CysLT1R modulation by Th1 Th2 and Th3 type cytokines in BSMC

Par
Karina Espinosa
Service d'immunologie
Département de pédiatrie

Mémoire présenté à la Faculté de Médecine
en vue de l'obtention du grade de
maître ès sciences (M.Sc.) en Immunologie

Juillet 2002
The author has granted a non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author’s permission.

L’auteur a accordé une licence non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de cette thèse sous la forme de microfiche/film, de reproduction sur papier ou sur format électronique.

L’auteur conserve la propriété du droit d’auteur qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

0-612-80583-2
Résumé

L'inflammation asthmatique chronique est caractérisée par le remodelage des voies aériennes, la dénudation de l'épithélium, l'épaississement des parois bronchiques, la fibrose sous-épithéliale, la métaplasie des glandes à mucus, ainsi que par l'hyperplasie et l'hypertrophie des muscles lisses bronchiques. Certaines études suggèrent un rôle possible pour les cystéinyl-leucotriènes (CysLTs) dans le remodelage des voies aériennes. Ce travail vise à caractériser la modulation du récepteur CysLT1 (CysLT1R) par des cytokines de type Th1, Th2 et Th3 dans un processus fonctionnel tel que le remodelage des voies aériennes. MÉTHODES: L'expression du CysLT1R dans des cellules de muscle lisse bronchique humaines (CMLB) provenant de cultures primaires a été évaluée par cytométrie de flux, avec un anticorps polyclonal anti-CysLT1R, après stimulation avec les cytokines pendant 24h. Une analyse par microscopie de fluorescence a été effectuée pour compléter les résultats obtenus par la cytométrie de flux. L'expression de l'ARNm de CysLT1R a été mesurée par RT-PCR à l'aide d'amorces spécifiques pour ce récepteur et pour GAPDH. La prolifération a été évaluée par une analyse colorimétrique dans des cellules traitées pendant 24h avec les cytokines puis pendant 72h avec LTD4. RESULTATS: L'expression de CysLT1R chez les CMLB a été modulée à la hausse par TGF-β, IL-13 et IFN-γ. IL-13 et IFN-γ, mais pas TGF-β, ont augmenté l'expression de l'ARNm de CysLT1R. La modulation à la hausse de CysLT1R
induite par TGF-β et IL-13 a augmenté la prolifération des CMLB en réponse à LTD₄. L'antagoniste de CysLT1R, Montelukast, a inhibé cet effet, ce qui suggère que l'effet de prolifération est sélectif pour CysLT1R. Ensemble, nos résultats soutiennent le rôle des CysLTs dans le remodelage des voies aériennes observé chez les patients asthmatiques.
## Table of Contents

Table of Contents .................................................................................................................. i
Diagrams and Tables ..................................................................................................... iii
Abbreviation list ............................................................................................................ iv
Résumé .................................................................................................................................

**Introduction** ................................................................................................................. 1

**Part 1 Asthma** ................................................................................................................. 1

1.1. Definition ...................................................................................................................... 1

1.2. The early and late reaction ........................................................................................... 1

The early phase reaction ..................................................................................................... 1

The late phase reaction ....................................................................................................... 2

**Part 2. Factors in the Development and Regulation of Airway Inflammation** ......................................................................................................................... 4

2.1. Airway smooth muscle cells in asthma ....................................................................... 4

The passive role .................................................................................................................. 4

The active role .................................................................................................................... 8

2.3. Biological actions of IFN-γ, IL-13 and TGF-β. ............................................................ 13

IFN-γ .................................................................................................................................. 13

TGF-β ............................................................................................................................... 15

IL-13 .................................................................................................................................. 19

2.4. Cysteinyi Leukotrienes ............................................................................................... 21

Synthesis ............................................................................................................................ 21
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biological actions</td>
<td>24</td>
</tr>
<tr>
<td>Cysteinyl Leukotriene Receptors</td>
<td>28</td>
</tr>
<tr>
<td>Objectives and strategies</td>
<td>32</td>
</tr>
<tr>
<td>Article</td>
<td>34</td>
</tr>
<tr>
<td>Introduction</td>
<td>37</td>
</tr>
<tr>
<td>Discussion</td>
<td>59</td>
</tr>
<tr>
<td>Conclusions</td>
<td>68</td>
</tr>
<tr>
<td>References</td>
<td>69</td>
</tr>
</tbody>
</table>
Diagrams and Tables

Diagram 1 ......................................................... 22

Table 1 ............................................................. 27

Table 2 ............................................................. 31
Abbreviation list

AHR: Airway hyperresponsiveness
ASMC: Airway smooth muscle cells
BAL: Bronchoalveolar lavage
BFGF: Basic fibroblast growth factor
BSMC: Bronchial smooth muscle cells
cAMP: Cyclic adenosine monophosphate
COX: Cyclo-oxygenase
CysLTs: Cysteinyi leukotrienes
CysLT1R: Cysteinyi leukotriene 1 receptor
CysLT2R: Cysteinyi leukotriene 2 receptor
ERK: Extracellular signal regulated kinase
Fc-ε-RII: Fragment crystallizable receptor type II
GM-CSF: Granulocyte monocyte-colony stimulating factor
GPCRs: G-protein coupled receptors
HASMC: Human airway smooth muscle cells
ICAM: Intercellular adhesion molecule
IFN-γ: Interferon gamma
LPS: Lipopolysaccharide
LT: Leukotriene
LTAs: Leukotriene antagonists
MAPK: Mitogen-activated protein kinase
MCP: Monocyte chemoattractant protein
OSM-R: Oncostatin M receptor
PAR2: Proteinase-activated receptor 2
PI3 K: Phosphatidylinositol 3-kinase
PGE2: Prostaglandin E2
RV: Rhinovirus
SES: Socio-economic status
STAT: Signal transducer and activator of transcription
Th: T helper
TNF-α: Tumor necrosis Factor alpha
TNFR: Tumor necrosis Factor receptor
VCAM: Vascular cell adhesion molecule
Introduction

Part 1 Asthma

1.1. Definition

Asthma is a chronic lung disease characterized by obstruction, inflammation, and hyperresponsiveness of the airway to a variety of nonspecific stimuli. The obstruction is recurrent, partially reversible due to airway remodeling (Fahy et al., 2000) and manifests clinically as dyspnea, wheezing and/or coughing (Monteleone and Sherman, 1997).

In asthma, there is an early and a late phase reaction;

1.2. The early and late reaction

The early phase reaction

The early asthmatic reaction develops within 20 min after exposure to allergen and is characterized by bronchospasm due to classical immediate-type hypersensitivity, triggered through allergen-induced cross-linking of specific IgE antibody bound to mast cells through high-affinity receptors (Host, 2000).
The activated mast cells release proinflammatory mediators which are divided into three groups: secretory granule preformed mediators (histamine, heparin, tryptase, chymase, cathepsin G and carboxypeptidase), cytokines produced upon activation (IL-3, IL-4, IL-5, IL-6 and TNF-alpha) and lipid mediators produced upon activation (Leukotrienes C4, D4, E4, Prostaglandin D2 and Platelet Activating Factor). These mediators are capable of inducing constriction of airway smooth muscle, increased secretion of mucus, and vasodilatation, as well as several important biological events, including modulation of the expression of adhesion molecules in endothelial and epithelial cells, and of inflammatory cell recruitment. Clinically, acute airway inflammation is characterized not only by a bronchoconstrictive response but also by a series of airway events that lead to enhanced bronchial hyperresponsiveness, mucus secretion and increased asthma severity (Abbas, A, 2000), (Nadel, 1998).

*The late phase reaction*

Cellular recruitment to the airway is a key factor in this process. There is an initial increase in neutrophils followed by the appearance of eosinophils. There is less evidence for the involvement of neutrophils in asthma than for eosinophils, but neutrophils may have a greater importance in the pathogenesis of severe asthma. Persistent neutrophil inflammation is present in bronchoalveolar lavage (BAL) fluid, biopsy specimens and sputum from patients with symptomatic severe asthma treated with high doses of glucocorticoids (Leckie, 2000).
Eosinophils show several biological effects that can modulate inflammatory cellular reactions and promote further inflammation. The preformed mediators in eosinophils include several proteins, such as Major Basic Protein, which is cytotoxic to epithelial cells, it has been identified in areas of bronchial denudation. Eosinophils also have specific granules that contain eosinophil peroxidase and Eosinophil Cationic Protein. They can synthesize other mediators including leukotrienes C₄, D₄, E₄ and lipoxins as well as proinflammatory cytokines (IL-4 and IL-13) that promote Th2 differentiation. Eosinophils can thus contribute to bronchoconstriction, mucus secretion and changes in vasopermeability (Nadel, 1998), (Abbas, A, 2000). Th2 lymphocytes are essential for the late-phase response, because they produce cytokines that promote allergic responses like IL-4, IL-13 and IL-5. In contrast, Th1 lymphocytes produce IFN-γ, IL-2 and TNF-β.
Part 2. Factors in the development and regulation of airway inflammation

2.1. Airway smooth muscle cells in asthma

Bronchial smooth muscle cells (BSMC) can have a passive and active role in asthma. For many years, researchers have considered BSMC as a passive tissue subjected to the actions of different cell mediators that could induce airway remodeling and bronchial smooth muscle contraction in asthmatic patients. But new findings demonstrate that BSMC could also have an active role. They can secrete several proinflammatory mediators and express different receptors that contribute to the chronic inflammation seen in the asthmatic airways.

The passive role

Airway remodeling occurs in chronic asthmatic inflammation, features of this response include epithelium denudation, wall thickening, subepithelial fibrosis, mucus metaplasia, myofibroblast hyperplasia, and myocyte hyperplasia and hypertrophy. Vascular abnormalities have also been described (Elias, 2000), (Wiggs et al., 1992), (Aikawa et al., 1992). The consequences of airway remodeling in asthma may include incompletely reversible airway narrowing, bronchial hyperresponsiveness, airway edema, and mucus hypersecretion.
Airway remodeling in asthma thus may predispose persons with asthma to asthma exacerbations and even death from airway obstruction caused by smooth muscle contraction, airway edema, and mucus plugging (Fahy et al., 2000).

