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Leukotriene E_4 activates human Th2 cells for exaggerated pro-inflammatory cytokine production in response to PGD_2

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Abstract

PGD_2 exerts a number of pro-inflammatory responses through a high affinity interaction with chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2) and has been detected at high concentrations at sites of allergic inflammation. Since cysteinyl leukotrienes (cysLTs) are also produced during the allergic response we investigated the possibility that cysLTs may modulate the response of human Th2 cells to PGD_2. PGD_2 induced concentration-dependent Th2 cytokine production in the absence of TCR stimulation. Leukotrienes D_4 (LTD_4) and E_4 (LTE_4) also stimulated the cytokine production but were much less active than PGD_2. However, when combined with PGD_2, cysLTs caused a greater than additive enhancement of the response, with LTE_4 being most effective in activating Th2 cells. LTE_4 enhanced calcium mobilisation in response to PGD_2 in Th2 cells without affecting endogenous PGD_2 production or CRTH2 receptor expression. The effect of LTE_4 was inhibited by montelukast but not by the $P2Y_{12}$ antagonistmethylthioadenosine 5′-monophosphate. The enhancing effect was also evident with endogenous cysLTs produced from immunologically activated mast cells since inhibition of cysLT action by montelukast or cysLT sythesis by MK886, an inhibitor of 5-LO-activating protein, reduced the response of Th2 cells to the levels produced by PGD_2 alone. These findings reveal that cysLTs, in particular LTE_4, have a significant pro-inflammatory impact on T cells and demonstrate their effects on Th2 cells are mediated by a montelukast-sensitive receptor.

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[3]Abbreviations used in this article: 2MeS, 2-methylthioadenosine 5′-monophosphate; 5-LO, 5-lipoxygenase; CRTH2, chemoattractant receptor-homologous molecule expressed on Th2 cells; cysLT, cysteinyl leukotriene; FLAP, 5-LO-activating protein; GPCR, G protein-coupled receptor; LTC_4, Leukotriene C_4; LTD_4, Leukotriene D_4; LTE_4, Leukotriene E_4; PTX, pertussis toxin;qPCR, real time quantitative PCR.

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Introduction

Both PGD_2 and cysteinyl leukotrienes (cysLTs) are products of the oxidative metabolism of arachidonic acid and have been detected in high concentrations at sites of allergic inflammation and play central roles in promoting airway inflammation and deterioration in lung function, often acting in concert (1,2).

PGD_2, produced by the activity of the cyclooxygenase enzymes, is the major prostanoid released from mast cells during an allergic response (3,4), although macrophages (5), dendritic cells (6), and $CD4^+$ Th2 lymphocytes (7,8) may contribute to PGD_2 production in some circumstances. A significant contribution of PGD_2 to the development of allergic inflammation has been suggested by the observations of enhanced eosinophilic lung inflammation and cytokine release in transgenic mice overexpressing PGD_2 synthase (9). Two distinct G protein-coupled receptors (GPCR) have been identified as PGD_2 receptors, D prostanoid receptor 1 (DP_1) and chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2). In recent years increasing evidence suggests that through its action on CRTH2, PGD_2 elicits many pro-inflammatory responses in leukocytes including chemotaxis of eosinophils, basophils, and Th2 cells (10,11), cytokine production by Th2 cells (12,13) and pro-inflammatory protein expression by eosinophils and Th2 cells (12,14). Our recent studies also demonstrated that activation of CRTH2 suppresses Th2 cell apoptosis (15), a process which is likely to impede the resolution of allergic inflammation. Allergic responses mediated by IgE, mast cells, Th2 cells and eosinophils are dramatically reduced in mice where CRTH2 is genetically ablated or by small molecule CRTH2 antagonists (16-19). Antagonism of CRTH2 is currently being considered as a potentially useful approach for the treatment of allergic diseases, including asthma, rhinitis and atopic dermatitis (20).

CysLTs, including cysteinyl leukotriene C_4 (LTC_4), D_4 (LTD_4) and E_4 (LTE_4), are derived from the 5-lipoxygenase (5-LO) pathway of the arachidonic acid metabolism (21,22). LTC_4 is formed by conjugation of LTA_4 with reduced glutathione and after extracellular export is converted to LTD_4 and then the stable metabolite LTE_4 by sequential enzymatic removal of glutamic acid followed by glycine. Two GPCR receptors for LTs have been cloned, characterised and designated as $CysLT_1$ and $CysLT_2$ (23-25). LTD_4 binds $CysLT_1$ with higher affinity than LTC_4 while $CysLT_2$ binds these cysLTs with equal affinity. LTE_4 has only weak activity on either $CysLT_1$ (23,24) or $CysLT_2$ (25,26) and has therefore been generally considered to be a stable inactive breakdown product although there is accumulating evidence that LTE_4 can stimulate inflammatory responses through mechanisms independent of $CysLT_1$ or $CysLT_2$ (27-31).

$CysLT_1$ mediates bronchoconstriction and also a range of pro-inflammatory effects including activation and migration of leukocytes (21,32,33), whereas $CysLT_2$ may mediate the vasoactive effects of LTC_4 and LTD_4. The leukotriene antagonists approved for use in asthma and allergic rhinitis, most notably montelukast, block the action of cysLTs (predominantly LTD_4) on $CysLT_1$ but do not inhibit $CysLT_2$-mediated effects. Monotherapy with montelukast thereby inhibits the $CysLT_1$-mediated bronchoconstrictor element of asthma but its anti-inflammatory activity and consequently, clinical efficacy, is modest compared to that of inhaled steroids (34). However, in patients with asthma not sufficiently controlled with inhaled steroids alone, add-on therapy with montelukast to a constant dose of inhaled steroid improves asthma control (35). Recent data suggests in primary care that leukotriene antagonists may have a more significant role as early therapy than previously considered since compliance of safe oral agent is better than with inhaled glucocorticoid (36).

We found that cysLTs markedly potentiated pro-inflammatory cytokine production from human Th2 cells in response to PGD_2. The potency of LTE_4 in the enhancing effect was significantly higher than that of LTD_4 or LTC_4 and this enhancing effect of LTE_4 was inhibited by montelukast. Although the CRTH2 antagonist TM30089 alone substantially inhibited IL-13 production in response to both exogenous and endogenous PGD_2 and LTE_4, a combination of TM30089 and montelukast was required to completely inhibit the response.

These data highlight an important interaction between PGD_2 and cysLTs in promoting Th2 cell activation at sites of allergic inflammation, and further point to the importance of TCR-independent mechanisms of T cell activation in inflammatory responses. Further study of these mechanisms may lead to improved therapies for allergic inflammation.

