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# Inhibition of PI3K and calcineurin suppresses chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2)-dependent responses of Th2 lymphocytes to prostaglandin D<sub>2</sub>

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## ABSTRACT

Interaction of prostaglandin D<sub>2</sub> (PGD<sub>2</sub>) with chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2) triggers chemotaxis and pro-inflammatory cytokine production by Th2 lymphocytes. We have investigated the role of inhibitors of various cell-signalling pathways on the responses of human CRTH2<sup>+</sup> CD4<sup>+</sup> Th2 cells to PGD<sub>2</sub>. Phosphatidylinositol 3-kinase (PI3K) and Ca<sup>2+</sup>/calcineurin/nuclear factor of activated T cells (NFAT) pathways were activated by PGD<sub>2</sub> in Th2 cells in a CRTH2-dependent manner. Inhibition of the PI3K pathway with LY294002 significantly reduced both PGD<sub>2</sub>-induced cell migration and cytokine (interleukin-4, interleukin-5 and interleukin-13) production. The inhibitory effect of LY294002 on cell migration is likely to be related to cytoskeleton reorganization as it showed a similar potency on PGD<sub>2</sub>-induced actin polymerization. The calcineurin inhibitors, tacrolimus (FK506) and cyclosporin A, had no effect on cell migration but completely blocked both cytokine production and the nuclear translocation of NFATc1 suggesting that Ca<sup>2+</sup>/calcineurin/NFAT is involved in CRTH2-dependent cytokine production but not chemotaxis. The promotion of NFAT nuclear location by PI3K activation may be mediated by negative regulation of glycogen synthase kinase-3 $\beta$  (GSK3 $\beta$ ), since the PGD<sub>2</sub>-stimulated increase in phospho-GSK3 $\beta$  was down-regulated by LY294002, and inhibition of GSK3 $\beta$  by SB216763 enhanced PGD<sub>2</sub>-induced Th2 cytokine production and reversed the inhibitory effect of LY294002. These data suggest that PI3K and Ca<sup>2+</sup>/calcineurin/NFAT signalling pathways are critically involved in pro-inflammatory responses of Th2 cells to PGD<sub>2</sub>.

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## 1. Introduction

Prostaglandin D<sub>2</sub> (PGD<sub>2</sub>) is a product of the arachidonic acid/cyclooxygenase pathway and is released from mast cells [1],

dendritic cells [2] and Th2 lymphocytes [3]. A significant contribution of PGD<sub>2</sub> to the development of allergic responses has been suggested based on the observations of enhanced eosinophilic lung inflammation and cytokine release in

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Abbreviations: PGD<sub>2</sub>, prostaglandin D<sub>2</sub>; CRTH2, chemoattractant receptor-homologous molecule expressed on Th2 cells (also named DP2); DP1, D prostanoid receptor 1; PI3K, phosphatidylinositol 3-kinase; NFAT, nuclear factor of activated T cells; GSK3 $\beta$ , glycogen synthase kinase-3 $\beta$ ; FK506, tacrolimus; IL-4, interleukin-4; IL-5, interleukin-5; IL-13, interleukin-13; CaM, calmodulin; TP, thromboxane-like prostanoid receptor; DK-PGD<sub>2</sub>, 13,14-dihydro-15-keto-PGD<sub>2</sub>; CsA, cyclosporin A; PLC $\beta$ , phospholipase C $\beta$ .  
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transgenic mice overexpressing PGD<sub>2</sub> synthase [4]. Two distinct G protein-coupled receptors have been identified as PGD<sub>2</sub> receptors, D prostanoid receptor 1 (DP1) and chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2; also named DP2). It has been reported that PGD<sub>2</sub> can induce mucin secretion, vasodilatation and inhibit apoptosis of eosinophils via the DP1 receptor [5–7]. Through its action on the CRTH2 receptor, PGD<sub>2</sub> elicits chemotaxis of eosinophils, basophils and Th2 cells [8,9], promotes cytokine production by Th2 cells [10,11] and up-regulates pro-inflammatory protein (CD40L and CD11b) expression by eosinophils and Th2 cells [10,12]. However, the mechanism mediating the effects of PGD<sub>2</sub> downstream of receptor activation have been largely unexplored.

It is well established that Th2 lymphocytes play an important role in modulating aspects of allergic inflammation. Accumulation of Th2 cells, in addition to that of basophils and eosinophils, is observed at sites of allergic inflammation [13]. Th2-derived cytokines induce IgE production by B cells, and growth and differentiation of mast cells and eosinophils [14]. In addition to their activation via TCR Th2 cells can be activated to produce cytokines by PGD<sub>2</sub> in the absence of antigen or co-stimulation [11]. This effect is CRTH2-mediated as is the ability of mast cell supernatants to promote the migration of Th2 cells [15]. Therefore, signalling through CRTH2, appears to play a key role in driving mast cell-dependent activation of Th2 cells in allergic disease. Although it has been shown that activation of CRTH2 leads to G $\alpha$ i-dependent intracellular Ca<sup>2+</sup> mobilization [9], little is known about the downstream signalling pathways that lead to chemotaxis and cytokine production by Th2 cells.

In this study, we have investigated potential signalling mechanisms by analysing the effect of selective inhibitors of different signal pathways on the inflammatory effects of PGD<sub>2</sub> in human CRTH2<sup>+</sup> CD4<sup>+</sup> Th2 cells. We have demonstrated that PGD<sub>2</sub> induced a phosphatidylinositol 3-kinase (PI3K)-dependent phosphorylation of Akt, and a PI3K inhibitor significantly inhibited chemotaxis and cytokine production in response to PGD<sub>2</sub>. Treatment with PGD<sub>2</sub> also stimulated the nuclear translocation of nuclear factor of activated T cells (NFAT), and this effect was blocked by calcineurin inhibitors. Calcineurin inhibitors completely inhibited production of Th2 cytokines but did not affect chemotactic responses. We have also shown that the effect of a PI3K inhibitor on NFAT activation and cytokine production may be mediated through regulation of the activation of glycogen synthase kinase-3 $\beta$  (GSK3 $\beta$ ).

## 2. Materials and methods

### 2.1. Reagents

PGD<sub>2</sub> was purchased from Biomol. BW245C, BW868C, SQ29548 and ramatroban were from Cayman Chemical. LY294002, U0126, SB203580, JNK inhibitor II, I $\kappa$ B kinase inhibitor peptide and ionomycin were obtained from Calbiochem. Tacrolimus (FK506) and rocaglamide were from Alexis Biochemicals. MACS CD4<sup>+</sup> T cell isolation kit II and anti-human CRTH2 MicroBead kit were from Miltenyi Biotec Ltd. X-VIVO 15

medium was purchased from Cambrex Bio Science. Recombinant human interleukin-4 (IL-4), QuantiGlo human IL-4 chemiluminescent immunoassay kit, quantikine human interleukin-5 (IL-5) immunoassay kit and quantikine human interleukin-13 (IL-13) immunoassay kit were purchased from R&D Systems Europe Ltd. RNeasy Mini kit, Omniscript RT kit and HotStar PCR kit were from Qiagen Ltd. The 96-well ChemoTx plates were purchased from Neuroprobe. All antibodies were supplied by New England BioLabs (UK) Ltd., except the antibody to active MAPK which was from Promega, the antibody to NFATc1 which was from Alexis Biochemicals and the antibody to  $\beta$ -tubulin which was from Santa Cruz. Other chemicals were from Sigma-Aldrich.