The number and size of bronchial blood vessels is increased in asthma, and these vessels may have an important role in regulating airway caliber, because an increase in vascular volume may swell the mucosa and narrow the airway lumen. Many inflammatory mediators cause vasodilation, a response that may be accompanied by increased permeability at the postcapillary venule, plasma extravasation, and airway mucosal edema (Laitinen et al., 1987), (McDonald, 1987). There is little evidence for bronchial narrowing due to the mechanical effect of peribronchial edema, or by swelling of the bronchial mucosa. However, edema foam may terminally cause grave airway obstruction (Snashall and Chung, 1991).

Compared with nonasthmatic subjects, airway wall thickness is increased from 50 to 300% in cases of fatal asthma and from 10 to 100% in cases of nonfatal asthma. There is also an increase of 50-230% and 25-150% in the area of airway smooth muscle (ASM) in fatal and nonfatal asthma, respectively (Elias, 2000). Increased thickness of the airway may have an important amplifying effect in the contractile response of ASM and may be a major mechanism contributing to airway responsiveness in asthma (James et al., 1989), (Wiggs et al., 1992).

The degree to which hyperplasia versus hypertrophy contributes to this response is controversial. Ebina and colleagues (Ebina et al., 1993), described two
differing responses; In Type I asthma the increase in muscle mass was associated only with hyperplasia (increase in cell number) and was restricted to large central airways. In Type II asthma, comparatively mild hyperplasia was seen in the large airways and hypertrophy (increase in cell size) was detected throughout the bronchial tree, particularly in small peripheral airways. IL-1 β and IL-6 can induce hyperplasia and hypertrophy of cultured guinea pig airway smooth muscle cells (De et al., 1995).

Several mediators can contribute to the proasthmatic changes in airway remodeling. For example, human mast cell mediators can cause human airway smooth muscle (HASM) proliferation, encourage fibrosis, and thereby contribute to airway remodeling and narrowing; they can cause contraction and increase the responsiveness of HASM to contractile mediators, also contributing to airway narrowing; and last but perhaps most important, these mediators can increase proinflammatory cytokine release and surface protein expression on HASM, thus favoring inflammatory cell infiltration and activation (Page et al., 2001). Studies have reported other differences between the airway smooth muscle from asthmatic and normal subjects. Data suggest that in asthmatic patients, the airway smooth muscle velocity of shortening is increased and the relaxation decreased (Que et al., 2000), (Solway, 2000). There are two important consequences of increased velocity of smooth muscle shortening. First, experiments demonstrate that sensitized tissues with increased velocity of shortening exhibit a greater extent of total shortening in response to contractile stimulation. Second, (which has not yet been proved experimentally) theory
suggests that a faster velocity of shortening should induce contractile hyperresponsiveness, by minimizing the bronchodilating effect of a deep inhalation (Black et al., 2001).

When a bronchospasm occurs, deep inspirations may be one of the most potent bronchodilating strategies, and they comprise the first line of defense. But in the spontaneous asthmatic attack, this potent bronchodilating mechanism fails and deep inspirations only make matters worse. The negative intrathoracic pressure associated with deep inspiration occurring in the context of increased leakiness of the airway vasculature may temporarily increase airway oedema and thus reduce luminal diameter in subjects with asthma (Burns and Gibson, 2002).

In asthma it exists a perturbed equilibrium of myosin binding (few cross-links attached at any moment, but cycling rapidly), where the fully activated muscle becomes much less stiff and much more viscous, almost as if the muscle had "melted" (Fredberg, 2000).

In asthma, not only the contractile responses of airway smooth muscle are altered, data show that airway smooth muscle from asthmatic patients have a higher proliferation rate compared with that of non-asthmatic patients (Johnson et al., 2001). There is a strong possibility, that this phenomenon is regulated by the ERK (extracellular signal regulated kinase) and PI3-K (phosphatidylinositol 3-kinase) pathways, because they appear to be major positive regulators of airway smooth muscle proliferation (Page and Hershenson, 2000).
The active role

As we mentioned before, BSMC not only have a passive role in asthma. They may also have an active immunomodulatory role by secreting chemokines, cytokines and expressing cell adhesion molecules or receptors that are important in modulating airway inflammation (Lazaar and Panettieri, 2001).

Production and expression of inflammatory molecules

Data suggest that HASM could express and release the C-X-C chemokine IL-8, (a major neutrophil chemoattractant involved in asthma exacerbations), induced by TNF-α and IL-1-β. This effect was inhibited by Th2 cytokines such as IL-4, IL-10, IL-13 and also by Dexamethasone (John et al., 1998), (Pang and Knox, 2000).

TNF-α induced IL-6 and RANTES secretion from HASM and this effect could be inhibited by [cAMP](i) elevating agents such as isoproterenol, PGE_2, the cAMP analog dibutyl-cAMP, or by the phospho diesterase inhibitors rolipram and cilomilast (Ammit et al., 2002).

Interleukin-13 and Interleukin-4 induced the release of Eotaxin by HASMC. This effect is synergistic with interleukin-1β and is mediated by the Interleukin-4 receptor alpha-chain (Hirst et al., 2002). Additional chemokines secreted by HASM are MCP-1, MCP-2 and MCP-3 (Pype et al., 1999), (Ghaffar et al., 1999).
We can also find several cytokines and their receptors expressed by BSMC, such as the receptors for TGF-β1 (de Boer et al., 1998). RT-PCR studies showed that HASM cells express transcripts for IL-4, IL-13RI, and IL-13RII, but not for the common IL-2R chain. JAK1, JAK3, and Tyk2 are expressed in cultured HASM cells, whereas JAK2 protein is not. IL-4 and IL-13 stimulation both lead to STAT-6 and ERK MAP kinase phosphorylation, but the time course of activation of STAT-6 differs for the two cytokines. IL-13 reduces the ability of isoproterenol to decrease HASM cell stiffness and to increase cAMP formation, whereas IL-4 does not (Laporte et al., 2001).

Human ASM endogenously express both Th2-type (IL-5, GM-CSF) and Th1-type cytokines (IL-2, IL-12, and IFN-γ) and their respective receptors. These molecules are sequentially upregulated in the atopic asthmatic sensitized state and they act to upregulate and downregulate proasthmatic perturbations in ASM responsiveness, respectively (Lazzeri et al., 2001), (Chung et al., 1999), (Hakonarson et al., 1999a), (Hakonarson et al., 1999b).

Other groups have reported the presence of both 50-60 kDa type I TNF receptor (TNFR1) and 70-80 kDa type II TNFR (TNFR2) receptor subtypes in ASM cells and that this receptors selectively activated the stress kinases, c-Jun N-terminal kinase and p38 mitogen-activated protein kinase (p38 MAPK) (McFarlane et al., 2001), (Amrani et al., 2001a).
IL-6R, IL-11R, and OSM-R (oncostatin M receptor) are expressed in Human airway smooth muscle cells. The activation of these receptors by members of the IL-6 cytokine family induced the phosphorylation of STAT3 and increased COX-2 expression and/or PGE_2 release in HASM cells. OSM is produced by macrophages and neutrophils, hence, it is possible that it might contribute to COX-2 expression and PGE_2 release with illnesses characterized by airway inflammation, such as asthma and other chronic obstructive pulmonary diseases (Lahiri et al., 2001), (Elias et al., 1997).

*Interactions between T cells and ASM*

Findings suggest that the interaction between activated T cells and HASM could induce proasthmatic-like changes in ASM and may have significant implications for inflammatory diseases.

The adhesion of anti-CD3-activated T cells to the surface of naive human ASM cells, upregulated the cell surface expression of CD25 in both cell types, as well as the mRNAs and protein expression of the cell adhesion molecules (CAMs)/costimulatory molecules, CD40, CD40L, CD80, CD86, ICAM-1 (CD54) in HASM. This upregulation increased contractile responsiveness to acetylcholine and impaired relaxation responsiveness to isoproterenol in isolated rabbit ASM tissues. This effect was abrogated by the pretreatment of ASM cells and tissues with mAbs directed either against CD11a or the combination of CD40 and CD86 (Hakonarson et al., 2001).
Similar data found by Lazaar and colleagues (Lazaar et al., 1997), demonstrated that anti-CD3-stimulated peripheral blood T cells also adhere to ASM and markedly upregulate the expression of ICAM-1 and induce the expression of MHC class II on HASM (although they can not present alloantigen to CD4+ T cells).

Results found by the same group, showed that the expression of VCAM-1 in HASM cells is linked to multiple signaling pathways and may function to augment growth factor-induced responses (Lazaar et al., 2001). They have also demonstrated that cultured human ASM express CD40 and that this expression can be upregulated by treatment with TNF-α or IFN-γ (Lazaar et al., 1998).

Proteinase-activated receptor 2 (PAR2) expression was detected in primary cultures of human bronchial smooth muscle cells. PAR2 activation mobilizes intracellular Ca2+ and induces contraction. PAR2 agonists, including trypsin and tryptase, induce bronchoconstriction of human airway by stimulating smooth muscle contraction (Schmidlin et al., 2001).

Evidence suggests that human ASM tissue expresses Fc-εRII. Interestingly the expression of this receptor is selectively increased in atopic asthmatic ASM. This phenomenon is associated with IgE immune complex/Fc-εRII-mediated elaboration of IL-1β by the ASM itself (Heise et al., 2000).

Under non-inflamed conditions, bronchial epithelial and smooth muscle cells of both human and mouse lung expressed C3a and C5a receptor protein (C3aR
and C5aR) and mRNA. C3aR expression increased significantly on both bronchial epithelial and smooth muscle cells in mice treated with LPS (Drouin et al., 2001).

