD2 (DK-PGD2), BW245C, BW868C, and SQ29548 were obtained from Cayman Chemical. Ramatroban was synthesized by Evotec-OAI. MACS CD4 T cell isolation kit II and anti-human CRTH2 MicroBead kit were purchased from Miltenyi Biotech. QuantiGlo Human IL-4 Chemiluminescent Immunoassay kit, Quantiglo Human IL-5 Chemiluminescent Immunoassay kit, Quantikine Human IL-10 Chemiluminescent Immunoassay kit, and Quantikine Human IL-13 Chemiluminescent Immunoassay kit were purchased from R&D Systems Europe. RNeasy Mini kit, Omniscript RT kit, and HotStar PCR kit were obtained from Qiagen. HitHunter Cyclic AMP II CL kit was obtained from DiscoverX. Other chemicals were from Sigma-Aldrich.

#### Culture of Th2 cells

Th2 cells were prepared using a modified method described previously (22). Briefly, PBMC were isolated from buffy coats (National Blood Service) by Ficoll-Hypaque (Amershams Biosciences) density gradient centrifugation, followed by CD4-positive cell purification using MACS CD4⁺ T
cell isolation kit II. After a 7-day culture in X-VIVO 15 medium (Cambrex BioScience) containing 10% human serum, 50 U/ml rhIL-2, and 100 ng/ml rhIL-4, CRTH2-positive cells were isolated from the CD4+ culture by positive selection using anti-human CRTH2 MicroBead kit. The harvested CD4+ CRTH2+ cells were treated as Th2 cells and were further amplified in X-VIVO 15 medium containing 10% human serum and 50 U/ml rhIL-2 before use.

**Binding assay**

Membranes of human Th2 cells were prepared for the binding assay. The cells were harvested by centrifugation at 300 × g for 10 min. After washing, the cells were homogenized using a 1× HBSS supplemented with 10 mM HEPES (pH 7.3). The cells were resuspended in the same buffer and centrifuged at 16,000 × g for 1 h at 4°C. The resulting pellet was resuspended in a cold buffer, homogenized with an Ultra Turrax homogenizer (setting 4–5), and aliquots of 200–500 μl were stored at −80°C until use. The protein concentration was determined by the Bradford method.

To perform [3H]PGD2 (160 Ci/mmol) binding experiments were performed as described previously (22). Cell membranes (30–40 μg) were preincubated at room temperature with varying concentrations of competing ligand for 15 min in 80 μl of 1× HBSS buffer supplemented with 10 mM HEPES (pH 7.3). Twenty micromolars of [3H]PGD2 (5 nM, final concentration) was then added, and the incubation continued for an additional 1 h at room temperature. Reactions were terminated by the addition of 200 μl of ice-cold assay buffer to each well, followed by rapid filtration through Whatman GF/B glass fiber filters using a Unifilter Cell harvester (PerkinElmer Life Sciences). The filters were washed six times with 300 μl/well ice-cold buffer. The Unifilter plates were dried at room temperature for at least 1 h, and the radioactivity retained on the filters was determined using a Beta Trilux counter (PerkinElmer Life Sciences), following addition of 40 μl of Optiphase Hi-Safe 3 (PerkinElmer Life Sciences) liquid scintillation. Non-specific binding was defined in the presence of 10 μM unlabeled PGD2.

**Cytokine release assays**

The medium of Th2 cell cultures were collected after certain periods of culture with varying concentrations of agonists (PGD2, DK-PGD2, or BW245C) of PGD2 receptors in the presence or in the absence of inhibitor at 37°C and 5% CO2 as indicated in the results. The concentrations of IL-4, IL-5, IL-10, and IL-13 in the medium were assayed using ELISA kits according to the manufacturer’s instructions. The results were measured in a Victor® V-1420 multilabel HTS Counter (PerkinElmer Life Sciences).

**RT-PCR**

Total RNA of Th2 cells after different treatments was extracted using a RNeasy Mini kit. The DNA-free total RNA samples were quantitated using a GeneQuant Pro (Biochrom). cDNA of the samples was prepared from the same starting amount of RNA using Omniscript RT kit. PCR products were separated on an agarose gel and detected with a Fluor-S MAX2 Multimager (Bio-Rad). The intensity of ethidium bromide-stained bands was quantified using Quantity One software (Bio-Rad). mRNA level of cytokines was normalized with the level of GAPDH.

