Leukotriene D₄ and Interleukin-13 Cooperate to Increase the Release of Eotaxin-3 by Airway Epithelial Cells

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Abstract

Introduction: Airway epithelial cells play a central role in the physiopathology of asthma. They release eotaxins when treated with Th2 cytokines such as interleukin (IL)-4 or IL-13, and these chemokines attract eosinophils and potentiate the biosynthesis of cysteiny1 leukotrienes (cysLTs), which in turn induce bronchoconstriction and mucus secretion. These effects of cysLTs mainly mediated by CysLT₁ and CysLT₂ receptors on epithelial cell functions remain largely undefined. Because the release of inflammatory cytokines, eotaxins, and cysLTs occur relatively at the same time and location in the lung tissue, we hypothesized that they regulate inflammation cooperatively rather than redundantly. We therefore investigated whether cysLTs and the Th2 cytokines would act in concert to augment the release of eotaxins by airway epithelial cells.

Methods: A549 cells or human primary bronchial epithelial cells were incubated with or without IL-4, IL-13, and/or LTD₄. The release of eotaxin-3 and the expression of cysLT receptors were assessed by ELISA, RT-PCR, and flow cytometry, respectively.

Results: IL-4 and IL-13 induced the release of eotaxin-3 by airway epithelial cells. LTD₄ weakly induced the release of eotaxin-3 but clearly potentiated the IL-13-induced eotaxin-3 release. LTD₄ had no effect on IL-4-stimulated cells. Epithelial cells expressed CysLT₁ but not CysLT₂. CysLT₁ expression was increased by IL-13 but not by IL-4 and/or LTD₄. Importantly, the upregulation of CysLT₁ by IL-13 preceded eotaxin-3 release.

Conclusions: These results demonstrate a stepwise cooperation between IL-13 and LTD₄. IL-13 upregulates CysLT₁ expression and consequently the response to cysLTs. This results in an increased release of eotaxin-3 by epithelial cells which at its turn increases the recruitment of leukocytes and their biosynthesis of cysLTs. This positive amplification loop involving epithelial cells and leukocytes could be implicated in the recruitment of eosinophils observed in asthmatics.

Introduction

Asthma is characterized by airway inflammation and remodeling processes, leading to bronchial hyperresponsiveness [1]. Airway epithelial cells likely play a central role in the pathophysiology of asthma given their ability to release numerous soluble mediators implicated in the inflammatory response [1]. The Th2 cytokines interleukin (IL)-4 and IL-13 are found in the bronchial fluids of asthmatic subjects and stimulate airway epithelial cells to release significant levels of eotaxins, which are potent chemotactic factors for eosinophils [2,3].

Eotaxins represent a group of chemokines consisting of eotaxin-1 (CCL11), eotaxin-2 (CCL24) and eotaxin-3 (CCL26) [4]. The production of the different eotaxins is cell-type specific. Eotaxin-1 is secreted by eosinophils, macrophages, lymphocytes, fibroblasts, smooth muscle and endothelial cells, whereas eotaxin-2 and eotaxin-3 are mainly released by epithelial and endothelial cells [4]. Among these cell type, epithelial cells are the major source of eotaxins and principally release high levels of eotaxin-3 [2,5,6]. Moreover, the release of eotaxin is differentially modulated by cytokines. The Th2 cytokines IL-4 and IL-13 enhance the secretion of all eotaxins, whereas the Th1 cytokines interferon-γ and tumor necrosis factor-α exclusively promote the release of eotaxin-1 [7,8].

Blood eosinophils migrate in the tissue under the action of potent and specific chemoattractants such as 5-oxo-6,8,11,14-eicosatetraenoic acid and eotaxin-1 [3,9]. Once in the mucosa, eosinophils generate and release soluble mediators that activate resident cells. Notably, eosinophils are an important source of cysteiny1 leukotrienes (cysLTs), which induce bronchoconstriction and mucus secretion, and promote eosinophil trafficking into the bronchial mucosa [10]. The effects of cysLTs on epithelial functions are mostly uncharacterized.
CysLTs mediate most of their biological effects through at least two distinct receptors, namely CysLT1 and CysLT2 [11,12]. CysLT1 is expressed in several tissues, on myeloid and smooth muscle cells, and Th2 cytokines enhance its expression [13–16]. A recent study also showed that airway epithelial cells expressed CysLT1 and this expression was increased in asthmatic individuals [17]. CysLT2 is expressed on eosinophils, macrophages, endothelial and smooth muscle cells, in the heart, brain and the adrenals [11].