Two important receptors expressed in human airways, are CysLT1R and CysLT2R, the receptors for the cysteinyi leukotrienes LTC₄, LTD₄, and LTE₄. They are members of the G protein-coupled receptor family and they mediate the contractile and inflammatory actions in human airways (Lynch et al., 1999), (Heise et al., 2000), (Nothacker et al., 2000).

Evidence suggests that CysLTs may participate in the remodeling of human airways by potentiating the effects of BSMC growth factors. In this study we wanted to test the hypothesis that the interaction between cytokines increased in asthmatics patients (such as TGF-β, IL-13 and IFN-γ) and CysLTs can induce BSMC proliferation when exposed to LTD₄.
2.3. Biological actions of IFN-\(\gamma\), IL-13 and TGF-\(\beta\).

So far, we have discussed the passive and active role of BSMC in asthma. We have seen that this cell type can secrete several mediators that contribute to inflammation. Now, we will discuss the importance of other mediators released by T cells such as cytokines. There are many cytokines that play a role in asthma, but we will review the biological functions of the three cytokines used in this study; IFN-\(\gamma\) (Th1 type), IL-13 (Th2 type) and TGF-\(\beta\) (Th3 type).

\textit{IFN-\(\gamma\)}

Viral infections play an important role in the exacerbation of asthma. The production of interferons is well known to limit viral spread, but IFN-\(\gamma\) can also prime alveolar macrophages to release more inflammatory cytokines, such as tumor necrosis factor-alpha and macrophage inflammatory protein-1\(\alpha\). Thus, IFN-\(\gamma\) produced at times such as during viral infections, can prime alveolar macrophages for enhanced release of inflammatory mediators during allergic reactions (Dery and Bissonnette, 1999).

During rhinovirus (RV) infections, the immune response is considered to contribute to upper respiratory symptoms and may also be an important contributor to lower airway dysfunction in patients with asthma (Parry et al., 2000). Cells producing IFN-\(\gamma\) may contribute to RV-induced wheezing, possibly through induction of leukotriene release (van Schaik et al., 2000).
Another mechanism by which IFN-γ may promote the development of virus-induced bronchial hyperresponsiveness is by enhancing ASM contractile responses to LTD₄ (Amrani et al., 2001b). However, further clinical studies are necessary to determine whether leukotriene antagonists (LTAs) may be useful in treating virus-induced bronchial hyperresponsiveness and cough.

IFN-γ can also upregulate the expression of several mediators and receptors in HASM. For example, Lazaar and colleagues (Lazaar et al., 1998), demonstrated that this cytokine increases the expression of CD40 in human ASM. The CD40-mediated signal-transduction pathway in ASM results in protein tyrosine kinase-dependent calcium mobilization, NFκ-B activation, and IL-6 production. It is thus possible that cell-cell interactions between T cell and smooth muscle may potentiate airway inflammation.

IFN-γ can also potentiate RANTES mRNA expression and protein release by TNF-α in human ASM cells and this effect can be partly inhibited by the Th2-derived cytokines IL-4, IL-10, and IL-13, as well as dexamethasone (John et al., 1997).

Other results suggest an important role for IFN-γ in the chronic inflammation of asthma. As reviewed above, activated mast cells, T cells and their mediators participate in the asthmatic early- and late-phase reaction. The interactions between the two cell types can induce and enhance IFN-γ production by T cells up to 60-fold. Thus, mast cells may constitute a negative feedback system locally down-regulating allergen-induced Th2 responses (De Pater-Huijsen et al., 2002).
Although there is no unanimity on the matter, there is evidence showing that the expression levels of IFN-γ in asthmatic subjects are increased. Bufe and colleagues (Bufe et al., 2002), found differences between patients with allergic asthma and patients with nonallergic asthma with respect to the capacity to produce IFN-γ. Although atopy is thought to be associated with a Th2 cytokine response, IFN-γ release from blood cultures was not reduced in allergic children. In contrast, patients with allergic rhinitis showed a significant increase in IFN-γ release compared to patients with nonallergic asthma. Similar data showed that using a segmental bronchoprovocation model to mimic asthma exacerbations, IFN-γ levels in BAL fluid of asthmatics was significantly higher than that in healthy controls (Guo et al., 2000). Data demonstrate that there is also an increased frequency of IFN-γ producing CD4+ and CD8+ T cells in asthmatic compared with normal subjects (Cho et al., 2002).

Genetic studies, have demonstrated that several loci in chromosome 6q24-q25 are linked either to asthma or wheezing. Interestingly, this region contains some candidate genes such as the gene coding for the IFN-γ receptor ligand-binding chain (Alcais et al., 2001).

**TGF-β**

Th3 cells are CD4+ regulatory cells associated with immune mechanisms involving oral tolerance towards antigens. They form a unique T-cell subset which
primarily secretes transforming growth factor (TGF)-β, provides help for IgA and has suppressive properties for both Th1 and Th2 cells (Weiner, 2001).

There is compelling evidence to suggest that TGF-β is over-expressed in several respiratory disorders and that it may have a predominant role in airway remodeling;

Asthmatic individuals exhibit a greater expression of TGF-β mRNA and immunoreactivity in the airway submucosa than normal control subjects, and these increases were directly related to the basement membrane thickness and disease severity (Minshall et al., 1997).

Similar results showed that, in contrast to normal human lung, TGF-β is detected in increased concentrations in asthmatic BAL fluid before and after antigen challenge (Renauld, 2001). It is also over expressed in pulmonary fibrosis, cryptogenic fibrosing alveolitis, cystic fibrosis (Corrin et al., 1994). There are also several non-fibrotic diseases where the expression levels of TGF-β are increased, such as interstitial lung disease, extrinsic allergic alveolitis, giant cell interstitial pneumonia, smoking-induced chronic bronchitis, emphysema (Li et al., 2002), chronic sinusitis (Min et al., 1999) and neutrophil-mediated lung injury (Suzuki et al., 1994).

TGF-β is a potent profibrotic cytokine, produced in large quantities by eosinophils, fibroblasts and epithelial cells which may play a role in structural changes within the airways, including subepithelial fibrosis, as well as chronic
eosinophilic inflammation. Within asthmatic airways, it has been demonstrated that activated eosinophils are a major source of this cytokine. The exact mechanisms responsible for this tissue remodeling have to be established (Minshall et al., 1997).

TGF-β may contribute to the thickening of the reticular lamina by the deposition of collagen fibers (Renauld, 2001) and induce human ASM cell proliferation by increasing the expression of insulin-like growth factor binding protein-3 (IGFBP-3). The mitogenic action of TGF-β may result in the hyperplastic nature of ASM cells in chronic asthma and bronchopulmonary dysplasia (Cohen et al., 2000).

The biologically active TGF-β released by plasmin, a serine protease, induces ASM cells to synthesize collagen I in an autocrine manner. The autocrine induction of collagen expression by ASMCs may contribute to the increase in airway smooth muscle cell mass and connective tissue proteins in the smooth muscle layer of airways, as seen in the airways of some asthmatics (Coutts et al., 2001).

TGF-β may play an important role in the pathophysiology of asthma, by inducing the secretion of IL-8 and the expression of COX-2 and PGE₂ in human ASM (Fong et al., 2000).

Contraction of type I collagen gels is an in vitro model of tissue remodeling. In addition to fibroblasts, some epithelial cells can mediate this process. Alveolar
epithelial cells might contract extracellular matrices and have the potential to directly participate in the remodeling of the lung after alveolar injury. TGF-β is able to augment the contraction by human bronchial epithelial cells (HBEC) and A549 cells, plated on top of type 1 collagenous gels in a concentration-dependent manner (Umino et al., 2000). Some Th2 cytokines overexpressed in asthmatic patients, such as IL-4 and IL-13, can stimulate the mRNA expression levels and production of other TGF-β isoform (TGF-β2) by human bronchial epithelial cells in a time- and concentration-dependent manner. IFN-γ, in contrast, can inhibit TGF-β2 release both under basal conditions and following IL-4 or IL-13 stimulation. The ability of these cytokines to modulate TGF-β release may contribute to both normal airway repair and to the development of subepithelial fibrosis in asthma (Wen et al., 2002).
IL-13

The third cytokine used in this work was IL-13, a Th2 type cytokine. Cytokines produced by Th2 lymphocytes have been implicated in the asthmatic airway inflammation and airway hyperresponsiveness and their expression is increased in airway tissues of asthmatics and animal models of asthma (Wills-Karp et al., 1998). There is strong support for the concept that IL-13 can produce airway hyperresponsiveness (AHR) indirectly by promoting the recruitment of inflammatory cells or by direct effects on resident airway cells (Venkayya et al., 2002). Evidence shows that asthmatic individuals exhibit a greater expression of IL-13 than normal control subjects (Wills-Karp et al., 1998).

Lee and colleagues (Lee et al., 2001b) demonstrated that the global effects of IL-13 on gene expression in airway cells could contribute to the phenotypic features of asthma. By using Genechips that contained probes for approximately 6,500 human genes, the authors observed that, despite activating a common signaling pathway, IL-13 induced dramatically different patterns of gene expression in primary cultures of airway epithelial cells, airway smooth muscle cells, and lung fibroblasts, with little overlap among cell types. The most prominent effects of IL-13 were on bronchial smooth muscle, where it induced the expression of several signaling effectors (e.g., components of MAP kinase signaling pathways, phospholipase A2 and diacylglycerol kinase), signaling receptors (e.g., signaling molecules of the Src family, CXCR2 and the signaling IL-13R1 subunit), contractile proteins (e.g., sarcolipin, dystroglycan-associated protein, smooth
muscle myosin heavy chain, and cardiac myosin heavy chain), and ion channels (e.g., KCNQ2, KVLQT1 and CLCL3). IL-13 also induced expression of secreted factors in airway smooth muscle cells, such as the basic fibroblast growth factor (bFGF) that induces BSMC proliferation and the IL-6 family cytokine leukemia inhibitory factor, which could contribute to the asthma phenotype through autocrine or paracrine effects on other airway cells.