Materials and Methods

Reagents

PGD_2, LTC_4, LTD_4 and LTE_4 were purchased from Enzo Life Science; PGD_2–MOX enzyme immunoassay kits and LTE_4 enzyme immunoassay kits were purchased from Cayman Chemicals; TM30089 was supplied by ChemieTek; human $CD4^+$ T cell isolation kit II, anti-human CRTH2 MicroBead Kits, human CD34 MicroBead kits and T cell activation/expansion kits were from MiltenyiBiotec Ltd; human recombinant stem cell factor, human rIL-6 and human IL-4/5/13 immunoassay kits were purchased from R&D Systems; Iscove's modified Dulbecco's medium and X-VIVO 15 medium were purchased from Lonza; AIM V medium was from Invitrogen; human myeloma IgE, antibodies against human tryptase and chymase were purchased from Chemicon International; Lymphoprep was purchased from Axis-Shield UK; The MultiScreen filter plates for ELISPOT were obtained from Millipore; ELISPOT kits for human IL-4/IL-13 were purchased from Mabtech; Alkaline phosphatase conjugate substrate kit was from Bio-Rad; RNeasy Mini kit and Omniscript reverse transcription kit were supplied from Qiagen; Real time quantitative PCR (qPCR) Master Mix and probes were from Roche; FLIPR Ca^{2+} 5 dye was purchased from Molecular Devices; PathHuntereXpressβ-arrestin assay kit for human CRTH2 was supplied by DiscoveRx; and human rIL-2, human rIL-4, goat anti-human IgE, diclofenac and other chemicals were from Sigma-Aldrich.

Human mast cell culture and activation

Human mast cells were cultured from $CD34^+$ progenitor cells using a modified method described previously (37). Briefly, $CD34^+$ progenitor cells were isolated from human cord blood (National Blood Service, Oxford, UK) by using a human CD34 MicroBead kit. The cells were cultured at a density of 1×10^5 cells/ml with Iscove's modified Dulbecco's medium containing 10% human serum, 0·55 μM 2-ME, penicillin/streptomycin, human recombinant stem cell factor (100 ng/ml) and human rIL-6 (50 ng/ml) in 5% CO_2 at 37°C for 14-15 weeks. Half the culture medium was replaced twice weekly with fresh medium containing the same concentration of cytokines. The expression of tryptase and chymase of the cells was tested by immunostaining using the method described previously (37). The mast cells used in this study were tryptase-positive (> 80%) and chymase-negative (< 1%). The cells were pre-treated with 5 mg/ml purified human myeloma IgE for 4 days, washed and then sensitized passively with fresh IgE (5 mg/ml) for 2 h. The cells were washed once with medium in absence or presence of 10 μM diclofenac, 10 μM MK886 or both and then continued to be incubated with medium or challenged with goat anti-human IgE (1 μg/ml) in the presence or absence of diclofenac, MK886 or both for 1 h. The supernatants of the cells were collected and measured for PGD_2 using a PGD_2–MOX enzyme immunoassay kit and LTE_4 using a LTE_4 enzyme immunoassay kit according to the manufacturer's instructions.

The supernatants were aliquoted and stored at −80°C until used as mast cell supernatants for the treatment of Th2 cells.

Human CRTH2$^+$CD4$^+$ Th2 cell culture and treatment

Human CRTH2$^+$CD4$^+$ Th2 cells were prepared as described in our previous report (13). Briefly, PBMC were isolated from buffy coats (National Blood Service, Bristol, UK) by Lymphoprep density gradient centrifugation, followed by CD4$^+$ cell purification using a CD4$^+$ T cell isolation kit II. After 7 days' culture in AIM V medium containing 50 U/ml rIL-2 and 100 ng/ml rIL-4, CRTH2$^+$ cells were isolated from the CD4$^+$ cultures by positive selection using an anti-human CRTH2 MicroBead Kit. The harvested CD4$^+$CRTH2$^+$ cells were treated as Th2 cells and were amplified further by stimulation with the T cell activation/expansion kit and grown in X-VIVO 15 medium containing 10% human serum and 50 U/ml IL-2 before use.

The Th2 cells were treated with X-VIVO 15 medium containing 10% human serum in the presence or absence of PGD_2, cysLTs or other compounds or treated with various mast cell supernatants in the presence or absence of antagonist compounds, as indicated in the results at 37°C and 5% CO_2 for different periods of time for qPCR, ELISA, or ELISPOT. In some experiments, Th2 cells were pre-incubated with pertussis toxin (PTX) as indicated in the results.

ELISA

Th2 cells were treated with different compounds for 6 h and then the supernatants were collected. The concentrations of IL-4 and IL-13 in the supernatants were assayed using enzyme-linked immunoassay kits, according to the manufacturer's instructions. The results were measured in a Victor^2 V-1420 multi-label HTS Counter (PerkinElmer Life Sciences).

ELISPOT cytokine assays

Th2 cells (4×10^3 cell/well) were seeded in a MultiScreen Filter Plate (Millipore) coated with capture Ab against human IL-4 or IL-13 and treated with PGD_2, LTE_4 alone or in combination at 37°C 5% CO_2 for 15 h. The plate was washed extensively with PBS containing 0.05% Tween 20 and then immuno-assayed using ELISPOT kits following the manufacturer's instructions. Briefly, the plate was incubated with specific biotinylatedAb (anti-IL-4 or anti-IL-13) followed by labelling with Streptavidin-Alkaline phosphatase. Spots were developed by adding substrate and then analysed with an AID ELISPOT Reader System (AutoimmunDiagnostika GmbH).

qPCR

Th2 cells were treated for 2.5 h and the total RNA of the cells were extracted using an RNeasy Mini kit. cDNA of the samples was prepared from the same starting amount of RNA using a Omniscript RT kit. qPCR was conducted using Master Mix and Probe in a LightCycler 480 Real-Time PCR System (Roche). GAPDH was used as control gene. The changes of mRNA level compared with untreated sample were calculated according to the manufacturer's instructions.

Primers and probes used were as follows: IL-4, 5′-caccgagttgaccgtaacag-3′ and 5′-gccctgcagaaggtttcc-3′ with probe 16 generating a 72-bp fragment; IL-5, 5′-ggtttgttgcagccaaagat-3′ and 5′-tcttggccctcattctcact-3′with probe 25 generating a 66-bp fragment; IL-13, 5′-agccctcagggagctcat-3′ and 5′-ctccataccatgctgccatt-3′ with probe 17 generating a 84-bp fragment; CRTH2, 5′-cctgtgctccctctgtgc-3′ and 5′-tctggagacggctcatctg-3′ with probe 43 generating a 95-bp fragment; $CysLT_1$, 5′-actccagtgccagaaagagg-3′ and 5′-gcggaagtcatcaatagtgtca-3′ with probe 29 generating a 112-

bp fragment; $CysLT_2$, 5′-ctagagtcctgtgggctgaaa-3′ and 5′-gtaggatccaatgtgctttgc-3′ with probe 48 generating a 61-bp fragment; $P2Y_{12}$, 5′-tttgcctaacatgattctgacc-3′ and 5′-ggaaagagcatttcttcacattct-3′ with probe 27 generating a 65-bp fragment;and GAPDH, 5′-agccacatcgctcagacac-3′ and 5′-gcccaatacgaccaaatcc-3′ with probe 60 generating a 66-bp fragment.

