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

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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) D2. We demonstrate that primary murine bone marrow-derived mast cells (BMMCs) express the PGD2 receptor; chemoattractant receptor-homologous molecule expressed on Th class 2 cells (CRTh2). Activation of CRTh2 on BMMC by PGD2 or the CRTh2-specific agonist, 13,14-dihydro-15-keto-prostaglandin D2 (DK-PGD2), resulted in signaling response including Ca²⁺ mobilization and phosphorylation of the p42/p44 extracellular signal-regulated kinases (ERKs). Phosphorylation of the ERKs could be blocked by pertussis toxin, as well as a small molecule antagonist of CRTh2, Compound A. Activation of CRTh2 on BMMC also resulted in the up-regulation of CD23 and CD30 on the cell surface, as well as CD62L shedding. Finally, PGD2 and DK-PGD2 induced the migration of BMMC in vitro and in vivo in response to an intra-dermal DK-PGD2 injection. Both these processes were inhibited by the CRTh2 antagonist. These results raise the possibility that the functional consequences of the PGD2–CRTh2 interaction on mast cells may be relevant in allergic inflammation.

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⁺/CD117⁺ 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) C4 and LTB4 and prostaglandin (PG) D2. 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).

PGD2 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 PGH2 by cyclooxygenase, hematopoietic PGD2 synthase converts PGH2 into PGD2 (11). PGD2 is then exported from the cytosol to the extracellular space by a PG transporter...
Murine bone marrow-derived mast cells express CRTh2 protein (12). Two receptors so far have been identified for PGD2, prostanoid D1 (DP1) receptor and chemoattractant receptor-homologous molecule expressed on Tn, class 2 cells (CRTh2). Both these G protein-coupled receptors bind PGD2 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 CRTh2 shows high identity to chemoattractant G-protein-coupled receptors (GPCRs). DP1 is associated with pertussis toxin (Ptx)-resistant Gaαi3, whereas CRTh2 signals with Ptx-sensitive Gaαi proteins (14), similar to chemokine receptors (9). CRTh2 is expressed by both Tn2 cells and a subset of Tn1 Tn cells in the mouse, as well as eosinophils and basophils (15, 16). Human basophils express both DP1 and CRTh2, and treatment (Tx) with a DP1-selective agonist, BW245C, inhibited FceRI-dependent exocytosis. In contrast, the selective CRTh2 agonist, 13,14-dihydro-15-keto-prostaglandin D2 (DK-PGD2), potentiated FceRI-dependent exocytosis (16). Similarly, DK-PGD2, 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 PGD2.

As mast cells are the largest source of PGD2 in the body, we investigated whether they express the CRTh2 receptor. We hypothesized that expression of CRTh2 by mast cells may result in a positive feedback circuit where mast cell release of PGD2 could recruit more mast cells to the sites of PGD2-mediated inflammation. Moreover, CRTh2 expression may influence the activation state of mast cells upon PGD2 stimulation. We show here the evidence that bone marrow-derived mast cells (BMMCs) express the CRTh2 receptor, and using a specific and potent small molecule antagonist demonstrate that signaling through CRTh2 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. Compounds A is a proprietary, selective and highly potent CRTh2 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 CRTh2 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 CRTh2 primers (5’ primer, 5’ CTCATCTTTAGCTCTCCTGC 3’ and 3’ primer, 5’ TGAAGCTTACGCGAGGCTAA 3’) and actin primers (5’ primer, 5’ TCACCAGGCGCCCTTGAA 3’ and 3’ primer, 5’ GCACGGACTGTAACTCCCTC 3’). The PCR protocol used to amplify the CRTh2 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 analysis 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 MgCl2, 0.1% BSA) at a concentration of 4 × 10⁶ 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 [3H]-PGD2 (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 polystyrene 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 (Across 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-PGD2 for each experimental condition, and a range of [3H]-PGD2 (from 0.3 to 10 nM). Data analyses were performed using the Prism™ graphics program using a one-site competition model (GraphPad Software Inc., San Diego, CA, USA). PGD2 and DK-PGD2 were purchased from Cayman Chemical. Radiolabeled PGD2 (PGD2 [5,6,9,8,12,14,15-3H(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–Pharmingen) 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 cd30.1) or FITC-conjugated anti-CD23 mAb (clone B3B4; all antibodies obtained from BD Pharmingen).
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 PTL 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 PGD2 and PGE2 was used to stimulate the BMMC in the Ca++ 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 × 10^6 ml⁻¹) 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 NaH₂PO₄, 1.6 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES (pH 7.5), 0.05% gelatin and 0.1% glucose] at a concentration of 10 × 10^6 ml⁻¹. One hundred microliter aliquots were incubated at 37°C for 30 min, and the BMMCs were then treated with either antigen (DNP-BSA, 100 ng ml⁻¹) in the presence or absence of PGD₂, ΔK-PGD₂ or PGE₂ or the PGs alone. The stimulated cells were incubated for an additional 45 min. At this point, the supernatant was isolated by pelleting the cells by centrifugation, and the histamine levels were determined by ELISA according to the manufacturer's protocol (IBL, Hamburg, Germany).