### 2.2. Culture of CRTH2<sup>+</sup> CD4<sup>+</sup> Th2 cells

CRTH2<sup>+</sup> CD4<sup>+</sup> Th2 cells were prepared using a modified method described previously [11]. Briefly, PBMC were isolated from buffy coats (National Blood Service Bristol, UK) by Ficoll Hypaque (Amersham Biosciences) density gradient centrifugation, followed by CD4 positive cell purification using MACS CD4<sup>+</sup> T cell isolation kit II. After 7-day culture in X-VIVO 15 medium containing 10% human serum, 50 U/ml rhIL-2 and 100 ng/ml rhIL-4, CRTH2 positive cells were isolated from the CD4<sup>+</sup> culture by positive selection using an anti-human CRTH2 MicroBead kit. The harvested CD4<sup>+</sup> CRTH2<sup>+</sup> 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.

### 2.3. Chemotaxis assay

Chemotaxis was measured using a method described previously [15]. Briefly, Th2 cells were resuspended in X-VIVO 15 medium and then pre-incubated with various inhibitors for 45–60 min. A 25  $\mu$ l of cell suspension and 29  $\mu$ l test samples prepared in X-VIVO 15 medium were applied to the upper and lower chambers of a 5  $\mu$ m-pore sized 96-well ChemoTx plate. After incubation at 37 °C for 60 min, any cells remaining on top of the filters were wiped off and any cells in the lower chamber beneath the filters were collected and quantified using a FACSCalibur flow cytometer (BD Biosciences) with 30 s counting at 2  $\mu$ l/s speed. Background cell migration was determined by measuring the response to X-VIVO 15 medium alone.

### 2.4. Cytokine release assays

Th2 cells were pre-incubated with various inhibitors for 45 min followed by treatment of 100 nM PGD<sub>2</sub> in the presence or absence of inhibitor, or ramatroban, or inhibitor alone as indicated in the results for 5 h. The concentrations of IL-4, IL-5 and IL-13 in the media were assayed using ELISA assay kits according to the manufacturer's instructions. The results were measured in a Victor<sup>2</sup> V-1420 multilabel HTS Counter (Perkin-Elmer Life Sciences).

### 2.5. RT-PCR

RT-PCR was performed as described previously [11]. Briefly, total RNA of Th2 cells after different treatments were

extracted using an RNeasy Mini kit. The RNA samples were quantitated using a GeneQuant Pro (Biochrom Ltd., Cambridge, UK). cDNA of the samples were prepared from the same starting amount of RNA using a 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, CA). mRNA level of cytokines was normalized with the level of GAPDH.

Primers used were as follows: IL-4, 5'-GCTGCCTCCAAGAA-CACAAC-3' and 5'-CTCTGGTTGGCTTCCTTCAC-3' generating a 221 bp fragment; IL-5, 5'-CTGCCTACGTGTATGCCATC-3' and 5'-CTTTCCACAGTACCCCTTG-3' generating a 217 bp fragment; IL-10, 5'-CGAGATGCCTTCAGCAGAGT-3' and GCCTTGATGT-CTGGGTCTTG-3' generating a 188 bp fragment; IL-13, 5'-CCTCAATCCTCTCTGTTGG-3' and 5'-GTCAGGTTGATGCTC-CATACC-3' generating a 206 bp fragment; GAPDH, 5'-GCCACT-CAGAAGACTGTGGATGGCC-3' and 5'-GCAATGCCAGCCCCA-GCATCAAAGG-3' generating a 350 bp fragment.

## 2.6. Actin polymerization assay

Th2 cells were pre-incubated in X-VIVO 15 medium alone or media containing different inhibitors for 45–60 min followed by treatment with the same media in the presence or absence of 100 nM PGD<sub>2</sub> for 20 s at 37 °C. The cells were immediately fixed with 4% paraformaldehyde at 25 °C for 15 min. 0.1 M glycine was added for a further 5 min, and then polymeric F-actin in the cells was stained with 0.1 μM FITC labelled phalloidin and 100 μg/ml 1-α-lysophosphatidylcholine for 1 h at 25 °C. The samples were analyzed with a FACSCalibur flow cytometer.

## 2.7. Western blotting

For normal cell lysates, the cells were cultured in X-VIVO 15 medium overnight. After incubation with different inhibitors for 45–60 min, the cells were stimulated with 100 nM PGD<sub>2</sub> in the presence or absence of the same inhibitors for 15 min and then solubilized in lysis buffer (20 mM Tris-HCl, pH 7.4, 250 mM sucrose, 1 mM EDTA, 1 mM EGTA, 1 mM sodium orthovanadate, 10 mM sodium glycerophosphate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 0.1% 2-mercaptoethanol, protease inhibitor mixture and 1% Triton X-100). For nuclear extracts, the cells were cultured in X-VIVO 15 medium overnight. Cells were pre-incubated with different inhibitors for 45 min followed by treatment of 100 nM PGD<sub>2</sub> in the absence or the presence of the same inhibitors for 6 h. Cells were harvested, washed twice in cold PBS and suspended in 150 μl of buffer A (10 mM KCl, 10 mM HEPES, pH 7.9, 0.1 mM EGTA, 0.1 mM EDTA, 1 mM DTT, 1 mM sodium orthovanadate, protease inhibitor mixture and 0.5% Nonidet P-40) for 3 min on ice. After immediate centrifugation at 14,000 rpm, the supernatant was collected as cytoplasmic cell extracts (CE). The pellets were washed twice in buffer A and incubated with 80 μl of buffer B (420 mM NaCl, 20 mM HEPES, pH 7.9, 1 mM EGTA, 1 mM EDTA, 1 mM DTT, 1 mM sodium orthovanadate and protease inhibitor mixture) for 2 h with constant shaking at 4 °C. Nuclear cell extracts (NE) were collected by centrifugation at 14,000 rpm for 20 min. The samples were fractionated by

SDS-PAGE and then electrophoretically transferred to a nitrocellulose membrane, and probed with antibodies as indicated in the results. The intensity of immuno-positive bands was quantified using Quantity One software (Bio-Rad).

## 2.8. Statistics

Data were analyzed using one-way ANOVA followed by the Newman-Keuls test. Probability values of  $p < 0.05$  were considered as statistically significant.

