BIOCHEMICAL PHARMACOLOGY 73 (2007) 843–853

available at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/biochempharm

Inhibition of PI3K and calcineurin suppresses chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2)-dependent responses of Th2 lymphocytes to prostaglandin D_2

Luzheng Xue *, *Shân L. Gyles*, *Anna Barrow*, *Roy Pettipher*

Oxagen Limited, 91 Milton Park, Abingdon, Oxon OX14 4RY, United Kingdom

ARTICLE INFO

Article history:
Received 16 October 2006
Accepted 27 November 2006

Keywords:
CRTH2
Th2 lymphocytes
Chemotaxis
Cytokines
PI3K
Calcineurin

ABSTRACT

Interaction of prostaglandin D_2 (PGD_2) 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 $CRTH2^+$ $CD4^+$ Th2 cells to PGD_2. Phosphatidylinositol 3-kinase (PI3K) and Ca^{2+}/calcineurin/nuclear factor of activated T cells (NFAT) pathways were activated by PGD_2 in Th2 cells in a CRTH2-dependent manner. Inhibition of the PI3K pathway with LY294002 significantly reduced both PGD_2-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_2-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^{2+}/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-3β (GSK3β), since the PGD_2-stimulated increase in phospho-GSK3β was down-regulated by LY294002, and inhibition of GSK3β by SB216763 enhanced PGD_2-induced Th2 cytokine production and reversed the inhibitory effect of LY294002. These data suggest that PI3K and Ca^{2+}/calcineurin/NFAT signalling pathways are critically involved in pro-inflammatory responses of Th2 cells to PGD_2.

1. Introduction

Prostaglandin D_2 (PGD_2) 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_2 to the development of allergic responses has been suggested based on the observations of enhanced eosinophilic lung inflammation and cytokine release in

* *Corresponding author*. Tel.: +44 1235 443394; fax: +44 1235 443301.
E-mail address: l.xue@oxagen.co.uk (L. Xue).

Abbreviations: PGD_2, prostaglandin D_2; 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; GSK3β, glycogen synthase kinase-3β; FK506, tacrolimus; IL-4, interleukin-4; IL-5, interleukin-5; IL-13, interleukin-13; CaM, calmodulin; TP, thromboxane-like prostanoid receptor; DK-PGD_2, 13,14-dihydro-15-keto-PGD_2; CsA, cyclosporin A; PLCβ, phospholipase Cβ

0006-2952/$ – see front matter
doi:10.1016/j.bcp.2006.11.021

transgenic mice overexpressing PGD_2 synthase [4]. Two distinct G protein-coupled receptors have been identified as PGD_2 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_2 can induce mucin secretion, vasodilatation and inhibit apoptosis of eosinophils via the DP1 receptor [5–7]. Through its action on the CRTH2 receptor, PGD_2 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_2 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_2 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 Gαi-dependent intracellular Ca^{2+} 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_2 in human $CRTH2^+$ $CD4^+$ Th2 cells. We have demonstrated that PGD_2 induced a phosphatidylinositol 3-kinase (PI3K)-dependent phosphorylation of Akt, and a PI3K inhibitor significantly inhibited chemotaxis and cytokine production in response to PGD_2. Treatment with PGD_2 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-3β (GSK3β).

2. Materials and methods

2.1. Reagents

PGD_2 was purchased from Biomol. BW245C, BW868C, SQ29548 and ramatroban were from Cayman Chemical. LY294002, U0126, SB203580, JNK inhibitor II, IkB kinase inhibitor peptide and ionomycin were obtained from Calbiochem. Tacrolimus (FK506) and rocaglamide were from Alexis Biochemicals. MACS CD4+ 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 β-tubulin which was from Santa Cruz. Other chemicals were from Sigma–Aldrich.

2.2. Culture of $CRTH2^+$ $CD4^+$ Th2 cells

$CRTH2^+$ $CD4^+$ 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 $CD4^+$ 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 $CD4^+$ culture by positive selection using an anti-human CRTH2 MicroBead kit. The harvested $CD4^+$ $CRTH2^+$ cells were treated as Th2 cells and were further amplified in X-VIVO 15 medium containing 10% human serum and 50 U/ml rhIL-2 before use.

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 μl of cell suspension and 29 μl test samples prepared in X-VIVO 15 medium were applied to the upper and lower chambers of a 5 μm-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 μl/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_2 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^2 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′-GCTGCCTCCAAGAACACAAC-3′ and 5′-CTCTGGTTGGCTTCCTTCAC-3′ generating a 221 bp fragment; IL-5, 5′-CTGCCTACGTGTATGCCATC-3′ and 5′-CTTTCCACAGTACCCCCTTG-3′ generating a 217 bp fragment; IL-10, 5′-CGAGATGCCTTCAGCAGAGT-3′ and GCCTTGATGTCTGGGTCTTG-3′ generating a 188 bp fragment; IL-13, 5′-CCTCAATCCTCTCCTGTTGG-3′ and 5′-GTCAGGTTGATGCTCCATACC-3′ generating a 206 bp fragment; GAPDH, 5′-GCCACTCAGAAGACTGTGGATGGCC-3′ and 5′-GCAATGCCAGCCCCAGCATCAAAGG-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_2 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_2 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_2 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_2 elicits chemotaxis of human $CRTH2^+$ $CD4^+$ Th2 cells via the CRTH2 receptor. The chemotactic response to PGD_2 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^{2+} 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^{2+}/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_2 (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_2-treated Th2 cells (Fig. 2). Similar to the chemotactic response, the dose–response of F-actin level to PGD_2 stimulation was also ''bell-shaped'' and peaked at ~100 nM (data not shown). One micromolar ramatroban completely abolished the stimulatory effect of PGD_2 (Fig. 2A). In keeping with its inhibitory effect on cell migration, LY294002 (100 μM) reduced F-actin formation by 60%, while