Measurement of IL-6 production

Mast cells were loaded with antigen-specific IgE as described above in the histamine release assay section. Following two washes, the cells were re-suspended at a concentration of 1 × 10^6 cells ml⁻¹ in full medium. The mast cells were then stimulated with antigen (DNP-BSA, 10 ng ml⁻¹) in the presence or absence of PGD₂, ΔK-PGD₂ or PGE₂ for 20 hr at 37°C. The supernatant was collected and assayed by ELISA for IL-6 according to the manufacturer's directions (R&D Systems).

Western blot analysis

BMMCs were re-suspended in Tyrode's buffer. Stimulation with PGD₂ or ΔK-PGD₂ 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 1/5000 for 1 h at 25°C. Phospho-MAPK was visualized using the chemiluminescent peroxidase substrate SuperSignal 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-CRT₂,2 blot was hybridized with an anti-CRT₂,2 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).

Chemoattractant 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 × 10⁶ cells ml⁻¹. Twenty-five microliters of medium or medium containing PGD₂ or ΔK-PGD₂ (Cayman Chemical) at varying concentrations were added to the bottom of the chamber. A 5-μm pore size polycarbonate free polycarbonate filter (Osmotics, Livermore, CA, USA) was overlayed, and a 5% w/v non-fat dry milk (Cell Signaling Technologies, Boston, MA, USA) was placed at the top of the chamber. The cells 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 1/5000 for 1 h at 25°C. Phospho-MAPK was visualized using the chemiluminescent peroxidase substrate SuperSignal 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-CRT₂,2 blot was hybridized with an anti-CRT₂,2 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).

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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⁻¹) or delivery vehicle prior to having their dorsal skin shaved and receiving an intra-dermal (i.d.) injection of DK-PGD₂ (3 μg DK-PGD₂ dissolved in a 50 μl volume of PBS and controls received PBS only). A total of 2.5 × 10⁶ BMMCs suspended

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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 display the mean number of mast cells per hpf ± SEM, and statistical significance was determined by the one-way analysis of variance test.

Results

CRTh2 mRNA and protein are expressed by BMMCs

To investigate whether primary BMMC express the CRTh2 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 CRTh2 is transcribed and translated by primary BMMCs.

Pharmacological characterization of CRTh2 on BMMC

Saturation analysis and competitive binding assays were performed with BMMC using [3H]-labeled PGD2 and non-labeled DK-PGD2, which is a CRTh2-specific ligand. These experiments showed an expected Kd of 2.9 ± 1.1 nM, and the Bmax 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 CRTh2 on BMMC.

CRTh2 activation modulates expression of cell surface molecules

Murine primary BMMCs were cultured in the presence of 50 nM DK-PGD2 for 15 min, and levels of CD62L (l-selectin) was assessed by flow cytometry. As shown in Fig. 3(A), there was a reduced amount of cell surface CD62L following DK-PGD2 Tx, indicating the BMMC shed l-selectin. Incubating the cells with DK-PGD2 for longer time periods (30 and 45 min) did not appreciably increase the amount of l-selectin shedding. When BMMCs were cultured with 50 nM DK-PGD2 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-PGD2-mediated activation of CRTh2 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 PGD2 results in the mobilization of intracellular calcium

As CRTh2 has been shown to be Gα-linked GPCR, we examined BMMC for the ability to mobilize intracellular calcium stores as an early response in the signaling cascade upon PGD2 stimulation. As shown in Fig. 4, PGD2 stimulation resulted in a transient Ca2+ mobilization, and this response was blocked by Ptx pre-Tx (data not shown). No cross-desensitization was observed when BMMCs were first treated with PGD2 followed by the CCR3 agonist chemokine CCL5 (RANTES), or inversely, treated with RANTES prior to the addition of PGD2. In contrast to FcεRI-induced Ca2+ mobilization, the flux detected with either PGD2 or the chemokines RANTES and eotaxin are both transient and of relatively low magnitude. Additionally, the magnitude of the PGD2-induced Ca2+ flux can be explained by the relatively low CRTh2 expression on the cell surface, as determined by saturation analysis (Fig. 2).

