Murine bone marrow-derived mast cells express chemoattractant receptor-homologous molecule expressed on T-helper class 2 cells (CRTh2)

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Keywords: allergy, chemotaxis, inflammation, prostaglandin D\textsubscript{2}

Abstract

Mast cells are bone marrow-derived effector cells that can initiate inflammatory responses to infectious organisms or allergens by releasing a multitude of pro-inflammatory factors including prostaglandin (PG) D\textsubscript{2}. We demonstrate that primary murine bone marrow-derived mast cells (BMMCs) express the PGD\textsubscript{2} receptor; chemoattractant receptor-homologous molecule expressed on Th class 2 cells (CRTh2). Activation of CRTh2 on BMMC by PGD\textsubscript{2} or the CRTh2-specific agonist, 13,14-dihydro-15-keto-prostaglandin D\textsubscript{2} (DK-PGD\textsubscript{2}), resulted in signaling response including Ca\textsuperscript{2+} mobilization and phosphorylation of the p42/p44 extracellular signal-regulated kinases (ERKs).

Introduction

Mast cells are resident cells present in all organs of the body and are known to increase in number at sites of inflammation (1, 2). They play a role in host defense to helminthic parasites and certain bacteria and are major effectors for the induction of inflammation and allergic reactions (3). Mast cells arise from CD34\textsuperscript{+}/CD117\textsuperscript{+} pluripotent hematopoietic stem cells in the bone marrow and mature to committed progenitors upon growth factor stimulation (4). These progenitors are released into the circulation from the bone marrow and subsequently migrate to various tissues of the body where local tissue-specific factors and cell surface interactions determine the mature phenotype of the mast cell appropriate for the microenvironment (5, 6). Two major subtypes of mast cells have been identified, connective tissue mast cells that are found in the skin, around blood vessels and the peritoneal cavity. Mucosal mast cells are found in the gut and the upper airways (7, 8).

Mast cells bind IgE via expression of the high affinity IgE receptor FcεRI, and it is via FcεRI that mast cells are most commonly activated in allergic diseases. Aggregation of receptor-bound IgE molecules by polyvalent antigen triggers a signaling cascade resulting in mast cell activation and rapid degranulation of preformed pro-inflammatory mediators, which include histamine, proteases and proteoglycans. Protein mediators such as cytokines and chemokines are also synthesized and released, as are de novo synthesized lipids such as leukotriene (LT) C\textsubscript{4} and LTB\textsubscript{4} and prostaglandin (PG) D\textsubscript{2}. The generation and release of these factors by mast cells induces a strong inflammatory response and contributes to the pathogenesis of various allergic diseases, such as asthma, allergic rhinitis and atopic dermatitis (9, 10).

PGD\textsubscript{2} is an arachidonic acid metabolite that has been implicated in the development and progression of various allergic diseases. Following the conversion of arachidonic acid to PGH\textsubscript{2} by cyclooxygenase, hematopoietic PGD\textsubscript{2} synthase converts PGH\textsubscript{2} into PGD\textsubscript{2} (11). PGD\textsubscript{2} is then exported from the cytosol to the extracellular space by a PG transporter.
protein (12). Two receptors so far have been identified for PDG_{D2}, prostanoid D1 (DP1) receptor and chemoattractant receptor-homologous molecule expressed on T_{H} class 2 cells (CRTh_{2}). Both these G protein-coupled receptors bind PDG_{D2} with nanomolar affinity; however, they share little amino acid homology and have different tissue expression patterns and signaling pathways, suggesting distinct roles in allergic and immune responses (13). The amino acid sequence of DP1 shows homology to other prostanoid receptors, whereas CRTh_{2} shows high identity to chemoattractant G-protein-coupled receptors (GPCRs). DP1 is associated with pertussis toxin (Ptx)-resistant G_{a_{16}}, whereas CRTh_{2} signals with Ptx-sensitive G_{a_{i}} proteins (14), similar to chemokine receptors (9). CRTh_{2} is expressed by both T_{H} and a subset of T_{H}1 T_{H} cells in the mouse, as well as eosinophils and basophils (15, 16). Human basophils express both DP1 and CRTh_{2}, and treatment (Tx) with a DP1-selective agonist, BW245C, inhibited FcεRI-dependent exocytosis. In contrast, the selective CRTh_{2} agonist, 13,14-dihydro-15-keto-prostaglandin D_{2} (DK-PGD_{D2}), potentiated FcεRI-dependent exocytosis (16). Similarly, DK-PGD_{D2} but not BW245C, induced human eosinophil degranulation (17). Thus, expression of these two receptors on the same cell appears to be playing different and often opposing roles in regulating responsiveness toward PDG_{D2}.