# 3. Results

## 3.1. Inhibition of PI3K pathway attenuates CRTH2-mediated Th2 cell chemotaxis

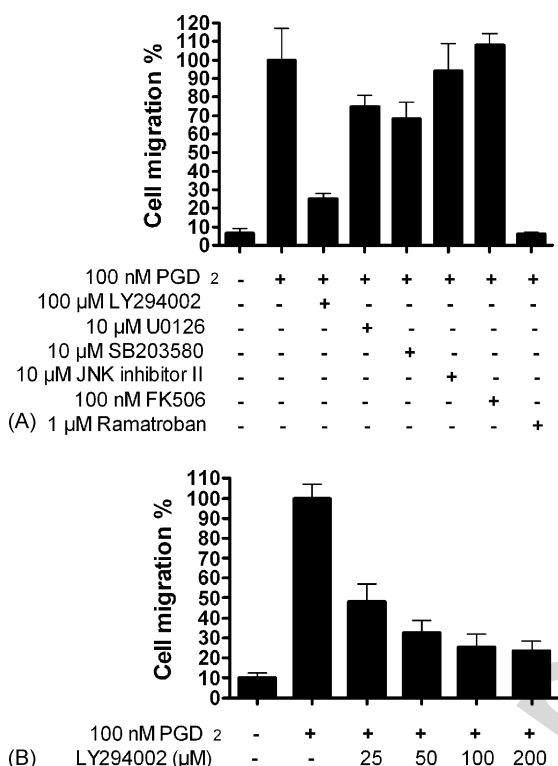
PGD<sub>2</sub> elicits chemotaxis of human CRTH2<sup>+</sup> CD4<sup>+</sup> Th2 cells via the CRTH2 receptor. The chemotactic response to PGD<sub>2</sub> followed a typical “bell” shaped dose-response curve and peaked at ~10–100 nM depending on the particular batch of cells (data not shown), consistent with previous reports [16]. To identify the signalling mechanisms used by CRTH2 to induce cell migration, we tested selective inhibitors of several signalling pathways for their ability to inhibit chemotactic responses. Since MAPK and PI3K pathways have been reported to be involved in chemotaxis in many different cell systems [17–21] and Ca<sup>2+</sup> is an important downstream signal of CRTH2 activation [8,9], the following inhibitors were employed in the chemotaxis assays: LY294002, an inhibitor of PI3K pathway; U0126, an inhibitor of ERK pathway; SB203580, an inhibitor of p38 MAPK pathway; JNK inhibitor II, an inhibitor of JNK pathway; and FK506, an inhibitor of Ca<sup>2+</sup>/calmodulin (CaM)/calcineurin pathway (Fig. 1A). Ramatroban, a dual CRTH2/thromboxane-like prostanoid receptor (TP) antagonist, used to demonstrate the CRTH2 dependency of this response [11,22] completely blocked PGD<sub>2</sub> (100 nM)-induced migration of Th2 cells. LY294002 (100 μM) was the most effective inhibitor and reduced cell migration by >75%. Ten micromolar U0126 and 10 μM SB203580 slightly reduced cell migration (~25% inhibition), whereas 10 μM JNK inhibitor II and 100 nM FK506 had no effect. A similar inhibitory effect of LY294002 was observed with 10 μM wortmannin, another PI3K inhibitor (data not shown). To further characterize the effect of PI3K inhibition, increasing concentrations of LY294002 were tested (Fig. 1B). The inhibition of cell migration increased with the concentration of the inhibitor from approximately 50% at 25 μM LY294002 to a maximum of ~80% at 100 μM.

It is well known that actin polymerization is an important cytoskeletal change that is required for cell motility [23]. To determine whether the inhibitory effect of these inhibitors on chemotaxis is via inhibition of cytoskeletal dynamics, we examined the effect of these inhibitors on actin polymerization in PGD<sub>2</sub>-treated Th2 cells (Fig. 2). Similar to the chemotactic response, the dose-response of F-actin level to PGD<sub>2</sub> stimulation was also “bell-shaped” and peaked at ~100 nM (data not shown). One micromolar ramatroban completely abolished the stimulatory effect of PGD<sub>2</sub> (Fig. 2A). In keeping with its inhibitory effect on cell migration, LY294002 (100 μM) reduced F-actin formation by 60%, while

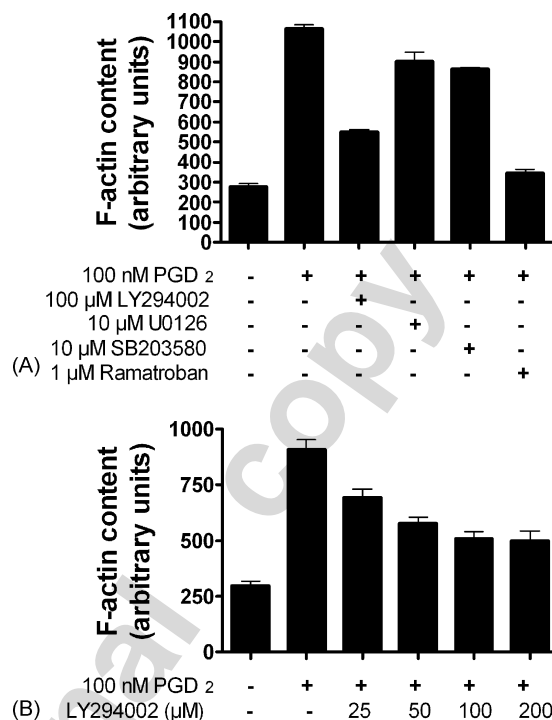


U0126 (10  $\mu$ M) and SB203580 (10  $\mu$ M) were less effective (~15–20%). The inhibitory effect of LY294002 was dose-dependent and reached maximum inhibition at ~100  $\mu$ M (Fig. 2B).

To further confirm the specificity of the inhibitors, we analyzed the activation of the pathways above in PGD<sub>2</sub>-treated Th2 cells by surveying phosphorylation of Akt, a substrate for PI3K, p38 MAPK and ERKs (Fig. 3A). PGD<sub>2</sub> (100 nM) stimulated phosphorylation of Akt, and this stimulation was completely blocked by 100  $\mu$ M LY294002 or 1  $\mu$ M ramatroban. However,