Ca^{2+} mobilization assay

CHO/CRTH2 cells (Evotec-OAI) (8×10^4 cells/well) were seeded into a poly-d-lysine coated 96-well plate and incubated at 37°C overnight. The culture medium was changed to HBSS before use. Th2 cells were washed once with HBSS and then seeded into a poly-d-lysine coated 96-well plate at a density of 2×10^5 Cells/well. The cells were incubated with FLIPR Ca^{2+} 5 dyein the presence or absence of PTX at 37°C 5% CO_2 for 1 h. The plate for Th2 cells was centrifuged at 600 rpm for 10 min before the assay. The changes in fluorescence after test compound loading (50 μl) were monitored over a 75 s time course with compound loading at 17 s, using a Flexstation (Molecular Devices).

β-arrestin recruitment assay

The assay was performed using a PathHuntereXpressβ-arrestin kit for human CRTH2 according to manufacturer's instructions. Briefly, CHO/CRTH2 EA-arrestin cells were re-suspended in OCC 2 media and seeded in a 96-well luminescence plate (white walled, clear bottom) for recovering at 37°C 5% CO_2 for 48 h. Treatment compounds were added for 90 min incubation at 37°C 5% CO_2, and then detection reagents were added for another 90 min incubation at room temperature. Luminescence was measured using a $Victor^2$ V-1420 multi-label HTS Counter (PerkinElmer Life Sciences).

Statistics

Data were analysed using one-way ANOVA followed by the Newman-Keuls test. Values of $p< 0.05$ were considered statistically significant.

Results

LTD_4 and LTE_4 are weak inducers of cytokine production by human Th2 cells compared to PGD_2

As previously reported, PGD_2 induced production of IL-13 when incubated with Th2 cells (13). Both LTD_4 and LTE_4 alone also induced IL-13 production in a dose-dependent manner with an EC_{50} similar to PGD_2 (75.6 nM for PGD_2, 107.8 nM for LTD_4 and 26.2 nM for LTE_4) (Fig. 1). However, the maximum responses achieved by LTD_4 and LTE_4 were only 23% and 41% respectively of that achieved by PGD_2.

CysLTs significantly enhance Th2 cytokine production in response to PGD_2

To define the effect of the combination of PGD_2 and cysLTs on Th2 cytokine production, Th2 cells were treated with PGD_2, LTD_4 or LTE_4 alone (at concentrations close to their relative EC_{50}) or in combination as indicated in Fig. 2 for 6 h. Interestingly, both LTD_4 and LTE_4 significantly enhanced IL-13 production in response to PGD_2 (Fig. 2A, Table 1). The enhancement was greater than additive ($p< 0.05$), and most marked with LTE_4 (Table 2). LTC_4 showed a similar enhancing effect of LTD_4 in the combination treatment (Table 1). ELISPOT analysis of IL-13 and IL-4 levels indicated that the enhancement occurred at the level of individual cells and not by an increased number of responder cells (Fig. 2B), as compared with the samples treated with PGD_2 alone, the combined use of PGD_2 and LTE_4 did not change the total spot numbers for both IL-4 and IL-13 significantly but enhanced the average intensities of the spots (Table 2).

To understand the nature of the enhancing effect of cysLTs on the Th2 cytokine production, the cells were stimulated with increasing concentrations of PGD_2 in the absence or presence of 50 nM cysLTs for 6 h as indicated in Fig. 2C. The addition of cysLTs increased the maximum response to PGD_2, but did not significantly affect the EC_{50} of PGD_2 (Fig. 2C).

To determine the effect of the treatment with the combination of PGD_2 and cysLTs at the gene transcription level, the levels of mRNA encoding IL-4, IL-5 and IL-13 in Th2 cells were measured by qPCR after different treatments of the cells for 2.5 h (Fig. 2D). The synergistic effect of PGD_2 and LTE_4 on the up-regulation of IL-5 and IL-13 mRNA was apparent although the effect on IL-4 at the level of mRNA was not significant (Fig. 2D, Table 2).

Effect of the CRTH2 antagonist TM30089 and montelukast on cytokine production in response to PGD_2 and cysLTs

The effects of the CRTH2 antagonist TM30089 and the $CysLT_1$ antagonist montelukast on cytokine production by Th2 cells in response to combinations of PGD_2 with LTD_4 (Fig. 3A, *LTD_4*) or LTE_4 (Fig. 3A, *LTE_4*) were investigated. TM30089 reduced cytokine production to the level produced by the leukotriene alone in the case of LTD_4 or even below in the case of LTE_4. The LTD_4- or LTE_4-mediated enhancement was inhibited by montelukast that effectively reduced responses to similar levels to those of PGD_2 alone. In both cases the combination of TM30089 and montelukast completely inhibited cytokine production by Th2 cells.

To further understand the potency of montelukast in the inhibition of enhancing effect of cysLTs in Th2 cytokine production induced by PGD_2, Th2 cells were treated with 100 nM PGD_2 and 50 nM LTE_4 in the presence of increasing concentrations of montelukast for 6 h (Fig. 3B). The inhibitory effect of montelukast was dose-dependent with IC_{50} = 0.2 nM. The enhancing effect of 50 nM LTE_4 was completely abolished by 10 nM montelukast.

LTE_4 does not affect PGD_2 production and CRTH2 expression in Th2 cells

It has been reported that activated Th2 cells are able to produce PGD_2 (7,8). To understand whether the enhancing effect of cysLTs is mediated by a secondary production of PGD_2 by Th2 cells, we examined the effect of diclofenac, a cyclooxygenase inhibitor, on cytokine production induced by LTE_4 (Fig. 4A). Diclofenac (10 μM) did not change the IL-13 production induced by LTE_4 demonstrating that PGD_2 synthesis is not involved in the enhancing effect of LTE_4. We also investigated the effect of LTE_4 on the expression of CRTH2 by qPCR (Fig. 4B). LTE_4 did not show any effect on the level of CRTH2 mRNA suggesting that the enhancing effect of LTE_4 is not mediated by an increase of transcription at the CRTH2 locus.

