

CysLT1 receptor upregulation by TGF- β and IL-13 is associated with bronchial smooth muscle cell proliferation in response to LTD₄

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Background: Airway remodeling is a feature of chronic asthma. It involves a number of structural changes, including bronchial smooth muscle cell (BSMC) hyperplasia and hypertrophy. Cysteinyl leukotrienes (cysLTs) have been suggested to play a role in airway remodeling in addition to their numerous other physiopathologic effects.

Objectives: This work was aimed at characterizing the potential modulation of CysLT1 receptor expression by cytokines and the eventual functional relevance of this modulation.

Methods: Expression of CysLT1 receptor was measured by flow cytometry and immunofluorescence microscopy. Transcripts were measured by RT-PCR and BSMC proliferation by crystal violet staining.

Results: When human BSMC were exposed to transforming growth factor (TGF)- β , IL-13, or IFN- γ , their expression of CysLT1 receptor was significantly augmented in a time- and concentration-dependent manner. Interestingly, IL-4 had no significant effect on CysLT1 receptor expression in BSMC.

Moreover, IL-13 and IFN- γ but not TGF- β were able to increase CysLT1 mRNA levels. Finally, when BSMC were pretreated with TGF- β or IL-13 but not IFN- γ , their responsiveness to LTD₄ was markedly enhanced in terms of BSMC proliferation. Whereas TGF- β , IL-13, or LTD₄ alone had little effect on BSMC proliferation, preexposure of the cells to TGF- β or IL-13 for 24 hours resulted in a significant increase in proliferation in response to LTD₄. The enhanced proliferation was totally prevented by pretreating the cytokine-primed BSMC with the selective CysLT1 receptor antagonist Montelukast.

Conclusions: Taken together, our findings indicate a synergy between certain cytokines and cysLTs, mediated by the augmented expression of the CysLT1 receptor and subsequent LTD₄-triggered BSMC proliferation. These findings support a role for cysLTs in the airway remodeling observed in asthmatic patients and may provide a rationale for preventive and therapeutic intervention. (*J Allergy Clin Immunol* 2003;111:1032-40.)

Key words: Bronchial smooth muscle, cysteinyl leukotrienes, leukotriene receptors, leukotriene D₄, airway remodeling, asthma, transforming growth factor- β , IL-13, IFN- γ

Airway remodeling occurs in chronic asthmatic inflammation, and features of this response include myocyte hyperplasia and hypertrophy. An increase of 50% to 230% and 25% to 150% is observed in the area of airway smooth muscle in fatal and nonfatal asthma, respectively.¹ Increased thickness of the airways may have an important amplifying effect in the contractile response of bronchial smooth muscle cells (BSMC) and may be a major mechanism contributing to airway hyperresponsiveness in asthma.^{2,3} A growing body of evidence suggests that cysteinyl-leukotrienes (cysLTs) may induce airway smooth muscle remodeling in animal models^{4,5} and potentiate the proliferation induced by growth factors.⁶

Cytokines are also thought to contribute to chronic inflammation and airway remodeling observed in asthmatic airways, and asthmatic individuals produce more transforming growth factor (TGF)- β 1 and IL-13 than normal subjects.^{7,8} TGF- β 1 is a potent profibrotic T_H3-type cytokine, which may induce structural changes within the airways, such as subepithelial fibrosis,⁷ thickening of the reticular lamina,⁹ and human airway smooth muscle proliferation by increasing the expression of insulin-like growth factor binding protein-3 (IGFBP-3).¹⁰ IL-13, a T_H2-type cytokine, may have important effects on gene expression in airway cells that contribute to the phenotypic features of asthma. IL-13 can induce the expression of secreted factors in airway smooth muscle cells, such as basic fibroblast growth factor (bFGF); bFGF induces BSMC proliferation, which could contribute to the asthma phenotype through autocrine or paracrine effects on other airway cells.¹¹ Data suggest that IL-13 causes subepithelial fibrosis by the production and activation of TGF- β 1 in lung macrophages.¹² Although there is no unanimity on the matter, there is evidence showing that the expression levels of IFN- γ , a T_H1-type cytokine, are increased in asthmatic subjects.^{13,14} There is an increased frequency of IFN- γ producing CD4+ and CD8+ T cells in asthmatic individuals compared with normal subjects.¹⁵ Genetic studies have demonstrated that several loci in chromosome 6q24-q25 are linked either to asthma or wheezing. Interestingly, this region contains the gene coding for the IFN- γ recep-

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Abbreviations used

BSMC: Bronchial smooth muscle cell
cysLT: Cysteinyl leukotriene
CysLT1R: cysLT receptor type 1

tor ligand-binding chain.¹⁶ In human bronchial airway smooth muscle, IFN- γ can enhance CysLT1R expression and increase the contractile responses to LTD₄ and thus possibly contribute to the airway hyperreactivity observed in asthma.¹⁷

Previous studies from our laboratory have demonstrated that CysLT1R mRNA and protein expression can be upregulated by T_H2-type cytokines (IL-13, IL-4, and IL-5) in monocytes, macrophages, and eosinophilic HL-60 cells.^{18,19} To our knowledge, however, there are no studies on interactions of cytokines (such as TGF- β /IL-13) and cysLTs, in terms of human BSMC proliferation.