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

To further confirm the specificity of the inhibitors, we analyzed the activation of the pathways above in PGD_2-treated Th2 cells by surveying phosphorylation of Akt, a substrate for PI3K, p38 MAPK and ERKs (Fig. 3A). PGD_2 (100 nM) stimulated phosphorylation of Akt, and this stimulation was completely blocked by 100 μM LY294002 or 1 μM ramatroban. However, surprisingly, both U0126 (10 μM) and SB203580 (10 μM) also partially (~50%) reduced PGD_2-induced phosphorylation of Akt. PGD_2 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_2-treated Th2 cells. The positive control treated with 20 ng/ml phorbol 12-myristate 13-acetate (PMA) and 1 μM ionomycin suggested that 10 μM U0126 was sufficient to inhibit ERK signalling, and so it seems that only the PI3K pathway is activated by PGD_2 in Th2 cells. To identify the receptor mediating PGD_2-induced Akt phosphorylation, we checked the effect of 13,14-dihydro-15-keto-PGD_2 (DK-PGD_2), a selective CRTH2 agonist and BW245C, a selective DP1 agonist on the phosphorylation of Akt in Th2 cells (Fig. 3B). DK-PGD_2 (1 μM) mimicked the effect of PGD_2, while BW245C (1 μM) exerted no effect further suggesting that phosphorylation of Akt is CRTH2-dependent.

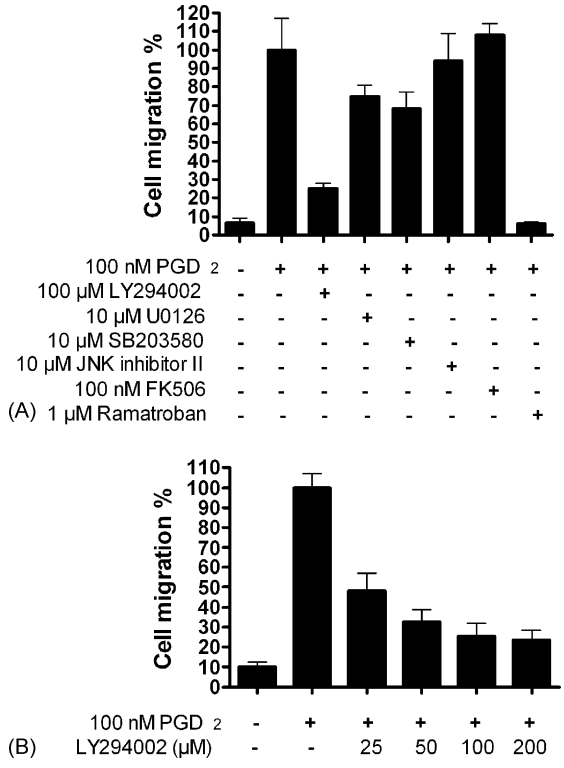

Fig. 1 – Chemotactic response of Th2 cells to PGD_2 was attenuated by LY294002, U0126, SB203580 but not by JNK inhibitor II and FK506. Th2 cells were pre-treated with medium, (A) 100 μM LY294002, 10 μM U0126, 10 μM SB203580, 10 μM JNK inhibitor II, 100 nM FK506 or (B) various concentration of LY294002 for 45 min followed by chemotaxis assay using medium or 100 nM PGD_2 in the absence or presence of same inhibitors or 1 μM 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_2 + 100 μM LY294002/100 nM PGD_2 + 1 μM ramatroban, 100 nM PGD_2 + 100 μM LY294002 vs. 100 nM PGD_2 + 1 μM ramatroban, 100 nM PGD_2 vs. 100 nM PGD_2 + 10 μM U0126/100 nM PGD_2 + 10 μM SB203580/100 nM PGD_2 + 10 μM JNK inhibitor II/100 nM PGD_2 + 100 nM FK506, 100 nM PGD_2 + 10 μM U0126 vs. 100 nM PGD_2 + 10 μM SB203580/100 nM PGD_2 + 10 μM JNK inhibitor II/100 nM PGD_2 + 100 nM FK506, 100 nM PGD_2 + 10 μM SB203580 vs. 100 nM PGD_2 + 10 μM JNK inhibitor II, 100 nM PGD_2 + 10 μM JNK inhibitor II vs. 100 nM PGD_2 + 100 nM FK506; $n = 3$.

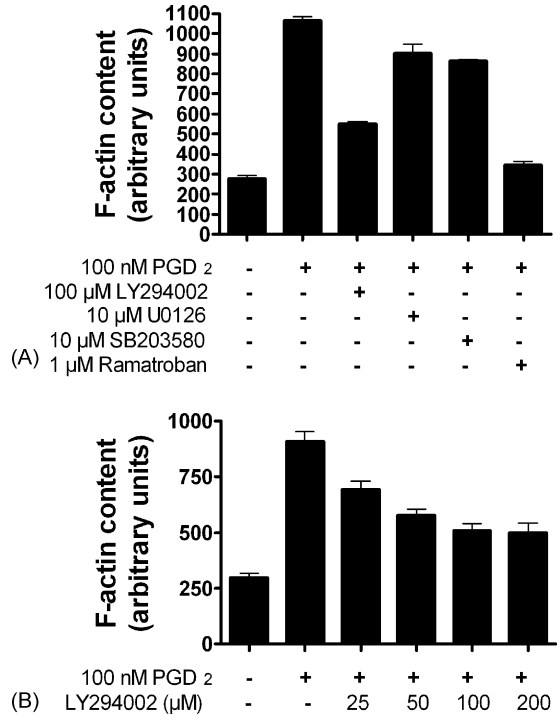

Fig. 2 – PGD_2-induced actin polymerization in Th2 cells was partially inhibited by LY294002, U0126 and SB203580. Th2 cells were pre-incubated with medium, (A) 100 μM LY294002, 10 μM U0126, 10 μM SB203580, 1 μM ramatroban or (B) various concentration of LY294002 as indicated for 45 min followed by stimulation with 100 nM PGD_2. 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_2 + 1 μM ramatroban, 100 nM PGD_2 + 10 μM U0126 vs. 100 nM PGD_2 + 10 μM SB203580; $n = 3$.