**Statistics**

Data were analyzed using Student’s one-tailed t test or one-way ANOVA followed by the Newman-Keuls test. Values of p < 0.05 were considered as statistically significant.

**Results**

**PGD2 induces production of IL-4, IL-5, and IL-13 but not IL-10 in human Th2 cells**

To test the effect of PGD2 on cytokine production from human Th2 cells, the cells were stimulated with increasing concentration of PGD2 for 4 h as indicated in Fig. 1. The treatment increased IL-4, IL-5, and IL-13 levels in the culture medium in a dose-dependent manner (Fig. 1). The EC50 of PGD2 for IL-4, IL-5, and IL-13 productions was 150, 63, and 54 nM, respectively. Interestingly, much higher concentrations of PGD2 were required to trigger production of IL-10 (EC50 > 3 μM). A time course of Th2 cytokine production responding to a single dose of PGD2 (100 nM) was also investigated (Fig. 2). No cytokine increase in the culture medium was detectable after 1-h stimulation. The levels of IL-4, IL-5, and IL-13 increased with time and peaked at 4 h (for IL-4) or 8 h (for IL-5 and IL-13) (Fig. 2B). The IL-4 level dropped quickly after pretreatment with 10 μM rolipram, the cells were incubated with various concentrations of compounds (PGD2, BW245C, or PGE2) in the presence of 0.5 mM IBMX for 30 min at 37°C. The cAMP level in the cells was determined using HitHunter Cyclic AMP II CL kit. The results were measured in a Victor® V-1420 multilabel HTS Counter.

**Primers used were as follows:** IL-4, 5′-GCTGCCCTCCAAGAACA CAAC-3′ and 5′-CTCTGGTGTGGCTCTTCAC-3′ generating a 221-bp fragment; IL-5, 5′-CTGGCTCAGTGTTACGGATC-3′ and 5′-CTTTC CACAGTACCCCCTTG-3′ generating a 217-bp fragment; IL-10, 5′-CGAGATCCTCCTCAAGAGAT-3′ and 5′CCTGGATGTCTGGTCTTT G-3′ generating a 188-bp fragment; IL-13, 5′-CCTCAATCCCTCCTCGT TGG-3′ and 5′-GTCAGGTGATGTGGCTCATACC-3′ generating a 206-bp fragment; and GAPDH, 5′-GCCACCTAGAAGAAGCTTGATGGCC-3′ and 5′-GCAATGCCGAGGCCCAGCATCAAGG-3′ generating a 350-bp fragment.
that of the protein. The mRNA encoding IL-4 peaked at 1 h declines toward baseline level at 8 h. However, the mRNA of IL-5 and IL-13 was only modestly increased at 1 h with maximal increase at 2 h and was maintained at higher levels than the baseline (−1.5-fold) after 24 h. There was no significant up-regulation of IL-10 mRNA under the same treatment (Fig. 2A), although a late weak increase (<2-fold peaked at 8 h) was detected at 1 μM PGD2 challenge (data not shown).

**Effect of a selective CRTH2 agonist and a selective DP agonist on Th2 cytokine production**

To elucidate the receptor mediating the effects of PGD2 on Th2 cytokine production, we examined the effects of DK-PGD2, a specific CRTH2 agonist, and BW245C, a specific DP agonist (Fig. 3). Both mRNA levels in the Th2 cells and cytokine levels in the medium were assayed after 4 h of treatment. DK-PGD2 played the same role as PGD2, triggering IL-4, IL-5, and IL-13 but not IL-10 gene transcription and protein secretion, although the potency of DK-PGD2 is slightly lower than that of PGD2. In contrast, 1 μM BW245C showed no effect on cytokine production (Fig. 3).

**Ramatroban but not selective TP and DP antagonists inhibits PGD2-mediated Th2 cytokine production**

To determine whether the effect of PGD2 on Th2 cytokine production is mediated by CRTH2, the effect of PGD2 was studied in the presence of ramatroban, a dual CRTH2/TP antagonist. Th2 cytokine production in response to PGD2 was markedly inhibited by ramatroban in a dose-dependent manner (Fig. 4). The IC50 of ramatroban for the inhibition of IL-4, IL-5, and IL-13 production induced by PGD2 (100 nM) was 103, 182, and 118 nM, respectively. The inhibition was also observed at the level of gene transcription (Fig. 5). In contrast to ramatroban, application of SQ29548, a selective TP antagonist, or BW868C, a selective DP antagonist, did not inhibit the effect of PGD2 on Th2 cytokine production at either mRNA or protein levels (Fig. 5).