Overexpression of IL-13 in the lungs causes a mononuclear and eosinophilic inflammatory response, mucus hypersecretion, deposition of Charcot-Leyden-like crystals, eotaxin production, airways obstruction, nonspecific AHR and airway fibrosis (Zhu et al., 1999). These results suggest that IL-13 could also have an effect in airway remodeling. Interestingly, data suggest that both IL-4 and IL-13 cause inflammation but only IL-13 causes subepithelial fibrosis which is mediated, to a great extent, by the production and activation of TGF-β1 in lung macrophages (Lee et al., 2001a). Similar data demonstrate that epithelial activation by IL-13 plays a critical role in initiating remodeling through release of TGF-β2. The latter can then activate the underlying myofibroblasts to secrete matrix proteins as well as smooth muscle and vascular mitogens to propagate remodeling changes into the submucosa (Richter et al., 2001).

Data support the role of IL-13 in airway contraction and narrowing. Liu and Colleagues (Liu et al., 2002) observed that IL-13 augmented the contraction of human airway smooth muscle cells embedded inside native type I collagen gels, in a concentration-dependent manner. This cytokine can also interfere with the
reduction in HASMC stiffness induced by the beta-agonist isoproterenol. This effect may contribute at least in part to the airway narrowing observed in patients with asthma (Laporte et al., 2001).

In conclusion, IL-13 may modulate airway smooth muscle activities leading to inflammation, remodeling and contraction. Therefore, it could play a role in the altered airway tissue which characterizes asthma.

2.4. Cysteiny1 Leukotrienes

Cysteiny1 leukotrienes (CysLTs) research began in 1940 when Kellaway and Trethewie demonstrated in a bioassay that the effluent from antigen-stimulated guinea pig lung tissue contracted gastrointestinal smooth muscle. This material was designated "slow reacting substance". Later, in 1960, it was renamed "slow reacting substance of anaphylaxis" by Brocklehurst (Brocklehurst, 1981).

The active substances were discovered in 1979 and named leukotrienes because the parent molecule was originally isolated from leukocytes, and its carbon backbone contained four double bonds (hence the 4 subscript), 3 of which are in a conjugated triene structure (Samuelsson et al., 1979).

Synthesis

Cysteiny1 leukotrienes are powerful proinflammatory mediators generated from arachidonic acid, an essential fatty acid component of phospholipids in membrane of all cells.
The enzyme 5-lipoxygenase metabolizes arachidonic acid to 5-hydroperoxyeicosatetraenoic acid (5-HPETE), which is spontaneously hydrolyzed to 5-hydroxyeicosatetraenoic acids (5-HETE) or further converted by 5-lipoxygenase into an unstable epoxide, the leukotriene A₄. The latter can be transformed into LTB₄ by a LTA₄ hydrolase or conjugated with the tripeptide glutathione (Cys-Glu-Gly) by LTC₄ synthase, leading to the formation of LTC₄, the first cysteinyll leukotriene. LTC₄ is then transported out of the cell where it is converted to LTD₄ and then to LTE₄. During this process, peptidases act on the glutathione side chain of LTC₄ to eliminate glutamic acid and then glycine. LTC₄ is converted into LTD₄ by a gamma-glutamyl-transpeptidase and LTD₄ into LTE₄ by a dipeptidase (Devillier et al., 1999). (Diagram 1)

Diagram 1.
The formation of leukotrienes and prostanoids (thromboxane A2 and prostaglandins) via arachidonic acid breakdown, with associated enzymes. FLAP=5-lipoxygenase activating protein. (Devillier et al., 1999).


**Biological actions**

The biological activities of CysLTs (Table 1), suggest a prominent role in the pathology of asthma, as well as in aspirin and exercise-induced asthma (Szczeklik et al., 2001),(Sun et al., 2002).

BSMC are one of the principal targets for CysLTs. They can induce smooth muscle contraction resulting in long-lasting bronchoconstriction with a potency at least 1000-fold greater than histamine (Schmidt and Rabe, 2000).

The bronchoconstriction that occurs during the asthmatic early and late response, is in large part caused by allergen-induced synthesis and release of these proinflammatory mediators. The CysLTs are also the most important mediators causing exercise-induced bronchoconstriction in individuals with asthma. Studies have demonstrated marked attenuation of exercise-induced bronchoconstriction after pretreatment with a variety of different Cys-LT1R antagonists, thereby blocking the action of the CysLTs on their receptors in human airways (O'Byrne, 2000).

The LTD₄-induced contractile responses in human airways elicit a small, transient change in intracellular calcium ion concentration, suggesting that the transduction mechanism linked to BSMC contraction is partly independent of Ca²⁺ and may involve the activation of PKC-α (Accomazzo et al., 2001).

CysLTs are important mediators in vascular permeability, vasoconstriction, increased mucus production (Buccellati et al., 2002) and bronchial
hyperresponsiveness (Leff, 2000) due to an enhanced responsiveness of ASM, particularly to LTC₄ (Schmidt and Rabe, 2000).

Growing data suggest that CysLTs may also induce ASM remodeling; Panettieri and colleagues (Panettieri et al., 1998) demonstrated that LTD₄, could potentiate the induction of DNA synthesis and proliferation induced by the mitogen, Epidermal Growth Factor on HASM. Similar results indicate that CysLTs may participate in the pathogenesis of smooth muscle hyperplasia and subepithelial fibrosis in a mouse model (Henderson et al., 2002).

In Brown Norway rats, the increase in bronchial responsiveness induced by inhaled antigen is directly related to an increase in the mass of BSMC, and both these increases are blocked by the LTAs MK-571, suggesting a mitogenic effect of CysLTs on smooth muscle (Salmon et al., 1999).

Epithelial denudation, in chronic inflammation of the asthmatic airway, may contribute to bronchial hyperresponsiveness by increasing access of stimuli to the bronchial mucosa and afferent nerve fibers, and by reducing the availability of bronchodilator and antiinflammatory mediators such as PGE₂. CysLTs may exacerbate the sensitizing effects of epithelial denudation by directly stimulating local afferent nerves to release tachykinins, leading to bronchoconstriction and plasma exudation (Holgate and Sampson, 2000).

Minoguchi and colleagues (Minoguchi et al., 2002) demonstrated that CysLTs have a role in airway eosinophilic inflammation in patients with asthma. These
mediators can significantly up-regulate Mac-1 expression and induce eosinophil chemotaxis in a dose-dependent manner (Fregonese et al., 2002).

The interactions between eosinophils from healthy volunteers and activated epithelial cells are associated with increased biosynthesis of CysLTs, suggesting a possible feedback in chronic eosinophil airway inflammation in asthma (Jawien et al., 2002).

In a complex reaction, such as the chronic inflammation observed in asthma, a number of mediators and modulators with different targets are likely to be present. The biologic response to an external or internal stimulus is not exerted by CysLTs alone, but rather represents the composite result of interactions in order to maintain homeostatic control in the intact organism. Vasodilating prostaglandins, lipoxins, and histamine are examples of endogenous substances that may interact with CysLTs in the inflammatory process (Hedqvist et al., 2000).

The proinflammatory effects of the CysLTs also play an important role in other disorders such as allergic rhinitis, chronic hyperplastic sinusitis with nasal polyposis (Borish, 2002) and atopic dermatitis (Chari et al., 2001). Recent evidence also suggest a role in interstitial cystitis (Bouchelouche et al., 2001) and cardiovascular disease (Folco et al., 2000).

In vitro and in vivo data have demonstrated that LTs may play a key role in atopic dermatitis (Chari et al., 2001), allergic rhinitis and chronic hyperplastic sinusitis
with nasal polyposis (CHS/NP) (Borish, 2002). There is a enhanced biosynthesis of Cys-LTs during atopic eczema/dermatitis syndrome exacerbations (AEDS) and preliminary clinical observations show the efficacy of leukotriene antagonists in alleviating the symptoms of this syndrome (Adamek-Guzik et al., 2002). Clinical evidence of the use of antileukotrienes in atopic dermatitis is limited, but initial results have been promising and these agents may one day serve as corticosteroid-sparing treatments for atopic dermatitis (Chari et al., 2001), and they might be an alternative to repeated surgical therapy in CHS/NP (Modrzynski et al., 2002). (Borish, 2002).

<table>
<thead>
<tr>
<th>SUMMARY OF BIOLOGICAL ACTIONS OF LEUKOTRIENES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agonist</td>
</tr>
<tr>
<td>--------</td>
</tr>
<tr>
<td>LTB₄</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>CysLT₁ receptor</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>LTC₄</td>
</tr>
<tr>
<td>LTD₄</td>
</tr>
<tr>
<td>LTE₄</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>CysLT₂ receptor</td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>

* Effects denoted in boldface have been characterized with respect to receptor involvement. Effects in italics remain to be further characterized.

Table 1.
Summary of the biological actions of Leukotrienes. (Dahlen, 2000).
**Cysteiny1 Leukotriene Receptors**

The receptors for CysLTs (CysLTR), are heterogeneous; at least two different classes have so far been recognized (Table 2), named CysLT1R which is blocked by the so-called classical antagonists, such as zafirlukast (Accolate, ICI 204,219), montelukast (Singulair, MK-476) and pranlukast (Onon, ONO-1078) and CysLT2R which is insensitive to the classical antagonists, but sensitive to BAY u9773 (Nicosia et al., 2000).