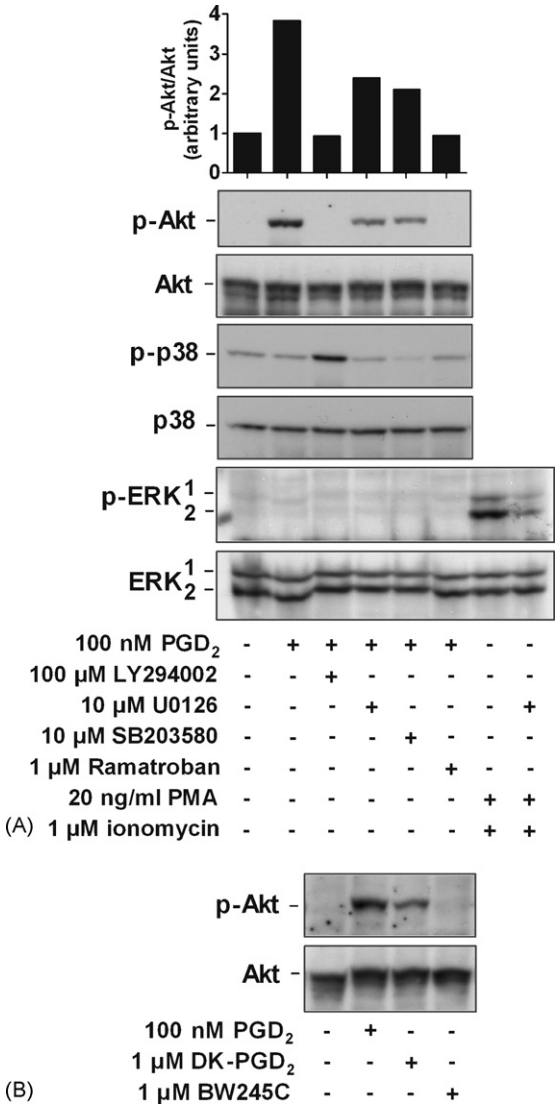

Fig. 3 – Stimulation of CRTH2 by PGD_2 induced phosphorylation on Akt but not ERK and P38 kinases in Th2 cells. (A) The cells were pre-incubated with medium, 100 μM LY294002, 10 μM U0126 or 10 μM SB203580 for 45 min, and then stimulated with 100 nM PGD_2 in the absence or presence of the same inhibitors or 1 μM ramatroban or (B) the cells were directly stimulated with 100 nM PGD_2, 1 μM DK-PGD_2 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_2-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_2-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_2-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_2-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_2-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_2 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_2 on nuclear translocation of NFATc1 was mimicked by DK-PGD_2 (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_2 was not affected by FK506 or CsA, indicating that activation of the PI3K pathway does not require the activity of Ca^{2+}/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_2-mediated activation of Th2 cells. As shown in Fig. 7A, PGD_2 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_2) and the DP1 selective agonist (BW245C) mimicked the stimulatory effect of PGD_2 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_2 (Fig. 7C). SB216763 also partially reversed the inhibitory effect of