As mast cells are the largest source of PDG_{D2} in the body, we investigated whether they express the CRTh_{2} receptor. We hypothesized that expression of CRTh_{2} by mast cells may result in a positive feedback circuit where mast cell release of PDG_{D2} could recruit more mast cells to the sites of PDG_{D2}-mediated inflammation. Moreover, CRTh_{2} expression may influence the activation state of mast cells upon PDG_{D2} stimulation. We show here the evidence that bone marrow-derived mast cells (BMMCs) express the CRTh_{2} receptor, and using a specific and potent small molecule antagonist demonstrate that signaling through CRTh_{2} results in extracellular signal-regulated kinase (ERK) kinase phosphorylation and mast cell chemotaxis both in vitro and in vivo.

Methods

Materials

All reagents were purchased from Sigma (St Louis, MO, USA) unless otherwise stated. The Institutional Animal Care and Use Committee approved all animal experimentation prior to implementation. Compound A is a proprietary, selective and highly potent CRTh_{2} small molecular weight antagonist that is developed as part of the Actimis Pharmaceuticals, Inc. portfolio of potent and selective compounds (WO/2004/096777).

Mast cell culture

Mast cells were cultured as described previously (18). In brief, bone marrow cells were flushed from femurs of the 6–10-week-old C57Bl/6 mice and cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FCS, 100 µM non-essential amino acids (Mediatech, Herndon, VA, USA), 50 µM 2-ME and 8% conditioned medium of IL-3 gene-transfected cells (BMMC medium). After 4 weeks of culture, >95% of the Trypan blue-excluding viable cells were mast cells. BMMCs used for experimentation were in culture for 4–6 weeks.

Reverse transcription–PCR assay

Messenger RNA from BMMCs was isolated using Oligotex mRNA isolation kit according to the manufacturer’s instructions (Qiagen Sciences, Valencia, CA, USA). Reverse transcription (RT)–PCR analysis of CRTh_{2} message expression was performed by standard techniques (19). Briefly, the RT reaction was carried out using isolated RNA, oligo dT 3’ primers and reverse transcriptase (Superscript III first strand synthesis system, Invitrogen). PCR was then performed on all the samples using CRTh_{2} primers (5’ primer, 5’ CCTCCCTTTAGCTCTCTGC 3’ and 3’ primer, 5’ TACCGAGGCCCTGTAAC 3’ and 3’ primer, 5’ GCACGCACGTGAATTCCT 3’). The PCR protocol used to amplify the CRTh_{2} and β-actin cDNA was 95°C, 30 s; 55°C, 60 s and 68°C, 2 min for 35 cycles, followed by 72°C for 5 min. The PCR products were visualized on a 1.5% agarose gel stained with ethidium bromide.

Saturation binding and competitive binding assay

Radioligand-binding analyses were performed according to the methods of Sugimoto et al. (20). For the competitive binding assay, BMMCs were re-suspended in binding buffer (50 mM Tris–HCl, pH 7.4, 40 mM MgCl_{2}, 0.1% BSA) at a concentration of 4 X 10^{6} ml^{-1} at room temperature. Fifty microliters of cell suspension was incubated for 1 h at room temperature with gentle shaking in the presence of 10 µl of [^{3}H]-PGD_{2} (3 nM final), 10 µl of competitor (10^{-12}–10^{-6} dose range) or buffer alone and the final volume adjusted to 100 µl with buffer. Incubations were performed in U-bottom polypropylene 96-well plates (Fisher Scientific, Pittsburgh, PA, USA) for 60 min at room temperature, and the cell suspension was transferred to filtration plates (MAFB, Millipore, Bedford, MA, USA), pre-wet with PEI (0.5%) buffer (Acros Organics, Morris Plains, NJ, USA). The cell pellets were washed three times with buffer and the radioactivity was counted on a TopCount (Packard Bioscience, Meriden, CT, USA). Saturation analysis was done using 10 µM DK-PGD_{D2} for each experimental condition, and a range of [^{3}H]-PGD_{2} (from 0.3 to 10 nM). Data analyses were performed using the PrizmTM graphics program using a one-site competition model (GraphPad Software Inc., San Diego, CA, USA). PGD_{D2} and DK-PGD_{D2} were purchased from Cayman Chemical. Radiolabeled PGD_{D2} ([^{3}H]-PGD_{2}; [5,6,9,12,15-^{3}H(N)]; specific activity 160 Ci mmol^{-1}) was purchased from Perkin Elmer (Waltham, MA, USA).