Two groups (Lynch et al., 1999), (Sarau et al., 1999) reported the molecular and pharmacological characterization of the cloned human CysLT1R. The full-length cDNA for this receptor has a 1014-bp sequence and encodes a protein of 337 amino acid residues. The CysLT1R gene was mapped to the long arm of the human X chromosome at bands 13-21(Xq13-Xq21). Analysis of the DNA sequence indicated homology of this polypeptide sequence to the seven-transmembrane-spanning G-protein coupled receptors (GPCRs). In addition, hydrophobicity plot analysis showed the existence of seven hydrophobic regions, each containing approximately 20 to 30 amino acids, which are likely to represent the membrane-spanning domains found among the GPCRs. The rank order of affinities of CysLT1R for the leukotrienes was LTD4 >LTE4 = LTC4 >LTB4. CysLT1R possesses 28% identity with the cloned LTB4 receptor. The sequencing of a genomic clone confirmed the DNA sequence and also revealed that the coding region of CysLT1R was intron-less.
CysLT1R mRNA of approximately 3.0 kb was revealed by Northern blots in spleen, peripheral blood leukocytes and lung. In normal human lung, expression of the CysLT1R mRNA was confined to smooth muscle cells and tissue macrophages (Lynch et al., 1999). In situ hybridization of the CysLT1R mRNA in human lung showed very confined localization to bronchial smooth-muscle and macrophages. There is also expression of CysLT1R mRNA and protein in most peripheral blood eosinophils and pregranulocytic CD34+ cells, and in subsets of monocytes and B lymphocytes (Figueroa et al., 2001).

Maekawa and colleagues (Maekawa et al., 2001) isolated the mouse CysLT1R from a mouse lung cDNA library and found two isoforms. A short cDNA isoform containing two exons encoded a polypeptide of 339 amino acids with 87.3% amino acid identity to the human CysLT1R. A long isoform has two additional exons and an in-frame upstream start codon resulting in a 13-amino acid extension at the N-terminus. The dominant mouse isoform with the N-terminal amino acid extension encoded by an additional exon, has the same ligand response profile as the spliced form and the human receptor.

The same group generated CysLT1R-deficient mice by targeted gene disruption (Maekawa et al., 2002). Their in vivo findings revealed a major role for the CysLTs-CysLT1R signal in innate and adaptive immune responses with enhanced vascular permeability during Zymosan-induced peritoneal inflammation and IgE-mediated, mast cell-dependent local anaphylaxis. They also
demonstrated that CysLT1R is the major functional CysLT receptor on peritoneal macrophages in terms of intracellular calcium mobilization.

Three groups (Heise et al., 2000) (Nothacker et al., 2000) (Takasaki et al., 2000) reported the cloning and characterization of a second CysLTR, CysLT2, a 346-amino acid protein with 38% amino acid identity to the CysLT1R. It is expressed in lung macrophages and airway smooth muscle, cardiac Purkinje cells, adrenal medulla cells, placenta, spleen, peripheral blood leukocytes, and brain. The receptor gene was mapped to chromosome 13q14, a region also linked to atopic asthma.

Evidence suggests that CysLT2R has biological significance in the cardiovascular system. High levels of CysLT2R mRNA were detected in the human atrium and ventricle and intermediate levels in the coronary artery (Kamohara et al., 2001). Furthermore, in situ hybridization revealed that CysLT2R mRNA was expressed in myocytes, fibroblasts, and vascular smooth muscle cells, but not in endothelial cells. CysLT2R may also be implicated in myocardial ischemia (Szczebeklik et al., 2002).
## IUPHAR Classification of LT Receptors

<table>
<thead>
<tr>
<th>Agonists</th>
<th>Class</th>
<th>Subclass</th>
<th>Antagonist</th>
</tr>
</thead>
<tbody>
<tr>
<td>LTB₄</td>
<td>BLT</td>
<td></td>
<td>LY-223982</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>LY-255283</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ONO-4057</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>SC-41930</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>RG-14893</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>RP-66668</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>SB-201993</td>
</tr>
<tr>
<td>LTC₄</td>
<td>Cys-LT</td>
<td>Cys-LT₁</td>
<td>ICI-198,615</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>MK-571</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ICI-204,219 (zafirlukast)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ONO-1078   (pranlukast)</td>
</tr>
<tr>
<td>LTD₄</td>
<td>Cys-LT</td>
<td></td>
<td>CGP-43715A (italukast)</td>
</tr>
<tr>
<td>LTE₄</td>
<td></td>
<td></td>
<td>MK-476 (montelukast)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>SKF-104353 (pobilukast)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>BAY u9773</td>
</tr>
</tbody>
</table>

Table 2.

IUPHAR Classification of the different leukotrienes receptors. (Nicosia et al., 2000).
**Objectives and strategies**

In the present work, we aimed at studying the modulation of CysLT1R expression in human BSMC by Th1, Th2 and Th3 type cytokines with potential relevance to airway remodeling.

First, we studied the modulation of expression of CysLT1R in BSMC by TGF-β, IL-13 and IFN-γ using flow cytometry and immunofluorescence microscopy. Our results indicated an up regulation of CysLT1R expression in BSMC induced by the three cytokines.

Moreover, we wanted to evaluate whether the increased protein expression was associated with augmented levels of CysLT1R mRNA. For this purpose, we used a RT PCR assay to compare the CysLT1R/GAPDH ratio of cytokine treated and control cells.

Finally, we wanted to know the functional relevance of the increased receptor expression in BSMC induced by TGF-β, IL-13 and IFN-γ. Using a colorimetric assay, we measured bronchial smooth muscle cell proliferation because it has been demonstrated that in asthmatic patients, chronic inflammation induces
airway remodeling. Evidence suggests that CysLTs may participate in this remodeling of human airways by potentiating the effects of BSMC growth factors.

However, no studies had demonstrated previously, the possible interaction between cytokines increased in asthmatics patients (such as TGF-β, IL-13 and IFN-γ) and CysLTs in the induction of BSMC proliferation. In our study, we demonstrated that TGF-β- and IL-13-pretreated BSMC showed increased proliferation when subsequently exposed to LTD₄. In order to demonstrate that this proliferation was dependent on CysLT1R, we used Montelukast, a selective CysT1R antagonist, and showed that proliferation was prevented.
Article

CysLT1 Receptor Upregulation by TGF-β and IL-13 is associated with Bronchial Smooth Muscle Cell Proliferation In Response To LTD4

Karina Espinosa BSc, Jana Stankova PhD and Marek Rola-Pleszczynski MD

Immunology Division, Department of Pediatrics, Faculty of Medicine, Université de Sherbrooke, Sherbrooke, QC Canada J1H 5N4

Correspondence: Marek Rola-Pleszczynski, M.D. Immunology Division, Department of Pediatrics, Faculty of Medicine, Université de Sherbrooke, 3001 N. 12th Avenue Sherbrooke, QC Canada J1H 5N4 Tel: 819-346-1110, ext 14851 Fax: 819-564-5215 E-mail: mrolaple@courrier.usherb.ca

Word count: 2737.
Abstract

Airway remodeling is a feature of chronic asthma and involves a number of structural changes, including bronchial smooth muscle cell (BSMC) hyperplasia and hypertrophy. Cysteiny1 leukotrienes (cysLTs) have been suggested to play a role in airway remodeling in addition to their numerous other physiopathological effects. The present work is aimed at characterizing the potential modulation of CysLT1 receptor expression by cytokines and the eventual functional relevance of this modulation. When human BSMC were exposed to TGFβ, IL-13 or IFNγ, 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 IFNγ, but not TGFβ, were able to increase CysLT1 mRNA levels. Finally, when BSMC were pretreated with TGFβ or IL-13, but not IFNγ, their responsiveness to LTD₄ was markedly enhanced in terms of BSMC proliferation. Whereas TGFβ, IL-13 or LTD₄ alone had little effect on BSMC proliferation, preexposure of the cells to TGFβ or IL-13 for 24 h resulted in a greater than 3-fold increase in proliferation in response to LTD₄. The proliferation was totally prevented by pretreating the cytokine-primed BSMC with the selective CysLT1 receptor antagonist, montelukast. 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 provide a rationale for preventive and therapeutic intervention.

*Keywords*: bronchial smooth muscle, cysteinyl leukotrienes, leukotriene receptors, leukotriene D4, airway remodeling, asthma, TGF-β, IL-13, IFN-γ.
Introduction

Airway remodeling occurs in chronic asthmatic inflammation and features of this response include myocyte hyperplasia and hypertrophy. An increase of 50-230% and 25-150% is observed in the area of airway smooth muscle in fatal and nonfatal asthma, respectively. Increased thickness of the airways may have an important amplifying effect in the contractile response of bronchial smooth muscle cells (BSCMC) and may be a major mechanism contributing to airway hyperresponsiveness in asthma. A growing body of evidence suggests that cysteiny1-leukotrienes (cysLTs) may induce airway smooth muscle remodeling in animal models and potentiate the proliferation induced by growth factors.

Cytokines are also thought to contribute to chronic inflammation and airway remodeling observed in asthmatic airways, and asthmatic individuals produce more TGF-β1 and IL-13 than normal subjects. TGF-β1 is a potent profibrotic Th3-type cytokine, which may induce structural changes within the airways, such as subepithelial fibrosis, thickening of the reticular lamina and human airway smooth muscle proliferation by increasing the expression of insulin-like growth factor binding protein-3 (IGFBP-3). IL-13, a Th2-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) that induces BSMC proliferation which could contribute to the asthma phenotype through autocrine or paracrine effects on other airway cells\textsuperscript{11}. Data suggest that IL-13 causes subepithelial fibrosis by the production and activation of TGF\(\beta\)1 in lung macrophages\textsuperscript{12}. Although there is no unanimity on the matter, there is evidence showing that the expression levels of IFN-\(\gamma\), a Th1-type cytokine, are increased in asthmatic subjects\textsuperscript{13, 14}. There is an increased frequency of IFN-\(\gamma\) producing CD4\(^+\) and CD8\(^+\) T cells in asthmatics compared with normal subjects\textsuperscript{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-\(\gamma\) receptor ligand-binding chain\textsuperscript{16}. In human BASM, IFN-\(\gamma\) can enhance CysLT1R expression and increase the contractile responses to LTD\(_4\) and thus possibly contribute to the airway hyperreactivity (AHR) observed in asthma\textsuperscript{17}.