Given that airway epithelial cells are an important source of eotaxins and are activated by Th2 cytokines and cysLTs, we hypothesized that the incubation of epithelial cells with both cysLTs and Th2 cytokines would enhance the release of eotaxins. The results presented in this study demonstrate a cooperation between LTD4 and IL-13 for the release of eotaxin-3 by airway epithelial cells.

Results

IL-13 stimulates airway epithelial cells to release eotaxin-2 and eotaxin-3

In a first series of experiments, we evaluated the effect of IL-13 and LTD4 on the release of eotaxins following a 24 hours incubation of A549 airway epithelial cells with these mediators. Vehicle- or LTD4-treated airway epithelial cells released minimal amounts of eotaxin-1, eotaxin-2, and eotaxin-3. In contrast, the treatment of airway epithelial cells with IL-13 induced a small accumulation of eotaxin-2 and a more substantial accumulation of eotaxin-3, without stimulating that of eotaxin-1 (Figure 1).

Since IL-13 greatly induced eotaxin-3 release (Figure 1) and that human epithelial cells mainly produce eotaxin-3 [2,5,6], we performed another series of experiments to further characterize the accumulation of eotaxin-3 in the supernatant of A549 cells treated with IL-13. The IL-13-induced eotaxin-3 accumulation was time-dependent. Eotaxin-3 was barely detectable after 6 hours (Figure 2 insert) but was observed after 24 hours and 48 hours of stimulation (Figure 2). Noteworthy, the accumulation of eotaxin-3 in the incubation media of IL-13-treated cells at 48 hours was greater than that observed at 24 hours by one order of magnitude. In these experiments and in agreement with the data presented in Figure 1, LTD4 alone did not induce the production or at least the accumulation of eotaxin-3 by airway epithelial cells. We did not perform experiments at longer time points, given the cells were forming multilayers. All together, these results show that IL-13 stimulated the production of eotaxin-3 from airway epithelial cells in a time-dependent manner.

LTD4 potentiates the IL-13-mediated release of eotaxin-3

When compared to pro-inflammatory cytokines or toll-like receptor agonists, LTs are weak inducers of cytokine and chemokine production and release [18]. However, they amplify the effects of cytokines and toll-like receptors agonists on gene expression in numerous experimental conditions [19–20]. We consequently investigated whether LTD4 could potentiate the effect of IL-13 on eotaxin-3 release by airway epithelial cells. As shown in Figure 3, the incubation of airway epithelial cells with the combination of IL-13 and LTD4 amplified the release of eotaxin-3 induced by IL-13 by 45%. This amplification could however only be observed at 24 hours when eotaxin-3 accumulation remain moderate. The lack of effect of LTD4 at 48 hours prompted us to perform additional experiments in which we assessed the half-life of LTD4. As shown in Figure 3B, LTD4 added to A549 cells was rapidly metabolized into LTE4, with a half-life of ~22 min. In contrast, LTE4 remained in our samples for a much longer period. However, LTE4 did not mimic the effect of LTD4 on IL-13-treated A549 cells (data not shown).