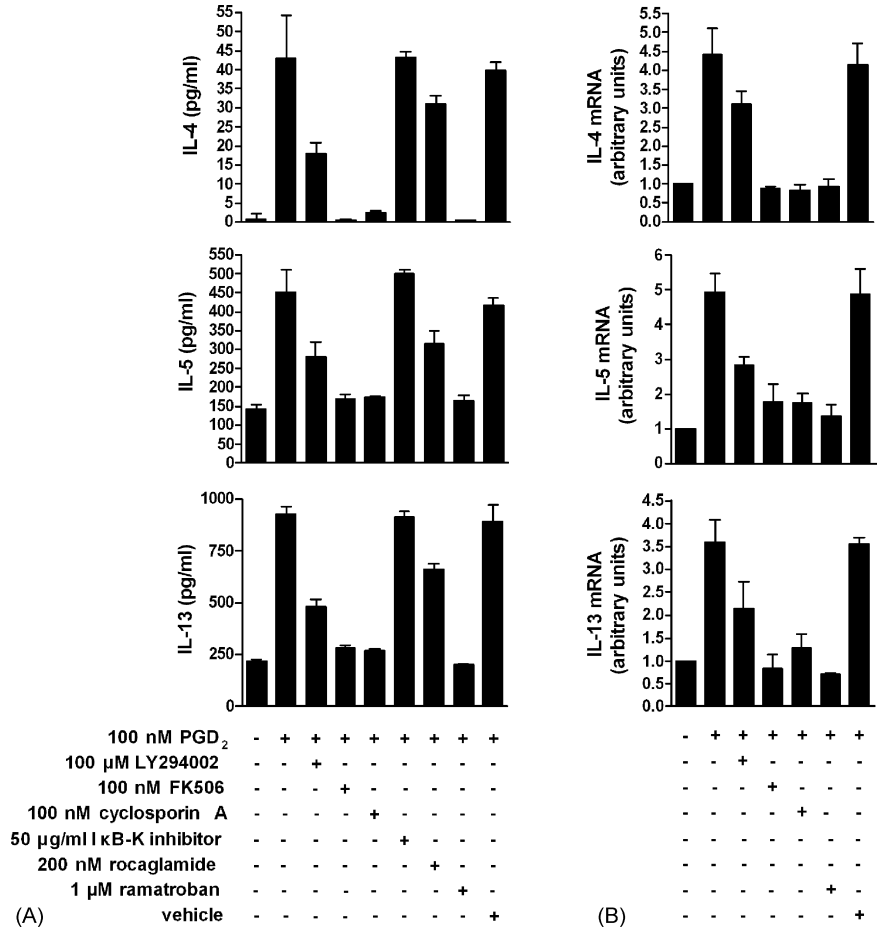

Fig. 4 – Inhibition of PI3K, calcineurin and CRTH2 but not NFκB suppressed PGD_2 triggered Th2 cytokine production. Th2 cells were pre-incubated with medium, 100 μM LY294002, 100 nM FK506, 100 nM cyclosporin A, 50 μg/ml IκB inhibitor peptide, 200 nM rocaglamide or 1 μM ramatroban for 45 min followed by treatment with medium or 100 nM PGD_2 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_2 + 100 nM FK506/100 nM PGD_2 + 100 nM cyclosporin A/100 nM PGD_2 + 1 μM ramatroban, 100 nM PGD_2 + 100 nM FK506 vs. 100 nM PGD_2 + 100 nM cyclosporin A/100 nM PGD_2 + 1 μM ramatroban, 100 nM PGD_2 + 100 nM cyclosporin A vs. 100 nM PGD_2 + 1 μM ramatroban, 100 nM PGD_2 vs. 100 nM PGD_2 + 50 μg/ml IκB-K inhibitor/100 nM PGD_2 + 200 nM rocaglamide (except IL-5/13)/100 nM PGD_2 + vehicle, 100 nM PGD_2 + 100 μM LY294002 (except IL-4/13), 100 nM PGD_2 + 50 μg/ml IκB-K inhibitor vs. 100 nM PGD_2 + 200 nM rocaglamide (except IL-5/13)/100 nM PGD_2 + vehicle, 100 nM PGD_2 + 200 nM rocaglamide/100 nM PGD_2 + vehicle (except IL-13); for (B) control vs. 100 nM PGD_2 + 100 nM FK506/100 nM PGD_2 + 100 nM cyclosporin A/100 nM PGD_2 + 1 μM ramatroban, 100 nM PGD_2 + 100 nM FK506 vs. 100 nM PGD_2 + 100 nM cyclosporin A/100 nM PGD_2 + 1 μM ramatroban, 100 nM PGD_2 + 100 nM cyclosporin A vs. 100 nM PGD_2 + 1 μM ramatroban, 100 nM PGD_2 vs. 100 nM PGD_2 + vehicle, 100 nM PGD_2 + 100 μM LY294002 vs. 100 nM PGD_2 (except IL-5/13)/100 nM PGD_2 + vehicle (except IL-5/13)/100 nM PGD_2 + 100 nM FK506 (except IL-4)/100 nM PGD_2 + 100 nM cyclosporin A (except IL-4)/100 nM PGD_2 + 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_2-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_2-treated $CRTH2^+$ $CD4^+$ Th2 cells (data not shown).

4. Discussion

CRTH2 is a Gαi-coupled receptor and plays important roles in mediating inflammatory responses of $CRTH2^+$ $CD4^+$ Th2 cells to PGD_2 [8,9,11]. However, the downstream signalling pathways following CRTH2 engagement are poorly understood. Our study has revealed that the PI3K and Ca^{2+}/calcineurin/NFAT pathways mediate the effects of CRTH2 activation in

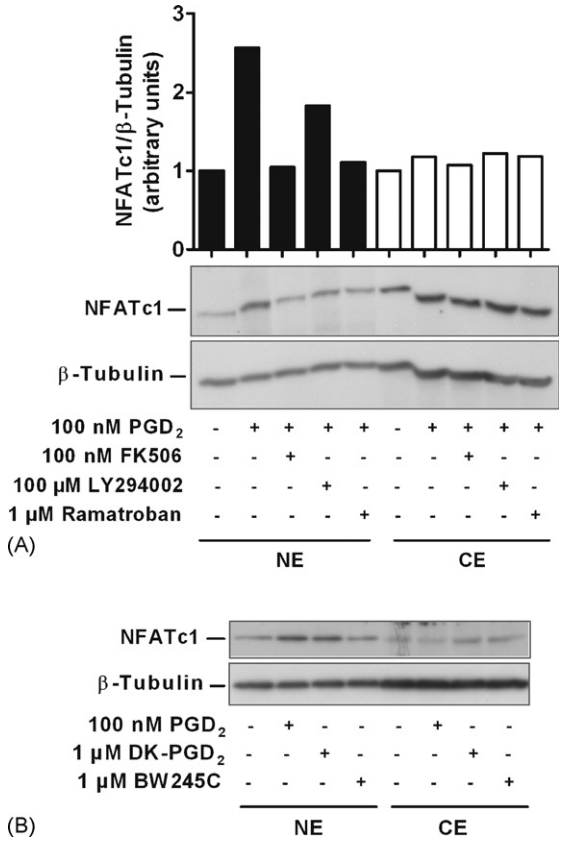

Fig. 5 – Stimulation of CRTH2 by PGD_2 increased nuclear translocation of NFATc1 in Th2 cells. (A) The cells were pre-treated with medium, 100 nM FK506 or 100 μM LY294002 for 45 min and than incubated with medium, 100 nM PGD_2 in the absence or presence of the same inhibitors or 1 μM ramatroban as indicated for 6 h, or (B) the cells were direct incubated with medium, 100 nM PGD_2, 1 μM DK-PGD_2 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.

