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Leukotriene E₄ Activates Human Th2 Cells for Exaggerated Proinflammatory Cytokine Production in Response to Prostaglandin D₂

Luzheng Xue,* Anna Barrow,† Vicki M. Fleming,‡ Michael G. Hunter,† Graham Ogg,*†§ and Roy Pettipher†

PGD₂ exerts a number of proinflammatory 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. Because 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 PGD₂. PGD₂ induced concentration-dependent Th2 cytokine production in the absence of TCR stimulation. Leukotrienes D₄ and E₄ (LTE₄) also stimulated the cytokine production but were much less active than PGD₂. However, when combined with PGD₂, cysLTs caused a greater than additive enhancement of the response, with LTE₄ being most effective in activating Th2 cells. LTE₄ enhanced calcium mobilization in response to PGD₂ in Th2 cells without affecting endogenous PGD₂ production or CRTH2 receptor expression. The effect of LTE₄ was inhibited by montelukast but not by the P2Y₁₂ antagonist methylthioadenosine 5'-monophosphate. The enhancing effect was also evident with endogenous cysLTs produced from immunologically activated mast cells because inhibition of cysLT action by montelukast or cysLT synthesis by MK886, an inhibitor of 5-lipoxygenase–activating protein, reduced the response of Th2 cells to the levels produced by PGD₂ alone. These findings reveal that cysLTs, in particular LTE₄, have a significant proinflammatory impact on T cells and demonstrate their effects on Th2 cells are mediated by a montelukast-sensitive receptor. The Journal of Immunology, 2012, 188: 000–000.

B

oth PGD₂ 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).

PGD₂, 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 PGD₂ production in some circumstances. A significant contribution of PGD₂ to the development of allergic inflammation has been suggested by the observations of enhanced eosinophilic lung inflammation and cytokine release in transgenic mice overexpressing PGD₂ synthase (9). Two distinct G protein-coupled receptors have been identified as PGD₂ receptors: D prostanoid receptor 1 and chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2). In recent years, increasing evidence suggests that through its action on CRTH2, PGD₂ elicits many proinflammatory responses in leukocytes, including chemotaxis of eosinophils, basophils, and Th2 cells (10, 11), cytokine production by Th2 cells (12, 13), and proinflammatory 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 that 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 in which 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 leukotrienes C₄ (LTC₄), D₄ (LTD₄), and E₄ (LTE₄), are derived from the 5-lipoxygenase pathway of the arachidonic acid metabolism (21, 22). LTC₄ is formed by conjugation of LTA₄ with reduced glutathione and after extra-cellular export is converted to LTD₄ and then the stable metabolite LTE₄ by sequential enzymatic removal of glutamic acid followed by glycine. Two G protein-coupled receptors for LTs have been cloned, characterized, and designated as CysLT₁ and CysLT₂ (23–25). LTD₄ binds CysLT₁ with higher affinity than LTC₄, whereas CysLT₂ binds these cysLTs with equal affinity. LTE₄ has only weak activity on either CysLT₁ (23, 24) or CysLT₂ (25, 26) and has therefore been generally considered to be a stable inactive breakdown product, although there is accumulating evidence that LTE₄ can stimulate inflammatory responses through mechanisms independent of CysLT₁ or CysLT₂ (27–31).

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Abbreviations used in this article: CHO, Chinese hamster ovary; CRTH2, chemoattractant receptor-homologous molecule expressed on Th2 cells; cysLT, cysteinyl leukotriene; LTE₄, leukotriene C₄; LTD₄, leukotriene D₄; LTE₄, leukotriene E₄; 2MeS, 2-methylthioadenosine 5′-monophosphate; PTX, pertussis toxin; qPCR, real-time quantitative PCR.