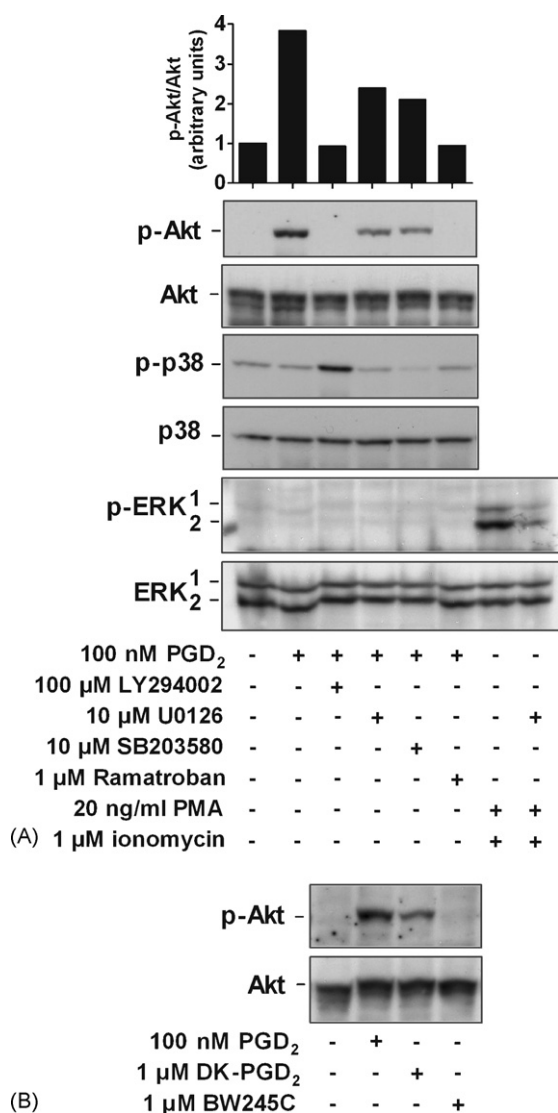


**Fig. 1 – Chemotactic response of Th2 cells to PGD<sub>2</sub> was attenuated by LY294002, U0126, SB203580 but not by JNK inhibitor II and FK506.** Th2 cells were pre-treated with medium, (A) 100  $\mu$ M LY294002, 10  $\mu$ M U0126, 10  $\mu$ M SB203580, 10  $\mu$ M JNK inhibitor II, 100 nM FK506 or (B) various concentration of LY294002 for 45 min followed by chemotaxis assay using medium or 100 nM PGD<sub>2</sub> in the absence or presence of same inhibitors or 1  $\mu$ M ramatroban as indicated. The graphs show a representative result of two to three independent experiments. Error bars show S.E.M. from triplicate samples. For (A),  $p < 0.05$  by ANOVA;  $p > 0.05$  by Newman-Keuls test for control vs. 100 nM PGD<sub>2</sub> + 100  $\mu$ M LY294002/100 nM PGD<sub>2</sub> + 1  $\mu$ M ramatroban, 100 nM PGD<sub>2</sub> + 100  $\mu$ M LY294002 vs. 100 nM PGD<sub>2</sub> + 1  $\mu$ M ramatroban, 100 nM PGD<sub>2</sub> vs. 100 nM PGD<sub>2</sub> + 10  $\mu$ M U0126/100 nM PGD<sub>2</sub> + 10  $\mu$ M SB203580/100 nM PGD<sub>2</sub> + 10  $\mu$ M JNK inhibitor II/100 nM PGD<sub>2</sub> + 100 nM FK506, 100 nM PGD<sub>2</sub> + 10  $\mu$ M U0126 vs. 100 nM PGD<sub>2</sub> + 10  $\mu$ M SB203580/100 nM PGD<sub>2</sub> + 10  $\mu$ M JNK inhibitor II/100 nM PGD<sub>2</sub> + 100 nM FK506, 100 nM PGD<sub>2</sub> + 10  $\mu$ M SB203580 vs. 100 nM PGD<sub>2</sub> + 10  $\mu$ M JNK inhibitor II vs. 100 nM PGD<sub>2</sub> + 100 nM FK506;  $n = 3$ .



**Fig. 2 – PGD<sub>2</sub>-induced actin polymerization in Th2 cells was partially inhibited by LY294002, U0126 and SB203580.** Th2 cells were pre-incubated with medium, (A) 100  $\mu$ M LY294002, 10  $\mu$ M U0126, 10  $\mu$ M SB203580, 1  $\mu$ M ramatroban or (B) various concentration of LY294002 as indicated for 45 min followed by stimulation with 100 nM PGD<sub>2</sub>. The F-actin levels in the cells were measured. The graphs show a representative result of 2–3 independent experiments. Error bars show S.E.M. from triplicate samples. For (A),  $p < 0.05$  by ANOVA;  $p > 0.05$  by Newman-Keuls test for control vs. 100 nM PGD<sub>2</sub> + 1  $\mu$ M ramatroban, 100 nM PGD<sub>2</sub> + 10  $\mu$ M U0126 vs. 100 nM PGD<sub>2</sub> + 10  $\mu$ M SB203580;  $n = 3$ .

surprisingly, both U0126 (10  $\mu$ M) and SB203580 (10  $\mu$ M) also partially (~50%) reduced PGD<sub>2</sub>-induced phosphorylation of Akt. PGD<sub>2</sub> treatment did not increase phosphorylation of p38 MAPK in Th2 cells. SB203580 further reduced the background phospho-p38 in the sample indicating the specificity and function of this inhibitor. In contrast, LY294002 enhanced the level of phospho-p38. Ramatroban and U0126 did not show any obvious effects on phospho-p38. No phospho-ERKs (p44/42) were detected in PGD<sub>2</sub>-treated Th2 cells. The positive control treated with 20 ng/ml phorbol 12-myristate 13-acetate (PMA) and 1  $\mu$ M ionomycin suggested that 10  $\mu$ M U0126 was sufficient to inhibit ERK signalling, and so it seems that only the PI3K pathway is activated by PGD<sub>2</sub> in Th2 cells. To identify the receptor mediating PGD<sub>2</sub>-induced Akt phosphorylation, we checked the effect of 13,14-dihydro-15-keto-PGD<sub>2</sub> (DK-PGD<sub>2</sub>), a selective CRTH2 agonist and BW245C, a selective DP1 agonist on the phosphorylation of Akt in Th2 cells (Fig. 3B). DK-PGD<sub>2</sub> (1  $\mu$ M) mimicked the effect of PGD<sub>2</sub>, while BW245C (1  $\mu$ M) exerted no effect further suggesting that phosphorylation of Akt is CRTH2-dependent.



**Fig. 3 – Stimulation of CRTH2 by PGD<sub>2</sub> induced phosphorylation on Akt but not ERK and P38 kinases in Th2 cells.** (A) The cells were pre-incubated with medium, 100 μM LY294002, 10 μM U0126 or 10 μM SB203580 for 45 min, and then stimulated with 100 nM PGD<sub>2</sub> in the absence or presence of the same inhibitors or 1 μM ramatroban or (B) the cells were directly stimulated with 100 nM PGD<sub>2</sub>, 1 μM DK-PGD<sub>2</sub> or 1 μM BW245C for 15 min, and the cells were lysed for Western blot analysis of active ERKs (A), phospho-p38 (A) or phospho-Akt (A, B). Sample loading was normalized by re-probing the blots with antibodies against ERKs, p38 or Akt, respectively. The cells treated with 20 ng/ml PMA and 1 μM ionomycin in the absence or presence of 10 μM U0126 were used as a positive control for active-ERK. The intensity of the bands for Akt in (A) was quantified.

### 3.2. Inhibition of PI3K and calcineurin/NFAT pathways suppresses CRTH2-mediated Th2 cytokine production

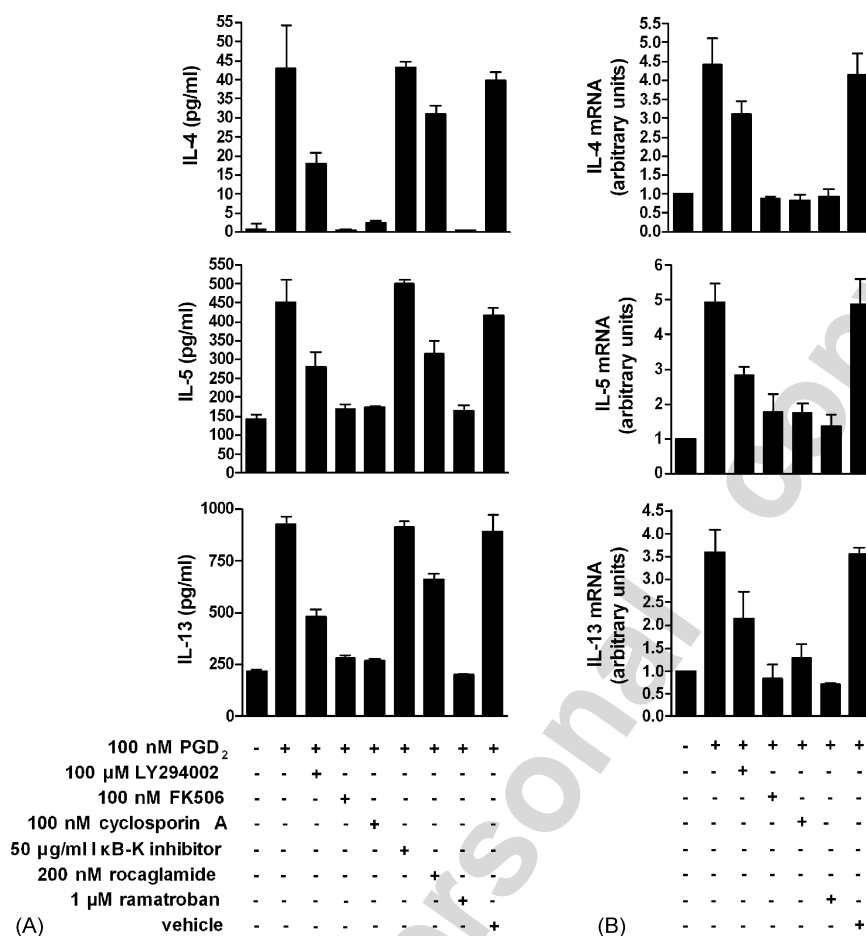
Our previous study showed that activation of CRTH2 causes preferential induction of pro-inflammatory Th2 cytokine

