Université de Sherbrooke

Interactions Between Fibroblast Growth Factor 2 and Distinct Asthma Mediators Enhance Bronchial Smooth Muscle Cell Proliferation

Par

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Département de pédiatrie, Service d'immunologie/allergologie

Thèse présentée à la Faculté de médecine et sciences de la santé

en vue de l'obtention du grade de

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Résumé

L'augmentation de la masse des muscles lisses autour des bronches et bronchioles est une caractéristique typique de l'asthme. L'hyperplasie des cellules musculaires lisses (CML) contribue de façon majoritaire à ce changement structural des voies respiratoires. Plusieurs mediateurs retrouvés dans les poumons des asthmatiques sont susceptibles d'influencer de façon concertée ou de façon antagoniste la prolifération des CML. Les travaux de cette thèse ont pour objet de quantifier le rôle de certains facteurs de croissance, de certaines cytokines de type T_H2 et des cystéinyl-leucotriènes (cys-LTs) dans la prolifération des CML bronchiques humaines *in vitro* en tenant compte de leurs séquences de surexpression dans les poumons des asthmatiques suite à une provocation allergique. Les résultats démontrent que le *transforming growth factor* (TGF)β1, l'interleukine (IL)-4 et l'IL-13, trois médiateurs tardivement régulés à la hausse suivant une provocation allergique, ont aucun effet sur la prolifération des CML lorsqu'ils sont administrés seul. Par contre, un pré-traitement avec le *fibrobast growth factor* (FGF)2, un facteur rapidement libéré dans la lumière des bronches suite à une provocation allergique, confère au TGFβ1, ainsi qu'à l'IL-4 et l'IL-13 des effets mitogéniques. Dans tous les cas, les synergies semblent partiellement dépendantes d'une boucle autocrine de facteurs de croissance dans la famille du *platelet-derived growth factor* (PDGF), où le FGF2 induit l'expression de la chaîne α du récepteur des PDGFs et le TGFβ1, l'IL-4 et l'IL-13 induisent l'expression des ligands dépendants de cette chaîne pour signaler, soient le PDGF-AA et le PDGF-CC. Les cys-LTs, tant qu'à eux, n'ont aucun effet direct sur la prolifération des CML bronchiques avec ou sans pré-traitement avec le FGF2. Cependant, ils stimulent la production du TGFβ1 par les cellules épithéliales. Dans des conditions *in vivo*, où les CML ont déjà été stimulées par le FGF2, les cys-LTs pourraient donc induire la prolifération par une boucle paracrine impliquant la production de TGFβ1 par les cellules épitheliales. En somme, les résultats suggèrent que plusieurs facteurs surexprimés dans les poumons des asthmatiques peuvent collaborer pour induire la prolifération des CML. Le FGF2, entre autre, est un facteur essentiel, puisqu'en plus de son effet direct sur la prolifération des CML, il confère un effect mitogénique au TGFβ1, ainsi qu'aux cytokines de type T_H2, IL-4 et IL-13. Étant donné que les séquences de stimulations utilisées dans ces travaux réflètent temporellement les évènements se produisant dans des conditions *in vivo*, les synergies prolifératives répertoriées dans cette thèse sont susceptibles de contribuer à l'hyperplasie des CML que l'on retrouve dans les voies respiratoires des sujets asthmatiques.

Mots clés: muscle lisse des voies respiratoires, mitogénèse, facteurs de croissance, cytokines, leucotrienes.

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Abstract

Increased bulk of smooth muscle mass arround the airways is a typical feature of asthma. Several mediators act in concert or antagonistically to regulate airway smooth muscle (ASM) cell proliferation. This thesis focuses on fibroblast growth factor (FGF)2 and transforming growth factor (TGF)β1, which are known to be sequentially upregulated in the lung following allergic challenge and have recently been shown to synergize together in ASM cell proliferation. Emphasis is put toward the conflicting studies documenting the mitogenic effect of TGFβ1 *in vitro* and to its seemingly potent effect *in vivo*. Thereafter, different asthma mediators, such as IL-4 and IL-13, are introduced and how their mitogenic potential toward ASM cells could be altered by FGF2 is presented. Finally, how the controversial issue between *in vitro* and *in vivo* data regarding the mitogenic effect of leukotrienes could be reconciliated and how it could be related to FGF2 and TGFβ1 proliferative synergism is discussed.

Key words : airway smooth muscle, mitogenesis, growth factors, cytokines, leukotrienes

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LIST OF ABBREVIATION

aa	Amino acid
AA	Arachidonic acid
Ab	Antibody
AHR	Airway hyperresponsiveness
ALK	Activin receptor-like kinase
AM	Alveolar macrophages
AR-Smad	Activin-receptor activated Smads
ASM	Airway smooth muscle
BALF	Bronchoalveolar fluid
BDP	Beclomethasone dipropionate
BLT	Leukotriene B_4 receptor
CC10	Clara cell 10-KD protein
Co-Smad	Co-mediator Smad
cys-LTs	Cysteinyl leukotrienes
CysLT1	Cys-LTs receptor 1
DG	Diacylglycerol
Dox	Doxycycline
ECM	Extracellular matrix
ECP	Eosinophil cationic protein
EGF	Epidermal growth factor
EMTU	Epithelial-mesenchymal trophic unit
ERK	Extracellular signal-regulated kinase

FBS	Fetal bovine serum
FEV_1	Forced expiratory volume in 1 sec
FGF	Fibroblast growth factor
FGFR	FGF receptor
FHFs	FGF homologous factors
FLAP	5-lipoxygenase activating protein
FRS2	FGF receptor substrate 2
GAG	Glycosaminoglycans
GGL	γ-glutamyl leukotrienase
γ-GT	gamma-glutamyl transpeptidase
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GPCR	G protein-coupled receptor
Grb2	Growth factor receptor-binding protein
GSH	Gluthathione
GTPase	Guanosine triphosphatase
HBGF	Heparin binding growth factor
HMW	Higher molecular weight
5-HPETE	5-hydroperoxyeicosatetraenoic acid
HSPG	Heparan sulfate proteoglycans
IGF	Insulin-like growth factor
IGFR	IGF receptor
IGFBP	IGF binding protein
IL	Interleukin

IP	Inositol phosphate
JAK-STAT	Janus kinase-Signal transduction and activator of transcription
LAP	latency-associated peptide
LAR	late asthmatic reaction
LBP	LPS binding protein
LMW	Low molecular weight
5-LO	5-lipoxygenase
LPA	Lysophosphatidic acid
LPS	Lipopolysaccharide
LRP1	Low-density lipoprotein receptor-related protein 1
LTBP	latency TGFβ binding protein
LTC_4S	LTC_4 synthase
LTRA	Leukotriene receptor antagonist
LTs	Leukotrienes
MAPK	Mitogen-activated protein kinase
MBP	Major basic protein
MMP	Matrix metalloproteinase
MT1-MMP	Membrane type 1-MMP
NLS	Nuclear localization sequence
OR	Odds ratio
OVA	Ovalbumin
$p70^{S6K}$	70-KD kinase of S6 Ribosomal protein
PDGF	Platelet-derived growth factor

PDGFR	PDGF receptor
PI3K	Phosphoinositide 3-kinase
PKC	Protein kinase C
PLC	Phospholipase C
PPAR	Peroxisome proliferator-activated receptor
pSmad	Phosphorylated Smad
PTB	Phosphotyrosine binding domain
RTK	Receptor tyrosine kinase
SAC	Segmental allergen challenge
SBE	Smad binding element
SH2	Src homology 2 domain
SNP	Single nucleotide polymorphism
Sos	Son of sevenless
TGF β	Transforming growth factor beta
TβR	TGF beta receptor
T_H	T helper lymphocyte
TNFα	Tumor necrosis factor alpha
tPA	Tissue plasminogen activator
uPA	urokinase plasminogen activator
VEGF	Vascular endothelial growth factor
VSM	Vascular smooth muscle

INTRODUCTION

Allergic asthma is cause by an immunological response toward innocuous inhaled environmental triggers and is characterized by recurrent breathing symptoms secondary to inflammation, airway hyperresponsiveness (AHR) and airway obstruction. In 1922, Huber and Koesler (Huber and Koessler, 1922) described for the first time an increase in ASM tissue in the airways of asthmatics. Since then, enlargement of peribronchial ASM tissue has become a histopathologic signature of asthma (Hirst et al., 2004). Other structural alterations of the lung, collectively called remodeling, also occur in asthma. It includes epithelial metaplasia, which is largely characterized by goblet cells hyperplasia, and hypertrophy of bronchial glands, which both lead to mucus hypersecretion. Altered deposition of extracellular matrix (ECM) is also observed and mostly translates into subepithelial fibrosis, furthering the thickness of the airway wall (Pascual and Peters, 2005). However, due to its contractile nature and its increased abundance in asthma, ASM tissue has alway been thought responsible for AHR. Current evidence for this contention includes: 1- Exagerated bronchial responsiveness of asthmatic patients to non-inflammatory contractile agonists, such as cholinergic stimuli, in periods of asthma remission where inflammation and mucus hypersecretion are largely attenuated (Seow and Fredberg, 2001); 2- Mathematical modeling established in the early 1990's , which suggested that among all features of airway remodeling found in asthma, increased bulk of ASM tissue arround the bronchi was likely the main contributor to AHR (reviewed in (Pare et al., 1997)); and 3- Bronchial thermoplasty, which alters the structure and the contractility of ASM tissue in the wall of conducting airways, and was shown to cause

long term improvement in AHR in human asthmatics (Brown et al., 2005; Cox et al., 2006; Danek et al., 2004).

In the last few years, new aspects of ASM cell biology have gained interest of researchers working in the field of asthma. Information emanating from these studies suggests that ASM cells are not only a contractile unit, but are also able to express adhesion molecules for inflammatory cell recruitment and are potent secretagogues for cytokines, chemokines and ECM components (Joubert and Hamid, 2005). Hence, beyond their role in bronchoconstriction, ASM cells are now believed to play a significant role in the initiation and/or perpetuation of airway inflammation, as well as in the architectural changes that arise in asthmatic airways.

These new emerging concepts, together with the ability of ASM to contract in response to spasmogens, highlight the possible deleterious consequences of an increased peribronchial ASM mass in the pathology of asthma. In the same direction, elucidation of the main factors involved in ASM cell hyperplasia may significantly enhance our understanding of asthma etiology and may, hopefully, culminate in the elaboration of improved therapeutics for the treatment of this prevalent disease.

This thesis aims to present the current evidence supporting the role of TGFβ1 and FGF2 in ASM cell hyperplasia, to document the lack of data concerning the effect of IL-4 and IL-13 in this altered phenotype and to underscore recent developments made in this field that may shed light on some of the paradoxical results published so far

concerning the mitogenic effects of TGFβ1 and leukotrienes on ASM cells. New evidence is given that FGF2 may be the cornerstone of ASM cell hyperplasia owing to its ability to confer mitogenic potential to different asthma mediators.

GENERAL AND SPECIFIC OBJECTIVES

Chapter 1

General Objective: To review published studies supporting the involvement of TGFβ1 on ASM cell hyperplasia characterizing asthma.

Specific Objectives: The first objective of this chapter is to give a global overview of the TGFβ1 cytokine, its cell-surface receptors and the intracellular signaling pathways that are responsible for transducing its biological effects. The second objective is to compute the results of published studies regarding the expression of TGFβ1 in asthma or experimentally-induced asthma. Finally, the last objectives of this chapter are to discuss the current *in vivo* evidence supporting a role for TGFβ1 in ASM cell hyperplasia and the rather controversial state of the literature regarding the effect of TGFβ1 on ASM cell proliferation *in vitro*.

Chapter 2

General Objective: To review the current literature documenting the potential role of FGF2 on asthmatic ASM cell hyperplasia.

Specific Objectives: The first objective was to give an overall picture of the FGF2 growth factor and to present the different cell-surface receptors onto which this ligand can bind. The intracellular signal transduction pathways that ensure its biological effects are also briefly discussed. Then, the consitent results obtained by different groups of investigators, revealing its increased expression in asthmatic airways, together with its unequivocal effect on ASM cell proliferation *in vitro* are reviewed.

However, its as yet unrecognized contribution to ASM tissue remodeling *in vivo* is also highlighted.

Chapter 3

General Objective: To document the mitogenic effect of TGFβ1 on primary human bronchial smooth muscle cells with or without a pre-treatment with FGF2.

Working hypothesis: FGF2 pre-treatment influences the mitogenic potential of TGFβ1, which would help to explain the conflicting results obtained so far concerning the effect of TGFβ1 on ASM cell proliferation *in vitro*.

Specific Objectives: To determine the time- and concentration-dependent effect of TGFβ1 on ASM cell proliferation with or without a 24 hour pre-treatment with increasing concentrations of FGF2. The most important finding included in this chapter is a striking synergism between FGF2 and TGFβ1 on ASM cell proliferation. The next objectives then turn toward elucidating the operational mechanisms involved in this proliferative synergism.

Chapter 4

General Objective: Discuss the results obtained in chapter 3 in relation to the collective proliferative synergism of ASM cells that might occur *in vivo* between any member of the TGFβ1 and FGF2 families.

Specific Objectives: The first objective of this chapter was to identify all other members of TGFβ1 and FGF2 families that have been shown to be upregulated in asthmatic airways. Since the different members of each of these families transduce their biological effects via similar signaling pathways, we postulate that they might

exert similar effects. If true, any member of one family potentially synergizes with any member of the other family in inducing ASM cell proliferation. This chapter underscores the potential contribution of other members of the TGFβ1 and FGF2 families to the enlarged ASM tissue found in the bronchi and bronchioles of asthmatic patients. Overall, it proposes the hypothesis that ASM cell hyperplasia occuring *in vivo* may not simply be the result of the individual proliferative synergism between FGF2 and TGFβ1, but rather to the potential additive action of all individual synergisms susceptible to occur between every upregulated members of the FGF2 family with any of the upregulated members of the TGFβ1 family.

Chapter 5

General Objective: To review the current state of the literarure in regard to the effect of IL-4 and IL-13 on ASM cell proliferation.

Specific Objectives: The first objective of this chapter was to introduce IL-4 and IL-13 cytokines in terms of their structures, their cognate cell-surface receptors and the intracellular signaling pathways that they use to transduce their biological effects. This chapter also aims to review the knowledge aquired so far concerning the role that each of these cytokines might have in asthma etiology and diathesis. It is concluded that even if either IL-4 or IL-13 were shown to be required, and sometime sufficient, in the development of many pathognomonic features of asthma, their respective role in ASM cell hyperplasia is still unknown.

Chapter 6

General Objective: To document the individual and the FGF2-combined effects of IL-4 and IL-13 on primary human bronchial smooth muscle cell proliferation.

Specific Objectives: To determine the concentration-dependent effect of IL-4 and IL-13 on ASM cell proliferation with or without a pre-treatment with increasing concentrations of FGF2. Similar to what we observed with TGFβ1 in chapter 3, the results suggest that both IL-4 and IL-13 have no mitogenic effect on their own, but synergize in a concentration-dependent manner with FGF2 to induce ASM cell proliferation. The next objectives were then to determine the operational mechanisms involved in this proliferative synergism.

Chapter 7

General Objective: To review the current state of knowledge concerning the effect of cys-LTs on ASM cell hyperplasia.

Specific Objectives: The first objectives of this chapter are to give a general overview of cys-LTs structure, their synthesis and their cognate cell-surface receptors, and to briefly discuss their well-established increased expression in asthma. Another objective is to revise the conflicting results between *in vitro* studies, which suggest very weak or no direct effect of cys-LTs on ASM cell proliferation, and the *in vivo* studies, which consistently report a trophic effect of these mediators in ASM cell hyperplasia. Owing to the great reminiscence of this last observation with the effect of TGFβ1 highlighted in chapter 1, the final objective of chapter 7 was to wrap up recent evidence suggesting that cys-LTs are potent inducers of TGFβ1 *in vivo*, as well as in different types of cells *in vitro*.

Chapter 8

General Objective: To document the potential paracrine influence of epithelial cell-derived, cys-LT-induced TGFβ1 on the proliferation of primary human bronchial smooth muscle cells.

Working hypothesis : Based on our previous results suggesting an important effect of TGFβ1 on ASM cell proliferation (chapter 3), we hypothesized that the mitogenic effect of cys-LTs reported *in vivo*, together with their lack of effect *in vitro*, may be related to their capacity to stimulate the production of this growth factor in airway epithelial cells, which will, in turn, act as a paracrine factor to induce ASM cell proliferation.

Specific Objectives: The initial objective of this chapter was to determine whether cys-LTs are capable to upregulate TGFβ1 expression in an epithelial cell line that overexpressed the high affinity receptor for leukotriene D_4, CysLT1. Another major concern was to determine whether this endogenously produced TGFβ1 is able to support ASM cell proliferation. This initial part of the work presented in chapter 8 reports that cys-LTs are potent inducers of TGFβ1 and that this cys-LT-induced TGFβ1 is able to support the proliferation of FGF2-pretreated ASM cells. It was thereof important to determine whether untransformed airway epithelial cells behaved in a similar manner. Hence, the hypothesis that CysLT1 is expressed on airway epithelial cells and that epithelial cells have the capacity to respond to cys-LTs in a CysLT1 specific manner in term of TGFβ1 production were tested. The cumulated data highlights the possibility that cys-LTs can induce TGFβ1 expression in airway epithelial cells. Altogether, these results shed light on the conflicting results reported

so far between *in vivo* and *in vitro* studies documenting the effect of cys-LTs on ASM cell proliferation, and suggest that the mitogenic effect observed *in vivo* may be related to a paracrine involvement of airway epithelial cell-derived ASM cell mitogens that are induced by cys-LTs.

CHAPTER 1

TGFβ1

1.1. TGFβ1

TGFβ1 was first isolated and characterized in platelets in 1983 (Assoian et al., 1983) and is now the prototypic member of a superfamily of cytokines, which actually counts 33 members in man (de Caestecker, 2004). TGFβ1 is encoded by a 7-exon gene localizes on chromosome 19q13 and several genetic studies have associated some of its common single nucleotide polymorphisms (SNP) with asthma phenotypes (Buckova et al., 2001; Hakonarson et al., 2001; Hobbs et al., 1998; Mak et al., 2006; Nagpal et al., 2005; Pulleyn et al., 2001). TGFβ1 protein has a short half-life (normally less than 3 min) in cell free systems (Wakefield et al., 1990). However, to overcome its lability, TGFβ1 is usually secreted in a latent form as a 180-210-KD multi-protein complex containing the glycosylated 125-190-KD latency TGFβ binding protein (LTBP), the 75-KD latency-associated peptide (LAP) and the 25-KD mature form of TGFβ1 (Harpel et al., 1992).

1.2. TGFβ1 activation

Activation of latent TGFβ1 occurs through different mechanisms including: 1- proteolytic dissociation from LAP by the urokinase plasminogen activator (uPA)/plasmin system (Khalil et al., 1996a; Lyons et al., 1988), or by other proteases such as metalloproteinase (MMP)-2 (McMahon et al., 2003), MMP-9 (Lee et al., 2001a; Yu and Stamenkovic, 2000) and the lysosomal serine protease cathepsin D (Lyons et al., 1988); 2- conformational alteration in its structure by thrombospondin

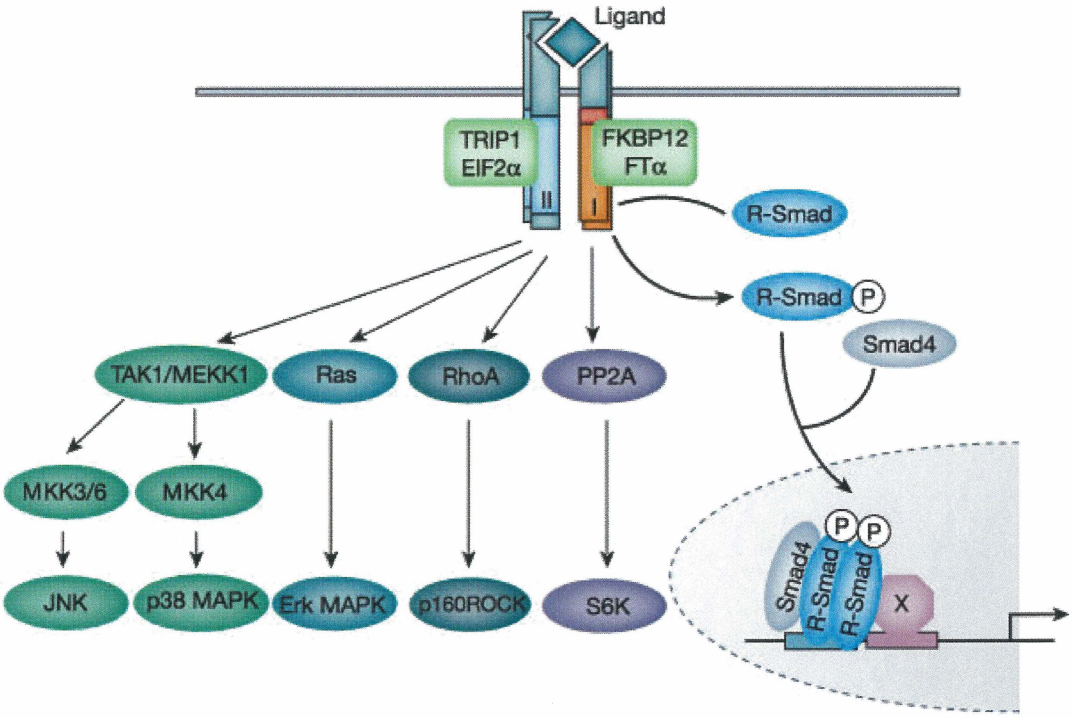
(Crawford et al., 1998) or integrin such as αvβ6 (Morris et al., 2003; Munger et al., 1999); 3- oxidation and nitrosylation (Barcellos-Hoff and Dix, 1996; Vodovotz et al., 1999); 4- removal of carbohydrate structure on LAP by glycosidases such as sialidase (Miyazono and Heldin, 1989); and 5- integrin αvβ8-mediated latent TGFβ1 recruitment to the cell membrane for membrane type 1 (MT1)-MMP-dependent proteolytic activation (Fjellbirkeland et al., 2003). Extracellular regulation of TGFβ1 activation is also influence by different cell-surface molecules and ECM constituents that bind TGFβ1 and ensure its activation in restricted localised compartment. For example, mannose 6-phosphate/insuline-like growth factor II (IGF-II) receptor (Gleizes et al., 1997; Kovacina et al., 1989) as well as integrins α8β1 (Lu et al., 2002) and αvβ1 (Munger et al., 1998) bind latent forms of TGFβ1 and are thus believed to target the latent complex on the surface of cells for subsequent proteolytic activation with ensuing binding to its signaling receptor. TGFβ1 is also a heparin binding growth factor (HBGF) (Miyazono et al., 1994a). Consequently, its binding availability for cell surface receptors is regulated extracellularly by heparan sulfate proteoglycans (HSPG). Whereas certain proteoglycans, such as betaglycan and endoglin (Cheifetz et al., 1992), facilitate TGFβ1 binding to its receptors; others, such as biglycan and decorin, sequester TGFβ1 in the ECM (Redington et al., 1998). In addition, because of their ability to release TGFβ1 from pericellular stores, certain enzymes such as thrombin, neutrophil elastase or mast cell chymase may be essential in the process of TGFβ1 activation, even if they cannot activate latent TGFβ1 directly (Taipale et al., 1992; Taipale et al., 1995).

1.3. TGFβ1 receptors and signaling

Six receptors have been identified for TGFβ1 (Huang and Huang, 2005), but the most studied are the 65-KD type I receptor (TβRI or ALK5), the 85-KD type II receptor (TβRII), the 280-KD type III receptor (TβRIII or betaglycan, a heparan sulfate/chondroitin sulfate proteoglycan), and more recently the 504-KD TβR5, which is also known as the low-density lipoprotein receptor-related protein 1 (LRP1). The canonic mechanisms by which TGFβ1 binds and activates its cognate cell surface receptors as well as the intracellular signaling pathways that transduce intracellularly the TGFβ1 message from the cell membrane to the nucleus have been reviewed extensively (Shi and Massague, 2003). Briefly, TGFβ1 initially binds to the single transmembrane, constitutively active, serine/threonine kinase TβRII homodimer. The formed complex subsequently recruits the single transmembrane, activable, serine/threonine kinase TβRI homodimer, which is concomitantly activated by TβRII-mediated phosphorylation of several threonine and serine residues in its intracellular GS juxtamembrane domain. This phosphorylated GS domain then serves as a docking site for activin-receptor activated Smads (AR-Smads; namely Smad2 and Smad3), which are, in turn, phosphorylated by TβR1. The phospho-AR-Smads (pSmad2 and pSmad3) then homo or hetero-oligomerize with each other and with at least one co-mediator Smad (Co-Smad; most often called Smad4) and the complex ultimately translocates to the nucleus where it binds Smad binding element (SBE)-containing promoters or interacts with other transcriptional partners to regulate gene expression. Apart from the Smad pathway, it is now clear that other intracellular signaling pathways such as mitogen-activated protein kinase (MAPK), the phosphoinositide 3-

kinase (PI3K), the PP2A phosphatase-mediated $p70^{S6K}$ inactivation and the Rho-family of small guanosine triphosphatase (GTPase) pathways are activated by TGFβ1 and transduce some of its biological activities (reviewed in (Derynck and Zhang, 2003) and (Moustakas and Heldin, 2005)) (Figure 1). In addition, Smads were shown to cross talk with other important signaling pathways such as Janus kinase-Signal transduction and activator of transcription (JAK-STAT) (Ulloa et al., 1999) and WNT (Nishita et al., 2000).

Figure 1: TGFβ1 signaling



Derynck and Zhang, Nature 2003; 425: 577-84.

1.4. Expression of TGFβ1 in asthma

Expression of TGFβ1 is altered in asthma and the current weight of evidence suggests that TGFβ1 is upregulated in human and animal asthmatic airways (summarized in Table 1 and 2). However, 5 studies performed with human tissues have shown no regulation of TGFβ1 expression in asthma. In contrast to their previous articles, in which they reported an increased expression of TGFβ1 in bronchoalveolar lavage fluid (BALF) before and after allergic challenge (Redington et al., 1997), Redington and coworkers (Redington et al., 1998) have demonstrated indistinguishable pattern of TGFβ1 immunohistochemical staining between asthmatic and control subjects. Aubert and coworkers (Aubert et al., 1994) had previously reported similar findings, but their results were contested since their control subjects were heavy smokers. The relative intensity of TGFβ1 immunostaining in the bronchial mucosa was also similar between asthmatics and healthy subjects in Hoshino and coworkers' study (Hoshino et al., 1998). More recently, Chu and coworkers (Chu et al., 2004) confirmed these results by documenting a lack of significant augmentation of TGFβ1 immunoreactivity in asthmatic epithelium. Agreeing with these studies, Balzar and coworkers (Balzar et al., 2005) have shown no difference in the number of cells staining positive for TGFβ1 in the submucosa of normal subjects and asthmatic patients suffering from different severity of the disease.

Reasons for these discrepancies are currently unknown. However, all conflicting results came from studies measuring TGFβ1 expression by immunohistochemical approach, using tissue specimens obtained by bronchial biopsies or lung resections.

Immunohistochemistry requires extensive tissue handling. All the steps before microscopic reading, including immediate precaution to preserve tissue integrity, reagent used for fixation or to embed the tissue, strength and specificity of the detection and the staining antibodies, and bleaching of the fluorochrome or attenuated chemiluminescence signal occurring during the procedures could all lead to erroneous results and false interpretations. It is thus reasonable to surmise that the conflicting results concerning the increased expression of TGFβ1 in the airways of asthmatics may be the result of technical artefacts. However, alternative hypothesis may explain this conundrum.