CysLTs do not interact with CRTH2 directly

To rule out the possibility that cysLTs bind CRTH2, the effects of a selective CRTH2 antagonist on the cytokine production induced by LTE_4 alone were examined (Fig. 5A). As expected, 1μM TM30089 completely inhibited responses to PGD_2 but not to LTE_4; vice versa, 1μM montelukast blocked the effect of LTE_4 to the background level but had no effect on PGD_2.

It has been established that two signal mechanisms are involved in the activation of CRTH2, Ca^{2+} influx and β-arrestin recruitment (10,38-40). We further analysed the effect of LTE_4 on the downstream signalling of CRTH2 (Fig. 5B,C). In CHO cells over-expressing human rCRTH2, PGD_2, but not LTE_4, induced Ca^{2+} mobilization (Fig. 5B) and β-arrestin recruitment (Fig. 5C) in a dose-dependent manner. Addition of LTE_4 did not enhance the

Ca^{2+} mobilization induced by PGD_2 in the recombinant cell system (Fig. 5B). However, in Th2 cells, both PGD_2 and LTE_4 induced Ca^{2+} mobilization in a dose-dependent manner although the efficacy of LTE_4 was much weaker (Fig. 5D). Addition of LTE_4 enhanced the Ca^{2+} mobilization induced by PGD_2 significantly, an effect that was completely inhibited by montelukast.

The enhancing effect of LTE_4 was not mediated by $P2Y_{12}$ receptorbut by a receptor that partially sensitive to PTX

It has also been reported that LTE_4 can act as an agonist at the $P2Y_{12}$ receptor (41,42) and that montelukast can antagonise someP2Y receptors (43) although the effect of montelukast has not been tested on $P2Y_{12}$.The expression of $P2Y_{12}$ can be detected in human Th2 cells but the level of this receptor is much lower than that seen with $CysLT_1$ (Fig. 6A).To address the role of $P2Y_{12}$ in LTE_4-induced enhancing effect, the effect of 2-methylthioadenosine 5′-monophosphate (2MeS), an inhibitor of the $P2Y_{12}$ (42), was investigated. 2MeS (100μM) had no significant effect on the IL-13 production induced by PGD_2 or LTE_4 alone or in combination suggesting that $P2Y_{12}$ receptor is not involved in mediating the effects of LTE_4 in this system (Fig. 6B).

To further understand the potential receptor mediating the effect of LTE_4 in Th2 cells, we examined the effects of PTX, an inhibitor of Gαi pathway, on the Ca^{2+} mobilization (Fig. 6C) and IL-13 production (Fig. 6D) induced by LTE_4 in Th2 cells. Both responses to LTE_4 were partially reduced by 1 μg/ml PTX but not by 100 ng/ml PTX.

Effect of mast cell supernatants containing endogenous PGD_2 and LTE_4 on cytokine production by Th2 cells

To analyse the impact of endogenously synthesized eicosanoids on Th2 function, the effect of supernatants collected from human mast cells activated with IgE/anti-IgE on the Th2 cytokine production was investigated (Fig. 7). Only low levels of PGD_2 (<0.1 ng/2×10^6 cell/ml) and LTE_4 (<10 ng/2×10^6 cell/ml) were detectable in the supernatant from the resting mast cells (supernatant 1 in upper panel of Fig. 7A). After 1 h activation with IgE/anti-IgE, mast cells produced high levels of both PGD_2 (>12 ng/2×10^6 cell/ml) and LTE_4 (>85 ng/2×10^6 cell/ml) (supernatant 2). Co-treatment of IgE/anti-IgE-activated mast cells with diclofenac (10 μM) during the period of anti-IgE stimulation abolished production of PGD_2 but enhanced production of LTE_4 (>135 ng/2×10^6 cell/ml) (supernatant 3). Co-treatment of the mast cells with MK886 (10 μM), an inhibitor of 5-LO-activating protein (FLAP), during the activation blocked production of LTE_4 but increased the level of PGD_2 (>15 ng/2×10^6 cell/ml) (supernatant 4). Co-treatment of the mast cells with both diclofenac and MK886 abolished production of both PGD_2 and LTE_4 (supernatant 5). Only very low levels of IL-13 (<200 pg/2×10^6 cell/ml) could be detected in any of these mast cell supernatants (lower panel of Fig. 7A).

Using these supernatants to treat human Th2 cells revealed, as expected, different capacities to stimulate IL-13 production depending on the levels of PGD_2 and LTE_4 contained in the supernatants (Fig. 7B). Supernatant 2 containing both PGD_2 and LTE_4 possessed the highest stimulatory capacity to induce IL-13 production. Treatment with diclofenac alone (supernatant 3) caused substantial (~76%) reduction of Th2 stimulatory activity as reported previously (37) and MK886 (supernatant 4) partially (~45%) reduced activity. Treatment of the mast cells with both diclofenac and MK886 (supernatant 5) reduced the level of stimulatory activity to the level produced by unactivated mast cell supernatant (supernatant 1).

The stimulatory activity of the mast cell supernatant containing both PGD_2 and LTE_4 (supernatant 2) on the Th2 cytokine production was reduced by treatment of Th2 cells with TM30089 (1 μM) or to a lesser extent by montelukast (1 μM) (Fig. 8). Treatment of the Th2 cells both TM30089 and montelukast reduced the level of stimulatory activity of supernatant 2 to that of the supernatant from unactivated mast cells (supernatant 1).

Discussion

Cytokine production from Th2 cells plays an important role in orchestrating allergic responses. High local concentrations of IL-4, IL-5 and IL-13 are detected in both airway and bronchial alveolar lavage fluids from patients with asthma and are present at increased levels following allergen challenge (44-46). It has been well established that PGD_2 induces Th2 cytokine production through activation of CRTH2 (12,13). In this study, we have revealed a previously unrecognised role of cysLTs in potentiating cytokine production by human Th2 cells in response to PGD_2. CysLTs alone are weak stimulators of Th2 cytokine production, butsignificantly enhanced cytokine production in response to PGD_2. The combined effects of PGD_2 and cysLTs at pathophysiological concentrations (similar to the concentrations produced by activated mast cells) were greater than additive (Table 2) and interestingly, LTE_4 was more potent than that of LTC_4 and LTD_4 in mediating this enhancing effect. The enhancing effect of cysLTs was inhibited by montelukast. A combination of TM30089 and montelukast was required to completely inhibit the synergistic effect of PGD_2 and cysLTs.