production [11]. To define the molecular mechanisms underlying this reaction, we investigated the effect of inhibitors of several signal pathways including the different MAPK pathways, PI3K, JAK/STAT, calcineurin/NFAT and NFκB pathways on PGD<sub>2</sub>-induced production of Th2 cytokines. Although these pathways have been reported to be involved in the regulation of gene expression in T cells [24–27], no significant effect of the inhibitors of ERK, p38 MAPK, JNK and JAK/STAT on PGD<sub>2</sub>-induced Th2 cytokine production was observed (data not shown). Similar to ramatroban (1 μM), the calcineurin inhibitors, FK506 (100 nM) and cyclosporin A (CsA) (100 nM), completely blocked cytokine (IL-4, 5 and 13) production from PGD<sub>2</sub>-stimulated Th2 cells (Fig. 4A). This blockade was also observed at the level of gene transcription (Fig. 4B). One hundred micromolar LY294002 reduced cytokine production by ~50% at both mRNA and protein levels (Fig. 4). IκB inhibitor peptide (50 μg/ml), an inhibitor of NFκB activation, did not show any effect on PGD<sub>2</sub>-triggered Th2 cytokine production (Fig. 4A), whereas rocaglamide (200 nM), another NFκB inhibitor [28], reduced cytokine production by ~30%.

To further characterize the potent inhibitory effect of calcineurin inhibitors on PGD<sub>2</sub>-induced Th2 cytokine production, we explored the effect of these inhibitors on the activation of NFATc1, a downstream transcription factor of calcineurin (Fig. 5). Nuclear translocation is a hallmark of NFAT activation [29]. Stimulation of Th2 cells with 100 nM PGD<sub>2</sub> for 6 h increased the level of NFATc1 in nuclear extracts (NE) but not in cytoplasmic extracts (CE), and this effect was clearly abolished by the presence of FK506 (100 nM) or ramatroban (1 μM) (Fig. 5A). The effect of PGD<sub>2</sub> on nuclear translocation of NFATc1 was mimicked by DK-PGD<sub>2</sub> (1 μM) but not by BW245C indicating that this response is CRTH2-mediated (Fig. 5B).

LY294002 (100 μM) also reduced nuclear translocation of NFATc1 by ~50% (Fig. 5A), thus implicating a link between the PI3K and calcineurin/NFAT pathways. To further understand the link, we looked at the effect of calcineurin inhibitors on the activation of the PI3K pathway. Phosphorylation of Akt induced by PGD<sub>2</sub> was not affected by FK506 or CsA, indicating that activation of the PI3K pathway does not require the activity of Ca<sup>2+</sup>/CaM/calcineurin (Fig. 6).

GSK-3β is one of the downstream targets of Akt and NFAT is a substrate for GSK-3β [30–31], so we suspected that it might be a possible link between the two pathways. Therefore, we examined the role of GSK-3β in PGD<sub>2</sub>-mediated activation of Th2 cells. As shown in Fig. 7A, PGD<sub>2</sub> stimulated phosphorylation of both GSK-3α (Ser21) and β (Ser9) in Th2 cells. One hundred micromolar LY294002 and 1 μM ramatroban inhibited phosphorylation of GSK-3β by ~60–70% (Fig. 7A). Interestingly, both the CRTH2 selective agonist (DK-PGD<sub>2</sub>) and the DP1 selective agonist (BW245C) mimicked the stimulatory effect of PGD<sub>2</sub> on the formation of phospho-GSK-3β (Fig. 7B). To further confirm the involvement of GSK-3β in the regulation of CRTH2-mediated Th2 cytokine production, SB216763, a specific GSK-3 inhibitor, was employed. SB216763 (30 μM) alone did not show any significant effect on IL-5 production but weakly increased IL-4 and IL-13 production (Fig. 7C). However, inhibition of GSK-3β markedly enhanced production of IL-4/5/13 in response to PGD<sub>2</sub> (Fig. 7C). SB216763 also partially reversed the inhibitory effect of



**Fig. 4 – Inhibition of PI3K, calcineurin and CRTH2 but not NFκB suppressed PGD<sub>2</sub> triggered Th2 cytokine production.** Th2 cells were pre-incubated with medium, 100 μM LY294002, 100 nM FK506, 100 nM cyclosporin A, 50 μg/ml IκB inhibitor peptide, 200 nM rocaglamide or 1 μM ramatroban for 45 min followed by treatment with medium or 100 nM PGD<sub>2</sub> in the presence or absence of the same inhibitors as indicated for 5 h. (A) The concentrations of IL-4, IL-5 and IL-13 in the media were measured by ELISA. (B) The mRNA level of IL-4, IL-5 and IL-13 from the cell pellets was determined and compared using semi-quantitative RT-PCR. The mRNA levels in untreated (medium alone) cells were treated as 1 unit. The graphs show a representative result of two independent experiments. Error bars show a range from duplicate samples or S.E.M. from triplicate samples.  $p < 0.05$  by ANOVA;  $p > 0.05$  by Newman–Keuls test for (A) control vs. 100 nM PGD<sub>2</sub> + 100 nM FK506/100 nM PGD<sub>2</sub> + 100 nM cyclosporin A/100 nM PGD<sub>2</sub> + 1 μM ramatroban, 100 nM PGD<sub>2</sub> + 100 nM FK506 vs. 100 nM PGD<sub>2</sub> + 100 nM cyclosporin A/100 nM PGD<sub>2</sub> + 1 μM ramatroban, 100 nM PGD<sub>2</sub> + 100 nM cyclosporin A vs. 100 nM PGD<sub>2</sub> + 1 μM ramatroban, 100 nM PGD<sub>2</sub> vs. 100 nM PGD<sub>2</sub> + 50 μg/ml IκB-K inhibitor/100 nM PGD<sub>2</sub> + 200 nM rocaglamide (except IL-5/13)/100 nM PGD<sub>2</sub> + vehicle, 100 nM PGD<sub>2</sub> + 100 μM LY294002 (except IL-4/13), 100 nM PGD<sub>2</sub> + 50 μg/ml IκB-K inhibitor vs. 100 nM PGD<sub>2</sub> + 200 nM rocaglamide (except IL-5/13)/100 nM PGD<sub>2</sub> + vehicle, 100 nM PGD<sub>2</sub> + 200 nM rocaglamide/100 nM PGD<sub>2</sub> + vehicle (except IL-13); for (B) control vs. 100 nM PGD<sub>2</sub> + 100 nM FK506/100 nM PGD<sub>2</sub> + 100 nM cyclosporin A/100 nM PGD<sub>2</sub> + 1 μM ramatroban, 100 nM PGD<sub>2</sub> + 100 nM FK506 vs. 100 nM PGD<sub>2</sub> + 100 nM cyclosporin A/100 nM PGD<sub>2</sub> + 1 μM ramatroban, 100 nM PGD<sub>2</sub> + 100 nM cyclosporin A vs. 100 nM PGD<sub>2</sub> + 1 μM ramatroban, 100 nM PGD<sub>2</sub> vs. 100 nM PGD<sub>2</sub> + vehicle, 100 nM PGD<sub>2</sub> + 100 μM LY294002 vs. 100 nM PGD<sub>2</sub> (except IL-5/13)/100 nM PGD<sub>2</sub> + vehicle (except IL-5/13)/100 nM PGD<sub>2</sub> + 100 nM FK506 (except IL-4)/100 nM PGD<sub>2</sub> + 100 nM cyclosporin A (except IL-4)/100 nM PGD<sub>2</sub> + 1 μM ramatroban (except IL-4)/control (except IL-4/5);  $n = 3$ .