Flow cytometric analysis

BMMCs were prepared and stained for flow cytometry as previously described (21). Briefly, the cells were incubated for 10 min with Fc block (Becton Dickinson–Pharmentin) to inhibit non-specific binding. The cells were subsequently incubated on ice and stained with FITC-conjugated anti-CD62L (clone Dreg 56) mAb, PE-conjugated anti-CD30 mAb (clone mCD30.1) or FITC-conjugated anti-CD23 mAb (clone B3B4; all antibodies obtained from BD Pharmentin).
The cells were washed in cold FACS buffer (PBS containing 1% BSA and 0.1% sodium azide) and analyzed using a FACSscan II analyzer (Becton Dickinson, Mountain View, CA, USA) and CellQuest software (Becton Dickinson).

Calcium mobilization assay

Murine BMMCs were labeled with 3 μM of indo-1AM dye (Invitrogen) for 60 min at 37°C. The cells were subsequently washed and re-suspended in 1 ml of HBSS containing 1% BSA (fraction V, Sigma). Calcium mobilization was measured using a PTI fluorometer (South Brunswick, NJ, USA) as previously described (22). Murine RANTES and eotaxin were obtained from R&D Systems (Minneapolis, MN, USA). A relatively high dose (10 μM) of PGD<sub>2</sub> and PGE<sub>2</sub> was used to stimulate the BMMC in the Ca<sup>2+</sup> mobilization experiments shown in Figs 4 and 5(D-G) in order to see a robust response under the experimental conditions used. Lower doses (1 μM) showed only a weak response and, in contrast to the other assays, no response was detected at concentrations <100 nM.

Histamine release assay

BMMCs were cultured overnight in full medium (2 × 10<sup>6</sup> ml<sup>-1</sup>) in the presence of the anti-DNP IgE antibody [H1(206)] (18). The cells were washed two times and re-suspended in Tyrode's buffer [112 mM NaCl, 2.7 mM KCl, 0.4 mM NaH<sub>2</sub>PO<sub>4</sub>, 1.6 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 10 mM HEPES (pH 7.5), 0.05% gelatin and 0.1% glucose] at a concentration 37°C and was halved by the addition of cold PBS. The cells were then centrifuged at 4°C and immediately re-suspended in 1× RIPA buffer (Pierce, Rockford, IL, USA) containing freshly added protease and phosphatase inhibitors. The cells were vortex mixed briefly, incubated on ice for 15 min and subsequently centrifuged to pellet-insoluble material. The supernatants were isolated, boiled for 5 min in 2× Laemmli sample buffer and loaded onto 10% Tris–glycine polyacrylamide gels (Invitrogen). Protein was transferred onto a nitrocellulose membrane using the iBlot dry blotting system (Invitrogen). Following transfer, the immunoblots were handled essentially as described previously (21). Briefly, the nitrocellulose membrane was incubated in 1× Tris-buffered saline [20 mM Tris–HCl (pH 7.6) and 137.5 mM NaCl], 0.1% Tween 20 with 5% w/v non-fat dry milk (Cell Signaling Technologies, Boston, MA, USA) for 1 h at 25°C to block non-specific protein-binding sites. The membrane was then incubated at 4°C overnight with p42/p44 anti-phospho-ERK antibody (Cell Signaling Technologies) diluted 1/1000. The immunoblots were subsequently washed in TTBS [20 mM Tris–HCl (pH 7.6), 137.5 mM NaCl and 0.1% Tween 20] and incubated with peroxidase-conjugated goat anti-rabbit IgG (Pierce) diluted 1/500 for 1 h at 25°C. Phospho-MAPK was visualized using the chemiluminescent peroxidase substrate Super Signal West Femto Maximum Sensitivity Substrate (Pierce). For subsequent blotting, the membrane was stripped using Restore Western Blot Stripping Buffer (Pierce) for 30 min at 25°C. The anti-MAPK blot was conducted in the same manner, except the primary antibody was a rabbit anti-ERK polyclonal antibody at 1/1000 (Cell Signaling Technologies). The anti-CRT<sub>2</sub>,<sub>2</sub> blot was hybridized with an anti-CRT<sub>2</sub>,<sub>2</sub> polyclonal antibody from Cayman Chemical. The western blot results were visualized using a Kodak Gel Logic 440 Imaging System (Eastman Kodak, New Haven, CT, USA).

Chemotaxis assay

All chemotaxis assays were performed in a 48-well modified Boyden chamber (Neuroprobe, Cabin John, MD, USA) as described (21). BMMCs were re-suspended in RPMI 1640 (Invitrogen) with 10% FBS (Lonza Biowhittaker, Portsmouth, NH, USA) at 2 × 10<sup>6</sup> cells ml<sup>-1</sup> at 37°C. Cells were allowed to migrate for 1 h at 37°C, after which time the filter was stained in Diff-Quik (Baxter, Deerfield, IL, USA). Migrating cells were quantified by microscopy, manually counting four fields per well under high power (×400). The assays were performed in duplicate, and the results are shown as the average number of cells enumerated per condition ±SEM.