CysLTs are produced by mast cells, eosinophils, and other cells during the allergic response and have been detectedin high concentrations in airways of asthmatics challenged with allergen (47), and LTE_4 is present in the urine of patients during spontaneous asthmatic exacerbations (48). CysLTs are the most potent known bronchoconstrictors (49,50), and promote airway hyperresponsiveness to histamine and PGD_2 when they are administered by inhalation to human subjects (1,2,51). At the cellular level, they induce chemotaxis of eosinophils and neutrophils, and prolong eosinophil survival (52,53). The involvement of cysLTs in Th2 cytokine production is suggested by the observations that Th2 cytokine production and consequent airway inflammation is reduced in LTC_4 synthase null mice (33) and cysLTs contribute to cytokine production by allergen-specific Th2 cells stimulated with GM-CSF (granulocyte macrophage-colony stimulating factor)-primed macrophages (54). The present study provides direct evidence that cysLTs are able to elicit cytokine production by Th2 cells in the absence of antigen or any other costimulation and highlights the possibility that PGD_2 and cysLTs act in concert to promote Th2 cytokine production.

Our previous report demonstrated the critical role of PGD_2 and its receptor CRTH2 in mediating cytokine production from Th2 cells in response to activated mast cells (37). The current study indicates that cysLTs, particularly LTE_4 can activate Th2 cells for exaggerated cytokine production in response to PGD_2. Both PGD_2 and LTE_4 were detected in high levels in supernatants from immunologically activated mast cells. Although the effect of the mast cell supernatants is predominantly through interaction of PGD_2 with CRTH2, a contribution of cysLTs to this response was demonstrated by selectively inhibiting mast cell cysLT production with the FLAP inhibitor MK886 or inhibiting cysLT action with montelukast. Removal of the cysLT-dependent component of the response to that produced by PGD_2 alone illustrates that endogenous cysLTs can activate Th2 cells for a heightened response to PGD_2.

The enhancing effect of cysLTs was not mediated by direct interaction with CRTH2, since in CHO cells over-expressing rCRTH2, LTE_4 had no effect on Ca^{2+} influx and β-arrestin recruitment that are downstream signalling events following CRTH2 activation (10,39,40). However, in human Th2 cells, LTE_4 elicits Ca^{2+} mobilization in a dose-dependent manner

and enhances Ca^{2+} mobilization in response to PGD_2 markedly, indicating that cysLTs enhance signalling pathways downstream of CRTH2 receptor activation. It has been reported that IFN-γ and TNF-αpotentiated PGD_2-induced eosinophil chemotaxis through up-regulation of CRTH2 (55). However, the enhancing effect of cysLTs on Th2 cytokine production was not mediated by enhanced production of PGD_2 or increased CRTH2 receptor expression as the inhibition of PGD_2 synthesis with diclofenac did not change the enhancing effect and LTE_4 did not change the mRNA level of CRTH2 in Th2 cells.

The receptor mediating the enhancing effect of cysLTs is unclear. On one hand the potent activity of LTE_4 suggests that the effect is not mediated by either $CysLT_1$ or $CysLT_2$although both receptors are expressed in human Th2 cells (Fig. 6A)sinceLTE_4 binds these receptors with only low affinity (23-25) and LTE_4 is a weak bronchoconstrictor in human airways compared to LTD_4 (56). However, although, LTE_4 has only weak affinity for $CysLT_1$, the enhancing effect of LTE_4 was inhibited by the $CysLT_1$ antagonist montelukast, suggesting that either LTE_4 can activate $CysLT_1$ when expressed endogenously in a Th2 cell environment or that montelukast has additional activities that are $CysLT_1$-independent. It has been proposed that both montelukast and LTE_4 may interact with the P2Y-like receptors, LTE_4 acting as an agonist (41,42) and montelukast as an antagonist (43). However, treatment of Th2 cells with 2MeS, a selective antagonist of $P2Y_{12}$, did not reduce significantly the enhancing effect of LTE_4 indicating that $P2Y_{12}$ does not mediate the effect of LTE_4. However, it is clear that LTE_4 can stimulate inflammatory responses in mice deficient in both $CysLT_1$ and $CysLT_2$ (57) so it is possible that the enhancing effect observed is mediated by a montelukast-sensitive receptor that is distinct from either $CysLT_1$ or $CysLT_2$and may fall in P2Y class of receptor but is not $P2Y_{12}$. Indeed, it has been established that LTE_4 can elicit inflammatory responses that are qualitatively distinct from other cysLTs (30,31). Inhalation of LTE_4, but not LTD_4, promotes airway eosinophilia in human volunteers (58) and interestingly, this persistent eosinophilia is suppressed by treatment with the $CysLT_1$ antagonist zafirlukast (27). Therefore further research is required to identify the receptor mediating the enhancing effect of LTE_4 in Th2 cells.Studies with PTX suggest that the effects of the putative receptor is not mediated by Gαi alone since both Ca^{2+} mobilization and cytokine production induced by LTE_4 in Th2 cells are partially inhibited by high concentrations (1 μg/ml) of PTX. It has been reported that $CysLT_1$-mediated Ca^{2+} mobilization and mitogen-activated protein kinase activation are PTX insensitive in some cell systems, whereas $CysLT_1$-mediated chemotactic response in these cells are PTX sensitive (23,59).

The “lukast” class of leukotriene receptor antagonists including montelukast and zafirlukast have clinical benefit in asthma. However, when used as monotherapy, the anti-inflammatory activity and consequently, clinical efficacy of these drugs are modest compared to inhaled steroids (34). The finding in this study may reveal a new application for montelukast and related drugs. In particular, the “unmasking” of the anti-inflammatory effect of such drugs may extend its utility beyond control of bronchoconstriction in mild asthma to control of airway inflammation and prevention of exacerbations in more severe disease when used in combination with a CRTH2 antagonist.

In conclusion, the present study highlights a novel function of cysLTs, particularly LTE_4, in potentiating inflammatory responses of Th2 cells to PGD_2 through a montelukast-senstive cysLT receptor with high affinity for LTE_4. These observations support the view that LTE_4 has significant pro-inflammatory activity and provide additional rationale for the combined use of montelukast with CRTH2 antagonists in allergic asthma and related disorders.

Acknowledgments

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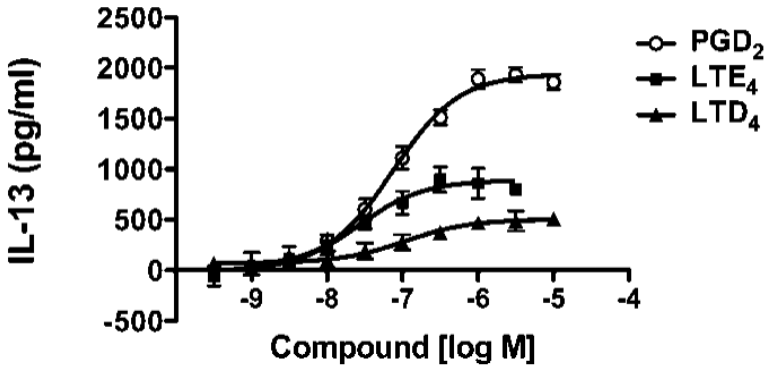

FIGURE 1. Stimulation of Th2 cells with PGD_2, LTD_4 or LTE_4 evokes IL-13 production

Th2 cells (1.5×10^6 cell/ml) were treated with various concentration of PGD_2 (○), LTD_4 (▲) or LTE_4 (■) for 6 h. The cell supernatant was collected and the concentrations of IL-13 were determined by ELISA. Data are expressed mean ± SEM of 2-5 independent experiments.