In the current work, we studied the modulation of CysLT1R expression in human BSMC by TGF- β , IL-13, and IFN- γ and the functional relevance of this modulation in terms of BSMC proliferation in response to LTD₄.

METHODS

Reagents

Human rIL-13, rIFN- γ , and TGF- β 1 were obtained from Pepro-Tech Canada, Inc (Ottawa, Ontario, Canada); all cytokine preparations contained <0.1 ng endotoxin per microgram (1 EU/ μ g); rabbit polyclonal anti-human C-terminal CysLT1R antibody was developed and characterized in collaboration with Cayman Chemical (Ann Arbor, Mich); rabbit IgG isotype control was obtained from Southern Biotechnology Associates (Birmingham, Ala); FITC-conjugated goat anti-rabbit IgG and Rhodamine (TRITC)-conjugated goat anti-rabbit IgG were obtained from Jackson Immuno Research Laboratories Inc (West Grove, Pa). LTD₄ was obtained from Cayman Chemical; Montelukast was obtained from Merck Research Laboratories (West Point, Pa). The p38 kinase inhibitor SB-203580 was from Calbiochem (San Diego, Calif). The MEK inhibitor PD-98059 and the PI-3 kinase inhibitor LY-294002 were from Biomol (Plymouth Meeting, Pa).

Cells

Primary cultures of normal human BSMC were purchased from the Clonetics Corporation (San Diego, Calif). The cells were cultured in 25-cm² flasks, in medium for human BSMC supplemented with 5% FBS, human recombinant epidermal growth factor (1 ng/mL), insulin (10 μ g/mL), human recombinant fibroblast growth factor (2 ng/mL), Gentamicin (50 μ g/mL), and Amphotericin B (0.05 μ g/mL) (SmGM-2 BulletKit; Clonetics) in an atmosphere of 5% CO₂ and 95% air at 37°C. When the cells became confluent, they were passaged with the use of 0.025% trypsin in 0.01% EDTA. Cells at passages 2 to 6 were used for experiments.

Flow cytometry

The expression of CysLT1R in BSMC was assessed with the use of a polyclonal anti-CysLT1R antibody directed against the carboxyl-terminal portion of the receptor.¹⁸ The antibody was raised against a peptide corresponding to amino acids 318-337 of the C terminus of human CysLT1R. For flow cytometry studies, BSMC cells were washed with PBS and fixed with 2% paraformaldehyde for 15 minutes at room temperature followed by permeabilization

with 0.1% saponin for an additional 15 minutes at room temperature. Cells were resuspended with PBS/2% BSA and labeled for 30 minutes at 4°C with anti-CysLT1R antibody (or with control, irrelevant antibody). Cells were then washed with cold PBS and incubated for 30 minutes at 4°C with FITC-conjugated goat anti-rabbit IgG. Finally, cells were washed again and resuspended in PBS before single-color immunofluorescence analysis of 5000 cells was performed on a FACScan flow cytometer (Becton Dickinson, San Jose, Calif). A 1:250 vol/vol dilution of the anti-CysLT1R antiserum and nonpertinent antibody was used in all cytometry studies.

Reverse transcriptase-polymerase chain reaction

Total RNA was extracted from BSMC with the use of Tri-Pure Isolation Reagent (Roche). Two micrograms of RNA was converted to cDNA by the reverse transcriptase enzyme reaction (AMV transcriptase-reverse; Promega, Madison, Wis) in a total volume of 20 μ L. PCR was performed in a final volume of 50 μ L containing 5 μ L of RT reaction product. Samples were placed in a Biometra T1 thermo cycler; (Montréal, Biotech Inc) for 34 cycles consisting of 2-minute denaturation at 94°C, 30-second annealing at 62°C, and 90-second extension at 72°C, followed by a final 5-minute extension at 72°C. CysLT1R was amplified with the primers derived from the published cDNA sequence for CysLT1R,²⁰ 5'-CGGGATCCGATGAAACAGGAAATC-3' as sense and 5'-CCGGAATTCATG GGTTTAACTATAC-3' as antisense. In each sample, the constitutively expressed, housekeeping gene transcript, GAPDH, was also amplified by using the following primers: 5'-GCTAGAGTAAGTAGTT-3' as sense and 5'-AACGACGAGCGTGAC-3' as antisense. A 10- μ L aliquot from each PCR was allowed to migrate by electrophoresis in a 1% agarose gel. The amplified CysLT1R fragment contained 1014 bp. The gel was then colored with ethidium bromide and photographed under UV transillumination. No PCR products were obtained when reverse transcriptase was omitted, indicating that there was no DNA contamination.