Temporal concerns

It is worth mentioning that collection of lung specimens offers promiscuous advantages for studying mRNA or protein expression at the tissue level. For example, staining of cross-sectional sections of these lung specimens by immunohistochemical approach or by *in situ* hybridization brings ample information regarding the tissue or the cellular sources of TGFβ1. Combined with laser microdissection, tissue specific expression of a particular gene can even be confirmed by more conventional technique such as RT-PCR (Kelly et al., 2005). Unfortunately, limits of these techniques are also prominent. As such, results obtained from these experiments must be interpreted with caution. Protein or mRNA detected in lung specimens reflect their expression levels at a particular time point. Asthma is a waxing and waning disease, where a period of exacerbation is usually followed by a period of remission and where the severity of symptoms is temporally associated with the degree of airway inflammation. Therefore, upregulation of asthma mediators, such as TGFβ1, is also

likely to be inducible and transient in nature. Correspondingly, TGFβ1 was shown to be increased at 24 h, but not at 10 min, following segmental allergic challenge (SAC) and its concentration returned to baseline level after 1 week (Batra et al., 2004; Redington et al., 1997). Whether TGFβ1 expression starts to increase earlier is unknown, but in animal models of acute or chronic antigen challenge, TGFβ1 expression in BALF is still unaffected 6 h following the last allergen exposure (Kumar et al., 2004). In contrast to Batra and coworkers (Batra et al., 2004), Redington and coworkers (Redington et al., 1997) also reported a statistically significant increase in TGFβ1 level in BALF of asthmatics at baseline compared to healthy controls (8 pg/ml vs 5.5 pg/ml), but whether this difference is physiologically relevant remains questionable. Tillie-Leblond and coworkers (Tillie-Leblond et al., 1999) have also reported no difference in the levels of latent and active form of TGFβ1 in BALF at baseline between mild asthmatics and healthy volunteers. In the same study, both the latent and active form of TGFβ1 were significantly increased in patients suffering from *status asthmaticus* compared to healthy controls or to patients presenting similar severity of the disease, but distant from an acute exacerbation period. Nomura and coworkers (Nomura et al., 2002) have substantiated these results by examining longitudinal changes that occur in the lung function (forced expiratory volume in 1 sec, % of predicted (%FEV1)) and the percentage of TGFβ1 positive cells in induced sputum samples of five asthmatic subjects. They demonstrated that during asthma exacerbation, %FEV1 decreased from 86.5 to 51.0% and that TGFβ1 positive cells rose from 1.9 to 55.4% during the same time period. These results confirmed the inducible and transient upregulation of TGFβ1 that have been

demonstrated by others in BALF following SAC (Batra et al., 2004; Redington et al., 1997). Based on results obtained with animal models of chronic allergen challenge-induced airway remodeling, it was also suggested that several allergen provocations must be required before the upregulation of TGFβ1 could be appreciated (Corbel et al., 2003).

These aformentioned findings suggest that the samples would need to be collected following bronchoprovocative challenge to observe the transient increase of TGFβ1 expression by immunohistochemistry. In all studies documenting no regulation of TGFβ1 expression in asthma, lung specimens had been taken at baseline, i.e. in a remission period where no sign of exacerbation was present or without prior experimentally-induced bronchoprovocation. In Hoshino and coworkers' study (Hoshino et al., 1998) for example, asthmatics presented daily symptoms, but based on their attack score, the number and severity of symptoms were very low, suggesting that subjects were not in an exacerbation period when biopsies were taken. In the immunohistochemical study carried out by Redington and coworkers (Redington et al., 1998), asthmatic subjects were presented as mildly symptomatic. However, they were clinically stable despite being restricted from use of oral or inhaled glucocorticoids for 4 weeks, indicating once again that no acute exacerbation was present at the time bronchial biopsies were taken. Hence, failure to demonstrate a significant upregulation may simply reflect the punctual expression of TGFβ1 measured in the two extreme poles of a transient response. Taken together, these results implied that TGFβ1 is not necessarily overexpressed in asthmatics at baseline,

but it is inducible upon allergen challenge. Determining the sequence and the kinetics of TGFβ1 expression may be important to increase our understanding of the role of this cytokine in asthma.

Spatial concerns

Along with the transient nature of TGFβ1 response following allergic challenge, failure to detect an increased expression of TGFβ1 in certain immunohistochemical studies may be related to the airway compartment studied. Expression of TGFβ was shown to be heterogenous within the same sample (Vignola et al., 1997) and its increased expression in asthma may occur exclusively in very localized compartments. For instance, periglandular tissues or sites of epithelial desquamation were shown to stain strongly for this cytokine (Kokturk et al., 2003; Vignola et al., 1997). On the other hand, Magnan and coworkers (Magnan et al., 1997) have demonstrated homogenous intensity of TGFβ immunostaining in ciliated and mucous cells as well as in areas of epithelial impairment, such as sites of deciliated cells or desquamated regions. However, apart from a homogenous staining in the epithelium within each sample, Magnan and coworkers (Magnan et al., 1997) suggested an altered compartmentalization of TGFβ expression in asthmatic airways. Whereas TGFβ immunoreactivity was strong in the epithelium of control subjects, negative or faintly positive staining was observed in this particular compartment of asthmatics. In contrast, asthmatics expressed higher amounts of TGFβ in the submucosa compared to healthy individuals. This epithelial to submucosal redistribution of TGFβ was in accordance with an increased number of inflammatory cells staining positive for

TGFβ in the submucosa of human asthmatics (Chakir et al., 2003; Chu et al., 2000; Flood-Page et al., 2003; Minshall et al., 1997; Ohno et al., 1996; Vignola et al., 1997).

Whatever the physiologic or pathophysiologic reason for this altered compartmentalization, the same trend of TGFβ1 relocalization was observed in murine models of allergic airway inflammation. In this regard, McMillan and coworkers (McMillan and Lloyd, 2004) have demonstrated that TGFβ1 expression was confined to the bronchiolar and alveolar epitheliums in control animals and was relocalized to the submucosal compartment in association with inflammatory infiltrates after repeated allergen challenges of sensitized animals. In this particular model, even smooth muscle became positive for TGFβ1 immunostaining during the chronic phase of allergen challenge. Interestingly, this altered compartmentalisation also occurred in other types of airway inflammation, such as the one induced by prolonged (4 wk) lipopolysaccharide (LPS) exposure (Savov et al., 2002). Initially, TGFβ1 expression was confined to the airway epithelium, but subsequent to LPS exposure, TGFβ1 immunostaining was mainly localised in the subepithelial area (Savov et al., 2002). Hence, in addition to look at the right time, investigators attempting to document an increased expression of TGFβ1 in asthma need to look at the right place.

With the use of techniques permitting to appreciate the overall expression of TGFβ1, such as in studies using BALF, serum or plasma, or with the use of animal models, which allow sufficient biologic materials to be homogenized, it is becoming clear that

TGFβ1 is upregulated in asthma following allergic challenge. But once again, controversies are reported and are related to different peculiarities of the studied populations. For example, Joseph and coworkers (Joseph et al., 2003) have reported an increase of TGFβ1 expression in the plasma of nonatopic, but not of atopic asthmatic patients. However, using atopic patients only, which were included based on skin prick test positivity and corroborating medical history of allergen-induced asthma, Karagiannidis and coworkers (Karagiannidis et al., 2006) reported a significant increase of TGFβ1 in the serum of asthmatics, attaining levels almost 7-fold higher than those measured in healthy controls. No clear explanation is currently ascribed to explain these contrasting results and the question of whether TGFβ1 expression is influence by the atopic phenotype will need further exploration.

Increased expression of TGFβ1 measured in the BALF must also be interpreted with caution. Epithelium desquamation is a characteristic feature of remodeled asthmatic airways. Epithelium denudation may give access to a certain amount of TGFβ1, which is otherwise masked by an intact epithelium in non-asthmatic individuals. Thus, the increased expression of TGFβ1 observed in BALF of asthmatics following challenge may simply be related to an easiest accessibility to TGFβ1 stores cause by epithelium desquamation. In support to this contention, increased concentration of TGFβ1 has been noted in BALF following a sham bronchoprovocation procedure, which is likely to be the result of epithelial damage (Redington et al., 1997). Moreover, positive correlation ($r = 0.89$) has been reported in the same study between concentration of TGFβ1 and the number of epithelial cells collected in BALF of saline-challenged site.

These results suggest that epithelium denudation renders a bulk of TGFβ1 normally sequestered in the subepithelial layer, such as the one associated with the basal lamina (Aubert et al., 1994; Redington et al., 1998), collectable by bronchoalveolar lavage. Of major concern, degranulation products of eosinophils such as major basic protein (MBP) and eosinophil cationic protein (ECP) (Frigas et al., 1980; Robinson et al., 1992), as well as mast cell proteases, such as tryptase and chymase (Redington et al., 1995), are damaging for the airway epithelium. Increased expression of TGFβ1 observed at 24 h, but not at 10 min, following SAC may consequently be caused by eosinophil-mediated epithelium desquamation, rather then a true *de novo* TGFβ1 protein synthesis. Together, these observations raise doubts on the techniques currently used to measure the expression of different mediators in the airways and for instance, to the increased expression of TGFβ1 in asthma.

Table 1: Expression of TGFβ1 in human asthma

Tissues	mRNA or protein	Description of the effect Asthmatics vs controls	References
Lung resections or necropsies	1- mRNA (expression relative to GAPDH) 2- Protein	1- 108 vs 100% in asthmatics vs controls, respectively (ns). 2- Neither different patterns nor different levels of expression. (P.S. control subjects were heavy smokers) - Connective tissues of the airway wall, alveolar macrophages and the epithelium were + for TGFβ1.	(Aubert et al., 1994)
Sputum	Protein	- Sputum was collected from asthmatics during moderate or severe attacks. - Active form was ND, but inactive form was 21.7 ng/ml. - There was no control group for comparison.	(Adachi et al., 1996)
Bronchial biopsies	mRNA	- 52.1 vs 10.5 + cells/mm^2 in severe	(Ohno et al.,

		asthmatics vs controls, respectively ($p < 0.02$), but was not elevated in mild asthmatics (1.0 + cell/mm^2) - In asthmatics, + cells were eo.	1996)
Alveolar macrophages	Protein	- Median of 435 vs 210 ng/million cells in asthmatics vs controls, respectively ($p = 0.005$).	(Vignola et al., 1996)
Bronchial biopsies	Protein*	- Altered compartmentalization: In asthmatics, TGFβ1 intensity of staining decreased in the epithelium ($p < 0.01$) and increased in the submucosa in association with an increase inflammatory cell infiltrate.	(Magnan et al., 1997)
Bronchial biopsies	Protein*	- Median of 4 vs 0 + cells/mm of BM in the epithelium and 31.5 vs 0 + cells/mm^2 in the submucosa in controls vs asthmatics, respectively ($p \leq 0.015$).	(Vignola et al., 1997)
BALF	Protein	- 8.0 vs 5.5 pg/ml in asthmatics vs controls, respectively ($p = 0.027$). - Increased expression following SAC: 31.3 vs 25.0 at 10 min ($p = 0.78$) and 46.0 vs 21.5 pg/ml at 24 h ($p = 0.017$) post-allergen- vs saline-challenged sites, respectively.	(Redington et al., 1997)
Bronchial biopsies	1- mRNA 2- Protein	- 18.5, 10.8 and 7.8 vs 3.5 + cells/mm of BM in severe, moderate and mild asthmatics vs controls, respectively ($p < 0.05$ for severe and moderate asthmatics). - 18.8, 12.3 and 9.2 vs 5.2 + cells/mm of BM in severe, moderate and mild asthmatics vs controls, respectively ($p < 0.05$ for all groups of asthmatics).	(Minshall et al., 1997)
Bronchial biopsies	Protein	- 18 vs 16% relative intensity in the bronchial mucosa of asthmatics vs controls (ns).	(Hoshino et al., 1998)
Bronchial biopsies	Protein	- Neither different patterns nor different levels of expression between asthmatics and controls. - Positive staining was observed in subepithelial connective tissues of the airway wall and in the bronchial epithelium.	(Redington et al., 1998)
Bronchial biopsies	Protein*	- 12.5 vs 6.6 + cells/mm^2 in the submucosa of asthmatics vs controls (ns, $p = 0.06$).	(Chu et al., 1998)
BALF	Protein	- Active TGFβ1: median of ~350 and 30 vs 60 pg/ml in *status asthmaticus* and stable asthmatic patients vs controls, respectively ($p < 0.05$).	(Tillie-Leblond et al., 1999)

		- Latent TGFβ1: median of ~900 and 75 vs 100 pg/ml in *status asthmaticus* and stable asthmatic patients vs controls, respectively ($p < 0.05$).	
Bronchial biopsies	Protein*	-Median of ~41.4 vs 7.8, 11.1, 15, and 8.1 cell/mm^2 in the submucosa of eo + severe asthmatics vs controls, mild asthmatics, moderate asthmatics and eo – severe asthmatics, respectively ($p = 0.0003$). - TGFβ + cells correlate with eosinophils, neutrophils and macrophages number ($p < 0.0001$).	(Wenzel et al., 1999)
BALF-enriched alveolar macrophages	mRNA	- Increased in mild atopic asthmatics vs controls ($p < 0.005$).	(Prieto et al., 2000)
1-Bronchial biopsies 2-Peripheral blood neutrophils	1- Protein* 2- Protein (spontaneous release *ex vivo*)	1- 108 vs 24 + cells/mm^2 of airway submucosa in asthmatics vs controls ($p = 0.002$). 2- 115 vs 46 pg/10^6 cells in asthmatics vs controls, respectively ($p = 0.007$).	(Chu et al., 2000)
Airway epithelial cells	Protein (*ex vivo*)	- ~4 vs 1.5 pg/10^4 cells in asthmatics vs controls ($p = 0.032$).	(Hastie et al., 2002)
Cells in sputum samples	Protein	- 23 out of 26 asthmatics demonstrated TGFβ1+ cells vs 0 out of 8 in normal volunteers.	(Nomura et al., 2002)
Bronchial biopsies	Protein*	- ~11 and 6 vs < 1 + cells/mm^2 of bronchial submucosa, in severe-to-moderate and mild asthmatics vs controls, respectively ($p < 0.05$). - Immunoreactivity was mainly localized in inflammatory cells.	(Chakir et al., 2003)
1- Bronchial biopsies 2- BALF	1- mRNA 2- Protein	1- Median of ~43 vs 5 + eo/mm^2 before vs after treatment with mepolizumab in mild atopic asthmatics, respectively ($p = 0.04$). - Median of ~20 vs 39 + eo/mm^2 before vs after placebo treatment, respectively. 2- Median of ~5 vs 4 pg/ml before vs after treatment with mepolizumab in mild atopic asthmatics, respectively ($p = 0.05$). - Median of ~4 vs 4 pg/ml before vs after placebo treatment, respectively. - In both cases, there was no control group for comparison.	(Flood-Page et al., 2003)
Bronchial biopsies	Protein	- 13.5 vs 3.2% of tissue area in ASM layer in persistent asthmatics vs controls ($p =$	(Berger et al., 2003)

		0.02), but was not elevated in intermittent asthmatics (2.7%) and was not affected in neither the epithelial nor the submucosal tissues.	
Plasma	Protein	- 2.5 vs 1.5 ng/ml in nonatopic asthmatics vs controls, respectively ($p = 0.002$), but was not different in atopic asthmatics (1.4 ng/ml).	(Joseph et al., 2003)
Bronchial biopsies	Protein	- Higher intensity of staining in the submucosa, but not in the epithelium, of asthmatics vs controls ($p < 0.05$). - TGFβ1 was mainly localized in association with connective tissue in all groups.	(Kokturk et al., 2003)
BALF	Protein	- Identical at baseline (~50 pg/ml) - Significantly increased after SAC in asthmatics only (~170 pg/ml) ($p < 0.05$).	(Batra et al., 2004)
Bronchial epithelium	Protein	- (1.0-2.4) vs (0.2-1.6)% of total epithelial area (interquartile 25-75% range) in asthmatic vs normal subjects, respectively (ns).	(Chu et al., 2004)
Bronchial biopsies	1- mRNA 2- Protein*	1- no difference between mild and severe (with or without persistent eosinophilia) asthmatics vs controls. 2- The majority of subjects in every groups expressed undetectable or very low number of + cells in the submucosa.	(Balzar et al., 2005)
1-Serum 2-$CD4^+$ T cells	1- Protein 2- mRNA	1- 11.7, 14.5 and 36.8, vs 5.5 ng/ml in severe asthmatics with inhaled and systemic GCs, moderate asthmatics with inhaled GCs and moderate asthmatics without any drug treatment vs healthy subjects, respectively ($p \leq 0.001$ for all groups of asthmatics vs controls). 2- Decreased expression in moderate asthmatics without any drug treatment compared to each of the 3 other groups ($p \leq 0.01$).	(Karagiannidis et al., 2006)
Exhaled breath condensate	Protein*	- 1.69-fold increase in asthmatics compared to healthy subjects ($p < 0.01$).	(Matsunaga et al., 2006)

*Antibody used did not discriminate between TGFβ1, 2 or 3 or is not specified.
Unless otherwise indicated, amounts of TGFβ1 represent the mean values.
Abbreviation: +, positive; BALF, bronchoalveolar lavage fluid; BM, basement membrane; eo, eosinophils; GCs, glucocorticoids; ns; not statistically significant; SAC, segmental allergen challenge.

Table 2: Increased expression of TGFβ1 in animal models of asthma

Species	Models	Tissues	mRNA or Protein	Extent of the increase	References
Female BALB/c mice	OVA sensitization and challenge	Whole-lung lavage	Active protein	- Increased in function of allergen concentration. From ~10, 60, 145 and 190 pg/ml in saline, 0.01, 0.1 and 1% OVA, respectively ($p < 0.01$).	(Tanaka et al., 2001)
C57BL/6 mice	IL-13 transgenic	1- Lung homogenates	1- mRNA	1- Increased expression in transgenic mice vs littermate controls.	(Lee et al., 2001a)
		2- Whole-lung lavage	2- Protein	2- ~294 vs 132, 1794 vs 59 and 2610 vs 88 pg/ml in transgenic mice vs littermate controls at 1, 2 and 3 mo of age, respectively ($p < 0.01$).	
			Active protein	- ~2.4 to 4.3-fold increases in luciferase activity when CCI-64 cells stably transfected with a luciferase reporter gene driven by the PAI-1 promoter were stimulated with BALF of transgenic compared to littermate control mice ($p < 0.001$).	
		3- Lung sections	3- mRNA and Protein	3- Expression was restricted to the airway epithelium and to some AM in littermate controls, but increased in AM and was appreciated in airway epithelium, type II pneumocytes and occasionally in eosinophils of transgenic mice.	
Male C3H/HeBF eJ mice	Endotoxin-induced asthma	Lung sections 1- Subepithelium area	Protein (Staining intensity was graded from 0 to 3)	1- 2.64 vs 0 relative intensity in air- vs LPS-exposed animals after 4 wk of LPS exposure ($p < 0.005$), but this difference did not persist after a 4 wk recovery period.	(Savov et al., 2002)
		2- Epithelium		2- 2.59 vs 0.83 relative intensity in air- vs LPS-exposed animals after 4 wk of recovery of the 4 wk LPS exposure ($p < 0.005$), but no difference (0.83 vs 1.14 in air- vs LPS-exposed animals) after the 4 wk of exposure.	
Male BP2 mice	OVA sensitization and challenge	- BALF and whole lung	Protein*	- Increased expression in both of these lung compartments 48 h after the last challenge of a 8 mo protocol of bronchoprovocation, but not after 48 h of a single challenge, compared to control mice.	(Corbel et al., 2003)
Female $WBB6F_1$, W/W^v and $S1/S1^d$ mice	OVA sensitization and challenge	Whole-lung lavage	Active protein	- 44.9, 41.8 and 583.6 pg/ml in control, sensitized only and sensitized/challenged $WBB6F_1$ mice, respectively ($p < 0.001$). - 38.4, 45.3 and 555.8 pg/ml in	(Masuda et al., 2003)

				control, sensitized only and sensitized/challenged W/W^v, respectively ($p < 0.001$). - 102.4 vs 464.6 pg/ml in sensitized only vs sensitized/challenged Sl/Sl^d, respectively ($p < 0.05$).	
Male C3HeB/FeJ (LPS-sensible) and C3H/HeJ (LPS-insensible) mice	Endotoxin-induced asthma	Whole-lung lavage	1- Protein 2- Active protein	1- ~865 vs 58 pg/ml in C3HeB/FeJ vs C3H/HeJ after 5 d of LPS exposure ($p < 0.05$), but was not different after 4 h of exposure or 96 h after an 8 wk of exposure. 2- ~111 vs 19 pg/ml in C3HeB/FeJ vs C3H/HeJ after 5 d of LPS exposure ($p < 0.05$), and ~22 vs 0 pg/ml in C3HeB/FeJ vs C3H/HeJ after 4 h of LPS exposure ($p < 0.05$), but not different 96 h after a 8 wk of LPS exposure.	(Brass et al., 2003)
Female C57BL/6 and congenic IP KO mice	OVA sensitization and challenge	Whole-lung lavage	Active protein	- 47.5, 57.9 and 178.0 pg/ml in control, sensitized only and sensitized/challenged C57BL/6, respectively ($p < 0.01$). - 39.1, 44.3 and 342.5 pg/ml in control, sensitized only and sensitized/challenged IP KO, respectively ($p < 0.01$).	(Nagao et al., 2003)
Female BALB/c, BALB/cJ, and BALB/cJ congenic IL-4 KO mice	OVA sensitization and challenge	Whole-lung lavage	Active protein	- 17.1 and 265.3 pg/ml in sensitized only and sensitized/challenged BALB/c, respectively ($p < 0.01$). - 27.5, 32.5 and 333.8 pg/ml in control, sensitized only and sensitized/challenged BALB/cJ, respectively ($p < 0.01$). - 27.9, 29.5 and 81.6 pg/ml in control, sensitized only and sensitized/challenged IL-4 KO, respectively ($p < 0.01$).	(Komai et al., 2003)
Female BALB/c mice	OVA sensitization and challenge	1- Lung sections 2- Lung homogenates	1- Protein 2- Active protein	1- Altered compartmentalization of TGFβ1 immunoreactivity, from airway epithelium to submucosal compartment. 2- ~66, 180, 160 and 66 vs 50 pg/ml in 25, 35, 55 days challenged and 80 days challenged, 1 month recovered vs control mice, respectively ($p < 0.05$ for 35 and 55 days challenged vs control mice).	(McMillan and Lloyd, 2004)
Female BALB/c mice	OVA sensitization and challenge	1- Whole-lung lavage fluid	Protein	1- 300 vs 135, 356 vs 156 and 369 vs 146 pg/ml in sensitized mice challenged vs unchallenged for 1, 3 and 6 mo, respectively ($p = 0.03$). - Reduction to 241 and 269 pg/ml with ISS treatment in sensitized mice challenged for 3 mo and 6 mo, respectively, ($p = 0.05$), but no reduction in the 1 mo challenged group.	(Cho et al., 2004b)

		2- Lung homogenates		2- 1946 vs 664 pg/mg of lung protein in challenged (3 mo) vs unchallenged sensitized mice, respectively ($p = 0.02$). - Reduction to 939 pg/mg of lung protein in challenged sensitized mice treated with ISS ($p = 0.05$)	
C57BL/6 and congenic IL-5 KO mice	OVA sensitization and challenge	1- Lung homogenates	1- Protein	1- 3740 vs ~1855 pg/ml in challenged vs unchallenged sensitized WT mice, respectively ($p < 0.01$). - 2170 vs ~1455 pg/ml in challenged vs unchallenged sensitized IL-$5^{-/-}$ mice ($p < 0.05$ when compared challenged mice with WT and IL-$5^{-/-}$ genotypes).	(Cho et al., 2004a)
		2- Lung sections (number of peribronchial cells + for TGFβ per bronchiole of 150-200 μm of internal diameter)	2- Protein*	2- 64.5 vs ~5 TGFβ + cells/bronchus in challenged vs unchallenged sensitized WT mice, respectively ($p < 0.001$). - 22.6 vs ~1 TGFβ + cells/bronchus in challenged vs unchallenged sensitized IL-$5^{-/-}$ mice ($p < 0.001$ when compared challenged mice with WT and IL-$5^{-/-}$ genotypes). - Peribronchial cells + for TGFβ were mainly eo (63%) and macrophages (35%), but increased expression of TGFβ was also observed in the epithelium of both WT and IL-$5^{-/-}$ challenged mice.	
Male C57BL/6 and congenic LBP KO mice	Endotoxin-induced asthma	Whole-lung lavage fluid	1- Protein	1- 266.1, 173.8 and 43.2 vs nd pg/ml in 4 wk exposed, 3 d recovered, 5 d and 4 h exposed vs control mice, respectively. - 115.7, 87.7 and 34.2 vs nd pg/ml in 4 wk exposed, 3 d recovered, 5 d and 4 h exposed vs control $LBP^{-/-}$ mice, respectively.	(Brass et al., 2004)
			2- Active protein	2- 4.3, 58.3 and 6.5 vs nd pg/ml in 4 wk exposed, 3 d recovered, 5 d and 4 h exposed vs control mice, respectively. - nd, 23.5 and 5.4 vs nd pg/ml in 4 wk exposed, 3 d recovered, 5 d and 4 h exposed vs control $LBP^{-/-}$ mice, respectively.	
Female BALB/c, congenic IL-$13^{-/-}$ and $CD4^+$-depleted mice	OVA sensitization and acutely challenge or chronically challenge	1- Lung sections	1a) Protein (Ab used was specific for the active form, but the latent form was revealed following proteinase K treatment) (staining	1a)- Intense staining in airway epithelium of naïve mice - Median values of 3 vs 2 in the epithelium of chronically challenged (6 wk), sensitized WT vs similarly treated IL-$13^{-/-}$ mice ($p < 0.01$). - Median values of 3 vs 3 in the epithelium of chronically challenged (6 wk), sensitized WT vs similarly treated $CD4^+$-depleted	(Kumar et al., 2004)

			intensity was graded from 0 to 3)	mice (ns).	
			1b) Active protein	1b)- Median values of 2, 1.5 and 0 in the epithelium at 3, 6 and 24 h following the last exposure, respectively, in chronically challenged, sensitized WT mice compared to 0, 0 and 0.5 in acutely challenged, non-sensitized and to 1, 1.5 and 0.5 in acutely challenged, sensitized WT mice at the same time points. - Median values of 2, 2 and 2 in the subepithelial zone at 3, 6 and 24 h following the last exposure, respectively, in chronically challenged, sensitized WT mice compared to 0, 0 and 0 in acutely challenged, non-sensitized and 2, 3 and 2 in acutely challenged, sensitized WT mice at the same time points. - Median values of 3, 2, 2 and 1 vs 0 in the subepithelial zone of sensitized WT mice, challenged for 8, 6, 4 and 2 wk vs naïve mice, respectively ($p < 0.05$ for 8 and 6 wk compared to naïve). - Median values of 2 vs 1 in the subepithelial zone of chronically challenged (6 wk), sensitized WT vs similarly treated IL-$13^{-/-}$ mice ($p < 0.05$). - Median values of 2 vs 0 in the epithelium of chronically challenged (6 wk), sensitized WT vs similarly treated IL-$13^{-/-}$ mice 3 h after the last exposure ($p < 0.01$). - Median values of 2 vs 0.5 in the subepithelial zone of chronically challenged (6 wk), sensitized WT vs similarly treated $CD4^{+}$-depleted mice ($p < 0.01$).	
		2- Whole-lung lavage	2-Active protein	2- 11.8, 6.5, 4.3 and 8.0 vs 5.1, ng/ml in non-sensitized acute, sensitized acute, non-sensitized chronic and sensitized chronic at 6 h following the last challenge vs naïve, respectively (ns). - 80% of TGFβ1 in the BALF was in an active form.	
Male BALB/c mice	OVA sensitization and challenge	Whole-lung lavage	Active Protein*	~300 vs 30 pg/ml in sensitized and challenged vs shammed animals, (ns).	(Peng et al., 2005)
Female BALB/c	OVA sensitization	1- Whole lung	mRNA	1- No difference between sensitized mice challenged with OVA or	(Kelly et al., 2005)