**CRTH2 is the dominant receptor mediating PGD2 signals in human Th2 cells**

To further confirm the receptor targeted by PGD2 in human Th2 cells, we measured the expression of CRTH2, DP, and TP in human Th2 cells. Although as reported by Tanaka et al. (19), both DP and CRTH2 receptors were detectable at mRNA level in human Th2 cells (data not shown), a competition binding assay using Th2 cell membranes revealed CRTH2 to be the dominant receptor for PGD2 binding in Th2 cells (Fig. 6A). PGD2 and DK-PGD2 competed with high affinity with [3H]PGD2 to bind to Th2 cell membranes with Ki values of 12 ± 2 nM (n = 9) and 36 ± 6 nM (n = 4), respectively. In contrast, BW245C did not displace [3H]PGD2 binding at concentrations up to 10 μM (n = 3) (Fig. 6A). Ramatroban displaced [3H]PGD2 binding with a value of Ki = 35 ± 8 nM (n = 4), while SQ29548 did not show any effect on binding to the Th2 membranes at concentration up to 10 μM (Fig. 6A).

Because the DP receptor is Gαi-coupled and the CRTH2 receptor is Gαs-coupled, we conducted calcium mobilization and cAMP assays to verify the dominant bioactive receptor for PGD2 in Th2 cells. Calcium mobilization in human Th2 cells was stimulated by CRTH2 agonists (PGD2 and DK-PGD2) but not by the DP selective agonist (BW245C) (22). PGD2 and BW245C both demonstrated low efficacy on cAMP signaling in Th2 cells (Fig. 6B). The level of cAMP in Th2 cells was not affected by PGD2 or BW245C at concentrations up to 1 μM.

**Discussion**

Although it has been shown previously that PGD2 can enhance Th2 cytokine production in the presence of costimulation (15, 19), our study has revealed that PGD2 alone has the remarkable ability to elicit cytokine production by Th2 cells in the absence of Ag or any other costimulation. Interestingly, PGD2 induced the production of the proinflammatory cytokines (IL-4, IL-5, and IL-13) preferentially with only modest effects on the anti-inflammatory cytokine (IL-10). The effect of PGD2 was mimicked by the selective CRTH2 agonist DK-PGD2 but not by the selective DP agonist BW245C. Further evidence for the involvement of CRTH2 in this response comes from the use of ramatroban, which has been shown recently to be a potent CRTH2 antagonist (21). This compound blocked the effects of PGD2 on Th2 cytokine production at similar concentrations required to inhibit CRTH2 (21), and this blockade is unlikely to be related to its TP activity because the selective TP antagonist SQ29548 was without effect. Furthermore, ligand binding studies with [3H]PGD2 on Th2 membranes and measurements of cAMP production established the functional presence of...
CRTH2 but not TP or DP. Taken together, these data lead us to the conclusion that the effect of PGD2 in causing Th2 cell activation is solely CRTH2 dependent. It has been suggested that DP is expressed by Th2 cells at low levels and may have a suppressive effect on Th2 activation (19), but we have found no evidence that the DP receptor on Th2 cells is functionally active at physiological concentrations of PGD2. Our RT-PCR data confirmed the presence of DP mRNA in Th2 cells, but the ligand binding studies suggested that CRTH2 expression at the functional level dominates that of DP quantitatively. Tanaka et al. (19) detected the ability of the DP agonist BW245C to inhibit Th2 function in CD3/CD28-stimulated Th2 cells, suggesting that there may be low levels of expression of functional DP. However, it is unclear whether DP plays a physiological role in regulating activation of Th2 cells because the DP antagonist BW868C did not enhance the ability of PGD2 to increase Th2 cytokine production (19). Furthermore, the function of DP could be amplified in CD3/CD28-stimulated Th2 cells because the CD3/CD28 stimulation down-regulates the expression of CRTH2 in Th2 cells (19).