Airway epithelial cells and primary human bronchial epithelial cells express CysLT1 but not CysLT2

Given that LTD4 preferentially activates CysLT1 and CysLT2 [11,12], we next addressed if these receptors were involved in the effects of LTD4 on eotaxin-3 release. We first screened whether CysLT1 and CysLT2 were expressed by resting airway epithelial or primary human bronchial epithelial cells. Eosinophils were used as positive controls since they express both receptors [29,30]. The expression pattern of CysLT receptors of airway epithelial and primary human bronchial epithelial cells were similar, as pictured in Figure 4. Indeed, only the mRNA for CysLT1 was detected in both cell types, indicating that the effect of LTD4 on eotaxin-3 release is likely the consequence of CysLT1 activation.
LTD₄ does not modulate the IL-4-induced eotaxin-3 release from airway epithelial cells

Since IL-4 is also known to induce the release of eotaxin-3 by airway epithelial cells [2] and that IL-4 and IL-13 share common receptor subunits [31], we also tested the effect of IL-4 alone or in combination with LTD₄ on eotaxin release after a 24 hr incubation. Similarly to IL-13, IL-4 induced the release of eotaxin-1 (28.3 ± 15.3 pg/ml \( p = 0.04 \)), eotaxin-2 (124.3 ± 50 pg/ml \( p = 0.05 \)) and eotaxin-3 (1694 ± 638.4 pg/ml \( p = 0.0006 \)). As opposed to IL-13-stimulated cells (Figure 3), the addition of LTD₄ to airway epithelial cells did not modulate the release of eotaxins induced by IL-4 (data not shown). Collectively, these results suggest that LTD₄ enhances the release of eotaxin-3 by airway epithelial cells activated by IL-13 but not by IL-4.

Differential effect of IL-4 and IL-13 on CysLT₁ expression by airway epithelial cells

The puzzling discrepancy between the effects of LTD₄ on IL-4- and IL-13-induced eotaxin-3 release by airway epithelial cells prompted us to investigate the putative underlying mechanism that could explain this difference. Since airway epithelial cells only express the mRNA for CysLT₁ (Figure 4), we investigated whether IL-4 and IL-13 had an impact on CysLT₁ cell surface expression which could explain, at least in part, the differential effect of LTD₄ on eotaxin-3 release observed in IL-4 or IL-13-activated airway epithelial cells. Flow cytometry analyses were therefore performed on airway epithelial cells following a 6 hours incubation with IL-4 or IL-13. Figure 5 shows that IL-13 significantly increased CysLT₁ cell surface expression whereas IL-4 was not significantly different from either vehicle or IL-13. The results obtained in these experiments are in line with the effects of LTD₄ on eotaxin-3 release presented in Figure 3.

The data presented herein indicate that the upregulation of CysLT₁ expression by IL-13 at early timepoints (6 hours) might play a role in the potentiating effect of LTD₄ on eotaxin-3 release by activated epithelia cells, which occurs at later time points (24 hours). To confirm this hypothesis and since the half-life of LTD₄ was very short in our experimental model (Figure 3B), we performed experiments in which LTD₄ was added following a 6 hour treatment of airway epithelial cells with IL-13, i.e. when CysLT₁ expression is increased. As shown in Figure 6, LTD₄ also increased the release of eotaxin-3 by airway epithelial cells treated with IL-13 for 6 hours. The effect of LTD₄ was similar to what we observed when LTD₄ was added simultaneously with IL-13 (Figure 3).

We performed additional experiments in which human primary bronchial epithelial cells were incubated with IL-13 alone or in combination with LTD₄ for 6 hours, and then eotaxin-3 expression were analysed by qPCR. As shown in Figure 7, the incubation of human primary bronchial epithelial cells with IL-13 for 6 hours induced a 3-fold increase of eotaxin-3 expression. The combination of IL-13 and LTD₄ amplified the expression of eotaxin-3 induced by IL-13 by 10 times. This potentiating effect of LTD₄ on eotaxin-3 release by airway epithelial cells is, to our knowledge, the first demonstration that LTD₄ acts as a positive modulator of the effect of IL-13 on airway epithelial cells.
LTD$_4$ on eotaxin-3 gene expression from human primary bronchial epithelial cells is consistent and more pronounced than the LTD$_4$ effect on IL-13-induced eotaxin-3 release from A549 airway epithelial cells (Figure 3). This supports a potentiating effect of LTD$_4$ on the expression of eotaxin-3 induced by IL-13 in human primary bronchial epithelial cells and suggests that both IL-13 and LTD$_4$ participate in the accumulation of eotaxin-3 in the lungs, which might be relevant in the physiopathology of asthma.