Western blot analysis

BMMCs were re-suspended in Tyrode's buffer. Stimulation with PGD<sub>2</sub> or DK-PGD<sub>2</sub> was conducted at 37°C and was halted by the addition of cold PBS. The cells were then centrifuged at 4°C and immediately re-suspended in 1× RIPA buffer (Pierce, Rockford, IL, USA) containing freshly added protease and phosphatase inhibitors. The cells were vortex mixed briefly, incubated on ice for 15 min and subsequently centrifuged to pellet-insoluble material. The supernatants were isolated, boiled for 5 min in 2× Laemmli sample buffer and loaded onto 10% Tris–glycine polyacrylamide gels (Invitrogen). Protein was transferred onto a nitrocellulose membrane using the iBlot dry blotting system (Invitrogen). Following transfer, the immunoblots were handled essentially as described previously (21). Briefly, the nitrocellulose membrane was incubated in 1× Tris-buffered saline [20 mM Tris–HCl (pH 7.6) and 137.5 mM NaCl], 0.1% Tween 20 with 5% w/v non-fat dry milk (Cell Signaling Technologies, Boston, MA, USA) for 1 h at 25°C to block non-specific protein-binding sites. The membrane was then incubated at 4°C overnight with p42/p44 anti-phospho-ERK antibody (Cell Signaling Technologies) diluted 1/1000. The immunoblots were subsequently washed in TTBS [20 mM Tris–HCl (pH 7.6), 137.5 mM NaCl and 0.1% Tween 20] and incubated with peroxidase-conjugated goat anti-rabbit IgG (Pierce) diluted 1/500 for 1 h at 25°C. Phospho-MAPK was visualized using the chemiluminescent peroxidase substrate Super Signal West Femto Maximum Sensitivity Substrate (Pierce). For subsequent blotting, the membrane was stripped using Restore Western Blot Stripping Buffer (Pierce) for 30 min at 25°C. The anti-MAPK blot was conducted in the same manner, except the primary antibody was a rabbit anti-ERK polyclonal antibody at 1/1000 (Cell Signaling Technologies). The anti-CRT<sub>2</sub>,<sub>2</sub> blot was hybridized with an anti-CRT<sub>2</sub>,<sub>2</sub> polyclonal antibody from Cayman Chemical. The western blot results were visualized using a Kodak Gel Logic 440 Imaging System (Eastman Kodak, New Haven, CT, USA).

In vivo chemotaxis assay

In vivo migration was carried out similar to that described previously (23). C57Bl/6 mice (4- to 6-week-old female) (Jackson Laboratories, Bar Harbor, ME, USA) were orally dosed with either Compound A (10, 1 and 0.1 mg kg<sup>-1</sup>) or delivery vehicle prior to having their dorsal skin shaved and receiving an intra-dermal (i.d.) injection of DK-PGD<sub>2</sub> (3 μg DK-PGD<sub>2</sub> dissolved in a 50 μl volume of PBS and controls received PBS only). A total of 2.5 × 10<sup>6</sup> BMMCs suspended
in 100 μl of Tyrode's buffer were then intravenously (i.v.) injected into the recipient animals. One hour after the i.v. transfer of BMMC, the dorsal skin surrounding the i.d. injection was isolated and snap frozen in OCT medium (Tissue Tech, Torrance, CA, USA). The skin samples were then sectioned and stained with Toluidine blue to allow visualization of mast cells. The number of mast cells was determined by counting the number of Toluidine blue staining cells per high-power field (hpf) (×400). A minimum of 9 hpf was counted for each skin section by a blinded reviewer. There were four mice in each Tx group. The results displayed the mean number of mast cells per hpf ± SEM, and statistical significance was determined by the one-way analysis of variance test.

Results

CRT\textsubscript{2} mRNA and protein are expressed by BMMCs

To investigate whether primary BMMC express the CRT\textsubscript{2} receptor, RNA isolated from these cells were subjected to RT-PCR analysis. As shown in Fig. 1(A), BMMC RNA shows a PCR product of the expected size (694 bp). Protein lysates made from BMMC were analyzed by western analysis. A specific 62 kDa band was observed at the expected molecular weight of the glycosylated form of the receptor (Fig. 1B). Collectively, these results demonstrated that CRT\textsubscript{2} is transcribed and translated by primary BMMCs.

Pharmacological characterization of CRT\textsubscript{2} on BMMC

Saturation analysis and competitive binding assays were performed with BMMC using [3H]-labeled PGD\textsubscript{2} and non-labeled DK-PGD\textsubscript{2}, which is a CRT\textsubscript{2}-specific ligand. These experiments showed an expected $K_d$ of 2.9 ± 1.1 nM, and the $B_{\text{max}}$ was 300.5 ± 50.1 pM (Fig. 2A and B). The receptor number per cell was calculated to be 1065 ± 167 receptor sites per cell. This data confirm functional cell surface expression of CRT\textsubscript{2} on BMMC.