Fluorescent microscopy

BSMC were grown to confluence in 6-well plates containing sterile coverslips. Supernatants were eliminated, and cells were fixed with 2% paraformaldehyde and permeabilized with 0.1% saponin. Cells were then incubated overnight with antibody against the C-terminus of CysLT1R¹⁸ (1:250 vol/vol), washed, and incubated for 2 hours with rhodamine-labeled goat anti-rabbit antibody (1:1000). Indirect immunofluorescence was examined without counterstain by using an Axioscop 2 fluorescent microscope (Carl Zeiss, Inc, Thornwood, NY) equipped with a band-pass filter for fluorescence of FITC (Ex. D480/30;Em. D535/40) or tetramethylrhodamine isothiocyanate (TRITC) (Ex. D560/40;Em. D630/60) (both from Chroma Technology Corp). Photomicrographs of 1315 $\times$ 1033 pixels were captured by using $\times$ 40 oil immersion objective and a cooled SPOT color digital camera (Diagnostic Instruments Inc, St Sterling Heights, Mich). The images were processed with the use of SPOT software.

Proliferation assay

BSMC were grown in 96-well plates (3000 cells/well), starved for 24 hours (medium 1% FBS), and incubated for 24 hours with cytokines or medium. Supernatants were removed, and washed cells were incubated for 72 hours with 10⁻⁷ mol/L LTD₄. To assess the specificity of the effect for CysLT1R, separate cell cultures were preincubated for 30 minutes with 10⁻⁶ mol/L Montelukast before incubation for 72 hours with 10⁻⁷ mol/L LTD₄. The cells were fixed in 70% ethanol at -20°C, dried, and stained with crystal violet²¹ (1% in water). After washing, stained cells were solubilized in 33%

acetic acid, and absorbance was determined in a Thermomax microplate reader (Molecular Devices) at 570 nm. The analysis was performed at least in quintuplicate in four independent experiments.

Statistics

Results are expressed as mean $\pm$ SEM of separate experiments, performed on different days with different cell cultures. Replicate measurements within an experiment were averaged before comparing the results of a group of experiments. Data were analyzed for statistical significance using, as appropriate, the Student *t* test for paired values or ANOVA. A value of *P* < .05 was considered significant.

RESULTS

Cytokine-induced CysLT1R protein expression

The expression of CysLT1R protein in cultured human BSMC was investigated by flow cytometry with a specific anti-CysLT1R antibody. BSMC were treated for 24 hours with graded concentrations of TGF- β , IL-4, and IL-13, ranging from 2.5 to 15 ng/mL, and concentrations of IFN- γ ranging from 50 to 200 U/mL. Whereas the basal expression of CysLT1R in cultured BSMC was low, exposure of the cells to TGF- β , IL-13, or IFN- γ markedly increased their expression of the receptor (Fig 1).

Basal expression levels of CysLT1R protein were adjusted to 100%, and CysLT1R expression is illustrated as percentage of untreated control cells. TGF- β and IFN- γ induced a >100% increase in the expression of CysLT1R as compared with nonstimulated cells, whereas IL-13 induced a 50% increase. The effects of TGF- β , IL-13, and IFN- γ were maximal at 10 ng/mL, 5 ng/mL, and 100 U/mL, respectively. Given that IL-4, a prototypic T_H2-type cytokine, could also contribute to chronic inflammation in asthma, we tested graded concentrations of this cytokine but failed to observe any significant effect on CysLT1R expression in human BSMC (Fig 1). Although BSMC also expressed basal levels of CysLT2R, no modulation of expression was observed with either of the cytokines (data not shown).

In kinetic studies, the effects of the three active cytokines on CysLT1R expression were found to be maximal after 24 hours of stimulation (data not shown).

Cytokine-induced CysLT1R mRNA expression

To assess whether increased CysLT1R protein expression was associated with augmented CysLT1R mRNA levels, BSMC were stimulated with TGF- β , IL-13, or IFN- γ respectively, and their steady-state levels of CysLT1R mRNA were analyzed (Fig 2). BSMC constitutively expressed low levels of CysLT1R mRNA. IL-13 and IFN- γ but not TGF- β induced an augmentation of transcript levels after 24 hours of stimulation. Exposure of BSMC to cytokines for a shorter time period (8 hours) failed to show any significant modulation of CysLT1R mRNA (data not shown). Scanning densitometry analysis showed that IL-13 and IFN- γ augmented CysLT1R mRNA levels 3-fold and 2-fold, respectively, over baseline levels (Fig 2, B). TGF- β did not significantly modulate CysLT1R mRNA expression.