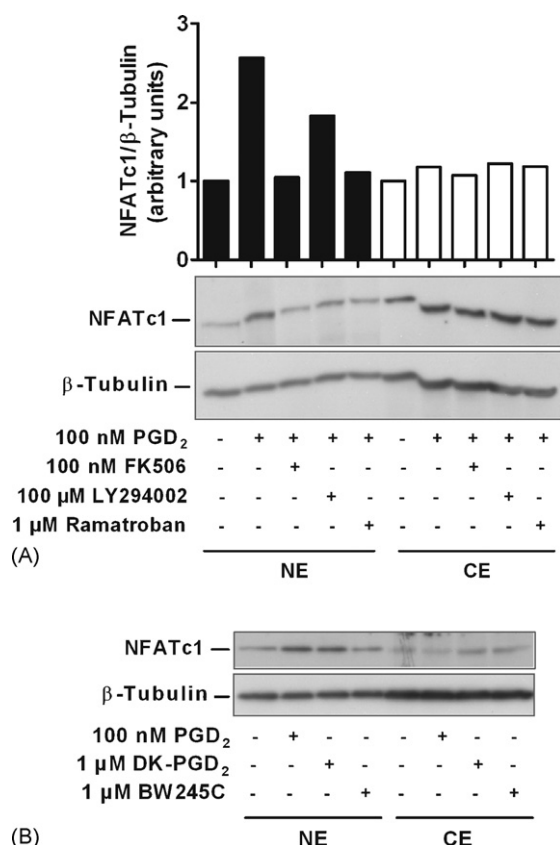
LY294002 on the cytokine response (Fig. 7C) and NFATc1 nuclear localization (Fig. 7D).

We also addressed the activation of the NFκB pathway. Although one of the NFκB inhibitors (rocaglamide) partially reduced PGD<sub>2</sub>-induced Th2 cytokine production (Fig. 4A), it appears to be a non-pathway-specific effect since no increase of phospho-IκB, a marker of NFκB activation, was detected in PGD<sub>2</sub>-treated CRTH2<sup>+</sup> CD4<sup>+</sup> Th2 cells (data not shown).

#### 4. Discussion

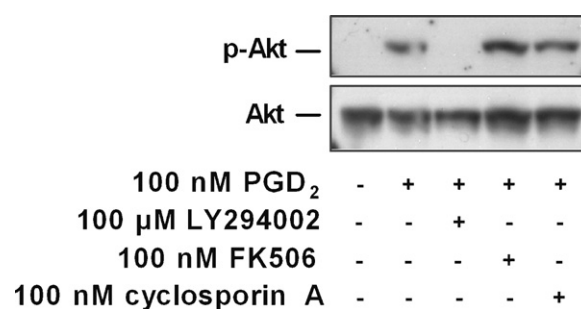
CRTH2 is a G<sub>α</sub>i-coupled receptor and plays important roles in mediating inflammatory responses of CRTH2<sup>+</sup> CD4<sup>+</sup> Th2 cells to PGD<sub>2</sub> [8,9,11]. However, the downstream signalling pathways following CRTH2 engagement are poorly understood. Our study has revealed that the PI3K and Ca<sup>2+</sup>/calcineurin/NFAT pathways mediate the effects of CRTH2 activation in





**Fig. 5 – Stimulation of CRTH2 by PGD<sub>2</sub> increased nuclear translocation of NFATc1 in Th2 cells. (A)** The cells were pre-treated with medium, 100 nM FK506 or 100 μM LY294002 for 45 min and then incubated with medium, 100 nM PGD<sub>2</sub> in the absence or presence of the same inhibitors or 1 μM ramatroban as indicated for 6 h, or **(B)** the cells were directly incubated with medium, 100 nM PGD<sub>2</sub>, 1 μM DK-PGD<sub>2</sub> or 1 μM BW245C for 6 h. Cytoplasmic extracts (CE) and nuclear extracts (NE) were separated from the cells for Western blot analysis of NFATc1. The intensity of the bands in **(A)** was quantified and normalized with the corresponding bands of β-tubulin.

human Th2 cells, and that inhibition of these pathways suppresses aspects of activation. PGD<sub>2</sub> enhanced the phosphorylation of Akt and the nuclear translocation of NFATc1 in a CRTH2-dependent manner as the effect of PGD<sub>2</sub> on these pathways was mimicked by DK-PGD<sub>2</sub>, a selective CRTH2 agonist but not by BW245C, a selective DP1 agonist. In addition, PGD<sub>2</sub>-induced activation of these pathways was completely abolished by ramatroban, a CRTH2/TP antagonist but not by SQ29548, a selective TP antagonist [11, data not shown]. The PI3K pathway is involved in both chemotaxis of Th2 cells and Th2 cytokine production induced by PGD<sub>2</sub> as inhibition of the PI3K pathway with LY294002 substantially down-regulated both responses. The regulation of Th2 cytokine production by PGD<sub>2</sub> is clearly Ca<sup>2+</sup>/CaM/calcineurin pathway-dependent, as FK506 and CsA inhibited cytokine production at both the protein level and gene transcriptional level. NFAT is likely to be a transcription factor activated downstream of calcineurin since CRTH2-mediated nuclear

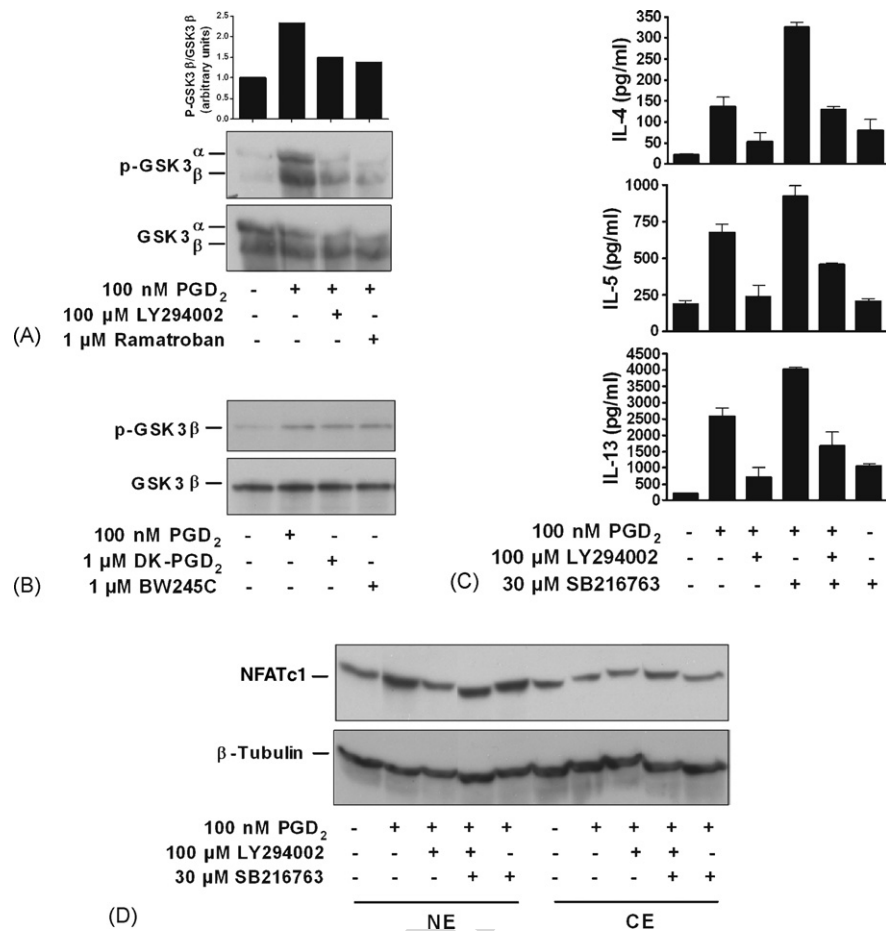


**Fig. 6 – Inhibition of calcineurin did not affect PGD<sub>2</sub>-induced Akt-phosphorylation in Th2 cells.** The cells were pre-incubated with medium, 100 μM LY294002, 100 nM FK506 or 100 nM cyclosporin A for 45 min followed by treatment with medium or 100 nM PGD<sub>2</sub> in the absence or presence of the same inhibitors as indicated for 15 min. The cells were lysed for Western blot for phospho-Akt. Protein loading was normalized relative to total Akt.