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CysLT₁ mediates bronchoconstriction and also a range of proinflammatory effects including activation and migration of leukocytes (21, 32, 33), whereas CysLT₂ may mediate the vasoactive effects of LTC₄ and LTD₄. The leukotriene antagonists approved for use in asthma and allergic rhinitis, most notably montelukast, block the action of cysLTs (predominantly LTD₄) on CysLT₁ but do not inhibit CysLT₂-mediated effects. Monotherapy with montelukast thereby inhibits the CysLT₁-mediated bronchoconstrictor element of asthma, but its anti-inflammatory activity and, consequently, clinical efficacy are modest compared with 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 suggest in primary care that a combination of montelukast and inhaled glucocorticoid (36).

We found that cysLTs markedly potentiated proinflammatory cytokine production from human Th2 cells in response to PGD₂. The potency of LTE₄ in the enhancing effect was significantly higher than that of LTD₄ or LTC₄, and this enhancing effect of LTE₄ was inhibited by montelukast. Although the CRTH2 antagonist TM30089 alone substantially inhibited IL-13 production in response to both exogenous and endogenous PGD₂ and LTE₄, a combination of TM30089 and montelukast was required to completely inhibit the response.

These data highlight an important interaction between PGD₂ 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
PGD₂, LTD₄, LTD₃, and LTE₄ were purchased from Enzo Life Science; PGD₂–MOX enzyme immunoassay kits and LTE₄ 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 Miltenyi Biotec; human recombinant stem cell factor, human rIL-6, and human IL-4/5/13 immunoassay kits were purchased from R&D Systems; IMDM and X-VIVO 15 medium were purchased from Lonza; AIM V medium was from Invitrogen; human myeloma IgE and Abs against human tryptase and chymase were purchased from Millipore; ELISPOT kits for human IL-4/IL-13 were purchased from Mabtech; alkaline phosphatase conjugate substrate kit was from Bio-Rad; BLNor05 MicroBead Kits, human CD34 MicroBead kits, and 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 PGD₂, 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% CO₂ for different periods of time for qPCR, ELISA, or ELISPOT. In some experiments, Th2 cells were preincubated 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 Victor² V-1420 multilabel HTS Counter (PerkinElmer Life Sciences).

ELISPOT cytokine assays
Th2 cells (4 × 10³ cell/well) were seeded in a MultiScreen Filter Plate (Millipore) coated with capture Ab against human IL-4 or IL-13 and treated with PGD₂, LTD₄, 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 PGD₂ using a PGD₂–MOX enzyme immunoassay kit and LTE₄ using a LTE₄ 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, U.K.) by Lymphoprep density gradient centrifugation, followed by CD4⁺ cell purification using a CD4⁺ T cell isolation kit II. After 7 d 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 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 PGD₂, 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% CO₂ for different periods of time for qPCR, ELISA, or ELISPOT. In some experiments, Th2 cells were preincubated with pertussis toxin (PTX) as indicated in the results.

qPCR
Th2 cells were treated for 2.5 h, and the total RNA of the cells was extracted using an RNeasy Mini kit. cDNA of the samples was prepared from the same starting amount of RNA using an Omniscript reverse transcription 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.

FIGURE 1. Stimulation of Th2 cells with PGD₂, LTD₄, or LTE₄ evokes IL-13 production. Th2 cells (1.5 × 10⁶ cell/ml) were treated with various concentration of PGD₂ (○), LTE₄ (●), or LTD₄ (▲) for 6 h. The cell supernatant was collected, and the concentrations of IL-13 were determined by ELISA. Data are expressed mean ± SEM of two to five independent experiments.
Primers and probes used were as follows: IL-4, 5'-GCCCTGCAGAAGGTTTCC-3' and 5'-GCCACATCGCTCAGACAC-3' with probe 60 generating a 66-bp fragment; IL-5, 5'-GGTTTGTTGCAGCCAAAGAT-3' and 5'-AGCCCGAGTTGACCGTAACAG-3' with probe 16 generating a 72-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; CysLT1, 5'-ACTCCAGTGCCAGAAAGAGG-3' and 5'-GCGGAAGTCATCAATAGTGTCA-3' with probe 29 generating a 112-bp fragment; CysLT2, 5'-CTAGAGTCCTGTGGGCTGAAA-3' and 5'-GTAGGATCCAATGTGCTTTGC-3' with probe 48 generating a 61-bp fragment; P2Y12, 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