LTD₄-induced proliferation of cytokine-primed BSMC

The functional relevance of increased CysLT1R expression in TGF- β -stimulated, IL-13-stimulated or IFN- γ -stimulated BSMC was investigated in terms of proliferation in response to LTD₄. In control cells, LTD₄ alone failed to induce any significant proliferation (Fig 3). However, when BSMC had been exposed for 24 hours to TGF- β or IL-13, a significant proliferation was induced by subsequent treatment with LTD₄ (Figs 3 and 4). Addition of the cytokines to the BSMC culture at the time of treatment with LTD₄, however, did not induce any proliferation (data not shown). Interestingly, IFN- γ -stimulated BSMC failed to respond to LTD₄ in terms of proliferation (Fig 4). Moreover, IL-4, which was not able to enhance CysLT1R expression on BSMC, was also unable to prime the cells for augmented proliferation in response to LTD₄ (data not illustrated). Finally, to evaluate whether the LTD₄-induced proliferation observed in TGF- β or IL-13-stimulated cells was mediated through the enhanced expression of CysLT1R, BSMC were preincubated for 30 minutes with the CysLT1R antagonist Montelukast (10⁻⁶ mol/L) before the addition of 10⁻⁷ mol/L LTD₄. As shown in Fig 4, Montelukast was capable of totally preventing the proliferation induced by LTD₄ in both TGF- β -pretreated and IL-13-pretreated cells.

To assess the potential involvement of MAP kinase and PI3 kinase signaling pathways in the proliferative response of BSMC to LTD₄, cells were pretreated for 30 minutes with selective inhibitors before the addition of 10⁻⁷ mol/L LTD₄. As shown in Fig 5, the PI3 kinase inhibitor LY-294002 completely prevented the proliferative effect of LTD₄ in cells primed with either TGF- β or IL-13. It also had a small but significant inhibitory effect on basal BSMC proliferation in these cells. The MEK kinase inhibitor PD-98059 was also effective in significantly reducing LTD₄-induced proliferation, whereas the p38 kinase inhibitor SB-203580 was ineffective. These findings suggested the involvement of PI3 kinase and MEK but not of the stress-activated kinase p38 in LTD₄-induced BSMC proliferation.

DISCUSSION

Our results demonstrate that TGF- β , IL-13, and IFN- γ can upregulate the expression of CysLT1R protein in BSMC. The effect was time- and concentration-dependent. Interestingly, another prototypic T_H2-type cytokine, IL-4, had no effect on CysLT1R expression in human BSMC.

IL-13-treated and IFN- γ -treated cells also showed enhanced levels of CysLT1R mRNA expression. In contrast, TGF- β increased receptor protein expression without significantly affecting CysLT1R mRNA expression. These results suggest that the increased protein expression induced by TGF- β may be due to a translational or posttranslational effect, mediated either by increased protein translation or lower protein degradation. Additional