mice	and challenge	2- Microdissected: a) Bronchial wall b) Epithelium c) ASM		saline at either 2 or 8 wk following the last exposure. 2- Increased at 2 wk, but not at 8 wk, following the last exposure in the bronchial wall ($p < 0.05$). - When the epithelium and the ASM cells of the bronchial wall were analysed separately, the increase observed at 2 wk after the last exposure was restricted to the epithelium ($p < 0.05$).	
BALB/c mice	OVA sensitization and challenge	Lung homogenates	mRNA	- Weak, but seemingly significant decrease at 2, but not 12 h, following two or four 1-h antigen challenge in mice recovered from a first period of challenge 228 days ago ($p \leq 0.01$).	(Karagianni dis et al., 2006)
BALB/c T-cell GATA-3 or T-bet transgenic mice	OVA sensitization and challenge	Lung homogenates	Protein	- ~6000 vs 7250 and 5250 pg/rat lung in WT vs GATA and T-bet transgenic naïve mice, respectively, compared to 5750 vs 8375 and 4425 in the same groups 1 d following the last challenge on sensitized mice ($p < 0.05$ for both transgenic mice vs WT after sensitization/challenge).	(Kiwamoto et al., 2006)
Female BALB/c mice	Occupational asthma (TDI-induced)	1- Lung homogenates 2- Whole-lung lavage	Protein	1- Increased expression 48 h after the last TDI inhalation. - PPARγ agonists rosiglitazone and pioglitazone, as well as transferred of adenovirus gene vector expressing PPARγ2 cDNA or BAY 11-7085 partially prevent TDI-induced TGFβ1. 2- nd vs ~120 pg/ml 48 h after the last TDI inhalation in control vs experimental group, respectively ($p < 0.05$). - PPARγ agonists rosiglitazone and pioglitazone, as well as transferred of adenovirus gene vector expressing PPARγ2 cDNA partially prevent TDI-induced TGFβ1 ($p < 0.05$).	(Lee et al., 2006a)
Female BALB/c mice	OVA sensitization and challenge	1- Whole-lung lavage 2- Lung sections	Protein	1- 236 vs 789 and 543 pg/ml in control vs sensitized and challenged mice treated or not with fluticasone ($p < 0.05$). 2- Increased positive cells in the peribronchial region of control vs sensitized and challenged mice, which was reduced by fluticasone treatment.	(Lee et al., 2006c)
Female BALB/c mice	OVA sensitization and challenge	Whole-lung lavage	Protein	- ~650 vs 450 pg/ml in OVA-challenged vs saline-challenged sensitized mice, respectively. - Continued to increase steadily in a chronic (38 additional days)	(Munitz et al., 2006)

				challenge protocol, up to ~900 pg/ml. - Correlated with eo counts in the lavage ($R^2 = 0.89$). - The increase was completely reversed with a bispecific antibody directed against CD300a that targeted CCR3+ cells (LC1) ($p < 0.01$).	
Brown Norway rats	OVA sensitization and challenge	- Lung sections (midlevel segmental bronchi)	- Protein*	- Staining intensity scores in airway epithelium and ASM tissue were significantly increased from isotype control in sensitized/challenged (4.71), but not in control rats (2.00). - Addition of pirfenidone in sensitized/challenged animal's diet may slightly decreased staining intensity (4.5).	(Mansoor et al., 2006)
Female C57BL/6	OVA sensitization and challenge	1- Whole-lung lavage (7 days after the last challenge) 2- Lung homogenates (7 days after the last challenge)	- Protein	1- ~125 vs nd pg/ml in OVA-challenged vs saline-challenged sensitized mice, respectively ($p < 0.05$). - Reduced to ~60, 65 and 70 vs 126 or 128 pg/ml in Montelukast-, Pranlukast- and anti-IL-11 Ab- vs vehicle- or control Ab-treated animals, respectively ($p < 0.05$). 2- Increased in OVA-challenged vs saline-challenged sensitized mice ($p < 0.05$). - Reduced in Montelukast-, Pranlukast- and anti-IL-11 Ab- vs vehicle- or control Ab-treated animals ($p < 0.05$).	(Lee et al., 2007)

*Antibody used did not discriminate between TGFβ1, 2 or 3 or is not specified. Unless otherwise indicated, amounts of TGFβ1 represent the mean values.
Abbreviation: AM, alveolar macrophages; d, day; eo, eosinophils; h, hour; IP, prostaglandin (PG)I_2 receptor; KO, knockout; LBP, LPS binding protein; nd, none detected; ns, not statistically significant; OVA, ovalbumin; PAI-1, plasminogen activator inhibitor-1; PPAR, peroxisome proliferator-activated receptor; TDI, toluene diisocyanate; wk, week; WT, wild type.

1.5. Cellular sources of TGFβ1 in asthma

Studies investigating the cellular source of TGFβ1 in asthma have also yielded inconsistent results. It is known that TGFβ1 is widely expressed throughout the body and every resident structural and immune cell in the lung, as well as every inflammatory cell mobilised to the airways during asthma exacerbation, are able to

express and secrete TGFβ1. In the lung of non-asthmatic human or animal, airway epithelium seems to be the major site of TGFβ1 expression (de Boer et al., 1998; Lee et al., 2001a; Magnan et al., 1994; Magnan et al., 1997; Pelton et al., 1991; Warshamana et al., 2001; Yamauchi et al., 1988). However, other stromal cells in the airways such as fibroblasts (Kelley et al., 1991a; Kelley et al., 1991b; Lee et al., 1995; Vignola et al., 1997), endothelial cells (Coker et al., 1996), vascular smooth muscle (VSM) cells (de Boer et al., 1998) and ASM cells (Berger et al., 2003; Black et al., 1996; Coutts et al., 2001; de Boer et al., 1998; Fukuda, 1993; Hamet et al., 1991; Kokturk et al., 2003; Lee et al., 2006b; Magnan et al., 1994; Majesky et al., 1991; McKay et al., 1998) are also potential sources of this cytokine, since they were all shown to express and produce detectable amount of TGFβ1. Due to its affinity to certain components of the ECM, latent forms of TGFβ1 in the lung have a tendency to accumulate in particular compartments of the airway wall. In fact, many immunohistochemical studies have localized TGFβ1 mainly in extracellular compartments in association with connective tissues of the airway wall (Aubert et al., 1994; Kokturk et al., 2003; Redington et al., 1998). However, the cellular sources of ECM-sequestered TGFβ1 are hard to infer. Contrasting these results, Magnan and coworkers (Magnan et al., 1997) were unable to identify TGFβ expression in extracellular space, but rather identified inflammatory cells infiltrating the submucosa and the epithelium as the major source of this cytokine. One could speculate that this controversy may be related to the use of a pan-TGFβ antibody (Ab) in Magnan and coworkers study, but the detection of all three forms of TGFβ instead of TGFβ1 only would be an additional reason to find TGFβ in extracellular spaces.

Neutrophils

In asthma, the cellular origin of TGFβ1 is less clear and numerous inflammatory cells as well as structural cells were shown to contribute. Blood- or airway-derived neutrophils in normal and asthmatic individuals were shown to express TGFβ (Chu et al., 2000; Grotendorst et al., 1989). Since, airway neutrophilia is particularly prominent in nonatopic asthma (Amin et al., 2000) and in more severe forms of the disease (reviewed in (Ennis, 2003)), neutrophils could contribute significantly to the increased expression of TGFβ in these types of asthma. In this regard, Chu and coworkers (Chu et al., 2000) have demonstrated that around 55% of TGFβ positive cells in the submucosal compartment of asthmatics and normal controls were neutrophils (Chu et al., 2000). However, only a fraction of neutrophils expressed TGFβ (29 and 20% in asthmatic and normal submucosa, respectively). In animals, increased TGFβ1 expression in the subepithelial area following prolonged (4 weeks) exposure to LPS was also neutrophil-dependent, as neutropenic animals did not develop this altered expression of TGFβ1 (Savov et al., 2002). Interestingly, upregulation of TGFβ1 expression in the airway epithelium observed after a recovery period of 4 weeks following exposure to LPS was also dependent on the presence of neutrophils during the LPS exposure period (Savov et al., 2002). Hence, it was concluded that neutrophils may be a direct source of TGFβ1 following their mobilisation into the submucosa, but may also alter the subsequent expression of TGFβ1 in other airway compartments, for instance the epithelium. In addition to the epithelium, Lee and coworkers (Lee et al., 2006b) have recently demonstrated that neutrophil elastase increased the expression of TGFβ1 in ASM cells, suggesting once

again that the mobilisation and activation of neutrophils into the airways may increase TGFβ1 via indirect mechanisms. Since TGFβ1 was also recognised as a potent trophic factor for granulopoiesis (Keller et al., 1991) and was shown to recruit, activate and prolong survival of neutrophils in other diseases (Fava et al., 1991), TGFβ1 upregulation and neutrophilia observed in asthma may mutually feedback each other, allowing the establishment of a vicious cycle potentially involved in disease exacerbation.

Eosinophils

In contrast to the aforementioned studies, Ohno and coworkers (Ohno et al., 1996) claimed that close to 100% of cells positive for TGFβ1 mRNA in mild and severe asthma were eosinophils, whereas this cell accounts for only 20.8% of total TGFβ1 mRNA positive cells in control subjects. Similarly, Flood-Page and coworkers (Flood-Page et al., 2003) have shown that 86% of cells positive for TGFβ1 mRNA in the bronchial mucosa were eosinophils, and that 76% of total eosinophil population in this tissue compartment was immunolabeled. These findings were substantiated by Minshall and coworkers study (Minshall et al., 1997), which reported a coefficient of determination (R^2) of 0.86 between TGFβ1 mRNA positive cells beneath the basement membrane and the degree of eosinophilia in the same compartment. In this latter study, 65% of the TGFβ1 mRNA positive cells were eosinophils and 75% of eosinophils were positive for TGFβ1 mRNA. The remaining TGFβ1 mRNA positive cells were identified as macrophages and fibroblasts (Minshall et al., 1997). Finally, Vignola and coworkers (Vignola et al., 1997) reported that eosinophils accounted for

80% of TGFβ1 mRNA positive cells in the submucosa, the other 20% being fibroblasts.

Many other studies performed in humans or in animals bear witness to the contention that eosinophils are a major cellular source of TGFβ1 in asthmatic lungs by demonstrating a linear relationship between the degree of eosinophilia and the expression of TGFβ1 in different lung tissues. For example, Nomura and coworkers (Nomura et al., 2002) found positive correlation between eosinophil counts and the number of cells staining positive for TGFβ1 in induced sputum samples of asthmatics. In a murine model of allergic asthma, Tanaka and coworkers (Tanaka et al., 2001) reported a positive correlation between the number of eosinophils and the level of TGFβ1 in whole-lung lavage fluid. It was also proposed that eosinophils from bronchial asthmatics are more competent in TGFβ1 secretion compared to eosinophils of control subjects, owing to their elevated TGFβ1 mRNA (Minshall et al., 1997) and protein (Ohno et al., 1992) levels per cell.

In inflammatory conditions affecting the upper airways, such as nasal polyps or allergic rhinitis, the cells expressing TGFβ1 gene in the mucosa specimens were also identified as being predominantly eosinophils (Ohno et al., 1992). The proportion of eosinophils positive for TGFβ1 gene was estimated to 50%. However, a great deal of TGFβ1 protein in these specimens was not cell-associated, but rather localized in the ECM associated with the vessels, the basement membrane or within the submucosa (Ohno et al., 1992). This later result was consistent with several

immunohistochemical studies investigating the expression of TGFβ1 expression in lung specimens (Aubert et al., 1994; Kokturk et al., 2003; Redington et al., 1998).

IL-5 knockout mice also support the role for eosinophils in TGFβ1 production. Compared to wild type animals, IL-5-deficient mice chronically exposed to allergic challenge showed a decreased expression of MBP positive cells, which paralleled the reduction in the number of TGFβ positive cells in the peribronchial region and a decrease of TGFβ1 expression in whole lungs (Cho et al., 2004a). These changes in TGFβ1 expression also correlated with fewer signs of airway remodeling. In accordance with this animal model, treatment of mild atopic asthmatics for 2 months with anti-IL-5 Ab (mepolizumab) was shown to be successful in reducing tissue eosinophilia, lung TGFβ1 expression and deposition of ECM components in the lamina reticularis (Flood-Page et al., 2003). Therefore, the fact that both IL-5-deficient mice (Cho et al., 2004a) and asthmatics treated with anti-IL-5 Ab (Flood-Page et al., 2003) demonstrated fewer eosinophils, less TGFβ1 expression and fewer signs of remodeling supports the notion that eosinophils are an important source of TGFβ1 in the lungs of asthmatics and that IL-5-dependent recruitment of eosinophils is a prerequisite for TGFβ1-mediated airway remodeling. However, it is worthy of mention that the statistically significant decrease in the median level of TGFβ1 in BALF reported by Flood-Page and coworkers (Flood-Page et al., 2003) following anti-IL-5 treatment was approximately 1 pg/ml, which is unlikely to be physiologically relevant.

Macrophages

In the above study with IL-5-deficient mice, Cho and coworkers (Cho et al., 2004a) also demonstrated that 35% of TGFβ1 positive cells in the peribronchial region were macrophages. In humans, macrophages derived from induced sputum samples also expressed TGFβ1 (Nomura et al., 2002) and alveolar macrophages from asthmatics released spontaneously higher amounts of TGFβ1 relative to alveolar macrophages derived from control subjects (Vignola et al., 1996). Similarly, Prieto and coworkers (Prieto et al., 2000) found higher level of TGFβ1 mRNA in alveolar macrophages of mild atopic asthmatics compared to healthy subjects. In Magnan and coworkers study (Magnan et al., 1997), TGFβ positive cells present in the submucosa of asthmatics were mainly identified as lymphocytes, macrophages and, to a lesser extent, eosinophils. In a transgenic model of asthma induced by lung overexpression of IL-13, TGFβ1 mRNA and protein were observed mainly in macrophages, but also in type II pneumocytes, airway epithelial cells and occasionally in eosinophils (Lee et al., 2001a). In a mice model of prolonged allergen challenge-induced airway remodeling, the main source of TGFβ1 was identified as mononucleated cells, likely macrophages (McMillan and Lloyd, 2004). Collectively, these studies suggest that macrophages are a likely source of TGFβ1 in asthma.

The higher levels of TGFβ1 mRNA in BALF-enriched alveolar macrophages (AM) observed by Prieto and coworkers (Prieto et al., 2000) in mild atopic asthmatics at baseline was not further increase following repeated low-dose allergen inhalation. Similarly, the increased release of TGFβ1 by alveolar macrophages derived from asthmatics demonstrated by Vignola and coworkers (Vignola et al., 1996) was at

baseline (i.e. without prior allergen bronchoprovocation). These results suggest that alveolar macrophages of asthmatics produce higher amounts of TGFβ1 spontaneously. It equally raise the possibility that the baseline overexpression of TGFβ1 observed in asthma by certain investigators (Kokturk et al., 2003; Matsunaga et al., 2006) is related to the increased production of this cytokine by alveolar macrophages. The teleologic advantage of an increased TGFβ1 production by these cells in asthma is unknown. However, it is worth mentioning that alveolar macrophages are predominant in the airways compared to other cells mobillised into the airways following allergen challenge. Consequently, the mediators they produce are susceptible to influence to a great extent the pathologic outcomes. Of interest, alveolar macrophages were shown to be protective against asthma development in a rat model of asthma (Careau and Bissonnette, 2004). Owing to its well known immunosuppressive activity, it is tempting to speculate that the increased expression of TGFβ1 observed in alveolar macrophages of asthmatics at baseline may represent a regulatory mechanism to mitigate the variable chronic ongoing inflammation during the stable phase of the disease. In addition, and in contrast to other airway compartments, expression of TGFβ1 may not be altered in this particular cell following allergen challenge.

Mast cells

Unfortunately, the contribution of mast cells in the upregulation of TGFβ1 expression in asthma is not clear either. Based on a study using mast cell-deficient mice (W/W^v and Sl/Sl^d), Masuda and coworkers (Masuda et al., 2003) have demonstrated that the overall contribution of mast cells in the upregulation of TGFβ1 expression in BALF

of sensitized and challenged mice was negligible. However, several groups have demonstrated that mast cells were capable of secreting TGFβ1 constitutively or upon stimulations in *in vitro* conditions (Baumgartner et al., 1996; Gordon and Galli, 1994; Pennington et al., 1991).

Epithelium

In contrast to Magnan and coworkers (Magnan et al., 1997) that reported a decrease expression of TGFβ in the epithelium of asthmatics, some studies have pointed toward this tissue to explain the increased expression of TGFβ1 in the airways of asthmatic individuals (Hastie et al., 2002; Vignola et al., 1997). Vignola and coworkers (Vignola et al., 1997) reported that TGFβ was faintly expressed in the airway epithelium of control subjects and was significantly elevated in asthmatic subjects. It was also demonstrated that the spontaneous release of TGFβ1 *ex vivo* was higher in airway epithelial cells derived from asthmatic subjects compared to that derived from non-asthmatic subjects (Hastie et al., 2002). In animal model of sensitized mice chronically challenged by inhalation of low doses of antigen, Kumar and coworkers (Kumar et al., 2004) have demonstrated that TGFβ1 expression increased in airway epithelial cells, but not in eosinophils or any other non-epithelial cells. In addition, using laser capture microdissection and real-time PCR to quantify TGFβ1 mRNA level in lung sections of mice, Kelly and coworkers (Kelly et al., 2005) demonstrated that the TGFβ1 mRNA upregulation observed 2 wk after chronic allergen exposure in sensitized animals was confined to the airway epithelium. Kumar and coworkers (Kumar et al., 2004) have also shown that TGFβ1 in airway epithelial cells of naïve animals is in its uncleaved, biologically inactive form. Following

chronic challenge of sensitized mice with low doses of antigen, the cleaved and biologically active form of TGFβ1 was found mainly in the subepithelial zone in association with connective tissue (Kumar et al., 2004). They suggested that the increased expression of TGFβ1 in the subepithelial zone simply reflects deposition of epithelial cell-derived TGFβ1 onto the subjacent ECM following its activation by antigen challenge. Hence, the concept that the increased expression of TGFβ1 in the submucosa originates from inflammatory cell infiltrates makes no unanimous consensus among investigators in the field. This result was consistent with observations made by Kokturk and coworkers (Kokturk et al., 2003) on human tissues, which confirmed the increased expression of TGFβ1 in the airway submucosa of asthmatics despite the lack of a simultaneous alteration in inflammatory cell infiltrate. However, only asthmatics that were free from symptoms for at least a month preceding the biopsy were included in this latter study. It is thus possible that remnant (i.e. non-utilised) inflammatory cell-derived TGFβ1 was stored in the ECM after secretion and, as a result, be responsible for the increased TGFβ1 expression observed at a time when cellular inflammation was resolved. Otherwise, increased TGFβ1 expression may be produced by airway structural cells such as the cells present in the submucosa, including (myo)fibroblasts and ASM cells (Kokturk et al., 2003; McMillan and Lloyd, 2004), or may be derived from the epithelium as suggested by Kumar and coworkers (Kumar et al., 2004). These later also suggested that the concentration of antigen and the number of antigen expositions are key elements determining which cells will preferentially produce TGFβ1 in allergic asthma. They concluded that eosinophils are the main TGFβ1-producing cells in acute models of

allergic asthma challenged with high doses of antigen, but the epithelium is the main source of this cytokine in sensitized animals chronically challenged with low doses of antigen.

This increased TGFβ1 production by the airway epithelium in asthma is consistent with numerous reports suggesting that epithelial cell-derived TGFβ1 could be upregulated upon different phlogogenic challenges *in vitro* (Dakhama et al., 2003; Hastie et al., 2002; Perng et al., 2006; Richter et al., 2001; Warshamana et al., 2001); albeit conflicting results have also been reported (Kwong et al., 2004). *Ex vivo* cultures of bronchiolar epithelial cells derived from smokers and from patients with COPD also secrete higher amounts of TGFβ compared from those of control patients (Takizawa et al., 2001). In addition, TGFβ1 upregulation in airway epithelial cells occurs by mechanical stress that mimics bronchocontriction (Tschumperlin et al., 2003), as well as in several *in vivo* conditions in addition to animal models of asthma (Cho et al., 2004a; Kelly et al., 2005; Kumar et al., 2004), including IL-13 transgenic mice (Lee et al., 2001a), and advanced pulmonary fibrosis (Khalil et al., 1996b) and COPD (Takizawa et al., 2001) in humans.

The decreased expression of TGFβ in the airway epithelium of asthmatics reported in some studies (Magnan et al., 1997) may reflect an active secretion of TGFβ. In this case, intracellular stores found in non-asthmatic epithelium would give higher staining intensity in immunohistochemical study, but the latter would be attenuated in asthmatics as soon as the intracellular stores are emptied from the cells during the

course of the disease. Physiologically, this active TGFβ secretion may be interpreted as an attempt by the epithelium to buffer excessive ongoing inflammation. Alternatively, a decreased immunoreactivity may represent a real decrease in *de novo* synthesis of TGFβ1 by the asthmatic epithelium. In this case, this may represent a well-regulated process that favours inception or perennialization of airway inflammation.

1.6. Cellular sources of TGFβ1 in other types of inflammation

In other types of airway inflammation mediated by allergy-independent mechanisms, such as the one induced by prolonged LPS exposure, TGFβ1 also increased in the whole-lung lavage, as well as in the epithelium and the submucosal compartments of the lung (Brass et al., 2003; Brass et al., 2004). Similarly, a single intratracheal delivery of an adenoviral vector containing the proinflammatory cytokine IL-1β was sufficient to increase the expression of TGFβ1 (Kolb et al., 2001a). Together, these findings suggest that TGFβ1 is induced downstream of many kinds of inflammation, probably acting as a counterregulatory cytokine to resolve inflammation and to initiate repair processes. If this conjecture is true, and because asthma is an inflammatory disease of the airways, it is expected that TGFβ1 would be upregulated in asthmatic airways at a later time-point following challenge. In addition, since it is a cytokine ubiquitously expressed, its cellular source in a particular disease may originate from the cells triggered by the inflammatory signals or by the inflammatory cells mobilised to the site of inflammation *per se*. Consequently, in the case of severe asthma where neutrophils predominate, neutrophils would be the main source of TGFβ1; and in the

case of mild to moderate asthma where eosinophils predominate, eosinophils would be the principal cells secreting this cytokine. This hypothesis would reconciliate many of the conflicting results published so far and simply suggested that TGFβ1 is upregulated as a general mechanism to circumvent inflammation and its secretion is ensured by any cells present at the site of inflammation. Therefore, inconsistencies surrounding the cellular source of TGFβ1 expression in asthma may be related to either the heterogeneity of asthma groups studied or to the particular states of the disease (exacerbation vs remission period) when the biopsies were taken.

Some weaknesses in the studies involved in the controversial issue concerning the cellular source of TGFβ1 in asthma are also worthy of mention: Firstly, the Ab used in Magnan and coworkers (Magnan et al., 1997), Vignola and coworkers (Vignola et al., 1997), and Chu and coworkers (Chu et al., 2000) did not discriminate between the 3 isoforms of TGFβ and thus, the staining distribution and intensity is additionally confounded by TGFβ2 and TGFβ3 expression. Secondly, the discrepancy may also be related to the control group of Magnan and coworkers (Magnan et al., 1997), half of which were smokers and all showed existing or suspected lung disease. Finally, absence of medication withdrawal in the asthmatic group before tissue collections in this same study could also have led to erroneous results.

1.7. Active TGFβ signaling in asthma

Active TGFβ signaling, measured by nuclear phosphorylated Smad2 (pSmad2) immunostaining, has also been observed in airways of animal (Lee et al., 2006c;

McMillan et al., 2005; Rosendahl et al., 2001) and human (Phipps et al., 2004; Sagara et al., 2002) asthmatics before and after allergic challenge. Whether it is a result of an increased expression of TGFβ, its desequestration from ECM or simply its activation could be debated, but active signaling surely testify that one or several of these processes are operative in asthma.

However, one should mention that this increased pSmad2 observed in asthma did not exclude the possible involvement of other TGFβ family members in the activation of AR-Smads (Smad2/3). Activin A, in particular, has recently gained interest in asthma pathophysiology. Both mRNA levels in total lung (Karagiannidis et al., 2006) and BALF concentrations (Cho et al., 2003) of Activin βA were upregulated following OVA sensitization and challenge in mice. In humans, serum levels of Activin A were shown to be elevated in moderate asthmatics (1.16 ng/ml) compared to healthy individuals (0.14 ng/ml) (Karagiannidis et al., 2006). These studies suggest that increased expression of pSmad2 following SAC may not be entirely related to TGFβ1, but also to other cytokines of the TGFβ superfamily that signal via the AR-Smads, such as Activin A. However, the serum levels of TGFβ1 were more than 20-fold higher than those of Activin A (Karagiannidis et al., 2006). In addition, this latter was 10-fold less potent than TGFβ1 to activate Smad2/3 complex, as measured by transfection of human lung fibroblasts (IMR-90) with a Smad2/3-responsive reporter gene (Karagiannidis et al., 2006). It is thus believed that TGFβ1 might outweigh the effect of Activin A, and consequently, may represent the main contributor of Smad2 phosphorylation following SAC.

TGFβ2 is also of particular interest in asthma. Two studies that failed to identify an increased expression of TGFβ1 in asthmatic airways have looked at TGFβ2 expression, and both revealed significant increases (Balzar et al., 2005; Chu et al., 2004). In one of these studies, Chu and coworkers (Chu et al., 2004) demonstrated a higher level of TGFβ2 in the airway epithelium of asthmatics compared to normal subjects. They further demonstrated that TGFβ2, but not TGFβ1, is increased in primary cultures of bronchial epithelial cells following IL-13 stimulation. This result was supported by two previous articles, in which both IL-13 and IL-4 increased TGFβ2 production in bronchial epithelial cells (Richter et al., 2001; Wen et al., 2002). In the second study, Balzar and coworkers (Balzar et al., 2005) quantified the number of cells staining positive for TGFβ in the submucosa. They demonstrated that among the 3 TGFβ isoforms, only TGFβ2 was increased in asthmatics. This increased expression was also restricted to the group of patients demonstrating the more severe form of the disease with persistent eosinophilia, which is surprisingly similar to the finding of Wenzel and coworkers (Wenzel et al., 1999) using a pan-TGFβ Ab. Moreover, they showed that tissue eosinophils from the severe group of patients expressed higher amounts of TGFβ2 compared to tissue eosinophils of control subjects or from patients suffering from a milder form of the disease. Hence, in addition to the increased production of TGFβ2 by the airway epithelium in response to T_H2 cytokines (Chu et al., 2004; Richter et al., 2001; Wen et al., 2002), eosinophils in the submucosa could contribute to the overall increase of TGFβ2 in the airways of severe asthmatics (Balzar et al., 2005).