Chinese hamster ovary (CHO) / CRTH2 cells (Evotec-OAI) (8 x 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 x 10^5 cells/well. The cells were incubated with FLIPR Ca2+ 5 dye in the presence or absence of PTX at 37˚C 5% CO2 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

**FIGURE 2.** Enhancement by cysLTs of the Th2 cytokine production in response to PGD2. A, Th2 cells were incubated with 100 nM PGD2, 50 nM LTD4, 50 nM LTE4 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 by a dashed line. B, Th2 cells were incubated with 100 nM PGD2, 50 nM LTE4 alone, or in combination as indicated for 15 h and then analyzed with ELISPOT for IL-3 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 analyzed. C, Th2 cells were treated with various concentration of PGD2 in absence (●) or presence of 50 nM LTD4 (○) or 50 nM LTE4 (△) 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 LTD4 (○), or 50 nM LTE4 (△) alone are included for comparison. D, Th2 cells were treated with medium alone or medium containing 100 nM PGD2, 50 nM LTE4, 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 three to nine independent experiments. *Significant differences between PGD2 + LTE4 or PGD2 + LTD4 and PGD2 alone (p < 0.05 by Newman–Keuls test). Other significant differences are not illustrated.

| Table I. IL-13 levels in the supernatants from Th2 cells after various treatments |
|------------------------------------------|-----------------|-----------------|-----------------|
| Treatment                               | IL-13 Level (pg/4 x 10^6 cell/ml) | Readout | Net Increase |
| Control                                 | 226.5 ± 54.8 (n = 10) |                  |               |
| 100 nM PGD2                              | 949.1 ± 219.6 (n = 11) |                  | 682.7 ± 202.9 |
| 50 nM LTD4                               | 302.4 ± 56.8 (n = 2) |                  | 35.9 ± 28.2   |
| 50 nM LTD4 + 100 nM PGD2                 | 1269.3 ± 119.9 (n = 2) |                  | 1002.9 ± 111.3 |
| 50 nM LTD4                               | 301.8 ± 22.7 (n = 4) |                  | 35.4 ± 24     |
| 50 nM LTD4 + 100 nM PGD2                 | 1226.6 ± 174.9 (n = 4) |                  | 960.1 ± 206.3 |
| 50 nM LTE4                               | 554.4 ± 181.8 (n = 9) |                  | 285 ± 121.2   |
| 50 nM LTE4 + 100 nM PGD2                 | 2571.2 ± 450.5 (n = 9) |                  | 2304.8 ± 300.3 |
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 PathHunter eXpress β-arrestin kit ( DiscoveRx) for human CRTH2 according to the manufacturer’s instructions. Briefly, CHO/CRTH2 EA-arrestin cells were resuspended in OCC 2 media and seeded in a 96-well luminescence plate (white walled, clear bottom) for recovering at 37°C 5% CO2 for 48 h. Treatment compounds were added for a 90-min incubation at 37°C 5% CO2, and then detection reagents were added for another 90-min incubation at room temperature. Luminescence was measured using a Victor® V-1420 multilabel HTS Counter (PerkinElmer Life Sciences).

Statistics

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

Results

LTD4 and LTE4 are weak inducers of cytokine production by human Th2 cells compared with PGD2

As previously reported, PGD2 induced production of IL-13 when incubated with Th2 cells (13). Both LTD4 and LTE4 alone also induced IL-13 production in a dose-dependent manner with an EC50 similar to PGD2 (75.6 nM for PGD2, 107.8 nM for LTD4, and 26.2 nM for LTE4) (Fig. 1). However, the maximum responses achieved by LTD4 and LTE4 were only 23 and 41%, respectively, of that achieved by PGD2.