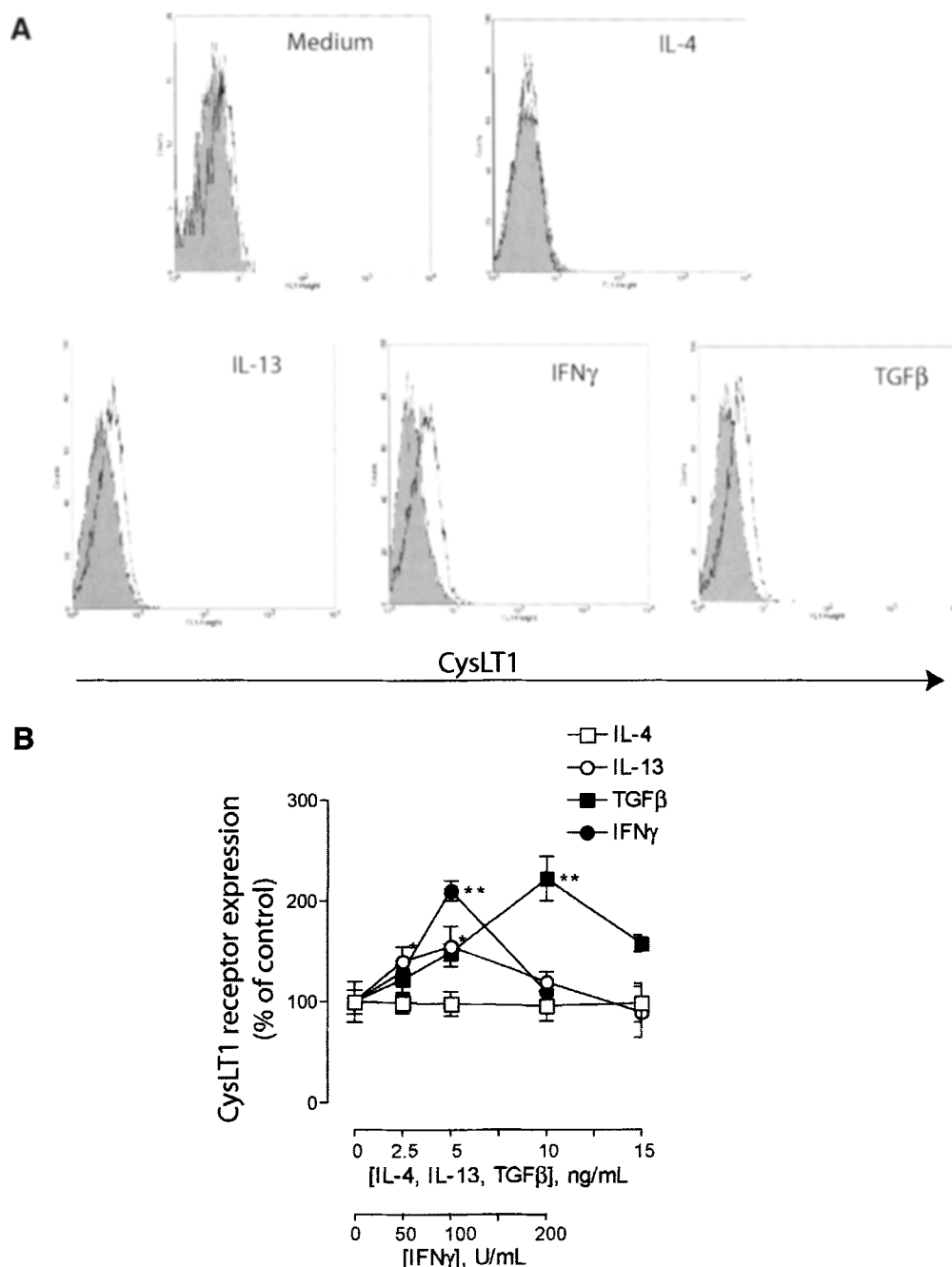


FIG 1. Flow cytometric analysis of CysLT1R expression in BSMC stimulated for 24 hours with IL-4, IL-13, TGF- β , or IFN- γ . **A**, Histograms of a single experiment, representative of three. *Shaded histograms* represent labeling with isotype control antibody. *Solid thick lines* represent labeling of medium-treated or cytokine-treated (IL-4, 10 ng/mL; IL-13, 5 ng/mL; TGF- β , 10 ng/mL; IFN- γ , 100 U/mL) cells, respectively, with anti-CysLT1R antibody. **B**, CysLT1R expression after BSMC treatment with graded concentrations of cytokines, with control normalized to 100% (n = 4; *P < .05; **P < .003).

studies will be needed to elucidate the mechanism(s) involved in CysLT1R upregulation induced by TGF- β .

Previous results obtained by our laboratory have demonstrated that CysLT1R expression levels are upregulated in eosinophilic HL-60 cells by IL-5 and in mono-

cytes and macrophages by IL-13 and IL-4 but not IFN- γ .^{18,19} In these myeloid cells, the increased expression of CysLT1R resulted in an enhanced responsiveness to LTD₄ in terms of Ca²⁺ transients and chemotaxis. In the current study, we showed that TGF- β , IFN- γ , and IL-13

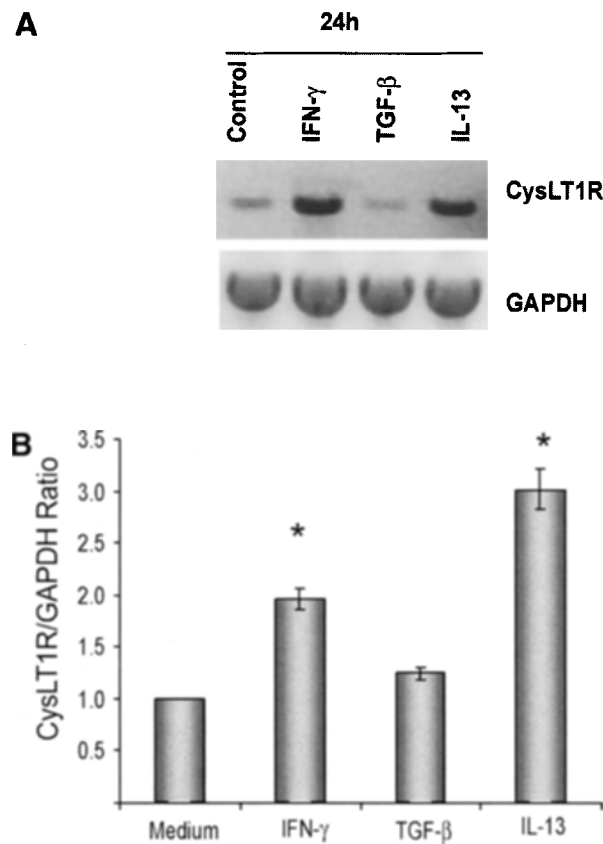


FIG 2. Effect of IFN- γ , TGF- β , and IL-13 on CysLT1R mRNA expression. **A**, BSMC were stimulated for 24 hours with 100 U/mL, 10 ng/mL, and 5 ng/mL of IFN- γ , TGF- β , and IL-13, respectively. RT-PCR reaction was performed with primers for CysLT1R and GAPDH. Reaction products were subjected to electrophoresis on 1% agarose gel. **B**, Scanning densitometry of gels normalized for medium. Data are mean $\pm$ SEM of 4 separate experiments (* P < .05).