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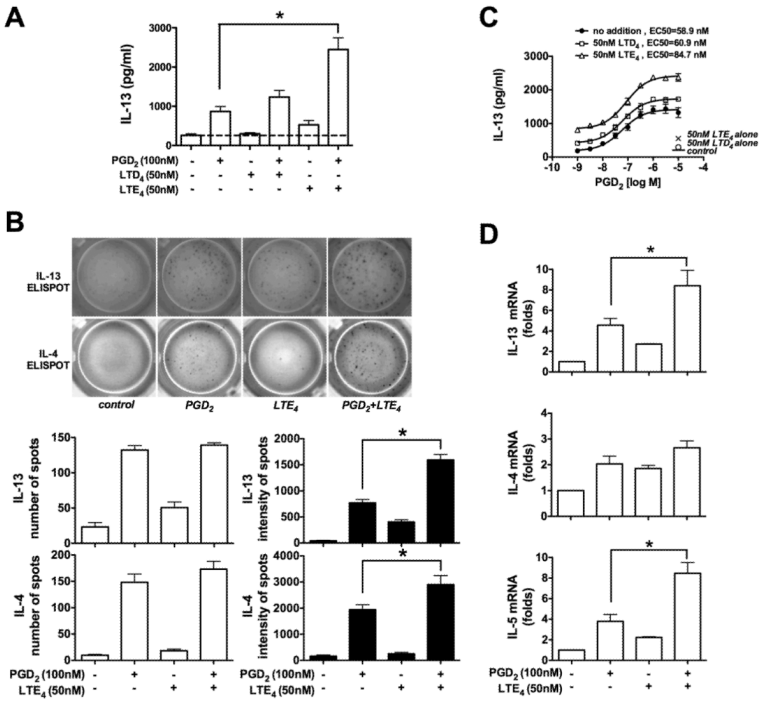

FIGURE 2. Enhancement by cysLTs of the Th2 cytokine production in response to PGD_2

A, Th2 cells were incubated with 100 nM PGD_2, 50 nM LTD_4, 50 nM LTE_4 alone or in combination as indicated for 6 h. The concentrations of IL-13 in the cell supernatants were determined with ELISA. The average background level of IL-13 in the control samples is indicated with a dashed line. *B*, Th2 cells were incubated with 100 nM PGD_2, 50 nM LTE_4 alone or in combination as indicated for 15 h and then analysed with ELISPOT for IL-13 or IL-4. Upper panels show representative images. The number (left side in the lower panels) and the average intensity (right side in the lower panels) of the spots were analysed. *C*, Th2 cells were treated with various concentration of PGD_2 in absence (●) or presence of 50 nM LTD_4 (□) or 50 nM LTE_4 (△) for 6 h. The concentrations of IL-13 in the supernatants were measured by ELISA. The IL-13 levels detected in the samples treated with control medium (—), 50 nM LTD_4 (○) or 50 nM LTE_4 (×) alone are included for comparison. *D*, Th2 cells were treated with medium alone or medium containing 100 nM PGD_2, 50 nM LTE_4 or both for 2.5 h. Total RNA was extracted from the cell pellets. The mRNA levels of IL-13 (upper panel), IL-4 (middle panel) and IL-5 (lower panel) were measured by using qPCR. The mRNA levels in untreated cells were treated as 1 fold. Data are expressed as mean ± SEM of 3-9 independent experiments. $p<0.005$ by ANOVA for all graphs. * indicate significant differences between PGD_2+LTE_4 or PGD_2+LTD_4 and PGD_2 alone ($p<0.05$ by Newman-Keuls test). Other significant differences are not illustrated.

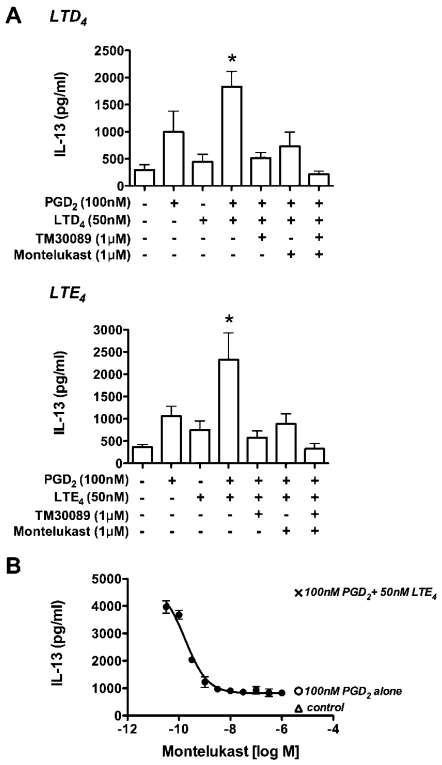

FIGURE 3. Inhibitory effect of TM30089 and montelukast on IL-13 production induced by PGD_2 and LTE_4

Th2 cells were treated (*A*)with 100 nM PGD_2, 50 nM LTD_4 (*A-LTD4*), 50 nM LTE_4 (*A-LTE4*) alone or in combination in the presence or absence of 1 μM TM30089, 1 μM montelukast or both as indicatedor (*B*) with 100 nM PGD_2 and 50 nM LTE_4 in the presence of various concentration of montelukast (●) for 6 h. The levels of IL-13 in the supernatants were determined by ELISA. The IL-13 levels detected in the samples treated with control medium (△), 100 nM PGD_2 alone (○) or combined with 50 nM LTE_4 (×) are included in *B* for comparison.Data are expressed as mean ± SEM of 3-7 independent experiments. For *A-LTD4*,p=0.002 and for *A-LTE4*,p=0.0002 by ANOVA. The significant differences between PGD_2+LTD_4 (*A-LTD4*) or PGD_2+LTE_4 (*A-LTE4*) and all other conditions are indicated with * (p<0.05 by Newman-Keuls test). Other significant differences are not indicated.