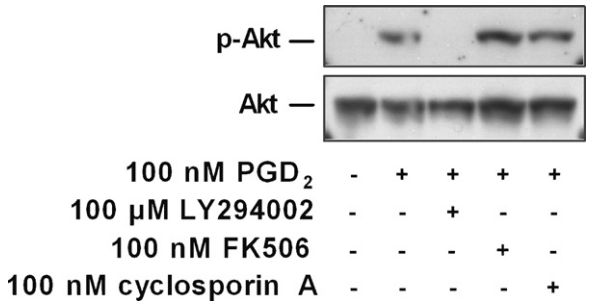

Fig. 6 – Inhibition of calcineurin did not affect PGD_2-induced Akt-phosphorylation in Th2 cells. The cells were pre-incubated with medium, 100 μM LY294002, 100 nM FK506 or 100 nM cyclosporin A for 45 min followed by treatment with medium or 100 nM PGD_2 in the absence or presence of the same inhibitors as indicated for 15 min. The cells were lysed for Western blot for phospho-Akt. Protein loading was normalized relative to total Akt.

human Th2 cells, and that inhibition of these pathways suppresses aspects of activation. PGD_2 enhanced the phosphorylation of Akt and the nuclear translocation of NFATc1 in a CRTH2-dependent manner as the effect of PGD_2 on these pathways was mimicked by DK-PGD_2, a selective CRTH2 agonist but not by BW245C, a selective DP1 agonist. In addition, PGD_2-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_2 as inhibition of the PI3K pathway with LY294002 substantially down-regulated both responses. The regulation of Th2 cytokine production by PGD_2 is clearly Ca^{2+}/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 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_2-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_2 stimulation. The enhancement of p38

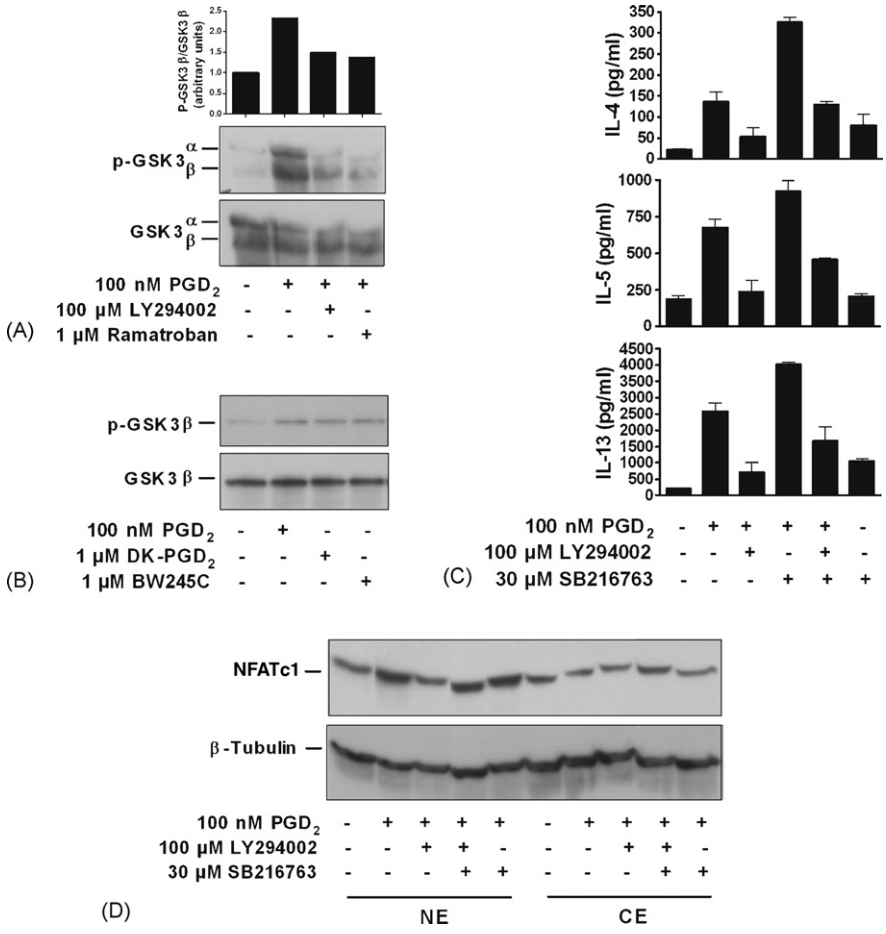

Fig. 7 – GSK-3β was involved in PGD_2-mediated Th2 cell activation. (A) The cells were pre-incubated with medium or 100 μM LY294002 for 45 min, and then stimulated with medium or 100 nM PGD_2 in the absence or presence of the same inhibitor or 1 μM ramatroban as indicated for 15 min or (B) the cells were directly treated with 100 nM PGD_2, 1 μM DK-PGD_2 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_2 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 than incubated with medium, 100 nM PGD_2 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_2 + 100 μM LY294002/30 μM SB216763, 30 μM SB216763 vs. 100 nM PGD_2 + 100 μM LY294002/100 nM PGD_2 + 100 μM LY294002 + 30 μM SB216763 (except IL-5/13), 100 nM PGD_2 vs. 100 nM PGD_2 + 100 μM LY294002 + 30 μM SB216763 (except IL-5/13)/100 nM PGD_2 + 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_2-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_2-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^{2+} 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^{2+} signalling [16, data not shown]. Elevation of intracellular free Ca^{2+} ($[Ca^{2+}]i$) 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^{2+} influx [41,42]. Several transcription factors including NFAT, NF-κB and AP-1 can be activated