translocation of NFATc1 was blocked by calcineurin inhibitors. The effect of PI3K activation on cytokine production appears to be through the regulation of GSK3β activity, which phosphorylates NFAT, since LY294002 reduced not only NFATc1 nuclear localization but also the formation of phospho-GSK3β. Furthermore, inhibition of GSK3β with SB216763 enhanced PGD<sub>2</sub>-triggered Th2 cytokine production and partially reversed the inhibitory effects of LY294002.

It has been noted that PI3K plays a pivotal role in chemotaxis in many cell systems. In particular, PI3Kγ, a class IB PI3K, has been found mainly expressed in white blood cells and has been identified to be downstream of the βγ complex of G-protein-coupled receptors [32]. Activated PI3Ks phosphorylate inositol phospholipids to produce phosphatidylinositol-3-phosphate (PI(3)P), phosphatidylinositol-3,4-bisphosphate (PI(3,4)P2), and phosphatidylinositol-3,4,5-trisphosphate (PI(3,4,5)P3). PI(3,4)P2 and PI(3,4,5)P3 are implicated in a wide variety of cellular processes including re-arrangements of the actin cytoskeleton [33]. During cell migration, a steep internal gradient of PI(3,4,5)P3 is created in response to external gradients of chemoattractants. Sites of high PI(3,4,5)P3 concentration are co-localized with the leading edge of the lamella, the site of maximal actin polymerization in the cells [18]. In PI3Kγ-deficient mice, the lymphocytes exhibited severe defects in activation and migration and failed to accumulate at sites of inflammation [34,35]. In our study, inhibitors of PI3K substantially inhibited both CRTH2-mediated cell migration and actin polymerization in Th2 cells confirming the importance of this pathway in mediating these responses. However, LY294002, while completely blocking Akt phosphorylation, only partially inhibited chemotaxis and actin polymerization suggesting a component of the responses is PI3K-independent.

It has been reported that ERK and p38 MAPK signalling may be involved in the chemotaxis of eosinophils, neutrophils, and macrophages [17,20,21]. Stubbs et al. also reported that inhibition of the p38 pathway modulated CRTH2-mediated eosinophil activation [36]. However, in our system, no increased phosphorylation of ERKs and p38 MAPK was detected after PGD<sub>2</sub> stimulation. The enhancement of p38



**Fig. 7 – GSK-3β was involved in PGD<sub>2</sub>-mediated Th2 cell activation.** (A) The cells were pre-incubated with medium or 100 μM LY294002 for 45 min, and then stimulated with medium or 100 nM PGD<sub>2</sub> in the absence or presence of the same inhibitor or 1 μM ramatroban as indicated for 15 min or (B) the cells were directly treated with 100 nM PGD<sub>2</sub>, 1 μM DK-PGD<sub>2</sub> or 1 μM BW245C for 15 min. Cell lysates were probed for phospho-GSK-3β. Protein loading was normalized by re-probing the blots with an anti-GSK-3β antibody. (C) The cells were pre-incubated with medium, 100 μM LY294002 or 30 μM SB216763 for 45 min, and then stimulated with medium or 100 nM PGD<sub>2</sub> in the absence or presence of the same inhibitors for 5 h. Cell supernatants were collected, and the levels of IL-4/5/13 were measured by ELISA. (D) The cells were pre-treated with medium, 100 μM LY294002 or 30 μM SB216763 for 45 min and then incubated with medium, 100 nM PGD<sub>2</sub> in the absence or presence of the same inhibitors as indicated for 6 h. Cytoplasmic extracts (CE) and nuclear extracts (NE) were separated from the cells for Western blot analysis of NFATc1. The graphs show a representative result of 2 independent experiments. For (C),  $p < 0.05$  by ANOVA;  $p > 0.05$  by Newman–Keuls test for control vs. 100 nM PGD<sub>2</sub> + 100 μM LY294002/30 μM SB216763, 30 μM SB216763 vs. 100 nM PGD<sub>2</sub> + 100 μM LY294002/100 nM PGD<sub>2</sub> + 100 μM LY294002 + 30 μM SB216763 (except IL-5/13), 100 nM PGD<sub>2</sub> vs. 100 nM PGD<sub>2</sub> + 100 μM LY294002 + 30 μM SB216763 (except IL-5/13)/100 nM PGD<sub>2</sub> + 30 μM SB216763 (except IL-4/13);  $n = 3$ .

phosphorylation by LY294002 was probably due to stress caused by the high concentration (100 μM) of the inhibitor. Considering the inhibitory effect of LY294002 on PGD<sub>2</sub>-induced Th2 activation, this enhancement further confirmed that the p38 pathway does not contribute to CRTH2-mediated activation of Th2 cells. Although inhibitors of ERK and p38 pathways caused a weak inhibition of PGD<sub>2</sub>-mediated cell migration, their inhibitory effect on phospho-Akt suggests that their effect on Th2 responses is likely to occur via non-specific inhibition of the PI3K pathway and not due to effects on ERK or p38 pathways.

Besides PI3Kγ, phospholipase Cβ (PLCβ) is also one of the downstream effectors of the βγ subunits from the Gi protein

complex [37,38]. PLCβ2 is the most highly expressed form of PLCβ (β1–4 isoforms) in leukocytes [39]. Activated PLCβ, by hydrolysing phosphatidylinositol-bisphosphate to generate inositol-trisphosphate, triggers intracellular Ca<sup>2+</sup> mobilization [40]. We have not directly addressed the effect of inhibition on PLCβ in this study. It has been well established that activation of CRTH2 results in pertussis toxin sensitive Ca<sup>2+</sup> signalling [16, data not shown]. Elevation of intracellular free Ca<sup>2+</sup> ([Ca<sup>2+</sup>]<sub>i</sub>) is an essential triggering signal for numerous biological functions. In activated T cells, the binding of antigen/MHC complexes to the TCR triggers activation of PLCγ leading to a strong Ca<sup>2+</sup> influx [41,42]. Several transcription factors including NFAT, NF-κB and AP-1 can be activated

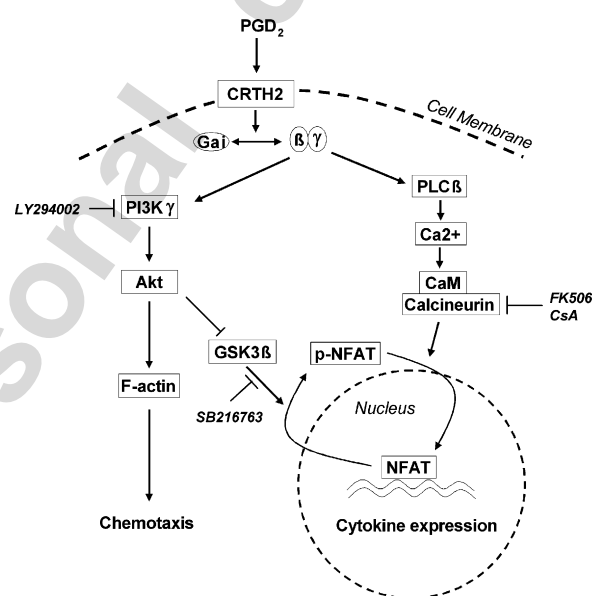
downstream from the TCR engagement in a  $\text{Ca}^{2+}$ -dependent manner [26,29,43]. In  $\text{PGD}_2$ -stimulated human Th2 cells, although NFATc1 is activated in a  $\text{Ca}^{2+}$ /calcineurin dependent manner, the activation of NF- $\kappa\text{B}$  and AP-1 was not detected (data not shown).