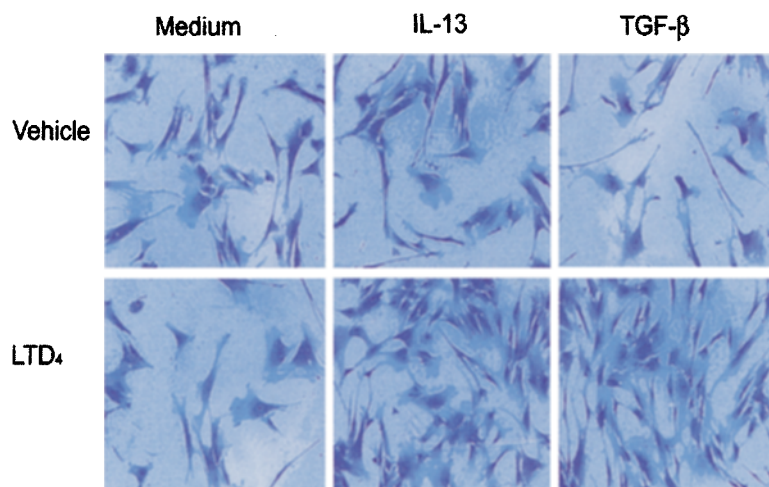


FIG 3. Proliferation assay. Photomicrographs of crystal violet-stained BSMC were taken before resuspending in 33% acetic acid. Cells were treated for 24 hours with medium (controls) or cytokines, followed by incubation for 72 hours with vehicle (*top row*) or with 10^{-7} mol/L LTD₄ (*bottom row*). Results of a single experiment, representative of 4 independent experiments performed in quintuplicate are illustrated.

but not IL-4 can also upregulate CysLT₁R expression in bronchial smooth muscle, suggesting that CysLT₁R expression is regulated differentially in a cell-specific manner. Moreover, a bell-shaped concentration response curve was observed in the cytokine-mediated effect. A bell-shaped isotherm is unusual in cytokine interactions with their receptors, although it is frequently observed with ligand interactions with G-protein-coupled receptors. In the latter, bell-shaped isotherms may be attributed to receptor desensitization at higher ligand concentrations, to different signal transduction systems activated by ligand binding at two independent receptors or to agonist-activated receptor coupling to multiple G proteins, which mediate opposing actions on a downstream effector.²² It is unclear, however, whether any such mechanisms are operative in our system. The current study is, to our knowledge, the first demonstration that the upregulation of CysLT₁R induced by TGF- β and IL-13 increases human BSMC proliferation in response to LTD₄. The CysLT₁R antagonist Montelukast ablated this effect, suggesting that the proliferation effect was selective for CysLT₁R. As previously reported in the literature,^{10,23} TGF- β could also induce BSMC proliferation by itself. Recently, Amrani et al¹⁷ reported that IFN- γ could upregulate CysLT₁R expression in human airway myocytes and that this upregulation increased their contractile responses to LTD₄. This may contribute to the airway hyperreactivity observed in asthma.^{24,25} In the Brown Norway rat model, the increase in bronchial responsiveness induced by inhaled antigen was reported to be directly related to an increase in the mass of BSMC, and both increases were blocked by the CysLT₁R antagonist MK571.⁵ However, in our study, human BSMC pretreated with IFN- γ did not show increased proliferation when exposed to LTD₄, although the expression of CysLT₁R was augmented by this cytokine. The reason for this discrepancy is not readily evident, and, despite the known inhibitory effects of IFN- γ on cell proliferation and protein synthesis,²⁴⁻²⁶ no decrease in basal BSMC proliferation was observed with IFN- γ .