CysLTs significantly enhance Th2 cytokine production in response to PGD2

To define the effect of the combination of PGD2 and cysLTs on Th2 cytokine production, Th2 cells were treated with PGD2, LTD4, or LTE4 alone (at concentrations close to their relative EC50) or in combination as indicated in Fig. 2 for 6 h. Interestingly, both LTD4 and LTE4 significantly enhanced IL-13 production in response to PGD2 (Fig. 2A, Table I). The enhancement was greater than additive (p < 0.05), and most marked with LTE4 (Table II). LTE4 showed a similar enhancing effect of LTD4 in the combination treatment (Table I). ELISPOT analysis of IL-13 and IL-4 levels indicated that the enhancement occurred at the level of individual cells and not by more responder cells (Fig. 2B); as compared with the samples treated with PGD2 alone, the combined use of PGD2 and LTE4 did not change the total spot numbers for both IL-4 and IL-13 significantly but enhanced the average intensities of the spots (Table II).

To understand the nature of the enhancing effect of cysLTs on the Th2 cytokine production, the cells were stimulated with increasing concentrations of PGD2 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 PGD2, but did not significantly affect the EC50 of PGD2 (Fig. 2C).

To determine the effect of the treatment with the combination of PGD2 and cysLTs at the gene transcription level, the levels of mRNA encoding IL-4, IL-5, and IL-13 in Th2 cells were measured.

<table>
<thead>
<tr>
<th>Compound</th>
<th>IL-4 mRNA (fold)</th>
<th>IL-5 mRNA (fold)</th>
<th>IL-13 mRNA (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGD2 alone</td>
<td>1.03 ± 0.07</td>
<td>0.27 ± 0.12</td>
<td>0.3 ± 0.05</td>
</tr>
<tr>
<td>LTD4 alone</td>
<td>0.12 ± 0.02</td>
<td>1.03 ± 0.09</td>
<td>0.56 ± 0.05</td>
</tr>
<tr>
<td>LTE4 alone</td>
<td>1.88 ± 0.10</td>
<td>0.27 ± 0.05</td>
<td>0.66 ± 0.08</td>
</tr>
<tr>
<td>PGD2 + LTD4</td>
<td>0.71 Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>PGD2 + LTE4</td>
<td>0.27 No</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>PGD2 alone + LTE4</td>
<td>1.03 Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>PGD2 alone + LTD4</td>
<td>1.49 Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>PGD2 + LTD4 + LTE4</td>
<td>1.03 Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>

The data are net increase and expressed as mean ± SEM of two to three independent experiments.

Significant difference between additive (sum of values from the treatments with PGD2 and LTE4 alone) and PGD2/LTE4 (values from combination treatment with PGD2 and LTE4) by Newman–Keuls test.

p 0.05 were considered statistically significant.

Table II. Comparison of the additive effect and synergistic effect of PGD2 and LTE4 in Th2 cytokine production

<table>
<thead>
<tr>
<th>Compound</th>
<th>IL-4 protein (pg/ml)</th>
<th>IL-13 protein (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGD2 alone</td>
<td>7.78 ± 1.9</td>
<td>662.7 ± 143.2</td>
</tr>
<tr>
<td>LTD4 alone</td>
<td>1.03 ± 0.3</td>
<td>1.95 ± 0.12</td>
</tr>
<tr>
<td>LTE4 alone</td>
<td>1.23 ± 0.07</td>
<td>0.78 ± 0.02</td>
</tr>
<tr>
<td>PGD2 + LTD4</td>
<td>13.56 ± 0.61</td>
<td>970.7 ± 250.4</td>
</tr>
<tr>
<td>PGD2 + LTE4</td>
<td>5.57 ± 2.51</td>
<td>2304.8 ± 500.3</td>
</tr>
</tbody>
</table>

The IL-13 levels detected in the samples treated with control medium (○) were considered statistically significant.

p 0.05 by Newman–Keuls test. Other significant differences are not indicated.