CRT\textsubscript{2} activation modulates expression of cell surface molecules

Murine primary BMMCs were cultured in the presence of 50 nM DK-PGD\textsubscript{2} for 15 min, and levels of CD62L (ι-selectin) was assessed by flow cytometry. As shown in Fig. 3(A), there was a reduced amount of cell surface CD62L following DK-PGD\textsubscript{2} Tx, indicating the BMMC shed ι-selectin. Incubating the cells with DK-PGD\textsubscript{2} for longer time periods (30 and 45 min) did not appreciably increase the amount of ι-selectin shedding. When BMMCs were cultured with 50 nM DK-PGD\textsubscript{2} for 12 h, there was a large increase in CD23, the low affinity IgE receptor (FcεRII) cell surface expression (Fig. 3B), and a modest increase in the tumor necrosis factor (TNF) receptor superfamily member CD30 expression (Fig. 3C). DK-PGD\textsubscript{2}-mediated activation of CRT\textsubscript{2} did not modulate the cell surface expression of FcεRI, CD40 ligand, OX-40 ligand, 4-1BB, CD28, CD11b, CD18 or CD117 (data not shown).

Activation of BMMC with PGD\textsubscript{2} results in the mobilization of intracellular calcium

As CRT\textsubscript{2} has been shown to be G\textsubscript{a}\textsubscript{16}-linked GPCR, we examined BMMC for the ability to mobilize intracellular calcium stores as an early response in the signaling cascade upon PGD\textsubscript{2} stimulation. As shown in Fig. 4, PGD\textsubscript{2} stimulation resulted in a transient Ca\textsuperscript{2+} mobilization, and this response was blocked by Ptx pre-Tx (data not shown). No cross-desensitization was observed when BMMCs were first treated with PGD\textsubscript{2} followed by the CCR3 agonist chemokine CCL5 (RANTES), or inversely, treated with RANTES prior to the addition of PGD\textsubscript{2}. In contrast to FcεRI-induced Ca\textsuperscript{2+} mobilization, the flux detected with either PGD\textsubscript{2} or the chemokines RANTES and eotaxin are both transient and of relatively low magnitude. Additionally, the magnitude of the PGD\textsubscript{2}-induced Ca\textsuperscript{2+} flux can be explained by the relatively low CRT\textsubscript{2} expression on the cell surface, as determined by saturation analysis (Fig. 2).

PGD\textsubscript{2} does not augment antigen-mediated degranulation or cytokine production

Activation of mast cells via polyvalent antigen cross-linking of IgE bound to FcεRI receptors results in degranulation and cytokine production and the prostanoid PGE\textsubscript{2} has previously been shown to enhance both responses (25–27). The PGE\textsubscript{2} receptor, EP3, and CRT\textsubscript{2} are both G\textsubscript{a}\textsubscript{16}-linked G protein-coupled receptors and we investigated whether PGD\textsubscript{2} could modify these responses also. As shown in Fig. 5(A), neither PGD\textsubscript{2} nor PGE\textsubscript{2} caused histamine release by mast cells when added in the absence of antigen. However, when given together with antigen to BMMC loaded with IgE, the co-administration of PGE\textsubscript{2} caused an increased release of histamine compared with antigen alone. On the other hand,
neither PGD$_2$ nor DK-PGD$_2$ had any significant effect on antigen-mediated histamine release. We investigated this further by next examining IL-6 production. IL-6 has been reported to be released by mast cells upon PGE$_2$ stimulation alone, as well as after FcERI-mediated activation (26). We observed that PGE$_2$ stimulation of BMMC resulted in IL-6 production, whereas PGD$_2$ or DK-PGD$_2$ did not (Fig. 5B). In contrast to PGE$_2$, the addition of PGD$_2$ together with antigen did not increase IL-6 production in IgE-loaded BMMCs when compared with antigen Tx alone (Fig. 5C). We also observed

Fig. 2. Pharmacological characterization of the CRTh2 receptor on BMMC. (A) Saturation analysis of $[^3$H]-PGD$_2$ binding to CRTh2 on BMMC. Cells were incubated with various concentrations of $[^3$H]-PGD$_2$ in the absence (total binding) or presence (non-specific binding) of DK-PGD$_2$. Each data point was repeated in duplicate. The specific binding is shown and this was calculated as the difference between total binding and non-specific binding. The $K_d$ and $B_{max}$ were determined using a one-site-binding model using GraphPad Prizm software. (B) Competitive binding analysis of BMMC incubated with $[^3$H]-PGD$_2$ in the presence of various concentrations of DK-PGD$_2$. Each data point was done in triplicate. Representative data from two independent saturation analysis experiments are shown, and the average ± SEM of two separate competitive binding assays are displayed.