The NFAT family of transcription factors encompasses five members (NFAT1–5), of which, NFAT1, NFAT2 and NFAT4 are believed to be mainly involved in immune system [29]. Upon stimulation, NFAT proteins are dephosphorylated by the  $\text{Ca}^{2+}$ /CaM-dependent phosphatase calcineurin, translocate to the nucleus, and become transcriptionally active. The DNA binding sites for NFATs have been found within the promoter regions of several cytokine genes including IL-2, IL-3, IL-4, IL-5, IL-8, IL-13, TNF $\alpha$  and IFN $\gamma$  [29]. It has also been observed in knockout mouse models that individual NFAT proteins may have selective roles in T-helper-cell differentiation. NFAT1 and NFAT4 are considered to promote Th1 switching [44,45] and NFAT2 is suggested to be required for Th2 cell differentiation [46,47]. In our study, NFAT2 (NFATc1) was activated in  $\text{PGD}_2$ -stimulated Th2 cells. Inhibition of calcineurin with FK506 or CsA resulted in blockade in the nuclear translocation of NFAT2 and IL-4/5/13 up-regulation, which not only confirmed the function of NFAT2 in Th2 polarization but also indicated a fundamental role of calcineurin-mediated NFAT2 activation in CRTH2-mediated inflammatory cytokine production [11].

It is also likely that PI3K signalling is involved in CRTH2-mediated Th2 cytokine production in an NFAT-dependent manner as LY294002 reduced  $\text{PGD}_2$ -induced NFAT2 nuclear translocation and cytokine production. However, this involvement is not under regulation of  $\text{Ca}^{2+}$ /CaM/calcineurin signals, as calcineurin inhibitors had no effect on  $\text{PGD}_2$ -triggered phospho-Akt. One possible link between PI3K and NFAT is GSK3 $\beta$ , a serine/threonine kinase that can phosphorylate several transcription factors including NFAT [48]. By phosphorylating NFAT, GSK3 $\beta$  inhibits NFAT DNA binding and promotes its nuclear export [31]. It is well known that GSK3 $\beta$  is one of the few protein kinases that are inactivated by phosphorylation. Several signalling pathways, including PI3K/Akt, can inhibit GSK-3 $\beta$  by phosphorylation at Ser9 [30,48]. Hence activation of the PI3K pathway, by inhibiting GSK3 $\beta$ , could potentially enhance NFAT transcription activity although such a pathway has not been directly observed in lymphocytes so far. In our study, in response to  $\text{PGD}_2$ , both Akt and GSK3 $\beta$  were phosphorylated in Th2 cells. Inhibition of the PI3K pathway caused a partial reduction of  $\text{PGD}_2$ -triggered GSK3 $\beta$  phosphorylation, NFAT2 nuclear translocation and cytokine production. Furthermore, inhibition of GSK3 $\beta$  by SB216763 enhanced CRTH2-mediated cytokine production and partially attenuated the inhibitory effect of LY294002. All this evidence suggests that GSK3 $\beta$  is involved in the regulation of cytokine production in  $\text{PGD}_2$ -treated Th2 cells, at least partially, by providing a link between Akt and NFAT2. The ability of SB216763 to promote cytokine production was much stronger when co-administered with  $\text{PGD}_2$  confirming that the negative regulation of GSK3 $\beta$  on gene transcription is post-NFAT activation. Phospho-GSK3 $\beta$  was increased by both CRTH2 and DP1 agonists in Th2 cells suggesting that phosphorylation of GSK3 $\beta$  is partially CRTH2- and PI3K-independent, which may explain the reason why LY294002

and ramatroban cannot completely abolish  $\text{PGD}_2$ -triggered GSK3 $\beta$  phosphorylation. GSK3 $\beta$  has been reported to negatively regulate the activity of a diverse array of other transcription factors including AP-1 [49],  $\beta$ -catenin [50], Myc [51], NF- $\kappa\text{B}$  [52], heat shock factor-1 [53] and CREB [54]. Additional studies are necessary to further understand whether any other transcription factors also mediate the effect of GSK3 $\beta$  in  $\text{PGD}_2$ -induced cytokine production.

Together, the findings in this paper lead us to conclude that PI3K and  $\text{Ca}^{2+}$ /calcineurin/NFAT pathways are involved in CRTH2-mediated activation of CRTH2 $^{+}$  CD4 $^{+}$  Th2 cells and inhibition of the pathways suppresses the activation. The PI3K pathway is involved in both chemotaxis and cytokine production, whereas  $\text{Ca}^{2+}$ /CaM/calcineurin-dependent NFAT activation mediates cytokine production. The summary



**Fig. 8 – Scheme summarizing the proposed signal pathways used by  $\text{PGD}_2$ /CRTH2 to activate human Th2 cells.** Activation of CRTH2 by  $\text{PGD}_2$  causes separation of GTP-bound Gai protein from the receptor and Gby subunits. Consequently Gby subunits stimulate effector molecules, which include PI3K $\gamma$  and PLC $\beta$ . Activation of PI3K $\gamma$  leads to phosphorylation of Akt and re-organization of cell skeleton including actin polymerization that is required by chemotaxis. Inhibition of the PI3K pathway by LY294002 attenuates downstream actin polymerization and chemotaxis. Gby-stimulated PLC $\beta$  generates IP3, which elicit  $\text{Ca}^{2+}$  influx into the cytosol. Liberated  $\text{Ca}^{2+}$  binds CaM that activates phosphatase calcineurin, which in turn dephosphorylates NFAT inducing its activation and translocation to the nucleus, and resulting in cytokine gene transcription. Inhibition of calcineurin with FK506 or CsA blocks NFAT nuclear translocation and cytokine production. PI3K signals are also involved in the regulation of NFAT via GSK-3 $\beta$ . Activated Akt phosphorylates and inactivates GSK-3 $\beta$ . Inhibition of GSK-3 $\beta$  phosphorylation promotes NFAT re-phosphorylation and nuclear export. Blockade of GSK-3 $\beta$  activity with SB216763 prolongs the duration of NFAT nuclear residence and enhances gene transcription.

scheme in Fig. 8 shows the proposed signalling pathways, utilised by PGD<sub>2</sub>/CRTH2 in the activation of human Th2 cells and potential inhibitory points of the pathways. The results presented here provide new insights into the mechanisms underlying CRTH2-mediated responses in Th2 cells, and may lead to the identification of new therapeutic targets for the treatment of Th2-mediated allergic disease.

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