Discussion

This study shows that both IL-4 and IL-13 stimulate the release of eotaxin-3 by airway epithelial cells, which is in complete agreement with earlier studies [2,32,33]. We also present novel findings showing that LTD$_4$ works in concert with IL-13, resulting in an amplification of expression and release of eotaxin-3 by airway epithelial cells.
airway epithelial cells most likely through the upregulation of CysLT1 expression by IL-13. Eotaxin-1 is principally produced by leukocytes and to a lesser extent by epithelial or endothelial cells. In contrast, eotaxin-2 and eotaxin-3 are mainly, if not almost exclusively, released by epithelial and endothelial cells and in much greater amount than eotaxin-1 [2,6,34]. Moreover, this cell type-specific regulation of eotaxins suggests a site-dependent function of these chemokines. Because of their localization in the bronchial mucosa, activated airway epithelial cells are likely implicated in eosinophilic inflammation by creating a chemotactic gradient of eotaxin-3 that attracts circulating and tissue eosinophils toward the airway lumen.

Moreover, the expression of each eotaxin is differentially regulated. For example, the production of eotaxin-1 is triggered by TGFβ1 and TGFβ2 cytokines, whereas that of eotaxin-3 is only modulated by TGFβ2 cytokines [7,8]. These differences probably account for their non-redundant roles in vivo. Eotaxin-1 could be secreted at inflammatory sites during the early phase of the allergic immune response where both TGFβ1 and TGFβ2 cytokines are present. In contrast, eotaxin-3 might be preferentially secreted at later time points when a TGFβ2-dominant inflammatory state is observed. This hypothesis is supported by studies demonstrating that eotaxin-3, but not eotaxin-1, expression in bronchial mucosa is increased after allergen challenge in asthmatic individuals [35,36].

CysLTs are not on mighty potent bronchoconstrictors [37] as they are also implicated in the regulation of gene expression in numerous cell types [18–28]. This latter role of cysLTs has indeed been observed in epithelial cells where LTD4 can upregulate the expression of the MUC2 gene and transforming growth factor-β1 in a CysLT1-dependent manner [38,39]. Importantly, the regulation of gene expression by cysLTs is often limited and regularly requires a co-stimulus [19–28]. For example, while having no effect by themselves, cysLTs increase the production of collagen, eotaxin-1 and CCL2 by transforming growth factor-β1-activated fibroblasts, IL-13-treated fibroblasts, and IL-4-treated monocytes, respectively [40–42]. In the present study, we showed that LTD4 alone did not induce the release of eotaxin-3 from airway epithelial cells, but significantly augmented the effect of IL-13 but not IL-4 (Figure 3), in agreement with the above-mentioned studies. The transcription of the eotaxin-3 gene induced by IL-4 or IL-13 involves the specific transcription factor signal transducer and activator of transcription (STAT) 6 [7,43–45]. The activity of this transcription factor is increased following its phosphorylation by janus kinases (JAK). STAT6 activation involves JAK1, JAK2, JAK3 and Tyk2, depending on which receptor is activated by IL-4 or IL-13 [31]. The activity of JAK proteins is negatively regulated by the suppressors of cytokine signalling (SOCS) proteins [31]. A recent study even reported that SOCS-1 and SOCS-3 are involved in the transcription of the eotaxin-3 gene [46]. Serezzoni and colleagues recently demonstrated that LTβ4 enhanced the expression of MUC5AC by downregulating SOCS-1 expression in murine alveolar macrophages [47]. The enhanced mRNA levels (Figure 7) we observed using the combination of LTD4 and IL-13 (compared to IL-13 alone) is in line with the above mentioned studies and suggest that LTD4 might also enhance eotaxin-3 translation by downregulating SOCS proteins. This however fosters additional studies to determine and confirm this hypothesis in our experimental model.