Fig. 3. DK-PGD$_2$ Tx induces CD62L shedding and CD23 and CD30 up-regulation in BMMC as determined by flow cytometry. (A) Mast cells were cultured with 50 nM DK-PGD$_2$ for 15 min, and cell surface expression of CD62L (L-selectin) was examined. The expression of CD62L following incubation with DK-PGD$_2$ is shown in bold, and no Tx shown in the light line. The gray filled area represents the isotype control antibody. Incubation of the mast cells with DK-PGD$_2$ for 30 or 45 min did not induce any further CD62L shedding. (B and C) Mast cells were cultured with 50 nM DK-PGD$_2$ for 12 h and the levels of CD23 (B) and CD30 (C) are up-regulated (bold lines) in comparison with untreated cells (light lines). The isotype control antibody staining is shown in gray. Representative FACS plots from three independent experiments are shown.
Representative results from three experiments are shown. Observed indicating that no receptor cross-desensitization occurred. The inverse experiment is shown on the right panel with consistent results.

Bone marrow-derived primary mast cells mobilize intracellular calcium in response to PGD2 stimulation. Mast cells were loaded with indo-1 AM and examined for the ability to release intracellular calcium in response to receptor activation. In the left panel, cells were stimulated first with PGD2 (10 μM) (lower trace). After ~90 s, the cells were re-stimulated with 100 nM CCL5 (RANTES) and as expected, an additive Ca2+ flux was observed indicating that no receptor cross-desensitization occurred. The inverse experiment is shown on the right panel with consistent results. Representative results from three experiments are shown.

We further examined whether the addition of PGD2 could modulate calcium mobilization induced by FcεRI-mediated activation. Figure 5(F) shows an antigen dose titration showing FcεRI-induced Ca2+ mobilization results in a robust and more sustained calcium flux than either of the prostanoids or chemokines examined. A possible reason for this difference is that G protein-coupled receptors linked to Gαq release only intracellular Ca2+ stores in response to activation. FcεRI, on the other hand, also induces a Ca2+ ion influx via SOCC channels, resulting in a much more robust and sustained flux (28, 29). As the amount of antigen decreased, the magnitude of the calcium flux both decreased and was delayed (Fig. 5F). We tested whether PGD2 or PGE2 could modulate calcium mobilization at the lowest dose of antigen tested. As shown in Fig. 5(G), the addition of PGE2 and 0.1 ng ml−1 antigen resulted in a more rapid calcium flux, where PGD2 had no significant effect. The inability of PGD2 to effect calcium mobilization by IgE-loaded BMMC is entirely consistent with the absence of a PGD2 effect on histamine release and IL-6 production. The non-responsiveness of BMMC loaded with IgE overnight may be due to CRTh2 down-regulation, as human Tp2 T lymphocytes down-regulate CRTh2 mRNA expression upon TCR ligation (30). As no anti-mouse CRTh2 antibody is available for FACS analysis, we examined CRTh2 mRNA levels in untreated BMMC and BMMC loaded with IgE for 18 h (Fig. 5H). RT-PCR analysis revealed that CRTh2 mRNA levels are dramatically decreased upon IgE binding, and this may explain, at least in part, the inability of PGD2 to modulate FcεRI-mediated histamine release and cytokine production.

We next asked whether Compound A could inhibit CRTh2 activation-dependent ERK phosphorylation. BMMC were pre-treated with various concentrations of Compound A for 10 min prior to the addition of 10−7 M PGD2. As shown in Fig. 6(C), Compound A inhibited ERK phosphorylation following an incubation with DK-PGD2 for 15 min, and the inhibition seen was dose dependent. The level of ERK phosphorylation was reduced to background levels following Tx of BMMC with 10−8 M of the CRTh2 antagonist. Similar results were seen when the BMMCs were stimulated with 10−7 M PGD2 (Fig. 6D). As Compound A is specific for CRTh2, in contrast with PGD2 which can bind and activate both the CRTh2 and DP1 receptors, these results...
demonstrate that BMMC ERK phosphorylation in response to PGD₂ stimulation occurs via CRTh₂-mediated pathway. CRTh₂ activation induces chemotaxis of bone marrow-derived mast cells