Previous studies from our laboratory have demonstrated that CysLT1R mRNA and protein expression can be up-regulated by Th2-type cytokines (IL-13, IL-4 and IL5) in monocytes, macrophages and eosinophilic HL-60 cells\textsuperscript{18, 19}. To our knowledge, however, there are no studies on interactions of cytokines (such as TGF-\(\beta\) IL-13) and cysLTs, in terms of human BSMC proliferation. In the present work, we studied the modulation of CysLT1R expression in human BSMC by TGF-\(\beta\), IL-13 and IFN-\(\gamma\) and the functional relevance of this modulation in terms of BSMC proliferation in response to LTD\(_4\).
Materials and Methods

Reagents

Human rIL-13, rIFN-γ and TGF-β1 were obtained from PeproTech Canada, Inc. (Ottawa, ON, Canada); all cytokine preparations contained <0.1 ng endotoxin per microgram (1 EU/μg); rabbit polyclonal anti-human C-terminal CysLT1R Ab was developed and characterized in collaboration with Cayman Chemical (Ann Arbor, MI); rabbit IgG isotype control was obtained from Southern Biotechnology Associates (Birmingham, AL); 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). Primers for CysLT1R and GAPDH were obtained from Invitrogen, (Burlington, ON, Canada). LTD$_4$ was obtained from Cayman Chemical; Montelukast was obtained from Merck Research Laboratories (West Point, PA).

Cells

Primary cultures of normal human bronchial smooth muscle cells were purchased from the Clonetics Corporation (San Diego, CA). The cells were cultured in 25cm$^2$ flasks, in medium for HBSMCs supplemented with 5% Fetal Bovine Serum (FBS), human recombinant Epidermal Growth Factor (0.5 μg/ml), Insulin (5 mg/ml), human recombinant Fibroblast Growth Factor (1 μg/ml),
Gentamicin (50 μg/ml), and Amphotericin B (0.05 μg/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 using 0.025% trypsin in 0.01% ethylenediaminetetraacetic acid (EDTA). Cells at passages 2 to 6 were used for experiments.

Flow cytometry

The expression of CysLT1R in BSMC was assessed using a polyclonal anti-CysLT1R Ab directed against the carboxyl-terminal portion of the receptor. The Ab 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 min at room temperature followed by permeabilization with 0.1% saponine for an additional 15 min at room temperature. Cells were resuspended with PBS/2% BSA and labeled for 30 min at 4°C with anti-CysLT1R Ab (or with control, nonpertinent Ab). Cells were then washed with cold PBS and incubated for 30 min 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 FACSscan flow cytometer (Becton Dickinson, San Jose, CA). A 1/250 v/v dilution of the anti-CysLT1R antiserum and nonpertinent Ab was used in all cytometry studies.
RT-PCR

Total RNA was extracted from BSMC using Tri-Pure® Isolation Reagent (Roche). Two μg of RNA were converted to cDNA by the reverse transcriptase enzyme reaction (AMV transcriptase-reverse; Promega, Madison, WI) in a total volume of 20 μl. PCR was performed in a final volume of 50 μl containing 5 μl of RT reaction product. Samples were placed in a Biometra T1 thermo cycler; (Montréal, Biotech Inc.) for 34 cycles consisting of 2 min denaturation at 94°C, 30s annealing at 62°C, and 90s extension at 72°C, followed by a final 5-min 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'-CCGGAATTCAATGGCTTTAAACTATAC-3' as antisense. Samples were subjected to parallel amplification of the constitutively expressed, housekeeping gene, GAPDH using the following primers: 5'-GCTAGAGTAAGTAGTT-3' as sense and 5'-AACGACGAGCGTGAC-3' as antisense. A 10-μl aliquot from each PCR was allowed to migrate by electrophoresis in a 1% agarose gel. The CysLT1R-amplified 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 six-well plates containing sterile coverslips. Supernatants were eliminated and cells were fixed with paraformaldehyde 2% and permeabilized with saponin 0.1%. Cells were then incubated overnight with antibody against the C-terminus of CysLT1R\textsuperscript{18} (1/250 v/v), washed and incubated for 2h with rhodamine-labelled goat anti-rabbit antibody (1/1000). Indirect immunofluorescence was examined without counterstain 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 x 1033 pixels were captured using 40x oil immersion objective and a cooled SPOT color digital camera (Diagnostic Instruments Inc, St. Sterling Heights, MI). The images were processed using SPOT software.
Proliferation assay

BSMC were grown in 96-well plates (3000 cells/well), starved 24h (medium 1% FBS) and then incubated for 24h with cytokines or medium. Supernatants were removed and washed cells were incubated for 72h with LTD$_4$ 10$^{-7}$ M. To assess the specificity of the effect for CysLT1R, separate cell cultures were pre-incubated for 30 min with Montelukast 10$^{-6}$ M before incubation for 72h with LTD$_4$ 10$^{-7}$ M. The cells were fixed in 70% ethanol at −20°C, dried and stained with crystal violet (1% in water). After washing, stained cells were solubilized in 33% acetic acid and the 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.
Results

Cytokine induced CysLT1R protein expression.

The expression of CysLT1R protein in cultured human BSMC was investigated by flow cytometry using a specific anti-CysLT1R Ab. BSMC were treated for 24h with graded concentrations of TGF-β 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 and IFN-γ, markedly increased their expression of the receptor (Fig 1-3).

Basal expression levels of CysLT1R protein were adjusted to 100% and CysLT1R expression is illustrated as % of the control cells. TGF-β (Fig 1) and IFN-γ (Fig 3) induced a 200% increase in the expression of CysLT1R as compared to non-stimulated cells, whereas, IL-13 (Fig 2) induced a 150% increase. The effects of TGF-β, IL-13 and IFN-γ were maximal at 10 ng/ml, 5ng/ml and 100 U/ml, respectively. Given that IL-4, a prototypic Th2-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 4).
In kinetic studies, the effects of the three active cytokines on CysLT1R expression were found to be maximal after 24h of stimulation (data not shown).

In order to complement the results obtained by flow cytometry and to evaluate the distribution of CysLT1R within BSMC, we assessed CysLT1R protein expression by immunofluorescence (Figure 5). Control cells expressed low levels of CysLT1R. In contrast, TGF-β, IL-13 or IFN-γ-treated cells showed an increased expression of the protein. The distribution of CysLT1R was observed throughout the cell. Although CysLT1R expression was increased by the three cytokines, some BSMC in each treatment group remained CysLT1R-negative. The reason for this phenomenon is unknown, but unpublished results from our laboratory show that other cell types such as monocytes and macrophages also show a partial and random distribution of CysLT1R.

Although BSMC also expressed basal levels of CysLT2R, no modulation of expression was observed with either of the cytokines (data not shown).

**Cytokine-induced CysLT1R mRNA expression.**

In order 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 6). BSMC constitutively expressed low levels of CysLT1R mRNA. IL-13 and IFN-γ, but not TGF-β, induced an augmentation of
transcript levels following 24h of stimulation. Exposure of BSMC to cytokines for a shorter time period (8h) failed to show any significant modulation of CysLT1R mRNA (data not shown). Scanning densitometry analysis of data from all experiments, showed that IL-13 and IFN-γ augmented CysLT1R mRNA levels 3-fold and 2-fold, respectively, over baseline levels (Fig 6B). 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, IL-13 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 7). However, when BSMC had been exposed for 24 h to TGF-β or IL-13, a significant proliferation was induced by subsequent treatment with LTD₄ (Fig 7 and 8). Interestingly, IFN-γ-stimulated BSMC failed to respond to LTD₄ in terms of proliferation (Fig 8). Finally, in order 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 pre-incubated for 30 min with the CysLT1R antagonist, Montelukast (10⁻⁶M), before the addition of LTD₄ 10⁻⁷M. As shown in figure 8, Montelukast was capable of totally preventing the proliferation induced by LTD₄ in both TGF-β- and IL-13-pretreated cells.
Fig 1. Flow cytometric analysis of CysLT1R expression in cells stimulated with TGF-β. BSMC were incubated with different concentrations of TGF-β for 24h. Cells were subsequently labeled with anti-CysLT1R or isotype-matched control Abs, followed by incubation with FITC-conjugated goat anti-rabbit IgG. (A) Histograms of a single experiment, representative of at least three. Solid thick lines represent labeling with isotype control Ab. Solid thick lines represent labeling of medium- and 10 ng/ml TGF-β-treated cells, respectively, with anti-CysLT1R Ab. (B) The graphic illustrates CysLT1R expression following graded concentrations of TGF-β, with the control normalized to 100%. (n=3) (*; p<0.005).
Fig 2. Flow cytometric analysis of CysLT1R expression in cells stimulated with IL-13. BSMC were incubated with different concentrations of IL-13 for 24h. Cells were subsequently labeled with anti-CysLT1R or isotype-matched control Abs, followed by incubation with FITC-conjugated goat anti-rabbit IgG. (A) Histograms of a single experiment, representative of at least three. Solid thin lines represent labeling with isotype control Ab. Solid thick lines represent labeling of medium- and 5 ng/ml IL-13-treated cells, respectively, with anti-CysLT1R Ab. (B) The graphic illustrates CysLT1R expression following graded concentrations of IL-13, with the control normalized to 100%. (n=3) (*:p<0.05).
Fig 3. Flow cytometric analysis of CysLT1R expression in cells stimulated with IFN-γ. BSMC were incubated with different concentrations of IFN-γ for 24h. Cells were subsequently labeled with anti-CysLT1R or isotype-matched control Abs, followed by incubation with FITC-conjugated goat anti-rabbit IgG. (A) Histograms of a single experiment, representative of at least three. Solid thin lines represent labeling with isotype control Ab. Solid thick lines represent labeling of medium- and 100 U/ml IFN-γ-treated cells, respectively, with anti-CysLT1R Ab. (B) The graphic illustrates CysLT1R expression following graded concentrations of IFN-γ, with the control normalized to 100%. (n=3) (*; p<0.003).
Fig 4. Flow cytometric analysis of CysLT1R expression in cells stimulated with IL-4. BSMC were incubated with different concentrations of IL-4 for 24h. Cells were subsequently labeled with anti-CysLT1R or isotype-matched control Abs, followed by incubation with FITC-conjugated goat anti-rabbit IgG. (A) Histograms of a single experiment, representative of at least three. Solid thin lines represent labeling with isotype control Ab. Solid thick lines represent labeling of medium- and 10ng/ml IL-4-treated cells, respectively, with anti-CysLT1R Ab. (B) The graphic illustrates CysLT1R expression following graded concentrations of IL-4, with the control normalized to 100%. (n=4).
Fig 5. Immunofluorescence assay. Cells were grown to confluence in six well plates containing sterile coverslips. Supernatants were eliminated and cells were fixed with paraformaldehyde 2% and permeabilized with saponine 0.1%. Cells were then incubated overnight with CysLT1R Ab, washed and incubated for 2h with rhodamine-labelled goat anti-rabbit Ab.
Fig 6. Effect of IFN-γ, TGF-β and IL-13 on CysLT1R mRNA expression. (A) BSMC were stimulated for 24h with 100 U/ml, 10ng/ml and 5ng/ml of IFN-γ, TGF-β and IL-13 respectively. Two micrograms of total RNA were used for the RT-PCR reaction with primers for CysLT1R and GAPDH. Reaction products were subjected to electrophoresis on 1% agarose gel. (B) Scanning densitometry of the gel normalized for medium. Data are means ± SEM of four different experiments. (*; p<0.05).
Fig 7. Proliferation assay. Photomicrographs of crystal violet-stained BSMC were taken before resuspended in acetic acid 33%. Top row shows cells treated for 24h with medium (controls) or cytokines followed by 72h with vehicle. Bottom rows shows cells treated for 24h with medium or cytokines followed by 72h with LTD$_4$ 10$^{-7}$M. Results of a single experiment, representative of four independent experiments performed in quintuplicata are illustrated.
Fig 8. Proliferation Assay. BSMC were grown in 96-well plates, starved for 24h (medium 1% FBS) and incubated for 24h with cytokines or medium. Supernatants were then removed and cells were incubated for 30 min with medium or Montelukast 10^{-6} M, followed by 72h with LTD_4 10^{-7}M. The cells were then fixed in 70% ethanol at -20 °C, dried and stained with crystal violet (1% in water). After washing, stained cells were solubilized in 33% acetic acid and the absorbance determined in a Thermomax microplate reader at 570 nm. The analysis was performed in quintuplicate in four independent experiments. (*; p<0.005).
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 Th2-type cytokine, IL-4, had no effect on CysLT1R expression in human BSMC.