Elevated expression of TGFβ2 following bronchoprovocation was further substantiated by Batra and coworkers (Batra et al., 2004). In that study, TGFβ2 expression was increased in BALF 24 h after SAC. However, these investigators also noted higher expression of TGFβ2 in non-asthmatics at baseline as well as 1 and 2 weeks after SAC compared to asthmatic subjects. In fact, only at 24 h post-SAC did TGFβ2 levels in BALF of asthmatics reach the concentration found in non-asthmatics. Collectively, these results suggest that TGFβ2 is increased in the airway epithelium of asthmatics (Chu et al., 2004), as well as in eosinophils of a subgroup of severe asthmatics (Balzar et al., 2005), and even if its baseline expression in the fluid harvested by bronchoalveolar lavage is lower compared to non-asthmatics, it is transiently increased after allergen challenge (Batra et al., 2004). Since the TGFβ2 isoform acts on the same receptors and signal via the same AR-Smads than TGFβ1, these results indicate that TGFβ2 is also a likely candidate to explain activation of Smad signaling in asthma.

However, TGFβ1 Ab was shown to prevent phosphorylation of Smad2 in a murine model of prolonged allergen challenge-induced asthma (McMillan et al., 2005). This finding suggested that TGFβ1 is responsible for the increased expression of pSmad2 in the airways of asthmatics and excluded the possible involvement of TGFβ2 or Activin A. Interestingly, this anti-TGFβ1 Ab was administered following the establishment of eosinophilic inflammation, and in addition to abrogating pSmad2 signaling *in situ*, it reduced total and proliferating ASM cell numbers, mucus

production and peribronchiolar ECM deposition. Collectively, these results suggest that anti-TGFβ1 therapy can be envisaged as a therapeutic approach (i.e. following the establishment and the diagnosis of the disease), not only to reverse fibrosis, but also to alleviate other features of airway disease in asthma.

Complementary to McMillan and coworkers' study (McMillan et al., 2005), Leung and coworkers have recently shown that SD-208, a pharmacological inhibitor targeting ALK5, administered both as a prophylactic or as a therapeutic, successfully reduce airway inflammation, goblet cell hyperplasia and ASM cell hyperplasia in a Brown Norway rat model of allergic asthma (Leung et al., 2006). This study suggests a potent pro-inflammatory action of TGFβ in asthma in addition to its well-recognized function in airway remodeling. This study also joined McMillan and coworkers' conclusions (McMillan et al., 2005), highlighting the beneficial effect of a strategy preventing TGFβ signaling in reversing established features of airway disease.

The anti-inflammatory effect observed with SD-208 are counterintuitive to the well-known immunomodulatory function of TGFβ1 (Leung et al., 2006). In fact, several evidences have shown that TGFβ1 counteracts excessive airway inflammation. Examples include the following: 1-TGFβ1 heterozygous mice, which express 30% of the TGFβ1 protein level observed in the wild type animal, develop a more severe form of the disease when exposed to an OVA sensitization/challenge protocol (Scherf et al., 2005); 2- T lymphocytes engineered to produce TGFβ1 or conditioned to secrete higher amounts of TGFβ by oral tolerance reverse and ameliorate, respectively, allergen-induced airway inflammation (Haneda et al., 1999; Hansen et

al., 2000); and 3- blocking TGFβ signaling in T cells by overexpressing Smad7 enhances allergen-induced airway inflammation (Sagara et al., 2002). However, the findings obtained with SD-208 indicate that considering TGFβ1 only as an immunosuppressive cytokine can be misleading. In support to the inflammatory role of TGFβ1 in asthma, others have shown that its release by structural cells in the airways contributes to inflammatory cell recruitment (Berger et al., 2003; Berger et al., 2005). In fact, TGFβ1 is a powerful chemotactic factor for monocytes/macrophages (Wahl et al., 1987), eosinophils (Luttmann et al., 1998), neutrophils (Thelen et al., 1995) and mast cells (Gruber et al., 1994) *in vitro*. Migration of monocytes/macrophages toward a gradient of TGFβ1 concentrations occurs at concentrations in the femtomolar range (Wahl et al., 1987). Additionally, TGFβ1 have been shown to rescue murine macrophages from apoptosis (Chin et al., 1999). *In vivo*, the number of mast cells in ASM bundles was positively associated with the ASM tissue expression of TGFβ1 (Berger et al., 2003). Given the important function of mast cells in the pathophysiology of allergic asthma (Brightling et al., 2002), increased secretion of TGFβ1 by structural cells in the airways following allergic challenge may foster, rather than attenuate, inflammation.

1.8. Speculative argument

With all data taken together, one might imagine the following scenario of TGFβ1 regulation in asthma and its potential role in the pathogenesis of the disease. In the first stage of the disease, structural cells-derived TGFβ1 may be release, or simply activated, to induce antigen presenting cell (APC, i.e. monocytes and dentritic cells)

and mast cell tissue infiltration. Both of these cells are required for an immunologic response to take place in the airways. APC capture and process the allergen and then migrate to regional lymph nodes to build a T- and B-lymphocyte immunologic response. On the other hand, mast cells homes the airway walls and will catch the B-cell-derived IgE to produce an allergen-specific reaction following subsequent allergen exposure. In this scenario, it is thus inferred that TGFβ1 is implicated in the inception of allergic asthma by fostering the sensitization process. In later stage of the disease (i.e. in already sensitized individuals), TGFβ1 synthesis by structural cells may stay downregulated to favor the establishment of lymphocytic and neutrophilic/eosinophilic inflammation. These mobilised inflammatory cells are first programmed to synthesize or secrete pro-inflammatory mediators and to sequentially express and secrete immunosuppressive cytokines, such as TGFβ1, to prevent excessive inflammation and damage. At later time points, when the bulk of inflammation is resolved, alveolar macrophages would maintain a higher secretion of TGFβ1 and the airway epithelium would start to expressed TGFβ1 again (Kelly et al., 2005). Increased expression of TGFβ1 by both of these cellular sources may aim to get rid of remnant inflammation and to pursue the healing response. In this scenario, when TGFβ1 action is well regulated, restitution of airway wall integrity would take place and airway function would be recovered. Otherwise, when TGFβ1 actions are uncontrolled, airway remodeling would be likely to occur.

1.9. TGFβ1 receptors in asthma

TGFβ receptors are also expressed ubiquitously on mammalian cells (Bassing et al., 1994; Miyazono et al., 1994b; Tucker et al., 1984; Wakefield et al., 1987). In the lung, TβRI and TβRII were identified in macrophages, as well as in epithelial, VSM, ASM and endothelial cells of both conducting airways and alveoli (de Boer et al., 1998). In contrast to its ligand, only few studies have documented the regulation of these receptors in asthmatic airways. Balzar and coworkers (Balzar et al., 2005) have demonstrated that TβR1 is downregulated in mild and severe asthma. Similarly, Barbato and coworkers (Barbato et al., 2003) have reported a decrease in the number of cells positive for TβRII in the subepithelium of children with asthma. According to the authors, these results may be indicative of active TGFβ signaling, which is associated with TGFβ receptor internalization. In contrast, wounding (Chen and Khalil, 2002) as well as granulocyte-macrophage colony-stimulating factor (GM-CSF) treatment (Chen et al., 2003) have been shown to increase TGFβ receptor expression in monocultures of ASM cells. Since ASM cells are subjected to different damaging stress and that GM-CSF is upregulated in the lungs of asthmatic patients (Marini et al., 1992; Sousa et al., 1993; Vignola et al., 1997), these *in vitro* observations will required further attention as they may actually subtantiate the biological effect of TGFβ1 on ASM cells *in vivo*.

1.10. Interim conclusions and perspectives

Despite being extensively studied, the expression of TGFβ1 in the airways of asthmatics still elicits more questions than answers. The current weight of evidence

suggests that TGFβ1 is upregulated in asthma. However, whether its expression is altered at baseline or it is upregulated in a transient fashion following bronchoprovocation is not clear and may depend on the particular cell and/or tissue studied. For instance, baseline expression of TGFβ1 seems to be increased in alveolar macrophages (Prieto et al., 2000; Vignola et al., 1996), but its expression in the airway lumen seem to be inducible. In support to the later contention, two groups of investigators that have collected BALF following SAC in human asthmatics have demonstrated that the TGFβ1 level is transiently increased (Batra et al., 2003; Batra et al., 2004; Redington et al., 1997). On the other hand, the kinetics of TGFβ1 expression in the airway epithelium of asthmatics seems to be more complex since both upregulation (Hastie et al., 2002; Vignola et al., 1997) and downregulation (Magnan et al., 1997) have been reported. Studies looking at the kinetic of TGFβ1 expression in this particular tissue following allergen challenge should shed light on these conflicting results.

As pointed earlier, all the studies that were unable to detect an altered expression of TGFβ1 in asthma were investigating its expression levels by immunohistochemistry. Owing to the static picture obtained by staining lung sections by immunohistochemical technique, the lack of increased expression of TGFβ1 reported by these groups of investigators can reflect the failure to capture the transient TGFβ1 upregulation and thus, be the origin of the conflicting results. On the other hand, limitations with BALF procedure were also highlighted and it may thus be to soon to reject the possibility that TGFβ1 in not overexpressed in asthma. Studies designed to

harvest tissues at multiple-time points following allergic challenge would be very useful to understand the kinetics of cytokine upregulations. Considering the invasive nature of trans- or endo-bronchial biobsies or even BALF, repeated measurements seems quite unrealistic with human subjects. Less invasive techniques of investigation, such as induced sputum (Adachi et al., 1996; Nomura et al., 2002) or exhaled breath condensate (Matsunaga et al., 2006), represent interesting alternatives for these multiple-time points studies and have previously been used successfully to assess TGFβ1 expression in human subjects. The question that remains is to which extent these techniques accurately reflect TGFβ1 expression in deeper airways. As such, the validity of these techniques needs to be tested. Otherwise, identification of other surrogates of lung TGFβ1 expression that could be readily measured by these less invasive techniques would be required. A last possibility to understand the kinetics of TGFβ1 expression and activation would be to consider the used of animal models.

In the mean time, increased expression of TGFβ1 have been shown to occur exclusively in restricted localization in the airways or only in particular subgroups of patients, such as in severe asthmatics demonstrating prominent eosinophilic inflammation (Wenzel et al., 1999). Hence, in addition to temporal concern, spatial concern needs to be considered. The fact that TGFβ1 may be differently involved in the pathogenesis (or in the remission) of asthma symptoms in phenotypicaly distinct group of asthmatics must also be appreciated. In addition, a debate still persists concerning the inflammatory cell that is mainly involved in the generation of TGFβ1

in asthma, as eosinophils, macrophages, neutrophils and epithelial cells have been pointed-out. Whether these conflicting results mirror the heterogeneity of the disease in term of triggering agents, individual genetic variability or history and severity of the disease, or whether it is simply related to the time points or the tissue chosen to measure TGFβ1 expression are still unresolved questions and will required further explorations.

Since TGFβ1 is released in an inactive form, its kinetics of activation will also be relevant to elucidate its biological or pathobiological functions in asthma. As highlighted earlier, several points of regulation can influence the final magnitude of the TGFβ1 response. All of these points of regulation are as important as the expression of TGFβ1 *per see* if one attempts to appreciate the overall contribution of this cytokine in airway pathogenesis that characterized asthma. Unfortunately, studies investigating these points of control are limited, so as the understanding of TGFβ1 activation in the airways. On the other hand, compiling studies brings evidence that TGFβ1 activity is increased in the airways of asthmatics by measuring intermediary end point of TGFβ1 signaling (pSmads), which testified that TGFβ1 has been activated and has bound to its cognate cell-surface receptor. These results suggested that if TGFβ1 is not upregulated in asthma, other points of control must be altered and translates into an increased TGFβ1 activity. However, these results do not exclude the involvement of other TGFβ superfamily members that signal via the same Smads. But in this regard, one study suggested that among these family members, TGFβ1 is likely the main contributor of this increased Smad signaling (McMillan et al., 2005).

Dissecting the kinetics of TGFβ1 regulation following bronchoprovocation will undoubtedly increase our understanding of the inflammatory and fibrotic processes that take place in asthma. Due to its large spectrum of biologic effect, it is also unfortunately to soon to distinguish whether TGFβ1 is the good guy or the nasty guy in asthma. However, results obtained recently with TGFβ1 Ab (McMillan et al., 2005) or with an ALK-5 inhibitor (SD-208) (Leung et al., 2006) on animal models of the disease are promising and suggested that targeting TGFβ1 may be beneficial in the treatment of human asthma.

1.11. In vivo links between TGFβ1 and ASM cell hyperplasia

Despite the conundrums surrounding the increased expression and the cellular sources of TGFβ1 in asthma, many recently gained insights highlight the potential contribution of TGFβ1 in ASM cell hyperplasia *in vivo*. The particular mechanisms explored in each of the following studies are not necessarily related to one another. However, they are a good reflection of the array of mechanisms currently explored to shed light on the understanding of asthma pathology.

To investigate the effect of IL-5 in airway remodeling, Cho and coworkers (Cho et al., 2004a) have measured lung expression of TGFβ1 and the thickness of the ASM layer (assayed directly by measuring the transverse diameter of ASM layer and by α-smooth muscle actin (α-SMA) immunostaining) in WT and IL-5-deficient mice exposed to a chronic protocol of allergic airway inflammation. In that study,

decreased expression of TGFβ1 in whole-lung homogenates and decrease in TGFβ positive cells in the peribronchial region of IL-5-deficient mice following 3 mo of allergen challenge correlated with a decreased thickness of the ASM layer.

In endotoxin-induced asthma, increases of TGFβ1 expression in whole-lung lavage of mice following graded periods of endotoxin exposure correlated temporally with an increased proliferation of cells in the submucosal compartment (Brass et al., 2004). In addition, mice deficient in LPS binding protein (LBP), a glycoprotein potentiating LPS binding to one of its cell surface receptors, CD14, demonstrated a significantly attenuated TGFβ1 induction and correspondingly less submucosal cellular proliferation when submitted to the same protocol of endotoxin-induced asthma (Brass et al., 2004). Enlargement of the submucosal area was seen in all airway sizes of the endotoxin-exposed wild type animals. However, the nature of the proliferating cells in the submucosa was not clear and may easily represent fibroblasts and/or myofibroblasts rather than ASM cells.

To investigate the role of the T_H2-biased transcription factor GATA-3 in asthma pathology, BALB/c mice overexpressing GATA-3 in their T lymphocytes were generated and submitted to a chronic protocol of allergic asthma. Increased expression of TGFβ1 in the lungs occurred in association with ASM mass enlargement in the transgenic animals sensitized and exposed to the allergen (Kiwamoto et al., 2006). Surprisingly, increased TGFβ1 expression did not occur in the non-transgenic mice in this particular model and this finding paralleled the lack of increase in ASM mass.

In a mouse model of occupational asthma induced by repetitive sensitizations and challenges with toluene diisocyanate, TGFβ1 expression in whole-lung lavages and in lung homogenates was shown to be increased in parallel with a thickening of the peribronchial smooth muscle layer (Lee et al., 2006a). Interestingly, treatment of these mice with peroxisome proliferator-activated receptor (PPAR)γ agonists, rosiglitazone and pioglitazone, partially prevents ASM tissue enlargement, which occurs in conjunction with a decreased induction of TGFβ1. In the same study (Lee et al., 2006a), intratracheal administration of an adenovirus gene vector carrying the PPARγ2 cDNA was equally potent to reduce both TGFβ1 expression and the increased layer of ASM tissue around the bronchi.

Increased expression of TGFβ1 in whole-lung lavage, as well as active TGFβ1 signaling in airway tissues (measured by pSmad2/3 staining in lung sections), were associated with an increased peribronchial immunostaining of αSMA in a murine model of allergic asthma chronically (3 mo) exposed to OVA (Lee et al., 2006c). Interestingly, treatment of these mice with fluticasone during the chronic phase of the challenge protocol (i.e. following the establishment of eosinophilic inflammation) reduced significantly αSMA expression, which occured in conjunction with a decrease in both TGFβ1 expression in the lavage and active Smad signaling in lung tissue. Surprisingly, fluticasone also increased lung tissue expression of the inhibitory Smad, Smad7.

Intraperitoneal injection of immunostimulatory sequences of DNA (ISS) into sensitized mice every other week during a chronic allergen challenge protocol reduced ASM layer thickness and peribronchial α-SMA staining (Cho et al., 2004b). These structural changes also paralleled the expression of TGFβ1 in the lavage and in the lung tissue, such that TGFβ1 was increased in chronically challenged mice and was reduced in the sensitized and chronically challenged mice treated with ISS (Cho et al., 2004b). These results suggest, once again, that TGFβ1 expression and increased peribronchial bulk of ASM tissue are two associated phenomenons.

In a murine model of chronic allergen challenge-induced airway remodeling, TGFβ1 increased acutely and continued to raise constantly during the chronic phase of the disease, which was associated with an increased thickness of the peribronchial ASM tissue on day 59 (Munitz et al., 2006). Interestingly, treatment of mice with a bispecific Ab directed against CD300a and targeting CCR3+ cells following the establishment of airway inflammation completely reversed the increased expression of TGFβ1, which occured in conjunction with a decreased thickness of peribronchial ASM tissue.

Other studies can be added to this list to support the positive correlation between TGFβ1 and ASM cell enlargement. For example, in a chronic murine model of pulmonary allergic inflammation, ASM cell hyperplasia occurred during the chronic phase of allergen challenge in conjunction with an increase in the active form of TGFβ1 in the lung homogenates (McMillan and Lloyd, 2004). Similarly, Corbel and

coworkers (Corbel et al., 2003) have reported an increase in peribronchial ASM mass and an increased expression of TGFβ expression in both BALF and whole-lung tissues in a mouse model of prolonged allergen challenge-induced airway remodeling.

However, all of the aforementioned studies correlate two phenomena occuring in asthma, namely increased expression of TGFβ1 and ASM cell hyperplasia, but do not support a cause and effect relationship between them. The following studies provide the proof of causality of this relationship and, thus, represent pivotal advancements in the understanding of ASM cell hyperplasia that occurs in asthma.

Several transgenic animals have been generated to investigate the *in vivo* role played by a particular cytokine on airway structure or physiology. Transgene constructs containing the Clara cell 10-KD protein (CC10) promoter is the most utilised to target the transgene of interest in the airways exclusively. Mice overexpressing the transgene in an airway-restricted fashion using this CC10 promoter approach have already been used successfully to investigate the respective roles of IL-4, IL-5, IL-13, IL-6 and IL-11 (Elias, 2000; Lee et al., 1997; Rankin et al., 1996). However, the TGFβ1 effect in the CC10 promoter transgenic mice required further engineering, since targeted overexpression of TGFβ1 in the lung in a constitutive manner induced fetal lethality (Zhou et al., 1996). This problem has been overcome by using an externally regulatable, triple transgenic system described earlier (Zhu et al., 2001). While being able to ablate leakage of the inducible transgene, this system allows TGFβ1 to be induced in a lung-specific fashion by adding an agent (Dox) in the

animal's diet. Using this innovating system, Lee and coworkers (Lee et al., 2004) studied the effects of TGFβ1 overexpression in the lungs of adult mice. Interestingly, TGFβ1 overexpression reproduced many of the remodeling features pathognomonic of human asthma, including ASM cell hyperplasia. This result was the first to suggest that overexpression of TGFβ1 in the lung, as it might occur in asthmatic subjects following allergic challenge, was sufficient to cause ASM cell hyperplasia.

The same group of investigators had previously demonstrated that the typical T_H2 cytokine IL-13 was capable of inducing and activating TGFβ1 *in vivo* when overexpressed in the lungs of mice (Lee et al., 2001a). Accordingly, the increased expression of active TGFβ1 expression in the epithelium and the subepithelial zone following chronic inhalation of antigen in sensitized mice was attenuated in IL-13-deficient mice (Kumar et al., 2004). The latent form of TGFβ1 was also reduced in the epithelium of IL-13-deficient mice, suggesting that the epithelial cells may represent a potent cellular source of TGFβ1 following increased expression of IL-13 in the airways. IL-13 is relevant here since it is upregulated in the airways of human asthmatics and is known as an effector cytokine in the establishment of many pathognomonic features of asthma in animal models of the disease (reviewed in (Chatila, 2004; Hershey, 2003; Izuhara and Arima, 2004; Mueller et al., 2002; Wills-Karp, 2001; Wills-Karp and Chiaramonte, 2003)). Lee and coworkers (Lee et al., 2001a) have documented that most of the fibrotic processes induced by lung overexpression of IL-13 are mediated by the induction and activation of TGFβ1. However, despite its capacity to induce and activate TGFβ1 *in vivo* and its capacity to

recapitulate most of the characteristic features of asthmatic airways, the effect of IL-13 on ASM cell proliferation is unknown (Elias, 2000). The only evidence supporting the proliferative effect of IL-13 on ASM cells comes from an review article by Wills-Karp and Chiaramonte (Wills-Karp and Chiaramonte, 2003), which demonstrated a thickened ASM layer in an airway of naïve mice following repeated tracheal instillation of recombinant IL-13. Based on these findings, one may still suggest that the growth-promoting effect of IL-13 *in vivo* may be related to its ability to induce and activate TGFβ1.

One of the best proof of concept that ASM cells are *in vivo* targets of TGFβ is the fact that ASM cells from airway sections derived from mice sensitized and chronically challenged with OVA strongly expressed pSmad2 (McMillan et al., 2005). The same group of investigators have equally shown that treatment of these mice with TGFβ1 Ab prevented phosphorylation of Smad2 in ASM tissue (McMillan et al., 2005), which concomitantly resulted in reduced number of total and proliferating ASM cells (McMillan et al., 2005). Taken together, these results clearly indicate that ASM cells are targeted by the increased expression and/or activation of TGFβ1 in asthmatic lungs and that active TGFβ1 signaling may be involved in ASM cell proliferation *in vivo*.

In addition to active TGFβ signaling (McMillan et al., 2005), Leung and coworkers (Leung et al., 2006) showed an increased expression of Smad2/3 in ASM tissue of Brown Norway rats sensitized and exposed to allergen. This result indicates that the

asthmatic state may prone ASM tissue to respond in an excessive manner to TGFβ. This altered expression of Smad2/3 was abrogated with oral administration of SD-208, a pharmacological inhibitor targeting ALK5, which suggests that TGFβ is involved in the upregulation of its own signaling intermediates that ensure its signal transduction. Interestingly, both preventive and curative treatments with SD-208 successfully abrogated ASM cell hyperplasia.

Overall, these results clearly indicate that TGFβ1 plays a significant role in ASM cell hyperplasia *in vivo*. However, the mechanisms that govern TGFβ1-induced ASM cell proliferation remain unknown. The following section will describe *in vitro* studies that aim to define the mechanisms by which TGFβ1 could foster ASM cell mitogenesis.

1.12. In vitro effect of TGFβ1 on ASM cell proliferation

Using bovine ASM cells, Black and coworkers (Black et al., 1996) initially reported a bimodal time-dependent effect of TGFβ1 on ASM cell mitogenesis. Whilst TGFβ1 inhibited DNA synthesis between 8 and 24 h post-stimulation, it increased DNA synthesis and cell counts at later time points. Increased DNA synthesis in bovine tracheal ASM cells between 24 and 48 h post-TGFβ1 stimulation was also observed by Okona-Mensah and coworkers (Okona-Mensah et al., 1998). This delayed proliferative response to TGFβ1 was consistent with the one observed in other mesenchymal cells (Battegay et al., 1990; Stouffer and Owens, 1994; Vivien et al., 1990). In the latter, the delayed effect was explained by an autocrine loop of growth factors, which were secreted following TGFβ1 stimulation. However, Chen and

Khalil (Chen and Khalil, 2006) have recently demonstrated that neutralizing Abs against FGF2, PDGF, epidermal growth factor (EGF) and IGF-1 have no effect on bovine ASM cell mitogenesis induced by TGFβ1. They also demonstrated that earlier assessment of DNA synthesis following TGFβ1 stimulation, such as between 20 and 24 h post-stimulation, was sufficient to appreciate the pro-mitogenic effect of this cytokine, which was in great contrast to the results presented by Black and coworkers (Black et al., 1996).

Black and coworkers (Black et al., 1996) also demonstrated that TGFβ1 could induce ASM cell hypertrophy. This result was subsequently confirmed by others (Goldsmith et al., 2006; McKay et al., 1998). Whether the increase in ASM mass in asthmatic airways is of hyperplastic (Heard and Hossain, 1973; Johnson et al., 2001; Woodruff et al., 2004) or hypertrophic (Benayoun et al., 2003) nature is still a contemporaneous debate as both phenotypes have been observed. Ebina and coworkers (Ebina et al., 1993) have suggested the existence of two types of asthma based on stereological assessment of ASM tissue in human lungs. Type 1 asthma was characterised by hyperplasia of ASM cells in the larger airways of the respiratory tract, whereas type 2 asthma was predominantly characterised with ASM hypertrophy in the smaller, peripheral airways of the respiratory tract with moderate hyperplasia observed in the major bronchi. Interestingly, the hyperplastic or hypertrophic response to TGFβ1 was shown to be animal specific in the study of Black and coworkers (Black et al., 1996). They reported a strong inverse correlation ($r = -0.97$) between changes in cell size and cell number after TGFβ1 stimulation. Whereas cell hypertrophy was observed in

ASM cells derived form some animals, the predominant effect of TGFβ1 on ASM cells from other animals was cellular hyperplasia. These results suggest that the individual response of asthmatics to TGFβ1 stimulation may determine whether they develop type 1 (ASM hyperplasia) or type 2 (predominantly ASM hypertrophy) asthma.

The first data concerning the effect of TGFβ1 on the proliferation of human ASM cells came from Panettieri's group in Philadelphia (Cohen et al., 1997; Krymskaya et al., 1997). In both of their studies, they showed that TGFβ1 had no effect on its own, but inhibited the growth-promoting effect of EGF and thrombin. They subsequently tried to elucidate the signaling mechanisms involved in this anti-mitogenic effect of TGFβ1, but neither MAPKs nor PI3K pathways, which were both activated and required for EGF-induced ASM cell proliferation, were affected by TGFβ1 treatment. In contrast, and despite failing to induce PI3K activity on its own, TGFβ1 increased by ~4-fold the PI3K activation induced by EGF (Krymskaya et al., 1997). However, the EGF-induced activation of $p70^{S6K}$, a downstream target of PI3K with well-recognized functions in cell cycle regulation, was unaffected by TGFβ1 (Krymskaya et al., 1997).

The anti-mitogenic trend of TGFβ1 on human ASM cells obtained with these two initial studies was subsequently reversed by two other studies published in 2000 (Cohen et al., 2000; Ediger and Toews, 2000). These results heralded the saga of another controversial issue concerning the effect of TGFβ1 in asthma. Cohen and

coworkers (Cohen et al., 2000) demonstrated that TGFβ1 enhanced the mitogenic effect of a growth medium containing FBS (5%), FGF2, EGF and insulin. In addition, they demonstrated that this growth-promoting effect of TGFβ1 was dependent on an autocrine loop of IGF binding protein (IGFBP)-3. Human ASM cells secrete impressive amount of IGFBP-3 protein in response to TGFβ1 stimulation. Cohen and coworkers (Cohen et al., 2000) reported an increase of IGFBP-3 levels attaining over 1 μg/ml in the conditioned medium of ASM cells following a 3-day stimulation with 1 ng/ml of TGFβ1 (Cohen et al., 2000). This tremendous increase was confirmed by Jarai and coworkers (Jarai et al., 2004), using gene chip array technology. Among ~12 500 genes analysed, IGFBP-3 was the most upregulated (54.1-fold over baseline) following 24 h stimulation of human ASM cells with 10 ng/ml of TGFβ1.