As CRTh₂ activation can induce signaling responses in primary BMMCs that are necessary for chemotaxis in other cell types (21), we examined whether PGD₂ or DK-PGD₂ stimulation can induce a chemotactic response. Chemotaxis assays, using a modified Boyden chamber, demonstrated that both ligands could elicit directed migration of BMMC in a dose-dependent manner (Fig. 7A). Interestingly, DK-PGD₂-mediated chemotaxis was maximal at 10⁻⁹ M, whereas maximal PGD₂-induced migration occurred at 10⁻⁸ M. The decreased efficacy seen with PGD₂ may be due to DP1 receptor expression on BMMC, as activation of DP1 has been shown to inhibit chemotaxis of other cells (31). In line with this, pre-Tx of BMMC with Ptx prior to CRTh₂ activation by either PGD₂ or DK-PGD₂ blocked all chemotaxis (Fig. 7A). As the DP1 receptor is linked to Gₐᵢ and is not Ptx sensitive, this provides further evidence that CRTh₂, which is Gₛ耦合的，is mediating the chemotaxis observed.
Inhibition of CRTh2 by the specific antagonist, Compound A, was able to block both PGD2- and DK-PGD2-induced chemotaxis (Fig. 7B). Compound A was effective at concentrations ranging from 1 to 100 nM, demonstrating that CRTh2 is the receptor responsible for regulating PGD2-mediated chemotaxis of BMMC.

We subsequently wanted to test whether i.v. injected BMMC could localize to areas of high DK-PGD2 concentration in vivo. As BMMCs migrated in response to CRTh2 activation in vitro, we hypothesized that they should be able to so
Further, Compound A would be expected to block this recruitment. To test this, C57Bl/6 mice were orally dosed with either drug vehicle or Compound A at various doses (10, 1 and 0.1 mg kg⁻¹) prior to receiving an i.d. injection of DK-PGD₂ into the dorsal skin (control mice received PBS i.d.). Primary BMMCs were then i.v. injected into the tail vein of the dosed recipient mice. After 1 h, the skin surrounding the i.d. injection site was excised, sectioned and stained with Toluidine blue. Toluidine blue stains mast cell granules and allows visualization of mast cells on the skin sections. Enumeration of mast cells showed that BMMC can indeed migrate from the bloodstream to the site of the i.d. DK-PGD₂ injection (Fig. 8A and B). Strikingly, this in vivo migration could be blocked by orally administered Compound A.

Discussion

This study shows that mast cells both express the CRTh₂ receptor and respond functionally to this receptor’s ligands, endogenous PGD₂ and the CRTh₂-specific ligand DK-PGD₂. CRTh₂ activation results in intracellular Ca²⁺ mobilization (Fig. 4), phosphorylation of the p42/p44 ERK kinases (Fig. 6), shedding of L-selectin (Fig. 3A) and chemotaxis (Fig. 7). If the responses of BMMCs are like those of mast cells in tissues in situ, then these responses may be part of a cascade necessary for in vivo migration of mast cells into tissues.

Activation of mast cells by polyvalent antigen cross-linking of cell surface-bound IgE molecules coupled with subsequent release of potent mediators make mast cells pivotal players in the initiation and maintenance of various inflammatory conditions as well as allergic responses. Furthermore, mast cell hyperplasia has been noted in various inflammatory conditions such as asthma (32), atopic dermatitis (33), allergic rhinitis (34), psoriasis (35) and rheumatoid arthritis (36). Whether this is due to mast cell proliferation or recruitment is not well understood. Neither is the process of mast cell recruitment to inflamed tissues nor the chemotactic factors regulating this process for specific inflammatory conditions have not been defined. Some chemoattractants that have the ability to induce in vitro chemotaxis of murine BMMC include stem cell factor, IL-3, MCP-1 (CCL2), MIP-1α (CCL3), RANTES (CCL5) and LTB₄ (23, 37, 38, 39). We demonstrate here that murine BMMCs were able to localize in the skin from the circulation in response to i.d. injected DK-PGD₂ (Fig. 8). Furthermore, the response could be inhibited by the oral administration of potent and selective CRTh₂ antagonist. Although the experiment carried out here represents an artificial experimental system as mature BMMCs are not ordinarily found in the circulation, these results can be taken to suggest that PGD₂ may be another chemoattractant utilized to recruit mast cells to the sites of inflammation where PGD₂ is released. This attribute may play a role in exacerbating diseases such as asthma, atopic dermatitis and allergic rhinitis.

Mast cells release the arachidonic acid metabolite PGD₂ following activation and are the greatest producers of PGD₂ in the body. This raises the notion that activation-induced release of PGD₂ may result in the recruitment of mast cells to the inflammatory sites. The ability of chemoattractants to attract cells expressing counter receptors into inflamed tissues has well been established. PGD₂ has been shown to be a chemotactic for eosinophils, basophils and TH₂ helper T cells, and this could inhibited by the antagonism of CRTh₂ (13, 20). Collectively, it is plausible that the PGD₂-CRTh₂ system acts as an autocrine feedback loop that can attract mast cells to the sites of inflammation and PGD₂ release, resulting in amplification of the inflammatory response.
CD30 is a cell surface receptor that belongs to the tumor necrosis receptor superfamily, and is up-regulated on BMMC treated with DK-PGD2 (Fig. 3C). Ligation of CD30 by CD30 ligand has been shown to result in the release of IL-8, MIP-1α, and MIP-1β, and this process was found to be independent of IgE (40). Hence, CRTh2-mediated recruitment of mast cells may also prime BMMC for CD30-induced cytokine and chemokine production.