Since LTD4 mainly activates the CysLT1 and CysLT2 receptors [11,12], we evaluated their expression by airway epithelial cells. We found that resting airway epithelial and human primary bronchial epithelial cells only expressed CysLT1 (Figure 4), indicating that the effect of LTD4 on eotaxin-3 release is likely the consequence of CysLT1 receptor activation. Even though both IL-4 and IL-13 were previously shown to up regulate the expression of CysLT1 [15,16,41,42,48], the potentiating effect of LTD4 on eotaxin-3 accumulation and CysLT1 expression was only significant could only be observed in IL-13-stimulated cells (Figure 3). Interestingly, while IL-13 upregulates CysLT1 expression on both leukocytes and structural cells (e.g. fibroblasts, smooth muscle and epithelial cells), IL-4 has only been described to do so in leukocytes [15,16,41,42,48]. Given that airway epithelial cells are structural cells, this might reflect the expression of distinct IL-4 and IL-13 receptor chains in airway epithelial cells. In this respect, the IL-4Rα and IL-13Rα1 subunits were shown to be expressed cell by A549 cells [49] and human primary bronchial epithelial cells [50,51], whereas the common γ chain and the IL-13Rα2 are expressed by human primary bronchial epithelial cells [51,52]. Importantly, these different receptor subunits differentially interact with JAK proteins (the target of SOCS) [53]. The putative regulatory role LTD4 might play at regulating SOCS proteins described above could explain why LTD4 potentiated eotaxin-3 expression upon treatment with IL-13 but not IL-4. This again fosters additional studies to elucidate whether this regulatory mechanism is also involved for the differential regulation of CysLT1 receptor expression between structural cells and leukocytes.

The synergistic effect between LTD4 and IL-13 on eotaxin-3 release was only observed after 24 hours of incubation when eotaxin-3 levels remained moderate but not after 40 hours when eotaxin-3 levels are very high. A loss of function of either IL-4 or IL-13 is unlikely since the accumulation of eotaxin-3 induced by these cytokines requires a continuous exposure to the cytokines and can be observed for up to 96 hours [2]. We did not perform experiments beyond 40 hours given our cells cultures were forming multiple layers after 40 hours. The ineffectiveness of LTD4 at later time points is likely explained by the short half-life of LTD4. We observed, suggesting that LTD4 acts rapidly upon its addition in the incubation media, its effect fading away at later time points.

In conclusion, the results presented in this study demonstrate a cooperation between LTD4 and IL-13 in the expression and the release of eotaxin-3 by airway epithelial cells. Both mediators could be seen as exerting a positive effect on the other, leading to a cascade of events amplifying the release of eotaxin-3. First, IL-13 rapidly activates numerous transcription factors involved in eotaxin-3 and CysLT1 receptor expression (Figure 5 and 7) while LTD4 activates CysLT1 on airway epithelial cells, resulting in an amplification of eotaxin-3 mRNA transcription and protein release possibly by SOCS inhibition (Figure 6 and 7). Given that eotaxin-3 levels and CysLT1 expression are elevated in bronchial epithelial cells from asthmatics [1,17] and that the biosynthesis of cysLTs is increased in asthma, we propose a new mechanism that could be implicated in the physiopathology of this disease. First, elevated levels of TGFβ2 cytokines such as IL-13 in the bronchial mucosa increase the expression of CysLT1 by epithelial cells and, consequently, their responsiveness to cysLTs. When cysLTs are released by activated tissue leukocytes (mast cells and eosinophils), this activate CysLTs on airway epithelial cells and, consequently, augment the release of eotaxin-3 induced by IL-13 which then activates pro-inflammatory functions of eosinophils, including the biosynthesis of cysLTs [54]. Further in vivo studies should be performed to confirm if this cycle would amplify and worsen the inflammatory state observed in asthma.
Materials and Methods