However, consistent with bovine ASM cells, the mitogenic effect of TGFβ1 on human ASM cells was delayed compared to other growth factors. The shorter time recorded for the pro-mitogenic effect of TGFβ1 on human ASM cells was reported in the study of Ediger and Toews (Ediger and Toews, 2000), where increased DNA synthesis was shown to occur during a 2-h pulse of radiolabeled thymidine begining 22 h post-stimulation.

TGFβ1 is also known to synergize with other mediators to induce ASM cell proliferation. For instance, the nonapeptide brakykinin, which otherwise has no mitogenic effect when administered alone, potentiates the weak mitogenic effect of TGFβ1 in bovine ASM cells by acting on its B_2 receptor (Gosens et al., 2006).

Lysophosphatidic acid (LPA), another GPCR ligand, equally amplifies the mitogenic effect of TGFβ1 on human ASM cells (Ediger and Toews, 2000). Similarly, receptor tyrosine kinase (RTK) ligands, such as EGF (Okona-Mensah et al., 1998), were shown to synergize with TGFβ1 to induce bovine ASM cell mitogenesis. This latter result was in marked contrast to the anti-mitogenic action of TGFβ1 on EGF-induced human ASM cell mitogenesis described by Panettieri's group (Cohen et al., 1997; Krymskaya et al., 1997). Cohen and coworkers (Cohen et al., 2000) also demonstrated that the supplemented medium is required for the proliferative effect of TGFβ1 to proceed, suggesting that factor(s) in the serum and/or one of the growth factors added in the growth medium (FGF2, EGF or Insulin) is(are) capable of conferring to TGFβ1 a mitogenic potential on human ASM cells (Cohen et al., 2000).

TGFβ1 could also potentiate ASM cell proliferation via indirect mechanisms. We previously demonstrated that LTD_4, a lipid mediator involved in asthma pathogenesis, was without any effect in ASM cell proliferation when administered alone. However, pre-treatment of human ASM cells with TGFβ1 was shown to confer to LTD_4 a significant mitogenic effect (Espinosa et al., 2003). The operational mechanism mediating this proliferative synergism involves the upregulation of CysLT1, the high affinity receptor for LTD_4, by TGFβ1 (Espinosa et al., 2003).

Taken all together, studies published in the last decade concerning the effect of TGFβ1 on ASM cell proliferation *in vitro* have yielded different, and sometimes contradictory results (summarized in Table 3). Some data report an anti-mitogenic

activity (Cohen et al., 1997; Krymskaya et al., 1997; Okona-Mensah et al., 1998), whereas other studies support the opposite, reporting a mitogenic activity for this cytokine, acting by itself (Black et al., 1996; Chen and Khalil, 2006; Ediger and Toews, 2000; Espinosa et al., 2003; Okona-Mensah et al., 1998) or in synergy with other mitogens (Bosse et al., 2006; Cohen et al., 2000; Ediger and Toews, 2000; Espinosa et al., 2003; Gosens et al., 2006). The effect of TGFβ1 on ASM cell proliferation *in vitro* may be influenced by a plethora of factors, such as: 1-the cell origin (the species, the level in the airway tree where the cells have been obtained, e.g. trachea vs bronchi, and the individual characteristics of the donor such as gender, age, race, existing lung diseases, smoking status and genetic background); 2- the peculiarities of culture conditions (the medium *per se*, whether the cells were growth-arrested or not and for how long, the presence of serum and its concentration, the level of cell confluency, and the matrix on which the cells were grown); 3-the conditions of stimulation (concentration of TGFβ1, the time spent after stimulation to assess proliferation, co-stimulation with other factors and their respective concentrations); and 4-the way to assess ASM cell proliferation (DNA synthesis vs cell numbers). Taking all this into account, the studies performed so far describing the mitogenic effect of TGFβ1 on ASM cell proliferation are seldom comparable. Hence, the results may not be conflicting after all. However, if all these inconsistencies were real and could be explained by cell or methodological peculiarities employed by different studies, one can only argue that the effect of TGFβ1 on ASM cell proliferation *in vitro* is context-dependent. The only trends that are worthy of mention in the two species investigated so far are the following: 1- In bovines, TGFβ1 seems to exert a weak

pro-mitogenic effect in confluent, tracheal ASM cells; 2-In humans, even if the picture is less clear, the weight of evidence suggests that TGFβ1 has no effect on its own, but synergises with other mediators to induce ASM cell proliferation. Unfortunately, based on the current litterature, no definitive conclusions can be drawn at the moment.

Table 3: Effect of TGFβ1 on ASM cell proliferation in vitro

Species and strains	Level in the airway tree	Cell passages	Confluency	Growth-arrested or not	Mitogenic assays	TGFβ1 concentration	Description of the effects	Ref.
Bovine	Trachea	1st	Non-confluent	24 h in 2% FBS Ham's F-12 media	1- [^{3}H]-thymidine incorporation at 24 and 48 h (pulse in the last 16 h of stimulation) 2- Cell counting (72 h post-stimulation)	From 0.01 to 10 ng/ml	1- Decreased and increased at 24 and 48 h, respectively, for concentration over 0.01 ng/ml ($p < 0.001$), but the response was variable from an animal to the other. 2- Variability of the response from an animal to the other, ranging from a ~30% increase over unstimulated cells to no effect at all. - In the high-responding cells the effect begans at 30 pg/ml.	(Black et al., 1996)
Human	Trachea	3rd to 5th	Confluent	48 h in serum-free F12 media supplemented with 5 μg/ml of insulin and transferrin	1- [^{3}H]-thymidine incorporation (pulse between 16 and 40 h post-stimulation) 2-Cell counting (48 h)	From 0.1 to 10 ng/ml	Dose-dependent inhibition of EGF- and Thrombin-induced ASM cell mitogenesis	(Cohen et al., 1997)
Human	Trachea	3rd to 4th	Confluent	48 h in serum-free F12 media containing 0.1% BSA	BrdU incorporation during the first 40 h of stimulation	1 ng/ml	Reduced cell cycle progression induced by EGF	(Krymskaya et al., 1997)
Bovine	Trachea	Up to 4th	1- Confluent (10 to 45 000 cells/cm^2) 2- Sparse (300 cells/cm^2)	24 h in serum-free M199 containing with 0.25% BSA	1- [^{3}H]-thymidine incorporation for confluent cells (pulse between 24 and 48 h post-stimulation) 2- Cell counting for sparse cells (5 d)	1 to 100 pM	1- Concentration-dependent increase, starting at 1 pM and increasing continuously up to ~300% above baseline level at 100 pM. - TGFβ1 (100 pM) synergized with EGF (70 nM) - Increased 0.5%, but not 10%, FBS-induced DNA synthesis ($p < 0.001$). 2- Decreased 10%, but not 0.5%, FBS-induced cell proliferation ($p < 0.01$).	(Okona-Mensah et al., 1998)
Human	Bronchi	ni	Confluent	No starving. Cells	MTT assay to	1 ng/ml	- Increased ASM cells proliferation in a time-	(Cohen

(from a 37 and a 17 years old healthy male donors)			(10 000 cells/cm^2)	were directly stimulated in SmGM containing 5% FBS and other growth factors such as EGF (10 ng/ml), FGF2 (2 ng/ml) and Insulin (5 mg/ml)	measure cell proliferation at 2, 5 and 7 days post-stimulation		dependent fashion ($p < 0.001$). - 90% increase at day 7 relative to SmGM alone ($p < 0.001$).	et al., 2000)
Human	Trachea	ni	Confluent	24 h in serum-free high-glucose (4.5g/L) DMEM containing 0.25% BSA	[^{3}H]-thymidine incorporation (pulse between 22 and 24 h post-stimulation)	1 ng/ml	- 6.1-fold increase over baseline. - Synergized with LPA (10 μM), increasing from 8.3 to 21.7-fold over baseline in LPA alone vs to LPA + TGFβ1.	(Ediger et al., 2000)
Human	Bronchi	2nd to 6th	Non-confluent (~10 000 cells/cm^2)	1- 24 h in 1% FBS SmBM 2- Pre-treated 24 h with TGFβ1 3- Washed 4- Stimulated for 72 h with LTD_4 (10^{-7}M) or the control vehicle (ethanol).	Crystal violet staining	10 ng/ml	- 24 h pre-treatment alone induced proliferation ($p < 0.05$). - TGFβ1 pre-treatment confered to LTD_4 a proliferative effect ($p < 0.05$).	(Espinosa et al., 2003)
Human	Bronchi	ni	Non-confluent (50% confluence)	ni, but experiments were conducted without serum.	Cell counting	ni (the isoform of TGFβ used was ni neither)	No effect, but the duration of the stimulation was ni.	(Goldsmith et al., 2005)
Bovine	Trachea	1st to 3rd	Confluent	72 h in serum-free DMEM supplemented with apo-transferrin (5 μg/ml, ascorbate (100 μM) and Insulin (1 μM)	1- [^{3}H]-thymidine incorporation (pulse between 4 and 28 h post-stimulation) 2- Alamar Blue® conversion, an metabolic assay (48 h post-stimulation)	2 ng/ml	1- Increased DNA synthesis to ~144% the baseline level ($p < 0.05$). -Additive, rather than synergistic effect occur with BK (10 μM), reaching ~170% the baseline level together vs ~122% with BK alone. 2- No effect on cell proliferation when used alone, but synergized with BK (10 μM), reaching ~123% of the baseline level together vs ~109% with BK alone ($p < 0.05$).	(Gosens et al., 2006)
Bovine	Trachea	1st to 5th	Confluent	3 days in one of the following media : 1- serum-free DMEM containing 0.2% BSA	1- [^{3}H]-thymidine incorporation (pulse between 20 and 24 or 44 and 48 h post-stimulation)	0.1 to 5 ng/ml	1- At 5 ng/ml, increased DNA synthesis in all three culture media occur: From ~14 to 55 x 10^3 DPM in 0.2% BSA ($p < 0.01$); from ~19 to 31 in 0.5% FBS ($p < 0.01$); and from 110 to 150 in 10% FBS (ns).	(Chen and Khalil, 2006)

				2- 0.5% FBS DMEM 3- 10% FBS DMEM	2-Cell counting with hemacytometer		- In 0.2% BSA medium, increased DNA synthesis in a concentration-dependent manner, starting at 0.1 and increasing continuously up to 5 ng/ml at both 24 and 48 h ($p < 0.05$), but at concentration higher than 1 ng/ml the effect was more pronounced at 48 h. -In 0.2% BSA medium, 1 ng/ml increased 10% FBS-induced DNA synthesis at 48 h ($p = 0.006$). Similar results were obtained with 1% FBS. 2- In 0.2% BSA medium, increased cell number in a concentration-dependent manner, starting at 0.1 and increasing continuously up to 5 ng/ml at both 24 and 48 h ($p < 0.05$), but no difference was observed between 24 and 48 h. - In 0.2% BSA medium, 1 ng/ml increased 10% FBS-induced cell number at 48 h ($p = 0.0002$). Similar results were obtained with 1% FBS.	
Human	Bronchi	4th	Non-confluent (~10 000 cells/cm^2)	24 h in 1% FBS SmBM with or without FGF2 (2 ng/ml)	1- Crystal violet (measured during 5 consecutive days following TGFβ1 stimulation) 2- [^{3}H]-thymidine incorporation (pulses in the last 4 h of 5 consecutive days)	0.1 to 20 ng/ml	1- No effect on cell proliferation when administered alone, at all concentrations and time points measured. - Increased FGF2-induced cell proliferation in a concentration-dependent manner, starting at 0.1 ng/ml and raising continuoulsy up to 20 ng/ml to attain a 6-fold increase over the proliferation induced by FGF2 alone at 4 days post-TGFβ1 stimulation ($p < 0.01$). - Increased FGF2-induced cell proliferation in a time-dependent manner, raising by more than 5-fold FGF2-induced proliferation at 5 days post-TGFβ1 (10 ng/ml) stimulation. 2- Neither affected DNA synthesis when administered alone, nor increased FGF2-induced DNA synthesis at the time window where FGF2 exerted its maximal effect, but prolonged by ~2 days FGF2-induced DNA synthesis ($p < 0.05$).	(Bossé et al., 2006)

Abbreviations: BK, Bradykinin; BrdU; bromodeoxyuridine; LPA, Lysophosphatidic acid; LTD_4, Leukotriene D_4; ni; not indicated; ns, not statistically significant; MTT, methylthiazolyldiphenyl-tetrazolium bromide; SmBM, smooth muscle basal medium; SmGM, smooth musle growth medium.

CHAPTER 2

FGF2

2.1. FGF family and biology

Fibroblast growth factor (FGF) proteins constitute one of the largest families of growth factors, counting 18 members and 4 homologues (FGF homologous factors/FHFs) in man, which are named FGF1 to FGF23 (Eswarakumar et al., 2005; Mohammadi et al., 2005). Their biology is unique as they often function together as intercellular signaling molecules ensuring reciprocal communication between adjacent tissues. They are best recognized for their trophic functions in embryonic development, including lung organogenesis (Arman et al., 1999; Celli et al., 1998; De Moerlooze et al., 2000; Hajihosseini et al., 2001; Peters et al., 1994; Xu et al., 1998), but also for their involvement in homeostasis and regenerative processes in adult tissues (Mohammadi et al., 2005). Deletion of *branchless* or *breathless*, genes encoding the drosophila orthologs of human FGF and FGF receptor (FGFR), respectively, gives rise to drosophila that fail to form tracheal intussusceptions and subsequent outgrowth of the branches that normally lead to an elaborated tree structure (Glazer and Shilo, 1991; Sutherland et al., 1996). These observations demonstrate the indispensable role of FGF signaling in lung branching morphogenesis.

2.2. FGF2 structure, secretion and extracellular localization

The most studied member of the FGF family in asthma pathophysiology is FGF2. This growth factor was first identified in bovine pituitary extracts, and together with

FGF1, they were named based on their capacity to stimulate the proliferation of the fibroblastic cell line NIH3T3 (Gospodarowicz, 1975). In humans, FGF2 is encoded by a 3-exon gene localized on chromosome 4q26-27. The cytoplasmic low molecular weight (LMW) isoform of FGF2 is a single-chain nonglycosylated protein of 18-kD (155 aa). Higher molecular weight (HMW) isoforms of 34, 24, 22,5 and 22-KD have also been identified and are derived from four translationally alternative CUG (leucine) initiation codons upstream of the AUG (methionine) initiation codon that give rise to the LMW isoform. The protein was previously known as basic FGF (bFGF) due to its high isoelectric point (p*I* = 9.6) owing to its large number of basic residues (Nugent and Iozzo, 2000).

Unlike most of the FGF family members, FGF2 lacks a recognizable N-terminal secretory signal sequence required for extracellular export (Mignatti et al., 1992; Ornitz and Marie, 2002). Consequently, rather than being secreted, it was previously thought that FGF2 acted as a cell-associated growth factor. More recently, it was demonstrated that the HMW isoforms of FGF2, which contain nuclear localization sequences (NLS) in their extended N-terminal sequence, are redirected into the nucleus after their release into the cytosol (reviewed in (Stachowiak et al., 2003)). However, FGF2 is also found anchored to ECM components at the extracellular surface of plasmalemma and within the basement membrane of different tissues (Folkman et al., 1988; Gonzalez et al., 1990; Sannes et al., 1992; Shute et al., 2004), suggesting that it can be released from the cell. In fact, FGF2 is the main growth factor stored in basement membranes (Folkman et al., 1988; Sannes and Wang, 1997).

Extracellular export of FGF2 was proposed to be mediated by an exocytotic mechanism independent of the classical endoplasmic-reticulum-Golgi secretory pathway and the multidrug resistance proteins (MRP) pathway (Mignatti et al., 1992).

Just as observed with TGFβ1, FGF2 in the ECM was shown to bind preferentially to glycosaminoglycan (GAG) side chains of HSPG, including cell-associated HSPG such as syndecans and glypicans, and ECM-associated HSPG such as agrin and perlecans (Nugent and Iozzo, 2000). In fact, FGF2 expression was shown to mirror the expression of perlecans in tracheal rings of rhesus monkeys (Evans et al., 2002). Anchorage of FGF2 onto HSPG and its accumulation in ECM provide a means by which this growth factor is protected from proteolysis and offer a reservoir of stable but inactive FGF2 that could be available upon requirement (Flaumenhaft et al., 1989; Folkman et al., 1988; Moscatelli, 1988; Vlodavsky et al., 1987a; Vlodavsky et al., 1987b). Accordingly, many investigators have suggested that its storage in basement membranes presumably offers a way for rapid cellular response to take place in the face of sudden changes in local environmental conditions (Bikfalvi et al., 1997; Nugent and Iozzo, 2000). However, the regulatory processes leading to FGF2 desequestration from the ECM *in vivo* are still unknown. *In vitro* evidence proposes that the release of stored pools of FGF2 could be mediated by the following mechanisms: 1- proteolytic cleavage of HSPG core protein by heparinases (Benezra et al., 1993; Folkman et al., 1988; Saksela and Rifkin, 1990); 2-glycosaminoglycans (GAG) degrading enzymes (Bashkin et al., 1989; Folkman et al., 1988; Ishai-Michaeli et al., 1990; Shute et al., 2004); and 3- the ability of heparin to elute FGF2 from

HSPG binding sites (Bashkin et al., 1989; Folkman et al., 1988; Shute et al., 2004). Release of FGF2 then allows it to bind one of its receptors on the plasma membrane.

2.3. FGFs receptors and co-receptors

Four high-affinity receptors with aa length ranging from 802 to 822 have been identified for the FGF family of growth factors. They were named FGF receptor (FGFR)1 through FGFR4. These receptors are type I transmembrane proteins with an ectodomain composed of 3 immunoglobulin-like domains (D1-D3) and a cytoplasmic tail bearing an intrinsic tyrosine kinase domain (EC 2.7.10.1). Several alternative splicing events take place during transcription of FGFR1, FGFR2 and FGFR3 and give rise to different isoforms with distinct functional properties. The mutually exclusive splicing of exon 8 (IIIb) or exon 9 (IIIc), which encode the C-terminal half of D3, with exon 7 (IIIa), which encodes the N-terminal half of D3, is of particular functional significance, because it confers specificity of binding to the different FGF ligands. In fact, this alternative splicing raises to 7 the number of functionally different receptors in the FGF family (Mohammadi et al., 2005). Interestingly, the splicing is not random, but rather tissue-specific. Whereas cells of epidermal origin express the IIIb isoform, cells of mesenchymal origin express the IIIc isoform. In turn, the production of ligands is also tissue-specific. The IIIb receptor-expressing tissue (epidermal) secretes the FGF ligands that bind to the IIIc isoforms of the receptors expressed on the neighboring tissue (mesenchymal) and *vice versa*. In addition, each subgroups mutually stimulates each other's production to form a reiterated paracrine loop. This continuous bidirectional communication is essential during embryogenesis

and is known to be of crucial importance for lung development during this ontologic stage. This epithelial-mesenchymal trophic unit (EMTU) is also thought to be reactivated in patients suffering from asthma and many investigators in the field believe that it may be the cause of airway remodeling (Holgate et al., 2003). As mentioned earlier, the basement membrane has a great propensity to accumulate FGF2. Considering that the basement membrane is the central structure in the EMTU, one can imagine that the rapid release of basement membrane-bound FGF2 following phlogogenic challenges may initiate this bidirectional communication between the epithelium and the mesenchymal compartments and ultimately foster ASM tissue enlargement.

In addition to regulating extracellularly the biology of FGFs, HSPGs also serve as low-affinity receptors for these ligands. Cell-associated syndecans and glypicans are of particular interest, but perlecans are also a likely candidate due to their close association with the cell surface (Nugent and Iozzo, 2000). HSPGs act by increasing the availability of FGFs to the plasmalemma and, thus, facilitate FGFs binding to FGFR by reducing ligand dimensionality. However, it was also recognised that HSPGs are required for the formation of the high-affinity binding sites and for the FGFs-mediated biological effects. The exact role of HSPGs in the elaboration of a functional receptor is a contemporaneous debate today, but the weight of evidence suggests that HSPGs stabilize the FGF receptor (FGFR) complex in an active conformation by binding to a basic canyon formed between the D2 ectodomain of the receptor and the receptor-bound FGF (reviewed in (Mohammadi et al., 2005)).

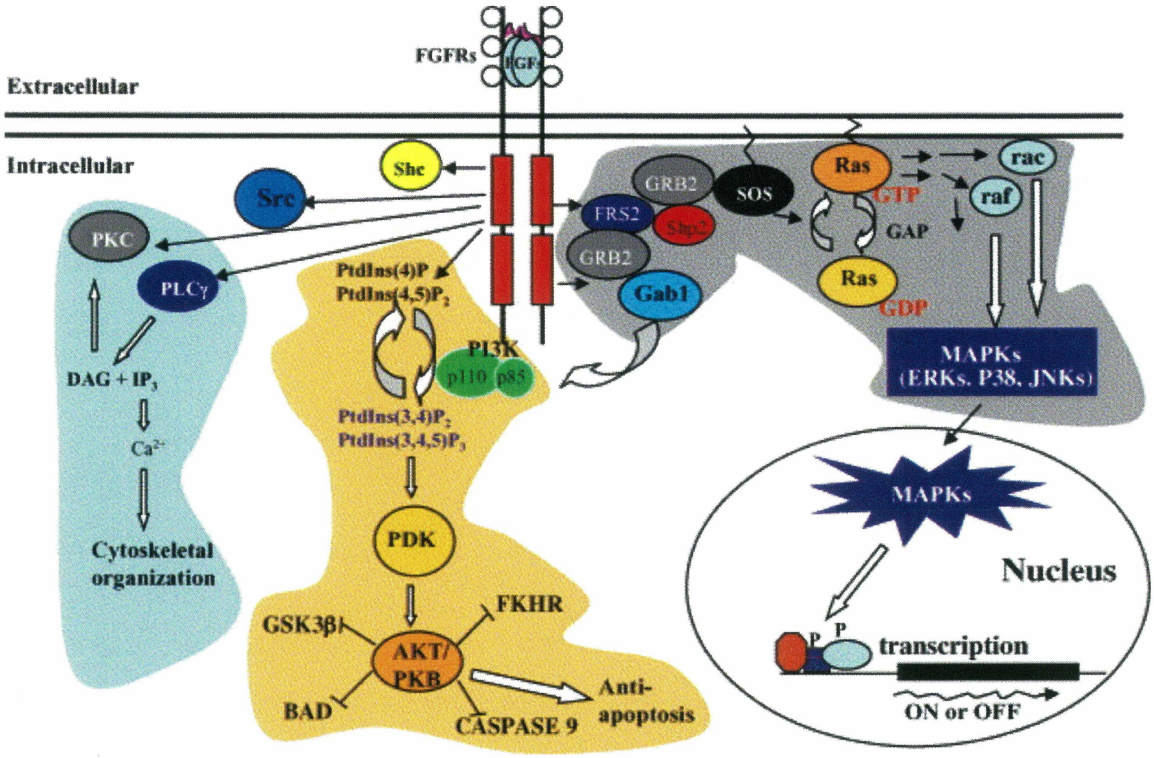
Horowitz and coworkers (Horowitz et al., 2002) also suggested that the cytoplasmic tail of certain HSPGs, for instance syndecan-4, participates in the activation of downstream intracellular signaling. In addition, this signal, which is generated by FGF2-induced cell surface clustering of syndecan-4, is required for the growth-promoting activity of FGF2. With 18 ligands and 7 functionally distinct high-affinity receptors, which raises to 126 the number of potential FGF-FGFR complexes, together with the different ligand and receptor isoforms known to exist and the requirement of HSPGs for the formation of a functional receptor complexes (Mohammadi et al., 2005; Wiedlocha and Sorensen, 2004), the family of FGF growth factors was already considered one of the most complex. This newly identified function of HSPGs, as a potent regulator of intracellular signaling and potent modifier of cell behaviour in response to FGFs, indicates that the biology of FGFs is even more complicated that what was previously thought.

2.4. FGF receptor signaling

As observed for the other growth factor receptors, FGFR dimerization is a prerequisite for the activation of downstream signaling pathways. FGF-induced FGFR dimerization brings the two chains of the receptor in sufficient proximity to permit the protein tyrosine kinase (PTK) domain of each chain to transphosphorylate several tyrosine residues on the other chain. Phosphorylated tyrosines then serve as docking sites for proteins containing src homology (SH)2 or phosphotyrosine binding (PTB) domains. Phospholipase (PL)Cγ1, Shc, Shb and Crk bind directly to the activated receptor (Wiedlocha and Sorensen, 2004). The scaffolding protein FGF receptor

substrate (FRS)2 also binds directly to the receptor via its PTB domain. However, in contrast to other proteins that bind to FGFR cytoplasmic tail, its association with the receptor occurs in a phosphotyrosine-independent manner (Ong et al., 2000). Upon receptor activation, the C-terminal portion of FRS2 is phosphorylated on several tyrosine residues, providing additional docking sites to recruit other adaptor proteins and enzymes into the signalosome. Of particular significance, the constitutive complex Growth factor receptor-binding protein (Grb)2-Son of sevenless (Sos) is recruited by FRS2 and initiates different signaling transduction pathways involved in mitogenesis, such as the Ras/MAPK and the PI3K/Akt pathways. FGFR dimerization also leads to the activation of PLCγ, which catalyses phosphatidylinositol 4,5-diphosphate into diacylglycerol (DG) and inositol 3-phosphate (IP). These latter second messengers subsequently activate conventional and novel forms of protein kinase (PK)C and increase the intracellular concentration of Ca^{2+} by binding to IP receptors on endoplasmic reticulum, respectively. Generally speaking, these enumerated pathways are the ones ensuring the signal transduction of activated FGFR and are thus responsible for the altered behaviour (proliferation, differentiation, migration, survival) adopted by a particular cell type in response to FGF stimulation (Figure 2). However, their respective contribution, as well as the role that other signaling pathways might play in the elaboration of this altered cellular behaviour, are hard to predict owing to the cell-type specific nature of these responses.

Figure 2: FGF2 signaling



Dailey *et al.*, Cytokine Growth Factor Rev 2005; 16(2): 233-47.

2.5. Increased FGF2 expression in asthma or in experimental animal models of asthma

FGF2 is almost ubiquitous (Cordon-Cardo et al., 1990; Hughes and Hall, 1993). In the lung, FGF2 is localized in the cytoplasm of airway epithelial cells (Cordon-Cardo et al., 1990; Kranenburg et al., ; Shute et al., 2004) and in the nuclei of cells within the subepithelial region (Shute et al., 2004), including ASM, VSM and endothelial cells (Kranenburg et al., 2002). ECM from the alveolar and bronchiolar epithelial basement membranes (Sannes et al., 1992; Shute et al., 2004), together with the pericellular matrix of endothelial cells (Shute et al., 2004) are also positive for FGF2 staining.

FGF2 is also localized by immunohistochemistry in lung specimens of other species, such as rodents (Sannes et al., 1992) and rhesus monkeys (Evans et al., 2002).

Compared to TGFβ1, interest in the potential role of FGF2 in the pathophysiology of asthma has just emerged. Studies documenting the expression of FGF2 in asthmatic tissues have started in the early years of this new millennium. Initially, BALF levels of FGF2 expression were shown to be higher in atopic asthmatics compared to control subjects (Redington et al., 2001). Elevated immunohistochemical reactivity for FGF2 was then demonstrated in the airway submucosa of asthmatic patients and in a non-human primate model of asthma (summarized in Table 4). In addition, FGF2 was shown to be further upregulated in BALF of atopic asthmatics following allergic challenge (Redington et al., 2001). However, in contrast to TGFβ1, which increases at 24 h but not at 10 min following allergic challenge (Redington et al., 1997), FGF2 is rapidly upregulated (at 10 min).