During the course of this study, we also examined whether PGD2 or DK-PGD2 could modulate the release of granule components, specifically histamine, or cytokine secretion, such as IL-6, TNF-α and IL-2, upon antigen-induced cross-linking of IgE-loaded BMMC. It has previously been shown that PGE2 could potentiate IgE-mediated cytokine release and in some reports been shown to induce IL-6 and GM-CSF production by PGE2 Tx alone (25–27). These effects were thought to be mediated by the Gαi-linked EP3 receptor. We found no effect of PGD2 or DK-PGD2 stimulation on either of these processes in contrast to PGE2, used a side-by-side control (Fig. 5A–C). Further, BMMC lost the ability to flux Ca++ in response to PGD2 when the cells were loaded with IgE overnight (Fig. 5D–G). Thus, the occupancy of the high affinity IgE receptor with IgE drastically decreased BMMC responsiveness to PGD2 in vitro. Our preliminary data indicate that this may reflect a down-regulation of CRTh2 mRNA and cell surface levels immediately following TCR ligation (30). Additionally, CRTh2 may act similarly to other Gαi-linked chemoattractant GPCRs in which activation elicits a chemotactic response with no apparent effect on FcεRI-mediated degranulation (41–43). In either case, if the observed decreased BMMC responsiveness to PGD2 in response to occupancy of the high affinity IgE receptor with IgE seen in vitro were to occur in human mast cells in vivo, then such mast cells may give little or no response to stimulation with PGD2. Taken together, our results suggest that CRTh2 activation may prime mast cells with no, or perhaps minimal, FcεRI occupancy with IgE for the release of inflammatory mediators, but PGD2 does not appear to control their release directly.

Fig. 8. In vivo migration of BMMCs in response to i.d. DK-PGD2 is inhibited by Compound A. (A) Mice received Compound A (0.1, 1 or 10 mg kg⁻¹) or delivery vehicle (saline) orally in a 100 µl volume, followed by an i.d. injection of DK-PGD2 (3 µg) and i.v. delivery of BMMCs. One hour after the i.d. DK-PGD2 injection, the skin surrounding the site was isolated and stained with Toluidine blue to allow for enumeration of mast cells. A blinded reviewer counted the number of mast cells from a minimum of 9 hpf per section. The average number of mast cells per hpf ± SEM is shown. Two independent replicates of this experiment are shown. (B) Toluidine blue-stained skin sections were isolated from the area where DK-PGD2 was i.d. injected. The mice received either PBS or BMMC injected i.v. 1 h prior to harvesting the skin.
In summary, these results show that primary BMMCcs express CRT\textsubscript{2} and that the endogenous ligand, PGD\textsubscript{2}, is a chemotactic factor both in vitro and in vivo via activation of the CRT\textsubscript{2} receptor. Mast cells are potent pro-inflammatory cells that play a key role in allergic diseases, such as atopic dermatitis and asthma, and their numbers are increased at the sites of inflammation. As mast cells also synthesize and release PGD\textsubscript{2} in response to activation, the CRT\textsubscript{2}-PGD\textsubscript{2} system may result in an autocrine feedback loop recruiting greater numbers of mast cells to the sites of inflammation. These observations together suggest that antagonism of CRT\textsubscript{2} may be a rational therapeutic course for the Tx of allergic diseases.

Acknowledgements
Conflict of interest statement: S.A.B., E.P.C., T.W.L. and K.B.B. are Actimis Pharmaceuticals shareholders. All other authors have no conflicts of interest.

Abbreviations

**BMMC** bone marrow-derived mast cell  
**CRT\textsubscript{2}** chemoattractant receptor-homologous molecule expressed on T\textsubscript{c} class 2 cell  
**DK-PGD\textsubscript{2}** 13,14-dihydro-15-keto-prostaglandin D\textsubscript{2}  
**DP\textsubscript{1}** prostanoid D1 receptor  
**ERK** extracellular signal-regulated kinase  
**GPCR** G-protein-coupled receptor  
**hpf** high-power field  
**i.d.** intra-dermal  
**i.v.** intravenous  
**LT** leukotriene  
**PG** prostaglandin  
**Ptx** pertussis toxin  
**RT** reverse transcription  
**TNF** tumor necrosis factor  
**Tx** treatment

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