Reagents and cells
Human recombinant IL-4 and IL-13 were obtained from Peprotech Inc., Rocky Hill, NJ, USA; LTD4 and rabbit polyclonal anti-human CysLT1 receptor from Cayman Chemical, Ann Arbor, MI, USA; rabbit Immunoglobulin Negative Control from Dako Canada Inc., Mississauga, ON, Canada; eotaxin-1, eotaxin-2 and eotaxin-3 Quantikine Kits from R&D Systems Inc., Minneapolis, MN USA; RNeasy Plus Mini Kit, One Step RT-PCR Kit, RT2 First Strand Kit and RT2 SYBR Green ROX qPCR Mastermix from Qiagen Inc., Mississauga, ON, Canada; Alexa Fluor® 488 goat anti-rabbit IgG antibody from Invitrogen Canada Inc., Burlington, ON, Canada; bovine serum albumin (BSA), foetal bovine serum (FBS), Hank’s buffered salt solution (HBSS) and Dulbecco’s Modified Eagle’s Medium (DMEM) from WISENT Inc., St-Bruno, QC, Canada. Human primary bronchial epithelial cells isolated from bronchial biopsies obtained from four subjects [55]. Human blood eosinophils were purified from three subjects by negative selection as previously described [56]. All subjects signed an informed consent form for the study with the approval from the local ethic committee named Comité d’éthique de la recherche de l’Institut universitaire de cardiologie et de pneumologie de Québec.

Airway epithelial cell culture, stimulation and analysis of eotaxins
A549 airway epithelial cells were cultured in DMEM supplemented with 10% FBS. Cells were grown in 6-well plates (10⁴ cells/well) until they reached 80% confluence, then serum-deprived overnight before stimulation. Airway epithelial cells were next incubated with IL-4 (10 ng/ml) or IL-13 (10 ng/ml) alone or in combination with LTD4 (100 nM) for 6, 24 and/or 48 hours. In selected experiments (Figure 6), airway epithelial cells were incubated with IL-13 alone for 6 hours, followed by the addition of 100 nM LTD4 for an additional 18 hours. Incubations were stopped by harvesting the supernatants. Supernatants were immediately spun to remove cell debris, aliquoted and kept at −80°C until the analysis of eotaxins by ELISA according to the manufacturer’s instructions. Following the removal of supernatants, cells were immediately harvested and processed for either RT-PCR or flow cytometry analyses.

Analysis of CysLT receptors by RT-PCR
Total RNA was extracted from purified human blood eosinophils, primary human bronchial epithelial cells and A549 airway epithelial cells using the RNeasy Plus Mini Kit. Both cDNA synthesis and PCR amplification were performed in a single tube using the One Step RT-PCR Kit. RT-PCR reactions were carried out on a Peltier Thermal Cycler PTC-200 (MJ Research, Watertown, USA). Specific primer for CysLT1 (NM_006072, from Qiagen Inc.) and RT2 SYBR Green ROX qPCR Mastermix from Qiagen Inc., Mississauga, ON, Canada; Alexa Fluor® 488 goat anti-rabbit IgG antibody from Invitrogen Canada Inc., Burlington, ON, Canada; bovine serum albumin (BSA), foetal bovine serum (FBS), Hank’s buffered salt solution (HBSS) and Dulbecco’s Modified Eagle’s Medium (DMEM) from WISENT Inc., St-Bruno, QC, Canada. Human primary bronchial epithelial cells isolated from bronchial biopsies obtained from four subjects [55]. Cells were cultured in DMEM/F12 supplemented with 10% FBS in 12-well plates until they reached 80% confluence. Human primary bronchial epithelial cells were next incubated with IL-13 (10 ng/ml) alone or in combination with LTD4 (100 nM) for 6 hours in DMEM/F12 with 1% FBS. Following its extraction with the RNeasy Mini Kit, total RNA (200 ng) will be converted into cDNA with Peltier Thermal Cycler PTC-200 using the RT2 First Strand Kit including built-in positive control elements for the detection of genomic DNA contamination and the efficiency of the reverse transcriptase and the polymerase chain reactions. qPCR amplification were done with commercially eotaxin-3 primers (Human CCL26 QuantiTeck Primer Assays, NM_006072, from Qiagen Inc.) and RT2 SYBR Green ROX qPCR Mastermix on 7900HT Fast Real Time PCR System (Applied Biosystems Canada, Streetsville, ON). A typical PCR amplification will be as follows: a denaturation step at 95°C for 5 min followed by 40 cycles (1-min denaturation at 95°C, 30-sec annealing at 60°C, and 1-min elongation at 72°C). Data acquisition and analyses will be performed with the SDS software (version 2.3). Eotaxin-3 expression was corrected for levels of 18 s rRNA as housekeeping gene.