IL-13- 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 post-translational effect, mediated either by increased protein translation or lower protein degradation. Additional 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 monocytes and macrophages by IL-13 and IL-4, but not IFNγ18,19. In these myeloid cells, the increased expression of CysLT1R resulted in an enhanced responsiveness to LTD4 in terms of Ca++ transients and chemotaxis. In the present study, we showed that TGFβ, IFNγ and IL-13, 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.

The present 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\textsuperscript{10,2,1}, TGF-β could also induce BSMC proliferation by itself. Recently, Amrani and colleagues reported that IFN-γ could upregulate CysLT1R expression in human airway myocytes and that this upregulation increased their contractile responses to LTD4\textsuperscript{17}. This may contribute to the airway hyperreactivity observed in asthma\textsuperscript{22,23}. 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 MK-571\textsuperscript{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. This effect could be explained by the inhibitory effects of IFN-γ on cell proliferation and protein synthesis\textsuperscript{24,22,23}.

It has been demonstrated that a significant increase in the area of airway smooth muscle occurs in chronic asthmatic inflammation\textsuperscript{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\textsuperscript{25}. Evidence suggest that LTD\textsubscript{4} can potentiate the induction of DNA synthesis and proliferation induced by the mitogen, Epidermal Growth Factor, in human BSMC\textsuperscript{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\textsuperscript{4}. In Brown Norway rats sensitized to ovalbumin, cysLTs induced airway smooth muscle and epithelial cell DNA synthesis as well as ASM thickening following repeated allergen exposure\textsuperscript{5}.

Studies also provide evidence that cytokines such as TGF-\(\beta\), a Th3-type cytokine, may also play a role in airway remodeling. Data obtained by Cohen and colleagues\textsuperscript{10} suggest that TGF-\(\beta\)1 can induce human BSMC proliferation by increasing the expression of Insulin-Like Growth Factor Binding Protein-3 (IGFBP-3). Hence, the mitogenic action of TGF-\(\beta\)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-\(\beta\)\textsuperscript{26}. The expression levels of this cytokine are dramatically increased in asthma and several other lung disorders\textsuperscript{27, 7}.
The Th2-type cytokine, IL-13, is also over-expressed in asthmatic patients\textsuperscript{28}. Interestingly, data suggest that both IL-4 and IL-13 cause inflammation, but only IL-13 causes subepithelial fibrosis\textsuperscript{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\textsuperscript{12, 29}. Data also support the role of IL-13 in airway remodeling by modulating the production of TGF-β2 from human bronchial epithelial cells. The release of TGF-β2 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-β2 induced by IL-13 in human bronchial epithelial cells\textsuperscript{30, 31}.

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 LTD\textsubscript{4} is increased by TGF-β and IL-13 (the levels of which are augmented in asthmatic airways). Increased receptor expression can lead to increased LTD\textsubscript{4} signaling resulting in BSMC proliferation. Although the exact proliferation mechanism is unknown, evidence suggest that ERK and PI3-kinase pathways may be involved, given that these pathways are the major positive regulators of airway smooth muscle proliferation\textsuperscript{32}. However, additional experiments are needed to understand the precise mechanisms involved in this process.
In conclusion, 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.
References


Acknowledgements

This work was supported by a studentship to K.E. from CONACYT, by a Medical School grant from Merck Frost and by a grant from the Canadian Institute for Health Research.
**Discussion**

Several medications are used for the treatment and prevention of asthma. \( \beta_2 \)-agonists have been used for more than 30 years. There has been some controversy surrounding the safety of \( \beta_2 \)-agonists in the treatment of asthma (McFadden, 1995), (Abramson et al., 2001). The use of \( \beta_2 \)-agonists has been associated to increased morbidity from asthma during regular and prolonged treatment, with increased symptoms, decreased pulmonary function, increased airways responsiveness and overall poorer control of asthma despite constant inhaled corticosteroid treatment. Several recent studies suggest a deleterious effect of frequent use of inhaled \( \beta \)-agonists, particularly higher potency preparations. Increased responsiveness to allergen is one possible mechanism for this effect, but whatever the mechanism, frequent use of \( \beta_2 \)-agonists appears to have caused a shift in the chronic severity of asthma, leading to increased morbidity and mortality (Sears, 1995), (Suissa et al., 1994).

Available evidence indicates that the high-dose preparations of isoproterenol and fenoterol are associated with increased mortality and were the major causes of the epidemics of asthma mortality observed in some countries (Beasley et al., 1999). A study in Saskatchewan, Canada, confirmed that the use of salbutamol
was associated with a substantial risk factor for asthma severity and, hence, mortality, especially at higher doses (Sears, 1995).

Bronchodilators such as Theophylline and β₂-agonists used in the treatment of airway disease have been shown to have a variety of cardiac effects that may contribute to the occurrence of life-threatening events such as cardiac arrhythmias and cardiac arrest. The use of these drugs administered orally or by nebulization should be avoided in subjects with significant cardiac disease or at high risk for such disease, especially acute coronary insufficiency and congestive cardiomyopathy (Suissa et al., 1996). Although epidemiologic studies cannot definitely identify the cause of the increased asthma mortality, they have stimulated widespread discussion and reevaluation of therapeutic strategies.

Corticosteroids have been routinely used in the treatment of asthma to reduce bronchial hyperresponsiveness. The mechanism of action of corticosteroids is predominantly through the blocking of inflammatory-cell activation and migration into the airways. In vivo studies suggest that corticosteroid treatment has a limited effect in reducing the increase in components of the extracellular matrix proteins. The interaction between the ASM and the allergic process may alter components of the airway wall in asthma, and corticosteroids may not prevent the fibrosis induced by resident cells within the airways (Johnson et al., 2000).
Vanacker et al. demonstrated that allergen-induced structural airway changes could not be reversed by treatment with inhaled corticosteroids, but concomitant treatment with fluticasone propionate could partly prevent these changes and improve airway responsiveness (Vanacker et al., 2001).

LTAs, or antileukotrienes, are a promising new group of anti-inflammatory drugs. Two major categories of antileukotriene drugs have been introduced: leukotriene receptor antagonists and leukotriene biosynthesis inhibitors. Both types of drugs provide comparable protection against bronchoactive stimuli (allergen, exercise, aspirin). Moreover, beneficial effects have been observed in the treatment of day-to-day asthma, resulting in amelioration of clinical symptoms and lung function parameters, with reduction of β2-agonist and corticosteroid use, suggesting antiinflammatory effects. Owing to these properties in combination with generally mild adverse effects, antileukotrienes are promising in the treatment of patients, including children with various forms of asthma.