downstream from the TCR engagement in a Ca^{2+}-dependent manner [26,29,43]. In PGD_2-stimulated human Th2 cells, although NFATc1 is activated in a Ca^{2+}/calcineurin dependent manner, the activation of NF-κB 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 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, TNFα and IFNγ [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 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 PGD_2-induced NFAT2 nuclear translocation and cytokine production. However, this involvement is not under regulation of Ca^{2+}/CaM/calcineurin signals, as calcineurin inhibitors had no effect on PGD_2-triggered phospho-Akt. One possible link between PI3K and NFAT is GSK3β, a serine/threonine kinase that can phosphorylate several transcription factors including NFAT [48]. By phosphorylating NFAT, GSK3β inhibits NFAT DNA binding and promotes its nuclear export [31]. It is well known that GSK3β is one of the few protein kinases that are inactivated by phosphorylation. Several signalling pathways, including PI3K/Akt, can inhibit GSK-3β by phosphorylation at Ser9 [30,48]. Hence activation of the PI3K pathway, by inhibiting GSK3β, 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 PGD_2, both Akt and GSK3β were phosphorylated in Th2 cells. Inhibition of the PI3K pathway caused a partial reduction of PGD_2-triggered GSK3β phosphorylation, NFAT2 nuclear translocation and cytokine production. Furthermore, inhibition of GSK3β by SB216763 enhanced CRTH2-mediated cytokine production and partially attenuated the inhibitory effect of LY294002. All this evidence suggests that GSK3β is involved in the regulation of cytokine production in 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 PGD_2 confirming that the negative regulation of GSK3β on gene transcription is post-NFAT activation. Phospho-GSK3β was increased by both CRTH2 and DP1 agonists in Th2 cells suggesting that phosphorylation of GSK3β is partially CRTH2- and PI3K-independent, which may explain the reason why LY294002 and ramatroban cannot completely abolish PGD_2-triggered GSK3β phosphorylation. GSK3β has been reported to negatively regulate the activity of a diverse array of other transcription factors including AP-1 [49], β-catenin [50], Myc [51], NF-κB [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 GSK3β in PGD_2-induced cytokine production.

Together, the findings in this paper lead us to conclude that PI3K and 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 Ca^{2+}/CaM/calcineurin-dependent NFAT activation mediates cytokine production. The summary

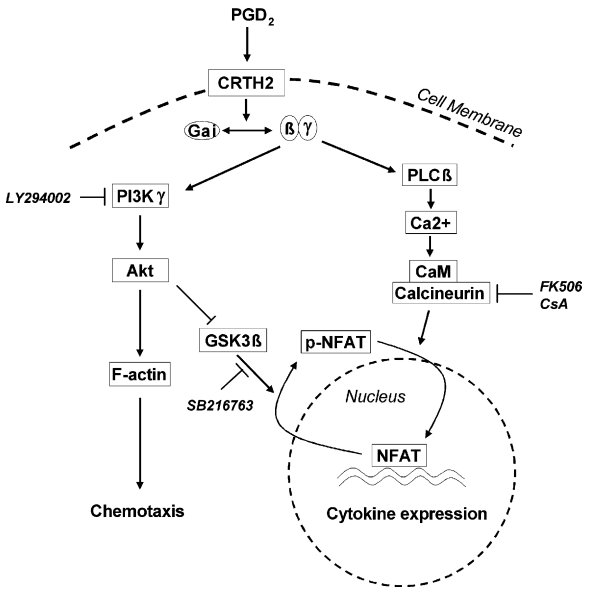

Fig. 8 – Scheme summarizing the proposed signal pathways used by PGD_2/CRTH2 to activate human Th2 cells. Activation of CRTH2 by PGD_2 causes separation of GTP-bound Gαi protein from the receptor and Gβγ subunits. Consequently Gβγ subunits stimulate effector molecules, which include PI3Kγ and PLCβ. Activation of PI3Kγ 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. Gβγ-stimulated PLCβ generates IP3, which elicit Ca^{2+} influx into the cytosol. Liberated 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-3β. Activated Akt phosphorylates and inactivates GSK-3β. Inhibition of GSK-3β phosphorylation promotes NFAT re-phosphorylation and nuclear export. Blockade of GSK-3β 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_2/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.

Acknowledgement

We are grateful to Dr. Aviva M. Tolkovsky for critical reading of the manuscript.