FIGURE 3. Inhibitory effect of TM30089 and montelukast on IL-13 production induced by PGD2 and LTE4. Th2 cells were treated with 100 nM PGD2, 50 nM LTD4 (A, top panel), or 50 nM LTE4 (A, bottom panel) alone or in combination in the presence or absence of 1 μM TM30089, 1 μM montelukast, or both as indicated or with 100 nM PGD2 and 50 nM LTE4 in the presence of various concentrations 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 PGD2 alone (△), or combined with 50 nM LTE4 (X) are included in B for comparison. Data are expressed as mean ± SEM of three to seven independent experiments. For A, top panel, p = 0.002, and for A, bottom panel, p = 0.0002 by ANOVA. The significant differences between PGD2 + LTD4 (A, top panel) or PGD2 + LTE4 (A, bottom panel) and all other conditions are indicated with an asterisk (*p < 0.05 by Newman–Keuls test). Other significant differences are not indicated.
was dose dependent, with IC50 = 0.2 nM. The enhancing effect of PGD2 alone. In both cases, the combination of TM30089 and montelukast completely abolished by 10 nM montelukast. LTE4 does not affect PGD2 production and CRTH2 expression in Th2 cells.

It has been reported that activated Th2 cells are able to produce PGD2 (7, 8). To understand whether the enhancing effect of cysLTs is mediated by a secondary production of PGD2 by Th2 cells, we examined the effect of diclofenac, a cyclooxygenase inhibitor, on cytokine production induced by LTE4 (Fig. 4A). Diclofenac (10 μM) did not change the IL-13 production induced by LTE4, demonstrating that PGD2 synthesis is not involved in the enhancing effect of LTE4. We also investigated the effect of LTE4 on the expression of CRTH2 by qPCR (Fig. 4B). LTE4 did not show any effect on the level of CRTH2 mRNA, suggesting that the enhancing effect of LTE4 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 LTE4 alone were examined (Fig. 5A). As expected, 1 μM TM30089 completely inhibited responses to PGD2 but not to LTE4; vice versa, 1 μM montelukast blocked the effect of LTE4 to the background level but had no effect on PGD2.

It has been established that two signal mechanisms are involved in the activation of CRTH2, Ca2+ influx, and β-arrestin recruitment (10, 38–40). We further analyzed the effect of LTE4...
on the downstream signaling of CRTH2 (Fig. 5B, C). In CHO cells overexpressing human rCRTH2, PGD₂, but not LTE₄, induced Ca²⁺ mobilization (Fig. 5B) and β-arrestin recruitment (Fig. 5C) in a dose-dependent manner. Addition of LTE₄ did not enhance the Ca²⁺ mobilization induced by PGD₂ in the recombinant cell system (Fig. 5B). However, in Th2 cells, both PGD₂ and LTE₄ induced Ca²⁺ mobilization in a dose-dependent manner, although the efficacy of LTE₄ was much weaker (Fig. 5D). Addition of LTE₄ enhanced the Ca²⁺ mobilization induced by PGD₂ significantly, an effect that was completely inhibited by montelukast.

The enhancing effect of LTE₄ was not mediated by P2Y₁₂ receptor but by a receptor that was partially sensitive to PTX.

It has also been reported that LTE₄ can act as an agonist at the P2Y₁₂ receptor (41, 42) and that montelukast can antagonize some P2Y receptors (43), although the effect of montelukast has not been tested on P2Y₁₂. The expression of P2Y₁₂ can be detected in human Th2 cells, but the level of this receptor is much lower than that seen with CysLT₁ (Fig. 6A). To address the role of P2Y₁₂ in an LTE₄-induced enhancing effect, the effect of 2-methylthioadenosine 5'-monophosphate (2MeS), an inhibitor of P2Y₁₂ (42), was investigated. 2MeS (100 μM) had no significant effect on the IL-13 production induced by PGD₂ or LTE₄ alone or in combination, suggesting that the P2Y₁₂ receptor is not involved in mediating the effects of LTE₄ in this system (Fig. 6B).