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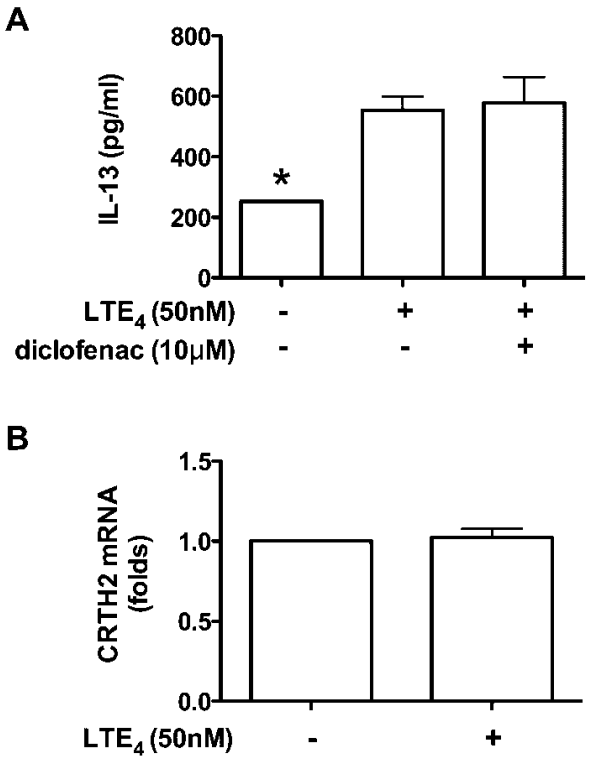

FIGURE 4. Effect of LTE_4 on the expression of PGD_2 and CRTH2 in Th2 cells

A, Th2 cells were incubated with 50 nM LTE_4 in the presence or absence of 10 μM diclofenac for 6 h. The IL-13 levels in the supernatants were measured by ELISA. *B*, Th2 cells were stimulated with 50 nM LTE_4 for 2.5 h. Total RNA was extracted from the cell pellets. The mRNA levels of CRTH2 were measured by using qPCR. The mRNA levels in untreated cells were treated as 1 fold. Data are expressed as mean ± SEM of 2 (for *A*) or 3 (for *B*) independent experiments. For *A*, p=0.01 and for *B*, p<0.0001 by ANOVA. The significant differences are not indicated.

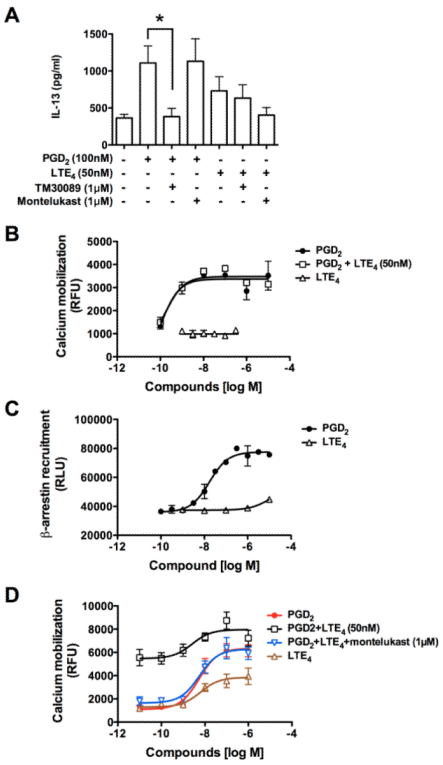

FIGURE 5. Effect of LTE_4 on responses mediated by CRTH2

A, Th2 cells were incubated with 100 nM PGD_2 or 50 nM LTE_4 in the presence or absence of 1 μM TM30089 or 1 μM montelukast for 6 h. The IL-13 levels in the supernatants were measured by ELISA. *B and D*, Calcium mobilization in response to various concentrations of LTE_4 (△) and PGD_2 alone (●) or in combination in the absence (□) or presence of 1μM montelukast (▽) in CHO-CRTH2 cells (*B*) or Th2 cells (*D*) was measured as described in the Materials and Methods. Data are reported in relative fluorescence unit (RFU). *C*, CHO-CRTH2 EA-arrestin cells were treated with various concentration of PGD_2 (●) or LTE_4 (△) for 90 min. β-arrestin recruitment in the cells was measured with a PathHunterеXpressβ-arrestin kit. Data are presented in RLU. Data are expressed as mean ± SEM of 2-8 independent experiments. For *A*, $p<0.01$ by ANOVA. Only significant difference between PGD_2 and PGD_2+TM30089 is indicated with * ($p<0.05$ by Newman-Keuls test). Other significant values are not indicated.

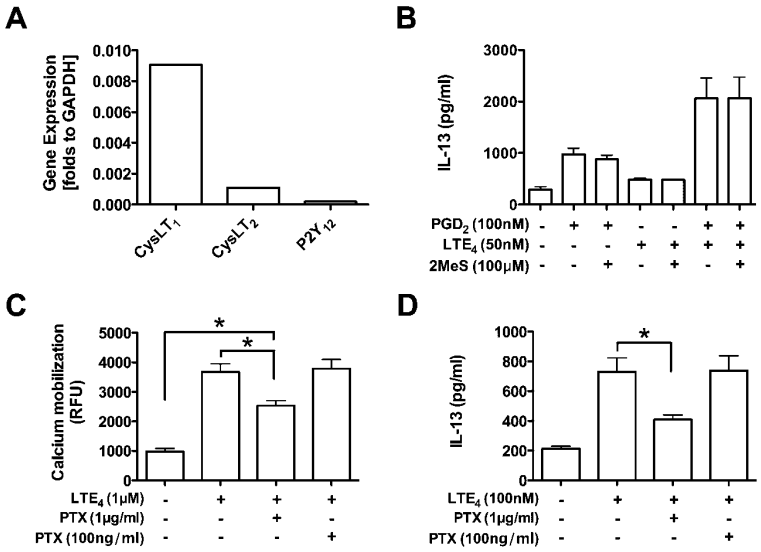

FIGURE 6. Effects of 2MeSand PTX on the responses induced by LTE_4

A, Total RNA was extracted from untreated Th2 cells. The mRNA levels of $CysLT_1$, $CysLT_2$ and $P2Y_{12}$ were measured by using qPCR. The mRNA level of GAPDH were treated as 1 fold. *B*,Th2 cells were incubated with 100 nM PGD_2 and 50 nM LTE_4 alone or in combination in the presence or absence of 100 μM 2MeS for 6 h. The levels of IL-13 in the supernatants were determined by ELISA. *C and D*, Th2 cells were pre-incubated with 1 μg/ml or 100 ng/ml PTX for 10 min (*C*) or 30 min (*D*) and then stimulated with 1 μM LTE_4 for calcium mobilization assay (*C*) or treated with 100 nM LTE_4 in the presence of PTX for 6 h for IL-13 ELISA (*D*).Datain *B, C* and *D* are expressed as mean ± SEM of 2-4 independent experiments. For *B*,$p<0.002$, for *C*, $p<0.0001$ and for *D*, $p<0.01$ by ANOVA.Only significant differences between control and LTE_4+1 μg/ml PTX, LTE_4 and LTE_4+1 μg/ml PTX are indicated with * ($p<0.05$ by Newman-Keuls test).