PGD2 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 PGE2 has previously been shown to enhance both responses (25–27). The PGE2 receptor, EP3, and CRTh2 are both Gα-linked G protein-coupled receptors and we investigated whether PGD2 could modify these responses also. As shown in Fig. 5(A), neither PGD2 nor PGE2 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 PGE2 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 Fc$_e$RI-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 CRT$_\alpha$ receptor on BMMC. (A) Saturation analysis of $[^{3}H]$-PGD$_2$ binding to CRT$_\alpha$ 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.
a similar lack of a PGD2 or DK-PGD2 effect on TNF-α and IL-2 production (data not shown). These results show a clear functional difference between the prostanoids PGD2 and PGE2 on IgE-loaded BMMC. To explore this further, we examined the ability of PGD2 and PGE2 to induce Ca2++ mobilization in IgE-loaded BMMC and as a side-by-side control, non-IgE-loaded BMMC. As shown in Fig. 4 (PGD2) and Fig. 5(D) (PGD2 and PGE2), stimulation of non-IgE-loaded BMMCs with either PGD2 or PGE2 resulted in mobilization of intracellular Ca2++. Strikingly, IgE-loaded BMMC only mobilized calcium in response to PGE2 and not PGD2 (Fig. 5E). We further examined whether the addition of PGD2 could modulate calcium mobilization induced by FceRI-mediated activation. Figure 5(F) shows an antigen dose titration showing FceRI-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. FceRI, 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, whereas 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-modulate 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 FceRI-mediated histamine release and cytokine production.

CRTh2 activation leads to the phosphorylation of ERK1 and ERK2 in primary BMMC

We next investigated whether CRTh2 stimulation could result in the phosphorylation of p42/44 ERK kinases. It has previously been shown for chemokine receptor activation and subsequent cellular migration that MEK-mediated ERK phosphorylation is a necessary event for actin rearrangement and chemotaxis (21). The PGD2–CRTh2 interaction has been demonstrated to induce the chemotaxis of T lymphocytes, eosinophils and basophils. As shown in Fig. 6(A), the addition of either PGD2 or DK-PGD2 induces ERK phosphorylation, whereas pre-Tx of the cells for 15 min with the MEK inhibitor PD98059 greatly reduced the levels of ERK phosphorylation. Kinetic analysis showed that the ERKs are phosphorylated within 1 min after the addition of DK-PGD2, reaching a maximum at 15 min (Fig. 6B). Dose titration analysis demonstrated that a concentration as low as 10−7 M of DK-PGD2 is sufficient for BMMC to phosphorylate the p42/44 ERK kinases, and the level of phosphorylation increased through 10−7 M. These results demonstrate that BMMC phosphorylation of the ERK kinases due to DK-PGD2 stimulation is both time and dose dependent, and this activation occurs via a MEK-dependent pathway.

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 DK-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.

**CRT₂ activation induces chemotaxis of bone marrow-derived mast cells**

As CRT₂ 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 Gi and is not Ptx sensitive, this provides further evidence that CRTh₂, which is Gi coupled, is mediating the chemotaxis observed.

Fig. 5. PGD₂ does not augment FcεRI-mediated histamine release or IL-6 production. (A) BMMCs were loaded overnight with an excess of antigen-specific IgE, and histamine release was measured after a 45-min incubation with either PGD₂ (1 µM), DK-PGD₂ (1 µM) or PGE₂ (1 µM) alone or in combination with polyvalent antigen (100 ng ml⁻¹). (B and C) IL-6 production was measured after BMMCs were incubated with PGD₂ (1 µM), DK-PGD₂ (1 µM) or PGE₂ (1 µM) for 20 h (B) or IgE-loaded BMMC treated with antigen (10 ng ml⁻¹) in the absence or presence of PGD₂, DK-PGD₂ or PGE₂ (C). (D–G) Calcium mobilization was measured in BMMC (D) or IgE-loaded BMMC (E) stimulated with PGD₂ (10 µM) and PGE₂ (10 µM). Panel (F) shows the calcium response of IgE-loaded BMMC in response to various doses of antigen, and (G) shows the response of IgE-loaded BMMC stimulated with 0.1 ng ml⁻¹ antigen and either PGD₂ (10 µM) or PGE₂ (10 µM). The BMMCs were stimulated with a 10 µM dose of either PGD₂ or PGE₂ in the Ca²⁺ mobilization experiments to induce a robust response under the experimental conditions used. (H) RT-PCR analysis of CRTh₂ expression in either BMMC or BMMC loaded with IgE overnight. Actin levels are shown as a control. All results shown are from representative experiments from a minimum of two independent experiments (n = 2–5).
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.

BMMC can migrate to DK-PGD2 in vivo and this response can be abrogated by Compound A

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...
Both the 10 and 1 mg kg\(^{-1}\) doses were effective at blocking BMMC migration to the exogenously administered DK-PGD\(_2\) in each experiment. Coupled with the \textit{in vitro} chemotaxis experiments, these studies show that primary BMMCs have the ability to respond functionally to CRT\(_{1,2}\) activation by migrating specifically to PGD\(_2\) or DK-PGD\(_2\).