It has been demonstrated that a significant increase in the area of airway smooth muscle occurs in chronic asthmatic inflammation.¹ A growing body of evidence suggests that CysLTs may have a role in airway remodeling. Chronic overproduction of relatively low levels of CysLTs by mast cells or eosinophils may have subtle effects on structural cells of the airways, leading to bronchial hyperresponsiveness and possibly influencing pathways involved in airway wall remodeling.²⁷ Evidence suggests that LTD₄ can potentiate the induction of DNA synthesis and proliferation induced by the mitogen epidermal growth factor in human BSMC.⁶ In a mouse model, CysLTs appeared to be responsible for the increase in airway smooth muscle after repeated allergen challenge. The allergen-induced increases in airway smooth muscle were significantly reduced by treatment with a CysLT₁R antagonist.⁴ In Brown Norway rats sensitized to ovalbumin, CysLTs induced airway smooth muscle and epithelial cell DNA synthesis

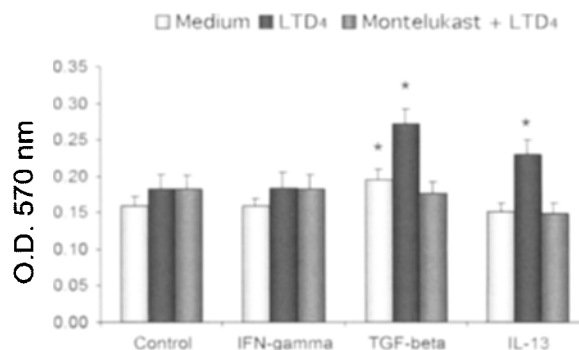


FIG 4. Proliferation assay. BSMC were grown in 96-well plates, starved for 24 hours (medium with 1% FBS), and incubated for 24 hours with cytokines or medium. Supernatants were then removed and cells were incubated for 30 minutes with medium or 10⁻⁶ mol/L Montelukast, followed by 72 hours with 10⁻⁷ mol/L LTD₄. BSMC proliferation was measured as detailed in the Methods section. Analysis was performed in quintuplicate in 4 independent experiments (**P* < .05).

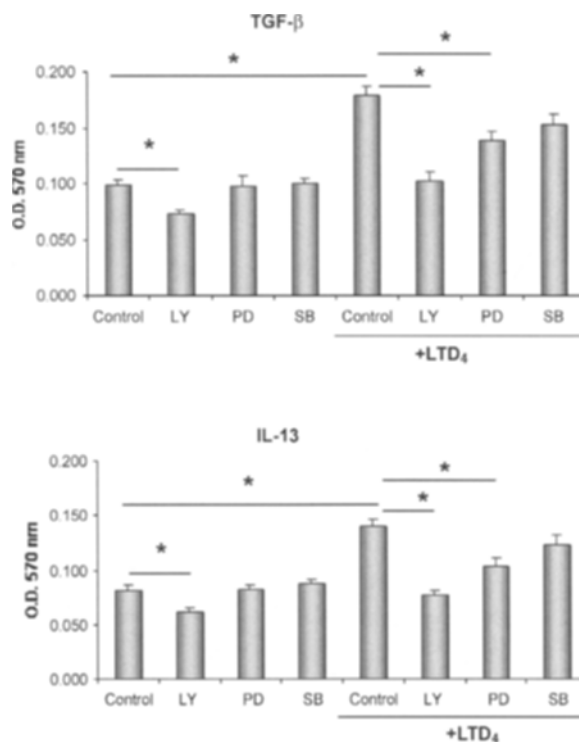


FIG 5. Proliferation assay. BSMC were grown in 96-well plates, starved for 24 hours (medium with 1% FBS), and incubated for 24 hours with TGF- β (upper panel) or IL-13 (lower panel). Supernatants were then removed and cells were incubated for 30 minutes with medium or kinase inhibitors, followed by 72 hours with 10⁻⁷ mol/L LTD₄. BSMC proliferation was measured as detailed in the Methods section. Analysis was performed in quintuplicate in 4 independent experiments (**P* < .05; ***P* < .01; ****P* < .001). LY indicates LY-294002 (PI3 kinase inhibitor; 1 μ mol/L); PD, PD-98059 (MEK kinase inhibitor; 50 μ mol/L); SB, SB-203580 (p38 kinase inhibitor; 10 μ mol/L).

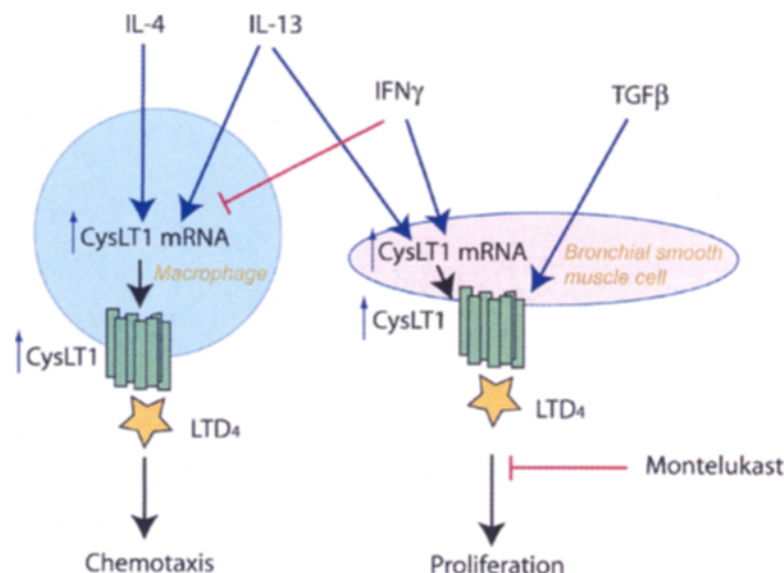


FIG 6. Schematic representation of modulation of CysLT1 receptor expression by cytokines in human macrophages¹⁹ and bronchial smooth muscle cells.