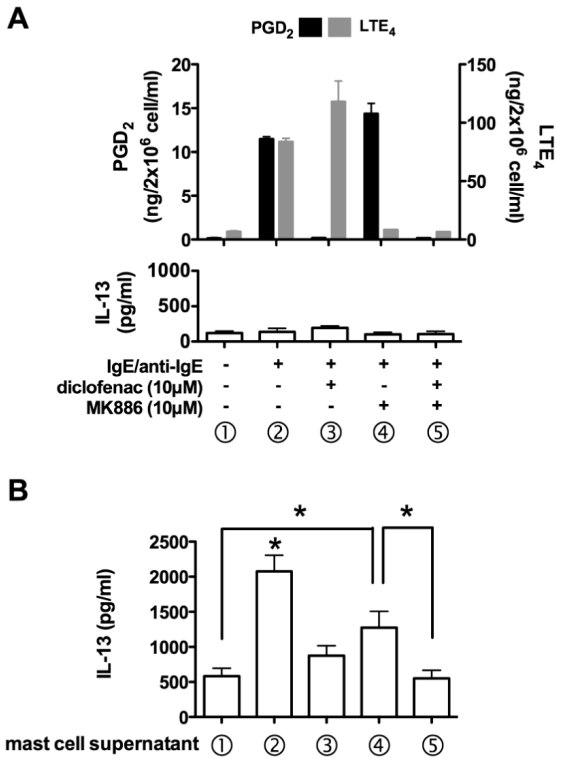

FIGURE 7. Effect of mast cell supernatants on the cytokine production by Th2 cells

A, Human mast cells were activated with IgE/anti-IgE as described in the Materials and Methods in the presence or absence of 10 μM diclofenac, MK886 or both for 1 h as indicated, and the supernatants were collected and assigned as mast cell supernatant 1 to 5. The levels of PGD_2, LTE_4 (upper panel of *A*) and IL-13 (lower panel of *A*) in the supernatants were measured by ELISA. *B*, Th2 cells were incubated with 1:1.5 diluted mast cell supernatants as indicated for 6 h. The IL-13 levels in the supernatants after the incubation were determined by ELISA. Data are expressed as mean ± SEM of 3-4 independent experiments. For upper panel in *A*, $p<0.0005$, for lower panel in *A*, $p<0.05$ and for *B*, $p<0.001$ by ANOVA. The significant differences in *B* are indicated with * ($p<0.05$ by Newman-Keuls test).

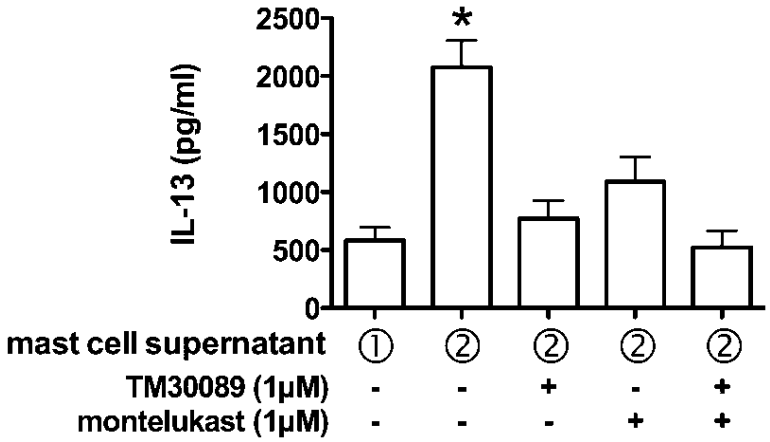

FIGURE 8. Inhibitory effect of TM30089 and montelukast on IL-13 production induced by mast cell supernatants

Th2 cells were incubated with 1:1.5 diluted mast cell supernatant 1 or 2 in the presence or absence of 1 μM TM30089, 1 μM montelukast or both for 6 h. The IL-13 levels in the supernatants after the incubation were measured by ELISA. Data are expressed as mean ± SEM of 4 independent experiments. p=0.0002 by ANOVA. * The values between supernatant 2 treatment and all other treatments are significant different (p<0.05 by Newman-Keuls test).

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Table 1

IL-13 levels in the supernatants from Th2 cells after various treatments

Treatment	IL-13 level (pg/4×10^6 cell/ml)	
	Read-out	Net increase
control	226.5±54.8 (n=10)	-
100nM PGD_2	949.1±219.6 (n=11)	682.7±202.9
50nM LTC_4	302.4±56.8 (n=2)	35.9±28.2
50nM LTC_4 + 100nM PGD_2	1269.3±119.9 (n=2)	1002.9±111.3
50nM LTD_4	301.8±22.7 (n=4)	35.4±24
50nM LTD_4 + 100nM PGD_2	1226.6±174.9 (n=4)	960.1±206.3
50nM LTE_4	554.4±181.8 (n=9)	288±121.2
50nM LTE_4 + 100nM PGD_2	2571.2±450.5 (n=9)	2304.8±300.3

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Table 2

Comparison of the additive effect and synergistic effect of PGD_2 and LTE_4 in Th2 cytokine production[a]

	100nM PGD_2	**50nM LTE_4**	**additive**	**PGD_2/LTE_4**	***p*<0.05[b]**
IL-4 protein (pg/ml)	7.78±1.9	5.57±2.51	13.36±0.61	24.39±0.71	yes
IL-5 protein (pg/ml)	-	-	-	-	-
IL-13 protein (pg/ml)	662.7±143.2	288±121.2	970.7±250.4	2304.8±300.3	yes
IL-4 mRNA (fold)	1.03±0.3	0.85±0.12	1.88±0.42	1.66±0.27	no
IL-5 mRNA (fold)	2.78±0.67	1.23±0.07	4.02±0.75	7.47±1.03	yes
IL-13 mRNA (fold)	3.56±0.66	1.72±0.28	5.27±0.63	8.42±1.49	yes
IL-4 Elispot intensity	1785.7±183.9	91.7±55.35	1877.3±199.9	2737±350.6	yes
IL-5 Elispot intensity	-	-	-	-	-
IL-13 Elispot intensity	726.3±68.3	360.2±37.3	1086.5±84.2	1549.8±104	yes

[a]The data are net increase and expressed as mean ± SEM of 2-9 independent experiments.

[b]Significant difference between additive (sum of values from the treatments with PGD_2 and LTE_4 alone) and PGD_2/LTE_4 (values from combination treatment with PGD_2 and LTE_4) by Newman-Keuls test.