Evidence suggest that LTAs have been associated with the development of Churg-Strauss syndrome (CSS), a rare form of vasculitic angitis. However, to date, there is no compelling evidence that the development of CSS in asthmatic patients receiving LTAs results from a direct drug effect. Rather, it appears that tapering of corticosteroids in these patients unmaskst the multiorgan manifestations of the disease (Jamaleddine et al., 2002).
The definitive place of LTAs in asthma management will depend on the long-term effects on chronic airway inflammation, which determines the structural changes within the airways, and the subsequent severity of clinical symptoms (Jamaleddine et al., 2002), (Diamant and Sterk, 1998). Montelukast (MK-0476), is a potent and selective antagonist of LTD₄ and it has been proven to be an effective therapy in chronic asthmatics, allergic rhinitis, exercise-induced bronchoconstriction and in aspirin-intolerant asthmatics (Centanni and Santus, 2002), (Parnes, 2002). Treatment with Montelukast has anti-inflammatory effects on the airway in patients with asthma, and its bronchodilatory effect is not solely dependent on a decrease in airway eosinophilia (Minoguchi et al., 2002). According to our results, it is likely that LTAs may be also useful in inhibiting airway remodeling and as a consequence decrease AHR in asthmatic patients.

In the present work, we aimed at studying the modulation of CysLT1R expression in human BSMC by Th1, Th2 and Th3 type cytokines with potential relevance to airway remodeling.

Our results suggest that TGF-β, IL-13 and IFN-γ can upregulate the expression of CysLT1R protein in BSMC. Immunofluorescence analysis confirmed these results showing an increased expression of the CysLT1R within the cytoplasm and nucleus of BSMC.
IL-13 and IFN-γ treated cells also showed enhanced levels of CysLT1R mRNA expression. In contrast, TGF-β increased the protein expression but not the mRNA expression significantly. These results suggest that the increased protein expression induced by TGF-β is due to a translational or post-translational effect, mediated either by increased protein translation or lower protein degradation. However, additional studies have to be performed to know the mechanism involved in the receptor upregulation induced by TGF-β.

Previous results obtained by our laboratory have demonstrated the modulation of CysLT1R expression in HL-60/eos by IL-5, an important regulator of eosinophil function. IL-5 up-regulated CysLT1R mRNA expression, with consequently enhanced CysLT1R protein expression and function in HL-60/eos. CysLT1R mRNA expression was augmented 2- to 15-fold following treatment with IL-5 (1-20 ng/ml). The effect was seen after 2 h, was maximal by 4 h, and maintained at 8 h. Although CysLT1R mRNA was constitutively expressed in undifferentiated HL-60 cells, its expression was not modulated by IL-5 in the absence of differentiation. Differentiated HL-60/eos cells pretreated with IL-5 (10 ng/ml) for 24 h showed enhanced CysLT1R expression on the cell surface. They also showed enhanced responsiveness to LTD₄, but not to LTB₄ or platelet-activating factor, in terms of Ca²⁺ mobilization and chemotactic response (Thivierge et al., 2000).

Additional studies from our laboratory demonstrated the modulation of CysLT1R expression by the Th2 cytokines IL-13 and IL-4. IL-13 up-regulated CysLT1R
mRNA levels, and also enhanced CysLT1R protein expression and function in human monocytes and monocyte-derived macrophages. CysLT1R mRNA expression was augmented 2- to 5-fold following treatment with IL-13 and was due to enhanced transcriptional activity. The effect was observed after 4 h, was maximal by 8 h, and maintained at 24 h. IL-4, but not IFN-γ, induced a similar pattern of CysLT1R up-regulation. Monocytes pretreated with IL-13 or IL-4 for 24h showed enhanced CysLT1R protein expression. They also showed enhanced responsiveness to LTD₄, but not to LTB₄, in terms of Ca²⁺ mobilization, as well as augmented chemotactic activity. These findings suggested a possible mechanism by which Th2 cytokines can modulate CysLT1R expression on monocytes and macrophages, and consequently their responsiveness to LTD₄ (Thivierge et al., 2001). In the present work, we showed that IL-13 can also upregulate CysLT1R expression in BSMC.

Amrani and colleagues (Amrani et al., 2001b) demonstrated that IFN-γ upregulated CysLT1R expression in cultured HASM cells and that cell stiffness was increased in response to LTD₄. These data suggest that increased levels of IFN-γ in asthmatic individuals may promote airway hyperresponsiveness and asthma exacerbations by directly modulating contractile responses of HASM. In animal models, the increase in bronchial responsiveness induced by inhaled antigen is directly related to an increase in the mass of BSMC, and both increases were blocked by the CysLT1R antagonist MK-571 (Salmon et al., 1999). 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. This effect could be explained by the inhibitory effects of IFN-γ on cell proliferation and protein synthesis (Fiorentini et al., 2002), (Kikuchi et al., 2001), (Kominsky et al., 2000).

In the present work, we demonstrated for the first time that the upregulation of CysLT1R induced by TGF-β and IL-13 increases human BSMC response to LTD₄. The CysLT1R antagonist, Montelukast, ablated this effect suggesting that the proliferation effect was specific for LTD₄ and its receptor CysLT1.

It has been demonstrated that a significant increase in the area of ASM occurs in chronic asthmatic inflammation (Elias, 2000). Chronic overproduction of relatively low levels of CysLTs by mast cells or eosinophils may have subtle effects on structural cells of the airway, leading to bronchial hyperresponsiveness and possibly influencing pathways involved in airway wall remodeling (Holgate and Sampson, 2000).

Evidence suggest that LTD₄ can potentiate the induction of DNA synthesis and proliferation induced by the mitogen, Epidermal Growth Factor in HASM (Panettieri et al., 1998). In a mouse model, CysLTs seemed to be responsible of the increase in ASM, after repeated allergen challenge. The allergen-induced increases in ASM were significantly reduced by treatment with a CysLT1R antagonist (Henderson et al., 2002). In Brown Norway rats sensitized to
ovalbumin, CysLTs induced ASM and epithelial cell DNA synthesis as well as ASM thickening following repeated allergen exposure (Salmon et al., 1999).

Studies also provide evidence that cytokines such as TGF-β, a Th3 type cytokine, may also play a role in airway remodeling. Data obtained by Cohen and colleagues (Cohen et al., 2000) suggest that TGF-β can induce HASMC proliferation by increasing the expression of Insulin-Like Growth Factor Binding Protein-3 (IGFBP-3). Hence, the mitogenic action of TGF-β could be relevant in the hyperplastic nature of ASM cells in chronic asthma. In normal human lung, the bronchial epithelial compartment appears to be the main source of TGF-β (Magnan et al., 1994). The expression levels of this cytokine are dramatically increased in asthma and several other lung disorders (Minshall et al., 1997), (Corrin et al., 1994).

The Th2 type cytokine IL-13, is also over-expressed in asthmatic patients (Humbert et al., 1997). Interestingly, data suggest that both IL-4 and IL-13 cause inflammation, but only IL-13 causes subepithelial fibrosis which is mediated, to a great extent, by the production and activation of TGF-β in lung macrophages (Lee et al., 2001a), (Zhu et al., 1999). Data also support the role of IL-13 in airway remodeling by modulating the production of TGF-β2 from human bronchial epithelial cells. The release of TGF-β2 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-β2 induced by IL-13 in human bronchial epithelial cells (Wen et al., 2002),(Richter et al., 2001).

Taking all the above data in 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 is increased by TGF-β and IL-13 (The levels of which are augmented in asthmatic airways). Increased receptor expression can lead to increased LTD₄ signaling resulting in BSMC proliferation. Although the exact proliferation mechanism is unknown, evidence suggest that ERK and PI3-kinase pathways may be involved, given that these pathways are the major positive regulators of ASM proliferation (Page and Hershenson, 2000). However, additional experiments are needed to understand the precise mechanisms involved in this process.
Conclusions

In the present work, we studied the modulation of the CysLT1R by Th1, Th2 and Th3 type cytokines in a functional process relevant to airway remodeling, given that several previous studies had demonstrated that chronic inflammation induces airway remodeling in asthmatic patients.

We observed an up-regulation of the expression of CysLT1R in BSMC, induced by TGF-β, IL-13 and IFN-γ. The flow cytometry results were confirmed by Immunofluorescence analysis showing that the increased expression of the CysLT1R was distributed within the cytoplasm and nucleus of BSMC.

IL-13 and IFN-γ, but not TGF-β, were able to increase the expression of CysLT1R mRNA. These results suggest that the increased protein expression induced by TGF-β is due to a translational or post-translational effect.

We further demonstrated that BSMC stimulated for 24h with TGF-β or IL-13 showed increased proliferation when exposed to LTD₄. BSMC proliferation was CysLT1R-dependent, given that Montelukast, a CysT1R antagonist, ablated this effect. Taken together, our findings support a role for CysLT1R in airway remodeling seen in asthma.
References


hyperplasia with mucus accumulation in the airways of patients who died of severe acute asthma attack. *Chest* **101**:916-21.


Amrani Y, Moore PE, Hoffman R, Shore SA, Panettieri RA Jr. (2001b) Interferon-gamma modulates cysteiny1 leukotriene receptor-1 expression and


novel therapeutic opportunities in asthma. *Farmaco* 57:235-42.


muscle cells by IL-1beta and TNFalpha: effects of IL-10 and corticosteroids. *Br J Pharmacol* 127:1145-50.


de Boer WI, van Schadewijk A, Sont JK, Sharma HS, Stolk J, Hiemstra PS, et al.


Figueroa DJ, Breyer RM, Defoe SK, Kargman S, Daugherty BL, Waldburger K, et


Constitutive and cytokine-stimulated expression of eotaxin by human


Hedqvist P, Gautam N, Lindbom L. (2000) Interactions between leukotrienes and


Human airway smooth muscle cells express and release RANTES in
response to T helper 1 cytokines: regulation by T helper 2 cytokines and


role of cysteinyl leukotriene 1 receptor in the enhanced vascular permeability of mice undergoing acute inflammatory responses. *J Biol Chem*


Min YG, Lee CH, Rhee CS, Hong SK, Kwon SH. (1999) Increased expression of IL-4, IL-5, IFN-gamma, IL-6, IL-8, and TGF-beta mRNAs in maxillary mucosa of patients with chronic sinusitis. *Am J Rhinol* **13**:339-43.


Snashall PD, Chung KF. (1991) Airway obstruction and bronchial