as well as airway smooth muscle thickening after repeated allergen exposure.⁵

Studies also provide evidence that cytokines such as TGF- β , a T_H3-type cytokine, may also play a role in airway remodeling. Data obtained by Cohen et al¹⁰ suggest that TGF- β 1 can induce human BSMC proliferation by increasing the expression of IGFBP-3. Hence, the mitogenic action of TGF- β 1 could be relevant in the hyperplastic nature of BSMC in chronic asthma. In normal human lung, the bronchial epithelial compartment appears to be the main source of TGF- β .²⁸ The expression levels of this cytokine are dramatically increased in asthma and several other lung disorders.^{27,29}

The T_H2-type cytokine IL-13 is also overexpressed in asthmatic patients.³⁰ Interestingly, data suggest that both IL-4 and IL-13 cause inflammation, but only IL-13 causes subepithelial fibrosis.¹² The tissue remodeling induced by IL-13 is mediated, to a great extent, by the production and activation of TGF- β 1 in lung macrophages.^{12,31} Data also support the role of IL-13 in airway remodeling by modulating the production of TGF- β 2 from human bronchial epithelial cells. The release of TGF- β 2 can activate the underlying myofibroblasts to secrete matrix proteins and smooth muscle mitogens to propagate remodeling changes into the submucosa. Interestingly, IFN- γ reduced the release of TGF- β 2 induced by IL-13 in human bronchial epithelial cells.^{32,33}

Although IL-4 and IL-13 share a common receptor complex of IL-4Ra-IL-13Ra1 in airway smooth muscle cells³⁴ and common signaling pathways involving STAT6 and ERK mitogen-activated protein kinase activation,^{34,35} IL-4 neither upregulated CysLT1 receptor expression nor synergized with LTD₄ for BSMC proliferation, in contrast to IL-13. A differential effect of IL-4

and IL-13 was also reported by Laporte et al³⁴ in airway smooth muscle cells in which IL-13 but not IL-4 reduced β -adrenergic responsiveness. Conversely, Pype et al³⁶ found IL-4 to inhibit IL-1 β -induced MCP-1 and MCP-2 expression in BSMC, whereas IL-13 did not. Interestingly, gene chip experiments show a distinct although partially overlapping array of genes expressed by BSMC in response to IL-4 and IL-13.³⁷

Taking all the above data into consideration, it is likely that the smooth muscle proliferation seen in the asthmatic airways is due to a positive feedback interaction between cysLTs and increased levels of TGF- β and IL-13. In our model, the number of receptors for LTD₄ was increased by TGF- β and IL-13 (the levels of which are augmented in asthmatic airways). Increased receptor expression could lead to increased LTD₄ signaling, resulting in BSMC proliferation. Although such a cytokine-leukotriene synergy could be due to cytokine-upregulated CysLT1 receptor expression, it could also be due to selective interactions between the signaling pathways of IL-13 and TGF- β and LTD₄, independent of CysLT1 receptor density. Distinction between the two mechanisms, which are not mutually exclusive, is difficult, but the following findings should also be considered: First, eliminating the preincubation period with IL-13 or TGF- β and adding them at the time of addition of LTD₄ abolished the synergy. Second, preincubating with IL-4, which did not upregulate CysLT1 receptor expression, did not result in synergy with LTD₄. Since these observations suggest but cannot prove a causal relation between upregulated CysLT1 receptor expression and synergy between cytokines and LTD₄ in BSMC proliferation, this relation should be considered as an association rather than a cause and effect, at this time.

Although the exact proliferation mechanism is unknown, our data suggest that MAP kinase (ERK) and PI3-kinase pathways may be involved, given that these pathways are the major positive regulators of airway smooth muscle proliferation.^{38,39} However, additional experiments are needed to understand the precise mechanisms involved in this process.

In conclusion, as illustrated in Fig 6, we demonstrated that TGF- β , IL-13, and IFN- γ but not IL-4 can upregulate the protein expression of CysLT1R in human BSMC. These cytokines, except for TGF- β can also increase CysLT1R mRNA expression. We further demonstrated that TGF- β and IL-13 were able to increase the proliferation of human BSMC in response to LTD₄. BSMC proliferation was CysLT1R-dependent, given that Montelukast ablated this effect. Taken together, our findings support a role for CysLT1R in the airway remodeling observed in asthma and may provide a rationale for preventive and therapeutic intervention at this level.

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