It is well established that PGD2 is produced by mast cells at the sites of allergic inflammation, including the asthmatic lung (23) at sufficient concentrations to cause chemotaxis of eosinophils, basophils, and Th2 cells (24, 25). Our data suggest that the physiological concentration of PGD2 can also act as an independent trigger for production of Th2 cytokines. These cytokines in turn play a proinflammatory role in the pathophysiology of allergic disease (26). IL-4 and IL-13, especially, are potent for IgE isotype switching to promote mast cell-mediated inflammatory processes (27). IL-4, IL-5, and IL-13 alone also exert their inflammatory effects through various leukocytes, such as eosinophils, mast cells, and Th2 cells (28). In recent years, accumulating evidence from mice and humans has identified these three Th2 cytokines as major contributors to allergic inflammation (29). However, IL-10 has been defined previously as a cytokine synthesis inhibiting factor and represents one of the important immune-regulating cytokines (30). It is capable of inhibiting the synthesis of several cytokines from different cells and the function of several types of immune cells (31–33). In IL-10 gene-deficient mice, an overproduction of inflammatory cytokines and development of chronic inflammatory disease have been observed (34, 35). Our discovery of preferential up-regulation of IL-4, IL-5, and IL-13 but not IL-10 from human Th2 cells further supports the proinflammatory effect of PGD2 on Th2-mediated inflammatory responses. It has been reported that IL-10 may not be coordinately expressed with IL-4, IL-5, and

**FIGURE 5.** CRTH2 but not TP and DP antagonist inhibited PGD2-triggered cytokine production. The cells were treated with or without 100 nM PGD2 in the presence or in the absence of 1 μM ramatroban, SQ29548, or BW868C for 4 h. The mRNA level of IL-4 ( ), IL-5 ( ), IL-10 ( ), and IL-13 ( ) from the cell pellets was determined and compared using semiquantitative RT-PCR. The concentrations of IL-4/IL-5/IL-13 in the medium were measured by ELISA. The graphs show a representative result of three independent experiments. Error bars show a range from duplicate samples or SEM from triplicate samples. *p < 0.05 by ANOVA, except the data set of IL-10 mRNA (p = 0.3176); *p > 0.05 by Newman-Keuls test for 100 nM PGD2 vs 100 nM PGD2 + 1 μM SQ29548/100 nM PGD2 + 1 μM BW868C, control vs 100 nM PGD2 + 1 μM ramatroban; n = 2–3.

**FIGURE 6.** DP and TP are not functional receptors for PGD2 in Th2 cells. A. Competition by various compounds (PGD2; , DK-PGD2; , BW245C; , ramatroban; or SQ29548) for [3H]PGD2 binding to human Th2 cell membrane. Maximum binding was expressed as 100%. Each point is the mean of triplicate determinations. Similar results were obtained on three independent experiments. B. cAMP levels in Th2 cells after treatment with various concentrations of DP agonists (PGD2; , BW245C). A total of 10 μM PGE2 ( ), an E prostanoid receptor agonist, was used as a positive control. The graphs show a representative result of three independent experiments. Error bars show a range from duplicate samples.
IL-13 during Th2 differentiation and could be expressed by a special subset of T cells (36, 37). However, the Th2 cells used in the present study are capable of IL-10 production, although a nonphysiological concentration of PGD2 is required, suggesting that PGD2 is unlikely to be an important trigger for IL-10 production by human Th2 cells. Our RT-PCR results indicate that CRTH2 signaling targets the gene transcription of proinflammatory Th2 cytokines, although the signal pathway downstream of the receptor is still unclear. It has been found that the genes encoding IL-4, IL-5, and IL-13 are closely linked within a 160-kb genomic region of human chromosome 5 and could be regulated as a single transcriptional locus (38). Take moto et al. (39) recently showed in a mouse model that ectopic activation of Stat6 or GATA-3 induces remodeling of this gene cluster region into an accessible chromatin conformation during Th cell differentiation. This mechanism might be used by PGD2/Crth2 for the coordinate up-regulation of Th2 cytokines.

Ramatroban was developed initially as a potent TP receptor antagonist (20). Recent studies revealed that this drug is also a CRTH2 antagonist and capable of inhibiting CRTH2-mediated eosinophil migration (21). In this study, we have demonstrated for the first time that ramatroban blocks CRTH2-mediated Th2 activities, including cytokine production and chemotaxis (data not shown). The potency of ramatroban in Th2 cells (Ki = 35 nM for binding, IC50 = 100–180 nM for cytokine production) is similar to that in eosinophils reported previously (Ki = 100 nM for binding, IC50 = 30 nM for Ca2+ mobilization, IC50 = 170 nM for chemotaxis) (21).

In summary, the present study shows that PGD2 can elicit Ag-independent activation of Th2 cytokine production mediated by the cell surface receptor CRTH2. These observations are consistent with CRTH2 playing a central role in mediating mast cell-Th2 interactions in allergic diseases.

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Disclosures

The authors have no financial conflict of interest.

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