To further understand the potential receptor mediating the effect of LTE₄ in Th2 cells, we examined the effects of PTX, an inhibitor of the Go/i pathway, on Ca²⁺ mobilization (Fig. 6C) and IL-13 production (Fig. 6D) induced by LTE₄ in Th2 cells. Both responses to LTE₄ were partially reduced by 1 μg/ml PTX but not by 100 ng/ml PTX.

Effect of mast cell supernatants containing endogenous PGD₂ and LTE₄ on cytokine production by Th2 cells

To analyze the impact of endogenously synthesized eicosanoids on Th2 function, the effect of supernatants collected from human mast cells activated with IgE/anti-IgE on Th2 cytokine production was investigated (Fig. 7). Only low levels of PGD₂ (<0.1 ng/2 × 10⁶ cell/ml) and LTE₄ (<10 ng/2 × 10⁶ 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 PGD₂ (>12 ng/2 × 10⁶ cell/ml) and LTE₄ (>85 ng/2 × 10⁶ cell/ml) (supernatant 2). Cotreatment of IgE/anti-IgE-activated mast cells with diclofenac (10 μM) during the period of anti-IgE stimulation abolished production of PGD₂ but enhanced production of LTE₄ (>135 ng/2 × 10⁶ cell/ml) (supernatant 3). Cotreatment of the mast cells with MK886 (10 μM), an inhibitor of FLAP, during the activation blocked production of LTE₄ but increased the level of PGD₂ (>15 ng/2 × 10⁶ cell/ml) (supernatant 4). Cotreatment of the mast cells with both diclofenac and MK886 abolished production of both PGD₂ and LTE₄ (supernatant 5). Only very low levels of IL-13 (<200 pg/2 × 10⁶ cell/ml) could be detected in any of these mast cell supernatants (Fig. 7A, lower panel).

Using these supernatants to treat human Th2 cells revealed, as expected, different capacities to stimulate IL-13 production depending on the levels of PGD₂ and LTE₄ contained in the supernatants (Fig. 7B). Supernatant 2 containing both PGD₂ and LTE₄ 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 PGD₂ and LTE₄ (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 with both TM30089 and montelukast reduced the level of stimulatory activity of supernatant 2 to that of the supernatant from unactivated mast cells (supernatant 1).
The supernatants after the incubation were measured by ELISA. Data are with 1:1.5 diluted mast cell supernatants 1 or 2 in the presence or absence of production induced by mast cell supernatants. Th2 cells were incubated by Th2 cells.

Inhibitory effect of TM30089 and montelukast on IL-13 production

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 supernatants 1 to 5. The levels of PGD2, LTE4 (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 four 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 an asterisk (*p < 0.05 by Newman–Keuls test).

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 PGD2 induces Th2 cytokine production through activation of CRTH2 (12, 13). In this study, we have revealed a previously unrecognized role of cysLTs in potentiating cytokine production by human Th2 cells in response to PGD2. CysLTs alone are weak stimulators of Th2 cytokine production but significantly enhanced cytokine production in response to PGD2. The combined effects of PGD2 and cysLTs at pathophysiological concentrations (similar to the concentrations produced by activated mast cells) were greater than additive (Table II), and interestingly, LTE4 was more potent than that of LTC4 and LTD4 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 PGD2 and cysLTs.

CysLTs are produced by mast cells, eosinophils, and other cells during the allergic response and have been detected in high concentrations in airways of asthmatics challenged with allergen (47), and LTE4 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 PGD2 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 are reduced in LTC4 synthase null mice (33), and cysLTs contribute to cytokine production by allergen-specific Th2 cells stimulated with GM-CSF–primed macrophages (54). The present study provides direct evidence that cysLTs are able to elicit cytokine production by Th2 cells in the absence of Ag or any other costimulation and highlights the possibility that PGD2 and cysLTs act in concert to promote Th2 cytokine production.