Analysis of LTD4 and IL-13 Promote Eotaxin-3 Release

The expression of CysLT1 by airway epithelial cells was assessed by flow cytometry as described before [15]. In brief, cells were washed with PBS, then fixed and permeabilized with the Cytofix/ CytopermTM solution (BD Biosciences, Mississauga, ON Canada) for 20 min at 4°C. Cells were resuspended in cold (4°C) HBSS containing 1% BSA and 0.1% saponin and incubated at 4°C for 45 min with the rabbit anti-human CysLT1 antibody or the appropriate control isotype. Cells were then washed with cold (4°C) HBSS containing 1% BSA and incubated at 4°C for 30 min with the Alexa Fluor® 488 goat anti-rabbit IgG antibody. Cells were washed again and finally resuspended in HBSS. Flow cytometric analysis was performed with an EPICS XL-MCL flow cytometer (Beckman-Coulter, Miami, FL).

Analysis of LTD4 and LTE4 by reversed-phase HPLC
A549 cells at 80% confluence were incubated with 100 ng of LTD4 in phenol red-free DMEM supplemented with 10% FBS for different times (see figure 3 legend). Incubation were stopped by adding 1 volume of cold (4°C) incubation medium. Samples (cells and media) then were harvested and denatured overnight with 0.5 volume of a cold (−20°C) stop solution (methanol/acetonitrile, 1/1) containing 12.5 ng of both 19-OH-PGB2 and PGB2 as internal standards. Samples were then centrifuged and the supernatants were collected for the analysis of cysLTs by reversed-phase HPLC as described before [57].

Analysis of eotaxin-3 expression by qPCR
Human primary bronchial epithelial cells were obtained as previously described [55]. Cells were cultured in DMEM/F12 supplemented with 10% FBS in 12-well plates until they reached 80% confluence. Human primary bronchial epithelial cells were next incubated with IL-13 (10 ng/ml) alone or in combination with LTD4 (100 nM) for 6 hours in DMEM/F12 with 1% FBS. Following its extraction with the RNeasy Mini Kit, total RNA (200 ng) will be converted into cDNA with Peltier Thermal Cycler PTC-200 using the RT2 First Strand Kit including built-in positive control elements for the detection of genomic DNA contamination and the efficiency of the reverse transcriptase and the polymerase chain reactions. qPCR amplification were done with commercially eotaxin-3 primers (Human CCL26 QuantiTeck Primer Assays, NM_006072, from Qiagen Inc.) and RT2 SYBR Green ROX qPCR Mastermix on 7900HT Fast Real Time PCR System (Applied Biosystems Canada, Streetsville, ON). A typical PCR amplification will be as follows: a denaturation step at 95°C for 5 min followed by 40 cycles (1-min denaturation at 95°C, 30-sec annealing at 60°C, and 1-min elongation at 72°C). Data acquisition and analyses will be performed with the SDS software (version 2.3). Eotaxin-3 expression was corrected for levels of 18 s rRNA as housekeeping gene.

Means and SEM were determined for continuous variables. The effect of IL-13 on the release of eotaxins from airway epithelial cells was analyzed using Dunnett’s method. The data obtained from kinetic experiments and qPCR analyses were evaluated by a Tukey-Kramer’s method. The cooperation of LTD4 with IL-13 on the release of eotaxin-3 and the expression of the CysLT1 receptor by flow cytometry were compared by using 2-way randomized block design. The results were considered significant if P values were <0.05. The data were analyzed using the statistical package program SAS version 9.1.3 (SAS Institute Inc., Cary, NC).
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Author Contributions

Conceived and designed the experiments: VP AL FC MRP JC NF ML. Performed the experiments: VP. Analyzed the data: VP AL FC MRP JC NF ML. Contributed reagents/materials/analysis tools: JC. Wrote the paper: VP AL FC MRP JC NF ML.

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