Table 4: Expression of FGF2 in human and in a non-human primate model of asthma

Species	Characteristic of the subjects or the models	Tissues	mRNA or protein	Description of the effects	Ref.
Human	Mildly atopic asthmatics	BALF	Protein	- Median of 0.22 vs 0.06 pg/ml at baseline in asthmatics vs controls, respectively ($p = 0.003$). - Median of 1.52 vs 0.3 pg/ml in allergen-challenge vs saline-challenge sites of asthmatics, respectively ($p < 0.002$).	(Redington et al., 2001)
Human	Atopic asthmatics	Bronchial biopsy specimens	Protein	- 53.7 vs 33.3 + cells/mm^2 in bronchial submucosa of asthmatics vs controls, respectively ($p < 0.01$).	(Hoshino et al., 2001)

Rhesus monkeys	Exposure to HDMA during the first postnatal 6 mo	Tracheal rings	Protein	- Increased expression in HDMA group in and around basal cells and within the BMZ - The intensity of immunoreactivy in the BMZ was stronger near the epithelial surface, suggesting that the basal epithelial cells may producing it.	(Evans et al., 2002)
Human	Mild asthmatics (6 out of 7 were atopic)	Bronchial biopsy specimens	Protein	- Median of 8.7 vs 5.3% of epithelial area staining + for FGF2 in asthmatics vs controls, respectively ($p < 0.05$). - Median of 36 vs 29.5% of endothelial area staining + for FGF2 in asthmatics vs controls, respectively (ns).	(Shute et al., 2004)
Human	11 out of 17 were atopic	Sputum	Protein	- 46.4 pg/ml vs 6.0 pg/ml in asthmatics vs controls, respectively ($p < 0.05$). - 1 yr of BDP treatment was without effect (45.4 pg/ml)	(Kanazawa and Yoshikawa, 2005)
Human	4 mild and 5 moderate asthmatics	Small (inner diameter < 2 mm) and medium (inner diameter between 2 to 5 mm) size airways	Protein	- 143.9 vs 75.8 and 106.2 vs 72.6 + cells/mm^2 in the inner and outer walls of small airways in asthmatics vs controls, respectively ($p < 0.05$ and ns). - 93.6 vs 75.5 and 51.2 vs 69.1 + cells/mm^2 in the inner and outer walls of medium airways in asthmatics vs controls, respectively (ns).	(Hashimoto et al., 2005)

Unless otherwise specified, numbers presented in the table represent mean values.
Abbreviations : BDP, beclomethasone dipropionate; BMZ, basement membrane zone; HDMA, house dust mite allergens; mo, month; ns, not statistically significant; yr, year.

2.6. Rapid release of FGF2 following allergen challenge

Proteolysis-mediated FGF2 desequestration from ECM

The rapid increase of FGF2 induced by allergen unlikely represents *de novo* protein synthesis. As mentioned earlier, desequestration of pre-formed FGF2 stored in ECM

may be involved. Among other proteases, mast cell tryptase (Broide et al., 1991; Jarjour et al., 1991; Wenzel et al., 1988), neutrophils elastase (Vignola et al., 1998), MMP-9 (Corbel et al., 2003; Dahlen et al., 1999; Tang et al., 2006), MMP-1 (Rajah et al., 1999), MMP-3 (Dahlen et al., 1999), thrombin (Terada et al., 2004) and kallikrein (Christiansen et al., 1992) are increased in lung tissues of asthmatics. Many of these were shown to be released and/or activated following allergic challenge and may likely be involved in the rapid desequestration of FGF2 from the basement membrane zone of the EMTU.

Mast cell degranulation

Identification of CD34-FGF2 double positive cells in the airways have prone investigators to suggest that inflammatory cells is also a likely sources of FGF2 in asthma (Hoshino et al., 2001). In fact, FGF2 can efficiently be secreted by macrophages (Baird et al., 1985; Henke et al., 1993; Hoshino et al., 2001), T lymphocytes (Blotnick et al., 1994; Hoshino et al., 2001), eosinophils (Hoshino et al., 2001) and mast cells (Hoshino et al., 2001; Inoue et al., 2002; Inoue et al., 1996; Qu et al., 1998; Qu et al., 1995). Considering the fast activation of mast cells in atopic reactions, this cell is a likely candidate to explain the rapid increase of FGF2 in BALF of asthmatics following allergic challenge (Redington et al., 2001).

The effect of mast cells in FGF2 upregulation may also be indirect. Mast cells are the only endogenous source of heparin in mammals and the latter is known to be released during the process of degranulation (Green et al., 1993). Due to heparin ability to elute FGF2 from its HSPGs binding sites *in vitro* (Bashkin et al., 1989; Folkman et

al., 1988; Shute et al., 2004), it is tempting to speculate that the *in vivo* release of heparin induced by allergen-triggering of sensitized mast cells may be a means by which FGF2 is rapidly freed from ECM following SAC (Redington et al., 2001). In addition, heparin structure is very similar to the heparan sulfate moiety of HSPGs and can efficiently substitute for the role of the latter as a coreceptor, conferring to FGF2 its high-affinity binding to FGFR and its subsequent biological effects (Mohammadi et al., 2005). Therefore, in addition to allowing FGF2 desequestration, heparin can also functionally potentiate the effect of FGF2 on ASM cell proliferation. Considering the close vicinity of mast cells and ASM cells in the airways of asthmatics (Brightling et al., 2002), a paracrine signaling involving concomitant secretion of FGF2 and heparin by mast cells, together with indirect desequestration of pre-formed stores of FGF2 by mast cell-derived heparin, may be of major significance in ASM cell hyperplasia.

In addition, other factors secreted by mast cells are known to support ASM cell proliferation *in vitro*. For example, tryptase, which is the main factor released by mast cells during degranulation (Schwartz, 1994), was documented as having a mitogenic effect on ASM cells by two groups of investigators (Berger et al., 2001; Brown et al., 2001). However, its mitogenic effect was delayed compared to other growth factors and was dependent on its proteolytic activity (Brown et al., 2001), suggesting that it was mediated by the release of growth factors sequestered on pericellular ECM. Interestingly, the mitogenic effect of tryptase was enhanced by heparin (Berger et al., 2001), which was in great contrast to the well-recognised anti-mitogenic action of

heparin (in particular to serum- and TGFβ1-induced ASM cell mitogenesis) (Johnson et al., 1995; Kanabar et al., 2005; Kilfeather et al., 1995; Okona-Mensah et al., 1998). On the other hand, the growth-promoting effect of heparin on tryptase-induced ASM cell proliferation is reminiscent to the role of heparin in conferring cell surface high-affinity binding and growth-promoting effect to ligands in the FGF family. Altogether, these observations suggest that the slow onset, protease-dependent mitogenic effect of tryptase on ASM cells may be related to its capacity to degrade ECM constituents, and consequently, to desequester one or several members of the FGF family. This conjecture as well as the potential relevance of other interplays between mast cells and ASM cells in the progression of ASM cell hyperplasia justifies further investigations.

Released following epithelium damage

Under pathologic conditions, such as asthma, it is also possible that FGF2 is released following epithelial damage. The airway epithelium is the sole source of the cytoplasmic, LMW isoform of FGF2 in the lungs (Kranenburg et al., ; Shute et al., 2004). Airway epithelial tissue is the primary target for different kinds of phlogogenic insults entering the airways (bacteria, virus, allergens, pollutants). Following such aggressions, cellular membrane integrity of the epithelium could be compromised, directly or through the subsequent mobilisation and activation of inflammatory cells into the airways, which could ultimately lead to cytoplasmic release of FGF2 into the airway lumen.

In support to this contention, Zhang and coworkers (Zhang et al., 1999) pioneered a three-dimensional co-culture system in which primary lung myofibroblasts were embedded in collagen gel and epithelial cells were seeded over it. This system allows elucidation of the paracrine influence of epithelial damage on the behaviour of underlying cells. Using this *in vitro* system, they demonstrated that different epithelial-damaging stimuli, such as mechanical scraping or poly-L-arginine treatment cause myofibroblast proliferation. Myofibroblast growth-promoting activity is released rapidly by epithelial cells following injuries (observed at the earlier time point measured: i.e. 4 h), suggesting that a pre-formed mitogenic factor, possibly FGF2, is liberated as a result of epithelial damage. Accordingly, this enhanced growth was temporally associated with FGF2 release in the supernatant of wounded cells. The highest FGF2 expression was observed at the earlier time point measured (i.e. 4 h following injury) and remained elevated thereafter for 24 h. Moreover, anti-FGF2 antibody (200 μg/ml) reduced by 32% myobibroblast proliferation induced by epithelial wounding. Other mitogenic factors, such as PDGF-AB, TGFβ2, IGF-1 and endothelin (ET)-1 were also upregulated, but their increased expression in the supernatant following epithelial injury were delayed compared to FGF2. The early increase in FGF2 expression in damaged epithelial cell supernatants prompted the authors to investigate whether MMPs are involved in ECM-bound FGF2 release. Whereas both mechanical and chemical injuries increased FGF2 release, only mechanical damage increased MMP-2 and MMP-9 activities, discarding the possibility that FGF2 is desequestrated after injury only by gelatinase-induced ECM degradation. Moreover, adding α2-macroglobulin, an inhibitor of MMPs, to the

epithelial culture did not prevent the early increase in FGF2 release induced by either mechanical or chemical damages. The authors thus suggest that intracellular stores of pre-formed FGF2 in the epithelium are rapidly liberated after damage and contribute to myofibroblast growth-promoting activity (Zhang et al., 1999). Noteworthy of mention, FGF2 is released prior to TGFβ following these types of airway epithelial cell aggression (Zhang et al., 1999). This sequence of growth factor production by the damaged epithelial cells is reminiscent to the kinetics of FGF2 (Redington et al., 2001) and TGFβ1 (Redington et al., 1997) upregulation observed in BALF of asthmatics following allergic challenge. In addition, since both growth factors bind to the same proteoglycans (HSPGs), the possibility is raised that *de novo* synthesis of TGFβ1 would be redirected in close vicinity of extracellular sites where FGF2 was previously stored before its desequestration, and consequently, these two growth factors may act sequentially on the same tissues in *in vivo* conditions.

The chemical used to damage the epithelium in Zhang and coworkers's study (Zhang et al., 1999) mimics the positively charged proteins liberated by eosinophils following their activation in the airways of asthmatics. It was recognised a long time ago that the degree of eosinophilia or their degranulation products correlate with the severity of different markers of asthma (Bousquet et al., 1990; Frick et al., 1989; Wardlaw et al., 1988). Airway eosinophilia induced by inhalation of rIL-5 was equally associated with an increased sputum level of ECP in asthmatic patients (Shi et al., 1998). These eosinophil-derived mediators were shown to be toxic for the airway epithelium (Robinson et al., 1992). Consequently, alteration of the plasma membrane of epithelial

cells by eosinophil-degranulation products, such as ECP and MBP, may be involved in the release of FGF2 by the epithelium.

In addition to acting as a potential tissue involved in the brisk release of FGF2 in asthmatic airways following allergic challenge, the epithelium of asthmatics was shown to express higher amount of FGF2 (Shute et al., 2004). In fact, the augmentation seems to be confined to the airway epithelium, since neither the pulmonary vascular endothelial expression, nor the response of stimulated release of FGF2 by heparin or heparitinase I (bacterial endoglycosidase) in endobronchial tissue specimens were significantly different between asthmatic and non-asthmatic subjects (Shute et al., 2004). Considering the following: 1- the localization of the epithelial cells relative to the airway lumen; 2- the demonstration that the epithelial cells are the sole source of the cytoplasmic LMW form of FGF2 in the airways; and 3- the increased expression of FGF2 in asthmatic epithelium, it is tempting to suggest that the alteration of epithelial cell plasmalemma integrity is responsible for the rapid increase of FGF2 expression observed in BALF of asthmatics following allergic challenge (Redington et al., 2001).

On the other hand, the involvement of eosinophils in the increased FGF2 expression has been requestioned recently. Kanazawa and Yoshikawa (Kanazawa and Yoshikawa, 2005) have shown that a one-year therapy with beclomethasone dipropionate (BDP) was effective in abrogating airway eosinophilia, but was inefficient to reduce the elevated level of FGF2 in induced sputum samples of

asthmatic subjects (Kanazawa and Yoshikawa, 2005). In addition, deficiency in MBP-1 in a mouse model of allergic asthma did not protect against allergen-induced asthma (Denzler et al., 2000). In contrast to FGF2, increased expression of VEGF in induced sputum of asthmatics (4270 pg/ml) compared to control subjects (1730 pg/ml) was responsive to BDP therapy (2530 pg/ml) (Kanazawa and Yoshikawa, 2005). This upregulation of VEGF in induced sputum of asthmatics and the reduced expression following corticosteroid treatment were in accordance with other studies (Asai et al., 2003; Asai et al., 2002). These data suggest that these 2 growth factors are differently regulated by corticosteroid therapy. Kanasawa and Yoshikawa (Kanazawa and Yoshikawa, 2005) also demonstrated that procollagen type III peptide (P-III-P), which is the N-terminal peptide of type III collagen precursor, increased in induced sputum of asthmatics and was also refractory to BDP therapy. In fact, FGF2 levels correlate positively and significantly with P-III-P levels before ($r = 0.84$) and after ($r = 0.89$) BDP therapy, suggesting that FGF2 could play a role in pro-fibrotic lesions in asthma (Kanazawa and Yoshikawa, 2005). Consistent with this result, Chakir and coworkers (Chakir et al., 2003) had demonstrated that fibrotic proteins such as collagens type I and III were unresponsive to the oral corticosteroid methylprednisolone. However, they attributed this lack of response to an increased TGFβ1 expression, which was also unaffected by corticosteroid treatment. Collectively, these studies suggest that the inability of corticosteroids to downregulate FGF2 and TGFβ1 expression may explain previous findings showing that airway fibrosis, characterized by collagen deposition in the lamina reticularis, was not effectively reduced by corticosteroid therapies (Chakir et al., 2003; Jeffery et al., 1992).

Overall, even if the cellular source of FGF2 is still unclear, its overexpression was consistently reported in the airways of asthmatics. Further studies will be required to identify the mechanisms involved in its increased release and to determine its functional relevance in asthma etiology. However, considering its growth-promoting effect toward cells of different origins and the well-known trophic functions of this family of growth factors in the EMTU during lung morphogenesis, FGF2 is a potential contributor to airway remodeling, including ASM cell hyperplasia.

2.7. FGF2 receptors on ASM cells

Unlike FGF1, which appears to be a universal FGFR ligand, FGF2 seems to possess a greater binding specificity for the IIIc splice form of FGFR1 to R3 and with the FGFR4 (Ornitz et al., 1996), but also demonstrates some activity toward the FGFR1b splice form. Being a mesenchymal tissue, ASM expresses the IIIc isoform of FGFRs, and consequently, is capable of responding to extracellular FGF2. However, it is not clear yet which FGFRs are expressed in the ASM cells. Among FGFRs, FGFR1 is the most widely distributed throughout the organism and its expression tends to be confined to tissue microvasculature, including lung vessels, and the epithelia, including epithelial cells from the respiratory tract (Hughes and Hall, 1993). Kranenburg and coworkers (Kranenburg et al., 2002) have detected FGFR1 in VSM cells, ASM cells and the airway epithelium. It is thus believed that the FGFR1c is expressed on ASM cells and may be responsible for the FGF2-mediated biological effects. However, due to insufficient studies on the matter, expression of the other

high-affinity receptors (FGFR2 to FGFR4) could not be excluded presently. In addition, no modulation of FGFRs has been reported so far in lung tissues of asthmatic subjects.

2.8. In vivo links between FGF2 and ASM cell hyperplasia

There is currently no published study documenting the effect of FGF2 on ASM cell hyperplasia *in vivo*.

2.9. In vitro effect of FGF2 on ASM cell proliferation

In contrast to TGFβ1, there is no controversy surrounding the effect of FGF2 on ASM cell mitogenesis. Whatever the origin of the cells and the cell culture conditions used, it is well established that FGF2 induces ASM cell proliferation *in vitro* (Bonacci et al., 2003; Bonacci and Stewart, 2006; Bonner et al., 1996; Bosse et al., 2006; Brown et al., 2002; Ediger and Toews, 2000; Fernandes et al., 2004; Fujitani and Bertrand, 1997; Ravenhall et al., 2000). Moreover, FGF2 was shown to synergize with PDGF-AA and PDGF-AB (Bonner et al., 1996; Bosse et al., 2006; Ediger and Toews, 2000), as well as with other RTK-acting ligands, such as insulin (Ediger and Toews, 2000). The synergism with some of the members of PDGF family was attributable to its ability to induce the expression of PDGF receptor α chain (PDGFRα) (Bonner et al., 1996; Bosse et al., 2006). Just as observed with TGFβ1, FGF2 can potentiate the mitogenic effect of the GPCR-acting ligand LPA (Ediger and Toews, 2000). ET-1, another GPCR-acting ligand, also synergized with FGF2 to induce guinea pig ASM cell mitogenesis (Fujitani and Bertrand, 1997). On the other hand, simultaneous

stimulation of human ASM cells with FGF2 and EGF induced no additive effect compared with each of the growth factors administered alone (Ediger and Toews, 2000). Since both of these growth factors act on distinct RTK cell surface receptors, these results suggest that they might use the same intracellular signaling pathways to transmit their mitogenic effect.

In contrast to other ASM cell mitogens, such as thrombin, FGF2 requires p38 MAPK to transduce its mitogenic signal into the nucleus (Fernandes et al., 2004). Accordingly, kinetic experiments demonstrated biphasic p38 activation, showing early (5 and 30 min) and late (20 h) phosphorylation following FGF2 stimulation without evidence of activation in middle time points (2 and 6 h). Upregulation of cyclin D1 mRNA and protein levels, as well as downregulation of the cyclin-dependent kinase (cdk) inhibitor $p21^{CIP1/WAF1}$ protein by FGF2 was unaffected by pre-treatment with the p38 pharmacological inhibitor SB203580. However, the extent of Retinoblastoma (Rb) protein phosphorylation was attenuated by SB203580 20 h post-FGF2 stimulation. These results suggest that late p38 activation induced by FGF2 participates in the late events of the G1 phase of the cell cycle and its activation is obligatory for G1 to S phase traversal, which subsequently leads to DNA synthesis and cell proliferation (Fernandes et al., 2004). Interestingly, treatment of mice with SD282, a selective inhibitor of p38, abrogated ASM cell hyperplasia in a model of allergic airway inflammation, which was further associated with a significant reduction in AHR to acetylcholine (Nath et al., 2006). This result may be the only clue so far for the involvement of FGF2 in ASM cell proliferation *in vivo*.

Interestingly, the mitogenic effect of FGF2 on ASM cells is inhibited by β2-adrenoceptor agonists (Bonacci et al., 2003) and by glucocorticoids (Bonacci et al., 2003; Bonacci and Stewart, 2006), which are the mainstay therapies for the treatment of asthma. The anti-migotenic action of glucocorticoids is maintained when ASM cells are cultured on laminin (Bonacci et al., 2003). However, its anti-mitogenic potential is lost when the cells are seeded on collagen type I (Bonacci et al., 2003; Bonacci and Stewart, 2006), suggesting that certain ECM components influence the response of ASM cells to glucocorticoids. On the other hand, the anti-mitogenic effect of β2-adrenoceptor agonists was conserved whatever the matrix on which the cells were seeded. Collectively, these results suggest that the drugs currently used to attenuate the symptoms of asthma may be efficacious to prevent ASM cell hyperplasia. However, the anti-mitogenic action of glucocorticoids may be compromised in remodeled airways of asthmatics associated with increased deposition of type I collagen (Chakir et al., 2003; Roche et al., 1989; Tormanen et al., 2005). Also of interest, Johnson and coworkers (Johnson et al., 2004) have shown that ASM cells from asthmatic subjects secrete higher amounts of collagen type I compared to ASM cells derived from non-asthmatic subjects, which may render asthmatic ASM cells less responsive to the anti-mitogenic effect of glucocorticoids.

The following chapter tend to demystify the conflicting results surrounding the mitogenic effect of TGFβ1 on human ASM cells *in vitro*, and to determine whether

initial pre-treatment of ASM cells with FGF2 could influence the mitogenic effect of this cytokine.

CHAPTER 3

MANUSCRIPT 1

Ynuk Bossé, Charles Thompson, Jana Stankova, Marek Rola-Pleszczynski. **FGF2 and TGFβ1 Synergism in Human Bronchial Smooth Muscle Cell Proliferation.** *American Journal of Respiratory Cell and Molecular Biology* 2006; 34(6): 746-53.

Contributions of first author:

All the experiments were performed by the first author.

CHAPTER 4

SYNERGISM BETWEEN FGF AND TGFβ FAMILY MEMBERS

We have good reasons to believe that this FGF2-TGFβ1 synergy has already been reported earlier in the literature, but without identifying the incriminated factor added to the Smooth muscle Growth Medium (SmGM) responsible for the mitogenic effect of TGFβ1. Effectively, Cohen and coworkers (Cohen et al., 2000) had demonstrated that TGFβ1 was able to induce human ASM cell proliferation, but this effect was silent if the growth factors (FGF2, EGF and Insulin) and the serum (5%) were omitted in the culture medium. To our view, the essential factor was not in the serum, as suggested by the authors, but was the FGF2 added to the culture medium. Without any effort for identifying this factor, Cohen and coworkers (Cohen et al., 2000) pursued their study and demonstrated convincingly that the IGFBP-3 induced by TGFβ1 was responsible for its mitogenic effect, offering an another perspective of what might be the mechanism explaining the mitogenic synergism between FGF2 and TGFβ1. However, considering the fact that TGFβ1 alone was sufficient in the absence of added serum or growth factors to induce IGFBP-3 (Cohen et al., 2000), but was unable to support ASM cell proliferation in the absence of added growth factors (Bosse et al., 2006), we could only expect that IGFBP-3 is one of the factor induced by TGFβ1 that mediates the proliferative synergism with FGF2.

One can argue that the concentrations of FGF2 and TGFβ1 required to induce the synergism were higher than what was recovered in BALF of asthmatic subjects, and is thus unlikely to occur under physiologic conditions. In fact, the synergism between

FGF2 and TGFβ1 was perceptible at 100 pg/ml of FGF2, which is 67-fold higher than the concentration reported in BALF of asthmatics following SAC (1.52 pg/ml) (Redington et al., 2001). Similarly, 100 pg/ml of TGFβ1 was sufficient to increase FGF2-induced proliferation, but still represents a 2-fold higher concentration than the one reported in BALF of asthmatic patients 24 h post-challenge (46 pg/ml) (Redington et al., 1997). However, 0.9% saline solution injected in the airways may not be adequate to harvest the totality of a particular protein depending on its predominant localization or to its binding properties with components of the airways. Since FGF2 is mainly produced by basal epithelial cells and is preferentially sequestered in the basement membrane zone after its extracellular release (Evans et al., 2002), the bulk of FGF2 may not be accessible by bronchoalveolar lavage. Moreover, based on the observations that FGF2 and TGFβ1 expression levels were calculated for the unconcentrated BALF in the two aforementioned studies, a considerable dilution factor must be taken into account (a total of 120 ml of saline have been injected in a particular segment of the airways). Since neither TGFβ1 nor FGF2 are found in the middle of the airway lumen, an issue of volume to surface ratio must also be considered. The concentration measured in BALF volume is actually derived from the luminal surface of the airway walls in *in vivo* conditions. Hence, BALF concentrations of FGF2 and TGFβ1 may not reflect the actual concentrations of these growth factors in the airway microenvironments in the vicinity of ASM tissue.

In addition, the time points where BALF have been collected may not represent the highest levels reached by FGF2 or TGFβ1 following allergen bronchoprovocation. However, the time points chosen to recover BALF (10 min for FGF2 and 10 min as well as 24 h post-challenge for TGFβ1) clearly indicate that FGF2 release in the airways occurs more rapidly compared to TGFβ1 and suggest that ASM cells are probably stimulated sequentially by these growth factors in this respective order following allergic challenge. Finally, antibodies used in the ELISA kit have usually been raised against the recombinant form of the protein of interest and the quantification assay is based on a standard curve obtained with the use of the recombinant protein. The values obtained by this technique surely correlate with the real amount of endogenous protein found in the samples, but may still underestimate it. For all these reasons, we assume that the concentrations of growth factors we used in our study were physiologically relevant. If not, the definition of an ASM cell mitogen will have to be reconsidered, because the proliferative synergism that we reported in our study is more than twice as potent as the stronger mitogen, namely PDGF-BB (10 ng/ml), to induce ASM cell proliferation (data not shown).

Even if TGFβ1 would not be upregulated in asthma, this FGF2-TGFβ1 synergy is likely to take place and cause ASM cell hyperplasia. The studies enumerated in Table 1, as well as de Boer and coworkers (de Boer et al., 1998) have demonstrated the constitutive nature of TGFβ1 expression in airway and alveolar epithelial cells. TGFβ1 mRNA was also observed in different cells of healthy human lungs, including bronchiolar epithelial, endothelial and mesenchymal cells, as well as in alveolar

macrophages (Coker et al., 1996). Hence, asthmatic or not, it is clear that TGFβ1 is expressed in the airways. Based on our results, TGFβ1 cannot trigger ASM cell proliferation in the absence of FGF2. However, following the release of FGF2 by allergic challenge, ASM cells may become primed to the proliferative effect of TGFβ1. Consequently, the constitutive expression of this later may become sufficient to cause ASM cell hyperplasia. In addition, TGFβ1 must be activated by post-translational modifications before binding to its cognate cell surface receptor and mediating its biological effect. Hence, even if its expression is not altered in asthma, its activation may occur in the face of asthmatic inflammatory reactions, and this will unmask its mitogenic action toward FGF2-primed ASM cells.

The synergy reported *in vitro* between FGF2 and TGFβ1 may also reproduce what is really occurring *in vivo* with other factors that act via similar receptors or signaling pathways. As mentioned earlier, Activin A, another activator of AR-Smads, is upregulated in asthma (Cho et al., 2003; Karagiannidis et al., 2006) and is a potent inducer of human ASM cell proliferation (Cho et al., 2003). Immunohistologic studies have co-localized Activin A with mast cell tryptase in asthmatics (Cho et al., 2003), suggesting that mast cells are potent sources of Activin A in asthma. Accordingly, increased expression of Activin A in a murine model of allergic asthma was severely impaired in mast cell-deficient mice (W/W^v) following allergic bronchoprovocation. Activin A secretion by mast cells following FcεRI cross-linking *in vitro* further corroborates these results (Cho et al., 2003). Interestingly, mast cells are interspersed in ASM cell bundles of asthmatics and are well-known modulator of ASM cell

functions (Brightling et al., 2002). Hence, considering the vicinity of mast cells and ASM cells *in vivo*, it is conceivable that mast cell-derived Activin A could be the main TGFβ superfamily member that synergizes with FGF2 (or other FGF family members) to induce ASM cell hyperplasia.

As deeply discussed previously, TGFβ2 is also upregulated in asthma (Balzar et al., 2005; Chu et al., 2004). Since the TGFβ2 isoform acts on the same receptors and signal via AR-Smads, TGFβ2 is also a likely candidate to synergize with FGF2, or other FGF family members, for inducing ASM cell hyperplasia in asthma.