REFERENCES

[1] Lewis RA, Soter NA, Diamond PT, Austen KF, Oates JA, Roberts 2nd LJ. Prostaglandin D2 generation after activation of rat and human mast cells with anti-IgE. J Immunol 1982;129:1627–31.
[2] Urade Y, Ujihara M, Horiguchi Y, Ikai K, Hayaishi O. The major source of endogenous prostaglandin D2 production is likely antigen-presenting cells. Localization of glutathione-requiring prostaglandin D synthetase in histiocytes, dendritic, and Kupffer cells in various rat tissues. J Immunol 1989;143:2982–9.
[3] Tanaka K, Ogawa K, Sugamura K, Nakamura M, Takano S, Nagata K. Cutting edge: differential production of prostaglandin D2 by human helper T cell subsets. J Immunol 2000;164:2277–80.
[4] Fujitani Y, Kanaoka Y, Aritake K, Uodome N, Okazaki-Hatake K, Urade Y. Pronounced eosinophilic lung inflammation and Th2 cytokine release in human lipocalin-type prostaglandin D synthase transgenic mice. J Immunol 2000;168:443–9.
[5] Wright DH, Ford-Hutchinson AW, Chadee K, Metters KM. The human prostanoid DP receptor stimulates mucin secretion in LS174T cells. Br J Pharmacol 2000;131:1537–45.
[6] Gervais FG, Cruz RP, Chateauneuf A, Gale S, Sawyer N, Nantel F, et al. Selective modulation of chemokinesis, degranulation, and apoptosis in eosinophils through the PGD_2 receptors CRTH2 and DP. J Allergy Clin Immunol 2001;108:982–8.
[7] Holgate ST, Broide D. New targets for allergic rhinitis—a disease of civilization. Nat Rev Drug Discov 2003;2:1–12.
[8] Hirai H, Tanaka K, Takano S, Ichimasa M, Nakamura M, Nagata K. Agonistic effect of indomethacin on a prostaglandin D2 receptor, CRTH2. J Immunol 2002;168:981–5.
[9] Nagata K, Hirai H. The second PGD_2 receptor CRTH2: structure, properties, and functions in leukocytes. Prostaglandins Leukot Essent Fatty Acids 2003;69:169–77.
[10] Tanaka K, Hirai H, Takano S, Nakamura M, Nagata K. Effects of prostaglandin D2 on helper T cell functions. Biochem Biophys Res Commun 2004;316:1009–14.
[11] Xue L, Gyles SL, Wettey FR, Gazi L, Townsend E, Hunter MG, et al. Prostaglandin D2 causes preferential induction of proinflammatory Th2 cytokine production through an action on chemoattractant receptor-like molecule expressed on Th2 cells. J Immunol 2005;175:6531–6.
[12] Monneret G, Gravel S, Diamond M, Rokach J, Powell WS. Prostaglandin D2 is a potent chemoattractant for human eosinophils that acts via a novel DP receptor. Blood 2001;98:1942–8.
[13] Robinson DS, Hamid Q, Ying S, Tsicopoulos A, Barkans J, Bentley AM, et al. Predominant TH2-like bronchoalveolar T-lymphocyte population in atopic asthma. N Engl J Med 1992;326:298–304.
[14] Romagnani S, Th1 and Th2 in human diseases. Clin Immunol Immunopathol 1996;80:225–35.
[15] Gyles SL, Xue L, Townsend ER, Wettey F, Pettipher R. A dominant role for chemoattractant receptor-homologous molecule expressed on T helper type 2 (Th2) cells (CRTH2) in mediating chemotaxis of $CRTH2^+$ $CD4^+$ Th2 lymphocytes in response to mast cell supernatants. Immunology 2006;119:362–8.
[16] Hirai H, Tanaka K, Yoshie O, Ogawa K, Kenmotsu K, Takamori Y, et al. Prostaglandin D2 selectively induces chemotaxis in T helper type 2 cells, eosinophils, and basophils via seven-transmembrane receptor CRTH2. J Exp Med 2001;193:255–61.
[17] Kampen GT, Stafford S, Adachi T, Jinquan T, Quan S, Grant JA, et al. Eotaxin induces degranulation and chemotaxis of eosinophils through the activation of ERK2 and p38 mitogen-activated protein kinases. Blood 2000;95:1911–7.
[18] Servant G, Weiner OD, Herzmark P, Balla T, Sedat JW, Bourne HR. Polarization of chemoattractant receptor signaling during neutrophil chemotaxis. Science 2000;287:1037–40.
[19] Sotsios Y, Whittaker GC, Westwick J, Ward SG. The CXC chemokine stromal cell-derived factor activates a Gi-coupled phosphoinositide 3-kinase in T lymphocytes. J Immunol 1999;163:5954–63.
[20] Hii CS, Anson DS, Costabile M, Mukaro V, Dunning K, Ferrante A. Characterization of the MEK5-ERK5 module in human neutrophils and its relationship to ERK1/ERK2 in the chemotactic response. J Biol Chem 2004;279:49825–34.
[21] Fujita T, Zawawi KH, Kurihara H, Van Dyke TE. CD38 cleavage in fMLP- and IL-8-induced chemotaxis is dependent on p38 MAP kinase but independent of p44/42 MAP kinase. Cell Signal 2005;17:167–75.
[22] Sugimoto H, Shichijo M, Iino T, Manabe Y, Watanabe A, Shimazaki M, et al. An orally bioavailable small molecule antagonist of CRTH2, ramatroban (BAY u3405), inhibits prostaglandin D2-induced eosinophil migration in vitro. J Pharmacol Exp Ther 2003;305:347–52.
[23] Affolter M, Weijer CJ. Signaling to cytoskeletal dynamics during chemotaxis. Dev Cell 2005;9:19–34.
[24] Rincón M, Flavell RA, Davis RA, The JNK. P38 MAP kinase signaling pathways in T cell-mediated immune responses. Free Radic Biol Med 2000;28:1328–37.
[25] Andrews RP, Ericksen MB, Cunningham CM, Daines MO, Hershey GK. Analysis of the life cycle of stat6. Continuous cycling of STAT6 is required for IL-4 signaling. J Biol Chem 2002;277:36563–9.
[26] Li Q, Verma IM. NF-κB regulation in the immune system. Nat Rev Immunol 2002;2:725–34.
[27] Quintana A, Griesemer D, Schwarz EC, Hoth M. Calcium-dependent activation of T-lymphocytes. Pflugers Arch 2004;450:1–12.
[28] Baumann B, Bohnenstengel F, Siegmund D, Wajant H, Weber C, Herr I, et al. Rocaglamide derivatives are potent inhibitors of NF-kappa B activation in T-cells. J Biol Chem 2002;277:44791–800.
[29] Rao A, Luo C, Hogan PG. Transcription factors of the NFAT family: regulation and function. Annu Rev Immunol 1997;15:707–47.
[30] Cross DA, Alessi DR, Cohen P, Andjelkovich M, Hemmings BA. Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. Nature 1995;378:785–9.
[31] Beals CR, Sheridan CM, Turck CW, Gardner P, Crabtree GR. Nuclear export of NF-ATc enhanced by glycogen synthase kinase-3. Science 1997;275:1930–4.