Our previous report demonstrated the critical role of PGD2 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 LTE4, can activate Th2 cells for exaggerated cytokine production in response to PGD2. Both PGD2 and LTE4 were detected in high levels in supernatants from immunologically activated mast cells. Although the effect of the mast cell supernatants is predominately through interaction of PGD2 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 PGD2 alone illustrates that endogenous cysLTs can activate Th2 cells for a heightened response to PGD2.

The enhancing effect of cysLTs was not mediated by direct interaction with CRTH2, because in CHO cells overexpressing rCRTH2, LTE4 had no effect on Ca2+ influx and β-arrestin recruitment that are downstream signaling events following CRTH2 activation (10, 39, 40). However, in human Th2 cells, LTE4 elicits Ca2+ mobilization in a dose-dependent manner and enhances Ca2+ mobilization in response to PGD2 markedly, indicating that cysLTs enhance signaling pathways downstream of CRTH2 receptor activation. It has been reported that IFN-γ and TNF-α potentiated PGD2-induced eosinophil chemotaxis through upregulation of CRTH2 (55). However, the enhancing effect of cysLTs on Th2 cytokine production was not mediated by enhanced production of PGD2 or increased CRTH2 receptor expression as the inhibition of PGD2 synthesis with diclofenac did not change the enhancing effect, and LTE4 did not change the mRNA level of CRTH2 in Th2 cells.

The receptor mediating the enhancing effect of cysLTs is unclear. On the one hand, the potent activity of LTE4 suggests that
the effect is not mediated by either CysLT1 or CysLT2, although both receptors are expressed in human Th2 cells (Fig. 6a), because LTE4 binds these receptors with only low affinity (23–25), and LTE4 acts as a weak bronchoconstrictor in human airways compared with LTD4 (56). On the other hand, although LTE4 has only weak affinity for CysLT1, the enhancing effect of LTE4 was inhibited by the CysLT1 antagonist montelukast, suggesting that either LTE4 can activate CysLT1 when expressed endogenously in a Th2 cell environment or that montelukast has additional activities that are CysLT1-independent. It has been proposed that both montelukast and LTE4 may interact with the P2Y-like receptors, LTE4 acting as an agonist (41, 42) and montelukast as an antagonist (43). However, treatment of Th2 cells with 2MeS, a selective antagonist of P2Y12, did not reduce significantly the enhancing effect of LTE4, indicating that P2Y12 does not mediate the effect of LTE4. However, it is clear that LTE4 can stimulate inflammatory responses in mice deficient in both CysLT1 and CysLT2 (57), so it is possible that the enhancing effect observed is mediated by a montelukast-sensitive receptor that is distinct from either CysLT1 or CysLT2 and may fall in the P2Y class of receptors but is not P2Y12. Indeed, it has been established that LTE4 can elicit inflammatory responses that are qualitatively distinct from other cysLTs (30, 31). Inhalation of LTE4, but not LTD4, promotes airway eosinophilia in human volunteers (58), and, interestingly, this persistent eosinophilia is suppressed by treatment with the CysLT2 antagonist zafirlukast (27). Therefore, further research is required to identify the receptor mediating the enhancing effect of LTE4 in Th2 cells. Studies with PTX suggest that the effects of the putative receptor are not mediated by Gαi alone because both Ca2+ mobilization and cytokine production induced by LTE4 in Th2 cells are partially inhibited by high concentrations (1 μg/ml) of PTX. It has been reported that CysLT1-mediated Ca2+ mobilization and MAPK activation are PTX insensitive in some cell systems, whereas CysLT2-mediated chemoattractant responses in these cells are PTX sensitive (23, 59).

The lukast class of leukotriene receptor antagonists, including montelukast and zafirlukast, has clinical benefit in asthma. However, when used as monotherapy, the anti-inflammatory activity and, consequently, clinical efficacy of these drugs are modest compared with 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 their 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 current study highlights a novel function of CRTH2 receptors in mediating chemotactic activation of CRTH2+ CD4+ T helper type 2 cells (CRTH2) in mediating chemotactic activation of CRTH2+ CD4+ T helper type 2 lymphocytes. Immunol. 121: 577–584.

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References