On the other hand, no members of the FGF family, apart FGF2, were reported to be upregulated in asthmatic tissues. However, FGF1 is overexpressed in other chronic inflammatory lung diseases (Kranenburg et al., 2002; Kranenburg et al., 2005) and was shown capable of inducing ASM cell proliferation *in vitro* (Kranenburg et al., 2005). In addition, IL-1β is structurally related to the family of FGF growth factors (Schmitz et al., 2005) and is known to be upregulated in asthma. Interestingly, and as observed for FGF2 (Bonner et al., 1996; Bosse et al., 2006), this pro-inflammatory cytokine synergizes with PDGF in mesenchymal cell proliferation through its ability to increase the expression of PDGFRα (Coin et al., 1996; Lindroos et al., 1995; Tsukamoto et al., 1991). Hence, as observed for FGF2, both FGF1 and IL-1β are likely candidates to prime ASM cells to proliferate in response to TGFβ family members that signal via AR-Smads.

It is also noteworthy to mention that each of the individual interactions that may take place *in vivo* between members of FGF and TGFβ family are not mutually exclusive. Instead, every extracellular cue from one family of growth factors may be cumulative and synergize with the culminated signal generated from simultaneous extracellular cues of several members of the other family of growth factors. ASM cell hyperplasia is a complex phenotype. It would be surprising that only a single synergism between two growth factors would explain this structural alteration. It is more likely that a concerted action of 2 families of growth factors, where every member of one family potentiates the mitogenic action of every member of the other family, would lead to ASM hyperplasia.

In the following two chapters, two other asthma mediators, namely IL-4 and IL-13, will be discussed in regard to their respective contribution to asthma pathogenesis, and the hypothesis that FGF2 could influence the mitogenic potential of these two cytokines was tested.

CHAPTER 5

IL-4 AND IL-13

5.1. IL-4 and IL-13 and their receptors

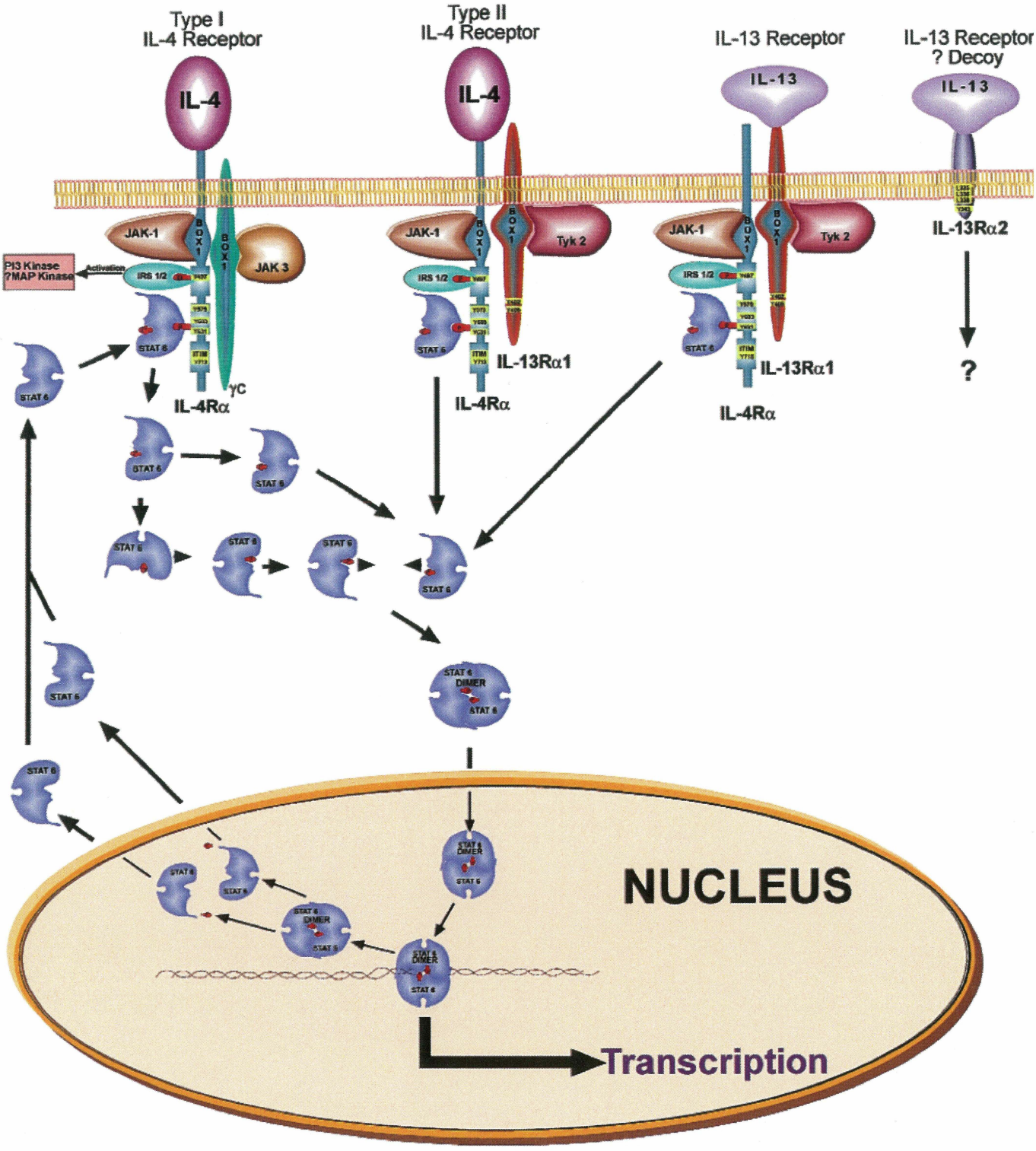
While TGFβ1 is considered the most important cytokine in the fibrotic processes that take place in asthma, IL-4 and IL-13 are cornerstones in the sensitization and effector phases of the disease, respectively (reviewed in (Chatila, 2004; Hershey, 2003; Izuhara and Arima, 2004; Mueller et al., 2002; Wills-Karp, 2001; Wills-Karp and Chiaramonte, 2003)). These cytokines are mainly produced by T_H2 lymphocytes, but eosinophils (Gessner et al., 2005; Schmid-Grendelmeier et al., 2002; Woerly et al., 2002), mast cells (Gessner et al., 2005; Kobayashi et al., 1998), macrophages (Hancock et al., 1998; Prieto et al., 2000), basophils (Gessner et al., 2005; Shimizu et al., 1998; Tschopp et al., 2006), dendritic cells (de Saint-Vis et al., 1998) and ASM cells (Grunstein et al., 2002) are also potential cellular sources. They are both encoded by a 4-exon gene cluster in the 5q31 chromosome, which has been associated with several asthma phenotypes (Izuhara and Arima, 2004). The primary sequences of the processed forms of IL-4 and IL-13 are 129 and 112 aa long, respectively. Their secondary and tertiary structures form in 4-helix bundle arranged in an up-up-down-down topology, just as the other cytokines acting on class I cytokine receptors (Grotzinger, 2002). Four receptor chains are involved in the binding of IL-4 and IL-13 onto the cell membrane (Chatila, 2004; Hershey, 2003) (Figure 3). Every chain possesses the conserved cytokine binding module (CBM) (consisting of two fibronectin type III domains with the conserved cysteine motif and the WSXWS sequence) in their ectodomain, a single transmembrane domain and the box domain in

the juxtamembrane region of the cytoplasmic tail. The latter is required for the constititive binding of members of the Janus kinase (JAK) family of cytoplasmic tyrosine kinases. The common γ chain, which is also required to initiate intracelullar signaling by IL-2, IL-7, IL-9, IL-15 and IL-21 cytokines, together with the IL-4Rα chain form the type 1 receptor. On the other hand, IL-4Rα with IL-13Rα1 form the type 2 receptor. Whereas dimerization of type 1 receptor is exclusively induced by IL-4 and leads to JAK1 and JAK3 activation, type 2 receptor is induced by both IL-4 and IL-13 and activates JAK1 and tyrosine kinase (Tyk)2, which is also one of the members of the JAK family. However, in both cases, engagement of type 1 or type 2 receptors culminates in the activation of the same signal transducer and activator of transcription (STAT), namely STAT6 (Figure 3). Consequently, many biological effects are shared between IL-4 and IL-13. IL-13 also binds to a second chain, IL-13Rα2, which was suggested to act as a decoy receptor owing to its short cytoplasmic tail, which is not coupled with downstream signaling pathways (Izuhara and Arima, 2004). This receptor chain is inducible by IL-4 or IL-13 stimulations and overexpressed in the case of bronchial asthma, suggesting that it may act as an autoregulatory mechanism to attenuate the effect of IL-13 (Izuhara and Arima, 2004).

5.2 Involvement of IL-4 and IL-13 in the development of asthma pathogenesis

The two initial studies documenting that each of these cytokines are sufficient alone to recapitulate most of the pathognomonic features of asthma in naïve mice have picked the curiosity of many investigators in the field and have generated an explosion of papers confirming their effects (Grunig et al., 1998; Wills-Karp et al., 1998). It was

Figure 3: IL-4 and IL-13 receptors and signaling



Hershey. JACI 2003; 111: 677-90.

thus demonstrated that IL-13 and IL-4 knockout mice are protected against allergen-induced AHR (Komai et al., 2003; Walter et al., 2001). Similarly, neutralization of IL-4 with Ab during the sensitization period (Corry et al., 1996) or with Ab against IL-13 before each challenge (Eum et al., 2005) prevent the development of AHR in murine models of allergic pulmonary inflammation. Furthermore, deficiency in IL-4Rα or in STAT6, which are the common receptor chain and signaling molecule borrowed by both IL-4 and IL-13 to propagate their biological effects, also render mice resistant to experimentally-induced allergic asthma (Akimoto et al., 1998; Grunig et al., 1998; Kuperman et al., 1998).

Targeting the IL-13-responsive cells in the airways by intranasal delivery of chimera cytotoxin, which was formed by anchorage of *Pseudomonas* exotoxin to IL-13, reduced features of airway disease, including AHR, lymphocytic inflammation, goblet cell hyperplasia and peribronchial fibrosis in a murine model of chronic asthma induced by *Aspergillus fumigatus* sensitization and conidia challenge (Blease et al., 2001). However, considering the amount of structural cells in the airways expressing IL-4Rα, IL-13Rα1 and IL-13Rα2, it is surprising that such a treatment did not induce airway destruction or, at least, severe inflammation.

In addition to mouse models, tracheal administration of IL-13 into the lungs of guinea pigs was also sufficient to induce AHR (Morse et al., 2002). Cellular inflammation was characterized by eosinophilia and a robust neutrophilia, but the number of macrophages and lymphocytes also increased significantly. In addition, double

intraperitoneal injections of 10 or 20 mg/kg of sIL-13Rα2 at 24 and 2 h before allergic challenge in sensitized guinea pigs prevented the establishment of AHR. Hence, similar to what was observed in murine models of asthma, AHR in guinea pigs following experimentally-induced allergic asthma requires IL-13.

Taken together, these results clearly indicate that IL-4 and IL-13 are required and sufficient for the development of asthma, at least in animal models of the disease. Interests have now turned toward the understanding of the downstream cellular and molecular mechanisms by which these two T_H2 cytokines mediate their actions (reviewed in (Wills-Karp and Chiaramonte, 2003)).

5.3 Cellular and molecular mechanisms mediating the effector funtions of IL-4 and IL-13

Based on recombination-activating gene 1 (Rag1)-deficient mice, it was suggested that IL-13 acts directly on the bronchial tissue through non-T cell and non-B cell mechanisms to induce the asthma phenotype (Grunig et al., 1998). Moreover, pre-treatment with vinblastine, a granulocyte-depleting agent, was ineffective in blocking IL-13-induced AHR (Singer et al., 2002), thus discarding the possible involvement of neutrophils, eosinophils and basophils in the asthma phenotype induce by IL-13. Correspondingly, IL-5 and CCL11 double knock-out mice, which are severely impaired in their ability to recruit eosinophils into the airways, still demonstrated AHR upon IL-13 exposure (Yang et al., 2001). In addition, a single dose of IL-13 or IL-4 was sufficient to induce AHR at an early time point (6 hours), when leucocyte

infiltration in the airways was still comparable to that of mice treated with saline alone (Venkayya et al., 2002). Intranasal administration of conditioned medium derived from T_H2-activated cells also induced AHR in wild-type mice. This effect was blunted in mice lacking either IL-4Rα or STAT6. Interestingly, AHR was still present in mast cell-deficient W/W^v mice in this particular setting (Venkayya et al., 2002), discarding the possible involvement of tissue residential mast cells in the asthma phenotype induced by IL-4 or IL-13. Finally, an elegant complementary study has confirmed all these observations by demonstrating with adoptive transfer experiments that IL-4Rα chain in the irradiated recipient, but not in the bone marrow donors, is a prerequisite for asthma development upon antigen exposure (Kelly-Welch et al., 2004). Hence, unequivocal evidence now suggests that T_H2 cytokines, at least in mice, exert their effector functions by acting primarily through airway structural cells.

Generation of trangenic mice overexpressing IL-13 in the airways using the CC10 promoter approach has been particularly useful in revealing the downstream cellular and molecular effector mechanisms driving IL-13-induced asthma-like phenotype. This transgenic model has evolved form a single (Zhu et al., 1999), to a double (Zheng et al., 2000) and then to a triple (Zhu et al., 2001) transgenic model to produce a mouse where the expression of IL-13 is exclusive to the airways and its expression is externally regulatable by adding dox to the animal's diet, just as the systeme used for the TGFβ1 transgenic mice (Lee et al., 2004). Collectively, these studies confirmed the results obtained with repeated intratracheal instillations (Wills-Karp et al., 1998) or intranasal administrations (Grunig et al., 1998) of rIL-13, in that it

reproduced reliably some of the pathognomonic phenotypes of human asthma, such as peribronchial eosinophilic and lymphocytic inflammations, subepithelial fibrosis, hypertrophy and metaplasia of the epithelium, basal airway obstruction and AHR (reviewed in (Elias, 2000; Zhu et al., 2001)). As expected, STAT6-deficient animals were protected from the asthma phenotype induced by IL-13 in this model (Kuperman et al., 2002). Kuperman and coworkers (Kuperman et al., 2002) subsequently demonstrated that reconstitution of STAT6 in the airway epithelium of $STAT6^{-/-}$ mice was sufficient to restore AHR and goblet cell hyperplasia, but not airway eosinophilia in this transgenic model. These results suggest that the airway epithelium is targeted by IL-13 and that active signaling of IL-13 in this tissue is involved in the development of some of the pathologic phenotypes mediated by IL-13 overexpression.

The use of the IL-13 transgenic model has also revealed important molecular insigths toward the understanding of IL-13-induced pathogenesis. By using knockout or inhibitor approaches, MMP9 and MMP12 (Lanone et al., 2002), TGFβ1 (Lee et al., 2001a), CCR2 (Zhu et al., 2002), adenosine (Blackburn et al., 2003), CCL6 and CCR1 (Ma et al., 2004), acidic mammalian chitinase (Zhu et al., 2004), IL-11 receptor α (Chen et al., 2005), ERK1/2 (Lee et al., 2006b), Erg-1 (Cho et al., 2006), CCR5 (Ma et al., 2006) and 5-lipoxygenase (Shim et al., 2006) were identified as downstream effector pathways of IL-13. Together, these results suggest that the pathology induced by IL-13 overexpression is as complex as the one induced by allergen sensitization and challenge.

5.4 Increased expression of IL-4 and IL-13 in asthma and experimental animal models of asthma

In humans, Huang and coworkers (Huang et al., 1995) provided the first *in vivo* evidence of IL-13 expression in human lungs. Constitutive expression levels of IL-13 in BALF of both asthmatic and normal subjects was in the range of 20 to 300 pg/ml. They further demonstrated that the IL-13 protein level was upregulated 18 to 24 h following SAC (6 out of 8 asthmatic patients demonstrated an increase in IL-13 expression), rising to the range of 0.1 to 2.5 ng/ml. In that study, IL-13 mRNA expression in BALF cells was also elevated by an estimated 100-fold following SAC to reach 0.55 pg of IL-13 transcript per μg of RNA. Non-asthmatic patients did not demonstrate this augmentation following the same allergic challenge. Mononucleated cells were identified as the cellular source of the IL-13 transcript, with none detected in the isolated eosinophil fraction. Similarly, Prieto and coworkers (Prieto et al., 2000) have demonstrated that mRNA expression of IL-13 was elevated in BALF-enriched alveolar macrophages of mild atopic asthmatics after repeated low-doses of allergen inhalation. Only 2 out of 8 asthmatic subjects expressed IL-13 mRNA before allergen exposure, confirming the inducible nature of IL-13 response rather than a constitutively higher expression level in asthmatics (Prieto et al., 2000). Effectively, no difference was observed in IL-13 mRNA expression of alveolar macrophages between asthmatic and healthy subjects before allergen bronchoprovocation (Prieto et al., 2000). T cells harvested from BALF of asthmatics also express higher levels of IL-13 after local allergen challenge (Bodey et al., 1999). More recently, Batra and

coworker (Batra et al., 2004) confirmed these observations, demonstrating no difference in BALF concentration of either IL-4 or IL-13 at baseline between asthmatic and control subjects, but the increases of both cytokines 1 day post-SAC. In addition, IL-13 induction following SAC was shown to occur during the late-phase (18 h), but not in the early-phase (10 min) of an airway allergic reaction (Tschopp et al., 2006). Hence, allergen-induced IL-13 seems to follow a kinetics similar to the one observed for TGFβ1 and is delayed relative to FGF2 upregulation.

At the tissue level, examination of endobronchial biopsy specimens by *in situ* hybridization has revealed that the number of positive-cells for both IL-4 and IL-13 mRNA per millimeter of basement membrane was elevated in the bronchial mucosa of atopic asthmatics compared to control subjects (Kotsimbos et al., 1996). In the latter study, they also demonstrated that 90% of IL-13 mRNA-positive cells were equally CD3-positive cells. RT-PCR performed on biopsy speciments also confirmed these results (Humbert et al., 1997), and further demonstrated that the asthmatic status, but not the atopic status, is a key determinant to predict the levels of IL-13 (Humbert et al., 1997) and IL-4 (Humbert et al., 1996) mRNA in lung tissues. These studies raised doubt concerning the aformentioned inducible nature of IL-4 and IL-13 expression identified by others. However, their results are readily explained by the higher number of T-lymphocytes in the airway of asthmatics. Altogether, these results are consistent with the increased expression of IL-4 and IL-13 observed in animal models of asthma and suggest that these two cytokines may be involved in the development of structural and functional alterations characterizing human asthma.

5.5 Effects of IL-4 and IL-13 on ASM cells

A large body of evidence also suggests that IL-4 and IL-13 act directly on ASM cells to mediate their effective functions. Using gene microarray analysis, it was established that among structural cells present in the airways, BSMC were the most responsive to IL-13 stimulation (Lee et al., 2001b). In addition, autocrine production of IL-13 was shown to increase airway responsivess to spasmogen-induced bronchoconstriction and to decrease β_2-agonist-induced bronchodilation (Grunstein et al., 2002), which remarkably mimics the contractile changes observed in airway tissues of asthmatics (Bai, 1990). Surprisingly, IL-13 alone induces a transient increase in intracellular Ca^{2+} concentration in murine ASM cells (Eum et al., 2005). It was thus suggested that IL-13 may act directly on ASM to induce AHR.

IL-13 and IL-4 also stimulate the secretion of different chemokines, cytokines and growth factors in ASM cells. In fact, both IL-4 and IL-13 stimulate the secretion of CCL11 (Faffe et al., 2005; Hirst et al., 2002), IL-5 (Grunstein et al., 2002), and VEGF (Faffe et al., 2006). Moreover, they synergized with IL-1β (Hirst et al., 2002) and oncostatin M (Faffe et al., 2005) to induce CCL11 release, and with tumor necrosis factor (TNF)α to induce CCL17 release (Faffe et al., 2003). Of particular interest in the present thesis, an experiment conducted with genechips containing probes for 6500 human genes has shown that a 6 h-stimulation of human ASM cells with IL-13 increased by 2.3-fold the mRNA expression of FGF2 (Lee et al., 2001b).

However, with all the accumulated results documenting the effects of IL-4 and IL-13 in asthma, it is surprising that ASM cell hyperplasia, which is one of the most invariable remodeling features of asthma, has been ignored. Even *in vitro*, the effects of IL-4 and IL-13 on ASM cell proliferation have never been reported.

CHAPTER 6

MANUSCRIPT 2

Ynuk Bossé, Charles Thompson, Karine Audette, Jana Stankova, Marek Rola-Pleszczynski. **The T_H2 Cytokines, IL-4 and IL-13, Enhance Human Bronchial Smooth Muscle Cell Proliferation.** Submitted in *Journal of Allergy and Clinical Immunology.*

Contributions of first author:

All the experiments except the microscopy in Fig. 3 were performed by the first author.

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TITLE: The Th2 Cytokines, IL-4 and IL-13, Enhance Human Bronchial Smooth Muscle Cell Proliferation
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Dear Dr. Marek Rola-Pleszczynski:

The Journal has received your submission. Thank you.

Best regards,

The Editors of
The Journal of Allergy and Clinical Immunology
Donald Y.M. Leung, MD, PhD
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Stanley J. Szefler, MD

FGF2 and TGFβ1 synergism in human bronchial smooth muscle cell proliferation

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ABSTRACT

Bronchial smooth muscle cell (BSMC) hyperplasia is a typical feature of airway remodeling and contributes to airway obstruction and hyperresponsiveness in asthma. Fibroblast growth factor 2 (FGF2) and transforming growth factor β1 (TGFβ1) are sequentially up-regulated in asthmatic airways after allergic challenge. Whereas FGF2 induces BSMC proliferation, the mitogenic effect of TGFβ1 remains controversial and the effect of sequential FGF2 and TGFβ1 co-stimulation on BSMC proliferation is unknown. This study aims to assess the individual and sequential cooperative effects of FGF2 and TGFβ1 on human BSMC proliferation and define the underlying mechanisms. Mitogenic response was measured using crystal violet staining and [^{3}H]-thymidine incorporation. Protein and steady state mRNA levels were measured by Western blot, ELISA and semi-quantitative RT-PCR, respectively. TGFβ1 (0.1-20 ng/ml) alone had no effect on BSMC proliferation, but increased the proliferative effect of FGF2 (2 ng/ml) in a concentration-dependent manner (up to 6-fold). Two distinct platelet-derived growth factor receptor (PDGFR) inhibitors, AG1296 and Inhibitor III, as well as a neutralizing Ab against PDGFRα partially blocked the synergism between these two growth factors. In this regard, TGFβ1 increased PDGF-A and PDGF-C mRNA expression as well as PDGF-AA protein expression. Moreover, FGF2 pre-treatment increased the mRNA and protein expression of PDGFRα and the proliferative effect of exogenous PDGF-AA (140%). Our data suggest that FGF2 and TGFβ1 synergize in BSMC proliferation and this synergism is partially mediated by a PDGF loop,

where FGF2 and TGFβ1 upregulate the receptor (PDGFRα) and the ligands (PDGF-AA and PDGF-CC), respectively. This powerful synergistic effect may thus contribute to the hyperplastic phenotype of BSMC in remodeled asthmatic airways.

Keywords: asthma, airway remodeling, smooth muscle, hyperplasia, TGFβ1, FGF2, PDGF

INTRODUCTION

Bronchial smooth muscle cell (BSMC) hyperplasia has been recognised as a feature of airway remodeling in asthmatic patients almost a century ago (1). Since then, several groups of investigators have suggested that BSMC hyperplasia is the major histologic alteration contributing to bronchial hyperresponsiveness (2, 3). BSMC are also active participant in the inflammatory response during asthma exacerbation by their ability to synthesize and secrete a diverse array of mediators involved in recruitment, activation and enhanced survival of inflammatory cells (4). Thus, elucidating the factors involved in BSMC hyperplasia is of major significance to the understanding of airway wall thickening and hyperresponsiveness, but is also likely relevant to the elaboration of improved therapeutics for the treatment of airway inflammation that arises in asthmatic patients.

Numerous individual mediators have been shown to induce BSMC proliferation *in vitro*, including growth factors, proteases, spasmogenes, reactive oxygen species (ROS) and cytokines (5). Most of these mitogens are up-regulated during asthma exacerbation and the order in which the expression of certain growth factors increase following phlogogenic challenge have been documented. In this regard, Redington and coworkers (6) have reported a rapid (10 min) FGF2 increase in bronchoalveolar lavage fluid (BALF) after a segmental allergen challenge, whereas TGFβ1 upregulation was observed only 24 h post-challenge (7).

Despite the lack of secretory signal peptide (8), the low molecular weight (LMW; 18 KDa) cytoplasmic isoform of FGF2 was found in the extracellular milieu (8, 9) and has been shown to support BSMC proliferation *in vitro* (10, 11). The airway epithelium is the main source of this LMW isoform in the lung (9), but inflammatory cells such as mast cells (12), macrophages (13) and eosinophils (14) can also secrete FGF2. Its elevated expression in asthmatic airways has been confirmed by immunohistochemistry in humans (9, 14) and in a non-human primate model (15).

Increased TGFβ1 expression in human asthmatics (7, 16-23) and in animal models of airway inflammation (24-26) has been extensively documented as well. Various cells in the airway could contribute to TGFβ1 up-regulation. However, its role in BSMC proliferation is still questionable. Some *in vitro* data reported anti-mitogenic activity (27-29), whereas other studies supported the opposite, reporting a mitogenic activity for this cytokine, acting by itself (29-33) or in synergy with other mitogens (31, 33).

The aim of the current study was to clarify the individual mitogenic effect of TGFβ1, and to measure the combined effects of sequentially added FGF2 and TGFβ1 on the proliferation of human primary BSMC. Our results showed that TGFβ1 had no significant mitogenic effect, but synergized with FGF2-induced proliferation in a concentration-dependent manner. Moreover, the results suggested that part of this synergism involved an autocrine PDGF loop, where FGF2 increased

PDGF receptor α chain (PDGFRα) expression and TGFβ1 increased production of PDGFR ligands (PDGF-AA and PDGF–C).

MATERIALS AND METHODS

Cell culture

Human primary bronchial smooth muscle cells (BSMC) (BioWhittaker, Inc. Walkersville, MD) were used for all the experiments. BSMC were derived from a 5 week-old black male and a 1 year-old and a 21 year-old caucasian females. All donors, had negative history of smoking and were free of pre-existing lung disease. Upon reception, cryopreserved cells were cultured in T-75 flasks in Smooth muscle Growth Medium (SmGM) (SmGM-2 Bulletkit) provided by the manufacturer and consisting of Smooth muscle Basal Medium (SmBM), 5% fetal bovine serum (FBS) and a mixture of growth factors, including fibroblast growth factor 2 (FGF2) (2 ng/ml), epidermal growth factor (EGF) (0.5 ng/ml) and insulin (5 μg/ml), as well as a mixture of antibiotics, including Gentamicin (100 ng/ml) and Amphotericin B (0.1 ng/ml). Thawing, subculturing and harvesting procedures were performed according to manufacturer's instructions. Experiments were performed with cells at the 4^{th} passage.