[32] Stephens L, Smrcka A, Cooke FT, Jackson TR, Sternweis PC, Hawkins PT. A novel phosphoinositide 3 kinase activity in myeloid-derived cells is activated by G protein beta gamma subunits. Cell 1994;77:83–93.
[33] Rameh LE, Cantley LC. The role of phosphoinositide 3-kinase lipid products in cell function. J Biol Chem 1999;274:8347–50.
[34] Hirsch E, Katanaev VL, Garlanda C, Azzolino O, Pirola L, Silengo L, et al. Central role for G protein-coupled phosphoinositide 3-kinase gamma in inflammation. Science 2000;287:1049–53.
[35] Sasaki T, Irie-Sasaki J, Jones RG, Oliveira-dos-Santos AJ, Stanford WL, Bolon B, et al. Function of PI3Kgamma in thymocyte development, T cell activation, and neutrophil migration. Science 2000;287:1040–6.
[36] Stubbs VE, Schratl P, Hartnell A, Williams TJ, Peskar BA, Heinemann A, et al. Indomethacin causes prostaglandin D(2)-like and eotaxin-like selective responses in eosinophils and basophils. J Biol Chem 2002;277:26012–20.
[37] Wu D, Katz A, Simon MI. Activation of phospholipase C beta 2 by the alpha and beta gamma subunits of trimeric GTP-binding protein. Proc Natl Acad Sci USA 1993;90:5297–301.
[38] Clapham DE, Neer EJ. G protein beta gamma subunits. Annu Rev Pharmacol Toxicol 1997;37:167–203.
[39] Rebecchi MJ, Pentyala SN. Structure, function, and control of phosphoinositide-specific phospholipase C. Physiol Rev 2000;80:1291–335.
[40] Kiselyov K, Shin DM, Muallem S. Signalling specificity in GPCR-dependent Ca^{2+} signalling. Cell Signal 2003;15:243–53.
[41] Premack BA, Gardner P. Signal transduction by T-cell receptors: mobilization of Ca and regulation of Ca-dependent effector molecules. Am J Physiol 1992;263(6 Pt 1):C1119–40.
[42] van Leeuwen JE, Samelson LE. T cell antigen-receptor signal transduction. Curr Opin Immunol 1999;11:242–8.
[43] Beals CR, Clipstone NA, Ho SN, Crabtree GR. Nuclear localization of NF-ATc by a calcineurin-dependent, cyclosporin-sensitive intramolecular interaction. Genes Dev 1997;11:824–34.
[44] Hodge MR, Ranger AM, Charles de la Brousse F, Hoey T, Grusby MJ, Glimcher LH. Hyperproliferation and dysregulation of IL-4 expression in NF-ATp-deficient mice. Immunity 1996;4:397–405.
[45] Kiani A, Viola JP, Lichtman AH, Rao A. Down-regulation of IL-4 gene transcription and control of Th2 cell differentiation by a mechanism involving NFAT1. Immunity 1997;7:849–60.
[46] Ranger AM, Hodge MR, Gravallese EM, Oukka M, Davidson L, Alt FW, et al. Delayed lymphoid repopulation with defects in IL-4-driven responses produced by inactivation of NF-ATc. Immunity 1998;8:125–34.
[47] Yoshida H, Nishina H, Takimoto H, Marengere LE, Wakeham AC, Bouchard D, et al. The transcription factor NF-ATc1 regulates lymphocyte proliferation and Th2 cytokine production. Immunity 1998;8:115–24.
[48] Frame S, Cohen P. GSK3 takes centre stage more than 20 years after its discovery. Biochem J 2001;359:1–16.
[49] Boyle WJ, Smeal T, Defize LH, Angel P, Woodgett JR, Karin M, et al. Activation of protein kinase C decreases phosphorylation of c-Jun at sites that negatively regulate its DNA-binding activity. Cell 1991;64:573–84.
[50] Rubinfeld B, Albert I, Porfiri E, Fiol C, Munemitsu S, Polakis P. Binding of GSK3beta to the APC-beta-catenin complex and regulation of complex assembly. Science 1996;272:1023–6.
[51] Henriksson M, Bakardjiev A, Klein G, Luscher B. Phosphorylation sites mapping in the N-terminal domain of c-myc modulate its transforming potential. Oncogene 1993;8:3199–209.
[52] Bournat JC, Brown AM, Soler AP. Wnt-1 dependent activation of the survival factor NF-kappaB in PC12 cells. J Neurosci Res 2000;61:21–32.
[53] Bijur GN, Jope RS. Opposing actions of phosphatidylinositol 3-kinase and glycogen synthase kinase-3beta in the regulation of HSF-1 activity. J Neurochem 2000;75:2401–8.
[54] Grimes CA, Jope RS. CREB DNA binding activity is inhibited by glycogen synthase kinase-3 beta and facilitated by lithium. J Neurochem 2001;78:1219–32.