\textbf{Discussion}

This study shows that mast cells both express the CRT\(_{1,2}\) receptor and respond functionally to this receptor’s ligands, endogenous PGD\(_2\) and the CRT\(_{1,2}\)-specific ligand DK-PGD\(_2\). CRT\(_{1,2}\) activation results in intracellular Ca\(^{2+}\) 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 \textit{in situ}, then these responses may be part of a cascade necessary for \textit{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 \textit{in vitro} chemotaxis of murine BMMC include stem cell factor, IL-3, MCP-1 (CCL2), MIP-1\(_x\) (CCL3), RANTES (CCL5) and LTB\(_4\) (23, 37, 38, 39). We demonstrate here that murine BMMCs were able to localize in the skin from the circulation in response to \textit{i.d.} injected DK-PGD\(_2\) (Fig. 8). Furthermore, the response could be inhibited by the oral administration of potent and selective CRT\(_{1,2}\) 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 PGD\(_2\) may be another chemoattractant utilized to recruit mast cells to the sites of inflammation where PGD\(_2\) 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 PGD\(_2\) following activation and are the greatest producers of PGD\(_2\) in the body. This raises the notion that activation-induced release of PGD\(_2\) 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. PGD\(_2\) has been shown to be a chemotactic for eosinophils, basophils and Th\(_2\) helper T cells, and this could inhibited by the antagonism of CRT\(_{1,2}\) (13, 20). Collectively, it is plausible that the PGD\(_2\)-CRT\(_{1,2}\) system acts as an autocrine feedback loop that can attract mast cells to the sites of inflammation and PGD\(_2\) release, resulting in amplification of the inflammatory response.

\textbf{In vivo} 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 \textit{i.d.} injection of DK-PGD\(_2\) into the dorsal skin (control mice received PBS \textit{i.d.}). Primary BMMCs were then \textit{i.v.} injected into the tail vein of the dosed recipient mice. After 1 h, the skin surrounding the \textit{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 \textit{i.d.} DK-PGD\(_2\) injection (Fig. 8A and B). Strikingly, this \textit{in vivo} migration could be blocked by orally administered Compound A.

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure7.png}
\caption{Chemotaxis of BMMC in response to PGD\(_2\) and DK-PGD\(_2\) and this can be inhibited by Compound A. (A) BMMCs migrated in response to with various concentrations of PGD\(_2\) or DK-PGD\(_2\). As a control, BMMCs were pre-incubated with 100 ng ml\(^{-1}\) Ptx for 1 h before stimulation. BMMCs were incubated with DK-PGD\(_2\) (B) or PGD\(_2\) (C) in the presence of varying concentrations of Compound A (10\(^{-9}\)-10\(^{-7}\) M). For each experiment, each experimental condition was performed in duplicate, 4 hpfs were counted per Tx (8 hpfs per experimental condition), and the average number of migrated cells per hpf is shown \pm SEM. The experiment was repeated three independent times and representative chemotaxis assays are presented.}
\end{figure}
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 Gβγ-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 in such cells after exposure to IgE, at least at the mRNA level (Fig. 5H). Down-regulation of CRTh2 mRNA upon IgE binding in BMMC is similar to TTh2 T lymphocytes transiently down-regulating CRTh2 mRNA and cell surface levels immediately following TCR ligation (30). Additionally, CRTh2 may act similarly to other Ga,i-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. Three to four sections were made from each mouse, and there were four mice per Tx group. 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 CRTh2 and that the endogenous ligand, PGD2, is a chemotactic factor both in vitro and in vivo via activation of the CRTh2 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 PGD2 in response to activation, the CRTh2-PGD2 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 CRTh2 may be a rational therapeutic course for the Tx of allergic diseases.

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Abbreviations
BMMC bone marrow-derived mast cell
CRTh2 chemotactrant receptor-homologous molecule expressed on Tc class 2 cell
DK-PGD2 13,14-dihydro-15-keto-prostaglandin D2
DP1 prostanoid D1 receptor
ERK extracellularsignal-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

References
5 Nakano, T., Sonoda, T., Hayashi, C. et al. 1985. Fate of bone marrow-derived cultured mast cells after intracutaneous, intraperitoneal, and intravenous transfer into genetically mast cell-deficient W/Wv mice. Evidence that cultured mast cells can give rise to both connective tissue type and mcucosal mast cells. J. Exp. Med. 162:1025.
18 Nishimoto, H., Lee, S. W., Hong, H. et al. 2005. Costimulation of mast cells by 4-1BB, a member of the tumor necrosis factor receptor superfamily, with the high-affinity IgE receptor. Blood 106:4241.
by Fc epsilon R1 is not shared by a G protein-coupled receptor. J. Biol. Chem. 270:10960.