Cell proliferation: Crystal violet staining

Cells were subcultured into 96-well plates at 3000 cells/well in a starvation medium, consisting of SmBM + 1% FBS, with or without FGF2 (2 ng/ml). Cells were maintained in these conditions for 24 h before TGFβ1 (PeproTech Canada, Inc. Ottawa, ON) or PDGF-AA (PeproTech, Inc. Rocky Hill, NJ) (10 ng/ml; unless otherwise specified) stimulations. BSMC proliferation was measured 96 h after

stimulation using the DNA staining property of crystal violet (Sigma, Oakville, ON). Briefly, cells were washed once with HBSS solution containing 2 mM $CaCl_2$ and 10 mM Hepes, fixed 20 min with ethanol (70%) at –20°C, incubated 15 min in crystal violet dilution (1% w/v) at room temperature, washed 6 times under tap water and dissolved in acetic acid (33%). Optical density (OD) was determined at 550 nm with an ELISA reader (Bio-Rad Laboratory, Inc. Hercules, CA).

To confirm the validity of crystal violet staining as a surrogate of cell proliferation in the BSMC cell line, preliminary tests were performed where different numbers of cells were cultured in a 96-well plate (from 1 to 11 thousands). Simple regression model was then calculated ($Y = 8 \times 10^{-6} \bullet X + 0.12$) and demonstrated significant value ($p<0.001$) with a very high coefficient of determination ($r^2 = 0.997$), indicating that the variance in one variable (OD) would predict almost all the variance in the other variable (number of cells). Based on the slope of the linear regression model, OD variation of 0.01 unit corresponds to 1230 cells. Thus, the crystal violet staining method is a valid approach to quantify BSMC proliferation.

To investigate signaling pathways involved in BSMC proliferation, pharmacological inhibitors were added 1 h before TGFβ1 treatment at the following concentrations: phosphatidylinositol 3-kinase (PI3K) inhibitors, LY294002 (10 μM) and Wortmannin (1 μM) (Biomol. Plymouth Meeting, PA); Src kinase inhibitors, PP1 and PP2 (10 μM) (Biomol. Plymouth Meeting, PA); PDGF receptor tyrosine kinase inhibitor, tyrphostin AG1296 (10 μM; unless otherwise specified) (Biomol.

Plymouth Meeting, PA) and PDGF receptor inhibitor III (Calbiochem, San Diego, CA); and mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) kinase (MEK) inhibitor, PD98059 (10 μM) (Biomol. Plymouth Meeting, PA). DMSO (0.1%; unless otherwise specified) was used as the control vehicle. To investigate the contribution of an autocrine PDGF loop in the observed FGF2-TGFβ1 synergism, neutralizing mAb against human PDGFRα (R&D, Minneapolis, MN) was also evaluated. Anti-PDGFRα was administered 1 h prior to TGFβ1 treatment at concentrations of 0.2, 2 or 20 μg/ml.

DNA synthesis: [^{3}H]-thymidine incorporation

Thymidine incorporation assays were used to measure DNA synthesis. Cells were seeded in 96-well plates at a density of 3000 cells/well in SmBM 1 % FBS and stimulated thereafter at different intervals during a 5 day time-course. FGF2 (2 ng/ml) and TGFβ1 (10 ng/ml) treatment were administered alone or sequentially with a 24 h interval in their respective order. BSMC were pulsed with 2.5 μCi/ml of [methyl-^{3}H]-thymidine (91 Ci/mmol) (Amersham Biosciences. Piscataway, NJ) 4 hours before being placed at -20°C until further processed. DNA of individual wells was then transferred onto a Whatman membrane using a cell harvester (Titertek Cell Harvester. Rockville, Md) and placed in scintillation vials for radioactivity quantification using a 1215 Rackbeta II liquid scintillation counter (LKB Wallac, Turku, Finland).

RT-PCR

Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) was used to measure mRNA expression. After reaching confluency in 6-well plates, cells were starved for 24 h in SmBM 1% FBS prior to TGFβ1 (10 ng/ml) stimulations in fresh medium. Cells were then harvested, centrifuged and resuspended in TriPure solution (Roche Diagnostics Canada, Laval, QC) to perform mRNA extraction as described by the manufacturer. To avoid DNA contamination and mRNA degradation, mRNA extracts were treated for 15 min with dexoxyribonuclease (DNAse 1 from Amersham Biosciences. Piscataway, NJ) and ribonuclease inhibitor (RNasin from Promega, Madison, WI) at 37°C. RT reactions were performed using 1 μg of mRNA extract and M-MLV reverse transcriptase kit (BioCan Scientific, Inc. Mississauga, ON) and PCR was performed with a Taq polymerase kit (New England BioLabs, Ltd. Pickering, ON). For all experiments, GAPDH mRNA was used as an internal house keeping gene control. The PCR primers used were: PDGF-A forward, 5′-gaccaggacggtcatttacg-3′; PDGF-A reverse, 3′-cctcacatccgtgtcctctt-5′; PDGF-C forward, 5′-ttcagcaacaaggaacagaac-3′; PDGF-C reverse, 3′-ctgaaggggtagctctgaa-3′; PDGFRα forward, 5′-gaagctgtcaacctgcatga-3′; PDGFRα reverse, 3′-atcgaccaagtccagaatgg-5′; tPA forward, 5′-cccagatcgagactcaaagc-3′; tPA reverse, 3′- tggggttctgtgctgtgtaa-5′; GAPDH forward, 5′-gatgacatcaagaaggtggtgaa-3′; GAPDH reverse, 3′-gtcttactccttggaggccatgt-5′.

Protein determination: ELISA

Cells were allowed to reach confluence in 6-well plates in SmGM before being washed once in PBS and starved for 24 h in SmBM 1% FBS. TGFβ1 (10 ng/ml) was then added in fresh starvation medium and the conditioned medium (CM) was recuperated 24 h later. Protein quantification in the CM was determined by the human/mouse PDGF-AA Quantikine® ELISA kit (R&D, Minneapolis, MN).

Western analysis

Cells reached confluence in 6-well plates in SmGM before being starved for at least 24 h in SmBM 1% FBS. FGF2 stimulation was then initiated for 24 h by adding fresh medium in all wells. Cells were harvested as described above and lysed in RIPA solution (NaCl, 0.15M; Tris-HCl, 0.05M; Igepal 1%, v:v; Na-Deoxycholate 0.5%, v:v, SDS 0.1%, w:v; EDTA, 5mM) containing the following inhibitors: Aprotinin (2 μg/ml), Leupeptin, (1μM), Soybean trypsin inhibitor (10 μg/ml), AEBSF (0.1 mg/ml), NaF (10mM), and Na_3VO_4 (1mM). Lysates were subsequently exposed to reducing condition (2.86 M Mercaptoethanol in the 4X loading buffer) and boiled for 3 min before being submitted to electrophoresis in 7% SDS polyacrylamyde gel and transferred overnight onto nitrocellulose membranes. Following blocking (milk), membranes were immunoblotted with a mouse anti-human PDGFRα mAb (1 μg/ml) (R&D, Minneapolis, MN) and the secondary goat anti-mouse mAb conjugated with HRP (1:2500) (Amersham Biosciences, Baie D'Urfé, Qc) with appropriated washing after each step (TBS: 0.1% Tween 20, v:v). Thereafter, HRP substrate (ECL™ Western Blotting Detection Reagents from

Amersham Biosciences, Baie d'Urfé, Qc) was added and protein amount revealed by exposure on ECL detecting film (Hyperfilm™ ECL from Amersham Biosciences, Baie d'Urfé, Qc). To control for equal loading, membranes were stripped (SDS 2%, w:v; Mercaptoethanol, 100mM; Tris, 50mM, pH6.8) for 20 min at 50°C and reblotted following similar procedures with a mouse anti-human vinculin mAb (1:1000) (mouse monoclonal anti-vinculin clone VIN-11-5 from Sigma-Aldrich, Oakvill, On) as the primary antibody.

Data analysis

Results illustrated in the figures, as well as their statistical analysis, are compiled data obtained using BSMC derived from the 5 week-old donor. Unless otherwise specified, raw data were used for statistical analysis. ANOVA was first performed to determine overall significance of differences among conditions tested and was followed by Fisher's PLSD test to specify which conditions significantly differed from each other. In RT-PCR analysis, ratios over GAPDH were standardized in Z score within each experiment before the data obtained for every experiment were compiled and analysed as described above. In ELISA analysis, unpaired Student's t test was used to compare the protein expression levels between TGFβ1-treated vs untreated cells. $p \leq 0.05$ was arbitrarily considered sufficient to reject the null hypothesis.

RESULTS

TGFβ1 synergizes in a concentration-dependent manner with FGF2 to induce BSMC proliferation.

The proliferative effect of TGFβ1, alone or 24 h after FGF2 stimulation, in human primary BSMC was examined. Results illustrated in the figures are calculated data obtained using BSMC from the 5 week-old donor. As shown in Figure 1A, increasing concentrations of TGFβ1 on its own had no effect on BSMC proliferation. However, when FGF2 (2 ng/ml) was added to the culture medium 24 h before TGFβ1 stimulation, the latter induced a concentration-dependent increase in BSMC proliferation. At 10 ng/ml, TGFβ1 induced a 5-fold increase in BSMC proliferation relative to FGF2 (2 ng/ml) alone. Conversely, a fixed concentration of TGFβ1 (10 ng/ml) also increased the mitogenic effect of increasing concentrations of FGF2 (Figure 1B). Dose-response curves for FGF2-TGFβ1 proliferative synergism with the two other donors gave similar results, although the overall effect was smaller. At the concentrations used above (2 ng/ml for FGF2 and 10 ng/ml for TGFβ1) TGFβ1 increased FGF2-induced BSMC proliferation by 89 and 129% in cells from the 1 year-old and the 21 year-old caucasian female donors, respectively (supplementary Figure 1). Unless otherwise specified, the remaining experiments were performed with BSMC obtained from the 5 week-old black male donor.

TGFβ1 increases and prolongs DNA synthesis induced by FGF2.

[^{3}H]-thymidine incorporation assays, as a surrogate of DNA synthesis and cell cycle activation, were also performed following sequential FGF2 and TGFβ1 co-stimulation. As shown in Figure 2, the mitogenic effect of FGF2 (2 ng/ml) peaked at 48 hours and returned to baseline levels at 96 hours post-stimulation. As expected, TGFβ1 (10 ng/ml) alone had no effect on DNA synthesis from 24 to 96 hours post-stimulation. However, TGFβ1 increased (at 96 h), but most importantly prolonged (to at least 120 h), the mitogenic effect of FGF2. Time-course experiments were also performed using crystal violet staining during 5 consecutive days following TGFβ1 administration. As depicted in figure 2B, FGF2 alone increased BSMC proliferation, starting at day one and peaking at day 4. On the other hand, TGFβ1 alone did not affect BSMC proliferation over baseline conditions (FBS 1%) at any particular day. However, when added following a 24 h pre-treatment with FGF2, TGFβ1 strikingly increased FGF2-induced proliferation, starting at day 2 and continuing for at least 5 days post-stimulation. Based on a standard curve and a calculated simple linear regression model obtained by plotting OD values with known quantity of BSMC seeded (see Materials and Methods), the number of cells per well at day 5 in this particular experiment was estimated at 4968 for control, 4845 for TGFβ1, 14 193 for FGF2 and 51 954 for the sequential FGF2 and TGFβ1 co-stimulation.

Tyrosine kinase activity of PDGFR is involved in the FGF2-TGFβ1 synergism

BSMC were treated with different pharmacological inhibitors or the vehicle control DMSO (0.1%) for 1 hour before TGFβ1 administration. As shown in Figure 3A, the Src inhibitor PP1 abrogated the proliferative effect of FGF2 and of FGF2-TGFβ1 co-stimulation. On the other hand, tyrphostin AG1296 (10 μM), a PDGFR tyrosine kinase inhibitor, partially blocked FGF2-TGFβ1 synergism without any effect on FGF2-induced proliferation. To confirm this partial inhibition of FGF2-TGFβ1 synergy, increasing concentrations of the tyrphostin AG1296 and another potent and selective ATP-competitive inhibitor of PDGFR tyrosine kinase activity, namely PDGF Receptor Tyrosine Kinase Inhibitor III (refered herein as Inhibitor III) were tested (Figure 3B). Whereas 20 μM of AG1296 as well as 1μM of Inhibitor III did not affect the FGF2-induced proliferation (data not shown), the synergism between FGF2 and TGFβ1 was greatly reduced (66 and 52%, respectively). At higher concentrations (50μM and 10μM of AG1296 and Inhibitor III, respectively), both PDGFR inhibitors abrogated the synersism between FGF2 and TGFβ1 (Figure 3B). However, these concentrations also partially reduced FGF2-induced proliferation (data not shown), suggesting a non-specific effect of these inhibitors on BSMC proliferation when used at these higher concentrations. Proliferative synergism between FGF2 and TGFβ1 observed in cells derived from the other two donors was also reduced by AG1296 (10μM) (23 and 37% for the 1 year-old and the 21 year-old caucasian females, respectively) (data not illustrated). Additional experiments were performed using a neutralizing mAb against PDGFRα. At 0.2, 2 and 20 μg/ml,

the synergism between FGF2 and TGFβ1 was reduced by 5, 22 and 33%, respectively (data not shown).

TGFβ1 increases PDGFR ligand expression

The expression of PDGF-A, -B and -C mRNA was measured by semi-quantitative RT-PCR during time-course stimulations with TGFβ1 (10 ng/ml). Relative PDGF-A mRNA increased significantly after 1 h stimulation with TGFβ1 and persisted for 12 h post-stimulation (Figure 4A). At the protein level, conditioned medium (CM) from BSMC treated for 24 h with TGFβ1 (10 ng/ml) showed a ~5-fold increase in PDGF-AA expression compared to CM from non-treated cells (from 5.60 ± 1.71 to 29.29 ± 4.93 pg/ml, $p = 0.001$) (Figure 4B). PDGF-C was also up-regulated after TGFβ1 stimulation (Figure 4C). However, in contrast to the transient induction of PDGF-A, the increase in PDGF-C expression was maintained for at least 2 days. PDGF-B, on the other hand, was not detected in BSMC (data not shown).

FGF2 increases PDGFRα as well as the proliferation induced by exogenous PDGF-AA

Semi-quantitative RT-PCR revealed that FGF2 (2 ng/ml) time-dependently increased the mRNA expression level of PDGFRα, reaching a peak at 1 h post-stimulation (Figure 5A). To ensure that PDGFRα protein was upregulated at the time of TGFβ1 stimulation, expression of PDGFRα was measured by Western blot analysis in whole cell lysates 24 h following FGF2 administration. As demonstrated in figure 5B, levels of PDGFRα were elevated 24 h post-FGF2 stimulation relative

to levels found in unstimulated cells. In contrast, expression of the structural protein vinculin did not differ between baseline and stimulated conditions, indicating that equal amounts of protein had been loaded in each well.

The effect of exogenous PDGF-AA with or without a 24 h pre-treatment with FGF2 was then evaluated to confirm the functionality of the induced receptor. Recombinant PDGF-AA alone (1 to 50 ng/ml) tended to increase BSMC proliferation in a concentration-dependent manner (not statistically significant at any concentration tested) (Figure 5C). However, with a prior FGF2 stimulation (2 ng/ml), the proliferative effect of increasing concentrations of PDGF-AA was enhanced, reaching a statistically significant difference at 10 ng/ml and demonstrating a 140% increase at 50 ng/ml compared to FGF2 alone.

FGF2 increases tissue plasminogen activator (tPA) mRNA expression

To investigate whether FGF2 pre-stimulation would allow latent PDGF-CC activation after its potential secretion induced by TGFβ1, kinetic studies of FGF2 stimulation were performed to measure mRNA expression of the PDGF-CC activating protease tPA. As demonstrated in Figure 6A, FGF2 increased in a time-dependent fashion the mRNA expression of tPA, reaching its peak levels between 12 and 24 h of stimulation. In additional experiments, leupeptin (50μM), a serine and cysteine protease inhibitor which inhibits tPA activity, administered 1 h before TGFβ1 stimulation, reduced significantly (36%) the proliferative synergism induced by sequential FGF2 and TGFβ1 co-stimulation.

DISCUSSION

This is the first demonstration of FGF2 and TGFβ1 synergism in human BSMC proliferation. The mechanisms responsible for this synergy are partially elucidated here and indicate that members of the well-known PDGF family of mitogens are involved. In support of this hypothesis, our data demonstrated that: 1- inhibitors of PDGFR tyrosine kinase activity (AG1296 and Inhibitor III) reduced significantly the synergism between FGF2 and TGFβ1; 2- TGFβ1 induced PDGF-AA mRNA and protein as well as PDGF-C mRNA expression; 3- FGF2 increased PDGFRα mRNA and protein expression and potentiated the proliferative effect of exogenous PDGF-AA; 4- FGF2 also increased the mRNA expression of the PDGF-CC activating protease tPA, and the tPA protease inhibitor leupeptin partially blocked the FGF2-TGFβ1 synergy; and 5- neutralizing Ab against the receptor engaged by both PDGF-AA and PDGF-CC, namely PDGFRα, also reduced the proliferative synergism triggered by sequential FGF2 and TGFβ1 co-stimulation (Figure 7). The mechanisms involved in the remaining part of the synergism, which is not blocked by inhibitors of PDGFR signaling, are still unresolved and are the subject of current investigations in our laboratory.

For the last two decades, the effect of TGFβ1 on airway smooth muscle proliferation *in vitro* has been a matter of debate. (27-33). However, its potential contribution to BSMC hyperplasia *in vivo* has recently gained many insights: 1- TGFβ1 was consistently reported to be upregulated in animal (24-26) or human

asthmatic airways (7, 16-21, 23); 2- its expression further increased after allergic challenge in human asthmatics (7); 3- active TGFβ1 signaling (measured by nuclear phospho-Smad2 immunostainning) has been observed in airways of animal (34, 35) and human asthmatics before and after allergic challenge (22, 36); 4- targeted overexpression of TGFβ1 in the adult lung of mice induced BSMC hyperplasia (37); and 5- therapeutically administered anti-TGFβ1 antibodies prevented BSMC hyperplasia in a prolonged allergen sensitization/challenge mouse model (35). However, the mechanisms that govern TGFβ1-induced BSMC hyperplasia *in vivo* remain unknown and its controversial effect observed in *in vitro* condition necessitates elucidation.

In this regard, the temporal increase in TGFβ1 expression after phlogogenic challenge relative to other growth factors could potentially bring information regarding the aetiology of BSMC hyperplasia. Contrary to other rapidly released mediators such as histamine, leukotrienes or FGF2, the upregulation of TGFβ1 during an asthma attack is delayed (7). In this work, we studied the effect of TGFβ1, alone or with a prior stimulation with FGF2, on human BSMC proliferation.

In our proliferation assay, TGFβ1 alone had no detectable effect on BSMC proliferation. However, when administered 24 h after FGF2 stimulation, TGFβ1 markedly increased BSMC proliferation. The mechanisms involved were tested by screening the effect of different pharmacological inhibitors. Src inhibitors PP1 and

PP2 abrogated the proliferation induced by FGF2 alone. Considering that both signals from FGF2 and TGFβ1 are required for the synergy to occur, the blocking effect of Src inhibitors on the synergy was expected. More surprisingly, tyrphostin AG1296 (20μM) and Inhibitor III (1μM), which inhibit tyrosine kinase activity of the α and β chains of PDGF receptors, significantly blocked the synergism between FGF2 and TGFβ1 without affecting FGF2-induced proliferation.

Based on time-course proliferation assays with [^{3}H]-thymidine and crystal violet staining, TGFβ1 did not increase FGF2 mitogenicity, but rather prolonged DNA synthesis induced by FGF2. The delayed mitogenic effect of TGFβ1, together with the involvement of PDGFR tyrosine kinase activity in the FGF2-TGFβ1 synergy, was suggestive of an induced expression of PDGFR ligands by TGFβ1. Our findings indicate that this may indeed be the case, since expression of both PDGF-AA and PDGF-C was induced by TGFβ1. Moreover, neutralizing antibody against PDGFRα reduced in a concentration-dependent manner FGF2-TGFβ1 synergy. Collectively, these results suggest that PDGF-AA and PDGF-C are upregulated by TGFβ1 and could likely act as autocrine growth factors to increase BSMC proliferation.

Intriguingly, TGFβ1 alone was sufficient to increase PDGF-AA and PDGF-C expression, but was unable to induce BSMC proliferation. However, Bonner and coworkers (11) have already demonstrated that FGF2 could potentiate the mitogenic response of BSMC to exogenous PDGF-AA by its ability to increase PDGFRα

expression. This mechanism seems operational in our study as well, since we found FGF2 to increase PDGFRα mRNA and protein expression. In addition, based on the enhanced proliferative response of BSMC to exogenous PDGF-AA following a 24 h pre-treatment with FGF2, these newly synthesized receptors were shown to befunctional. The magnitude of this enhanced proliferation may however be underestimated compared to the role of PDGF in the FGF2-TGFβ1 synergy, since stimulations were performed with PDGF-AA only, rather than with both PDGF-AA and PDGF-CC. In any case, the lack of mitogenic effect of TGFβ1 on its own supports the notion that TGFβ1-induced PDGF-AA and PDGF-CC were insufficient to stimulate mitogenesis, but would require PDGFRα upregulation by FGF2 to exert their proliferative effect.

In contrast to PDGF-AA, PDGF-CC is secreted in a latent form. To be of any significance in the proliferative synergism between FGF2 and TGFβ1, latent PDGF-CC would need to be activated by proteolytic cleavage. The only protease known to activate latent PDGF-CC is tissue plasminogen activator (tPA) (38). Accordingly, we demonstrated that FGF2 induced tPA mRNA expression in BSMC. Moreover, the serine and cysteine protease inhibitor leupeptin partially blocked the synergism between FGF2 and TGFβ1. These results highlight the possibility that latent PDGF-CC induced by TGFβ1 would be cleaved by FGF2-induced tPA, which will subsequently permit binding to and dimerization-induced activation of its receptors. This also corroborates the lack of proliferative effect of TGFβ1 on its own.

In conclusion, we speculate that the proliferative synergism obtained in *in vitro* conditions with human BSMC following such sequential FGF2 and TGFβ1 co-stimulation represents potential etiologic events leading to BSMC hyperplasia in asthmatic individuals. Since this observed synergism has been reproduced in BSMC derived from two other donors with different age, sex and ethnicity, we propose that this proliferative response is a generalized mechanism leading to BSMC hyperplasia, regardless of the genetic heterogeneity among the population. Our results also suggest that the well known mitogenic receptor PDGFRα contributes significantly to this response via an autocrine agonist-dependent activation, but other mechanisms are likely operational as well.

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FIGURE LEGENDS

Figure 1. TGFβ1 and FGF2 synergized in a concentration-dependent manner to induce BSMC proliferation. BSMC were seeded with a fixed (A) or increasing (B) concentration of FGF2 (2 ng/ml) and stimulated 24 h later with increasing (A) or fixed (B) concentrations of TGFβ1. Cell proliferation was measured 4 days following TGFβ1 stimulation as described in Materials and Methods. Points are means ± SEM of quadruplicate measurements of at least 3 independent experiments. In Figure A, all concentrations over 0.25 ng/ml of TGFβ1 are significantly higher compared to FGF2 alone ($p<0.01$). In Figure B, the effects of TGFβ1 are significantly higher compared to respective concentration of FGF2 alone, starting at 0.25 ng/ml ($p\leq0.01$).

Figure 2. TGFβ1 prolonged mitogenicity when administered 24 h after FGF2 treatment. (A) Cells were stimulated with FGF2 (2 ng/ml) or TGFβ1 (10 ng/ml) individually or sequentially at 24 h intervals in their respective order during a 5 day time-course experiment. DNA synthesis was evaluated with a 4 h pulse of [^{3}H]-thymidine at the end of 4 consecutive days following TGFβ1 administration. Bars are means ± SEM of quadruplicate measurements of at least 3 independent experiments. $p<0.05$: * compared to baseline. (B) Five day time-course experiment performed with crystal violet staining to assess BSMC proliferation. Bars are means of quadruplicate measurements of a single experiment.

Figure 3. Tyrosine kinase inhibitors of PDGFR reduced the proliferative synergism obtained by sequential FGF2 and TGFβ1 co-stimulation. (A) Effects of different pharmacological inhibitors on FGF2 (2 ng/ml)- and FGF2 (2 ng/ml) + TGFβ1 (10 ng/ml)-induced proliferation. Pharmalogical inhibitors were administered 1 h before TGFβ1 treatment and, hence, 23 hours after FGF2 treatment. Results are expressed as percentage of baseline OD (i.e. without any stimulus, inhibitor orvehicle) where each bar represents means ± SEM of quadruplicate measurements of at least 4 independent experiments. $p \leq 0.05$: * compared to their respective vehicle (DMSO)-treated control. (B) The effect of increasing concentration of AG1296, Inhibitor III or DMSO on FGF2-TGFβ1 synergy. Results are expressed as percentage of the synergism (defined as the difference between FGF2- and FGF2 + TGFβ1-induced proliferation) for every concentration of AG1296, Inhibitor III or equivalent AG1296-concentration of DMSO tested. Each bar represents means ± SD of quadruplicate measurements of at least 3 experiments. $p \leq 0.05$: * compared to control FGF2-TGFβ1 synergism. Inhibitor III was used in one experiment in quadruplicate.

Figure 4. TGFβ1 increased PDGF-AA and PDGF–C expression. Representative kinetics (upper panels) and densitometric analysis (lower panels) of PDGF-A (A) and PDGF-C (C) mRNA expression in response to TGFβ1 (10 ng/ml) stimulation. Each point represents a compilation of at least 3 independent experiments. $p \leq 0.05$: * compared to time 0. (B) PDGF-AA expression level in CM from TGFβ1 (10 ng/ml)- or non-treated BSMC. Bars represent means ± SEM of duplicate

measurements of 6 independent experiments. $p \leq 0.05$: * compared to non-treated cells.

Figure 5. FGF2 increases PDGFRα expression as well as the proliferation induced by increasing concentrations of exogenous PDGF-AA. Representative kinetics (upper panels) and densitometric analysis (lower panels) of PDGFRα expression (A) in response to FGF2 (2 ng/ml) stimulation. Each point represents a compilation of at least 3 independent experiments. $p \leq 0.05$: * compared to time 0. (B) Western blot analysis of PDGFRα (~180 KDa) and vinculin (~113 KDa) expression at baseline and following 24 h stimulation with FGF2 (2 ng/ml). Molecular markers were loaded in the first lane. (C) BSMC were seeded with or without a fixed concentration of FGF2 (2 ng/ml) and stimulated 24 h later with increasing concentrations of PDGF-AA. Points are means ± SEM of quadruplicate measurements of at least 3 independent experiments. $p \leq 0.05$: * compared to FGF2 alone.

Figure 6. FGF2 increases tPA expression and protease activity is required for optimal proliferative synergism between FGF2 and TGFβ1. Representative kinetics (upper panels) and densitometric analysis (lower panels) of tPA mRNA expression (A) in response to FGF2 (2 ng/ml) stimulation. Each point represents a compilation of at least 3 independent experiments. $p \leq 0.05$: * compared to time 0. (B) FGF2-TGFβ1 synergism was investigated in the presence of leupeptin (50μM; administered 1 h before TGFβ1 stimulation). Points are means ± SEM of

quadruplicate measurements of 3 independent experiments. $p \leq 0.05$: * compared to FGF2-TGFβ1 without inhibitor.

Figure 7. Proliferative synergism between FGF2 and TGFβ1 involves a PDGF loop. Whereas FGF2 increases PDGF receptor α-chain (PDGFRα) and PDGF-CC activating protease (tPA) expression in human bronchial smooth muscle cells, TGFβ1 increases the expression of PDGFRα ligands, namely PDGF-AA and PDGF-CC. When FGF2 and TGFβ1 are administered sequentially at a 24 h interval in their respective order, as demonstrated in the schematic representation, a proliferative synergism occurs.

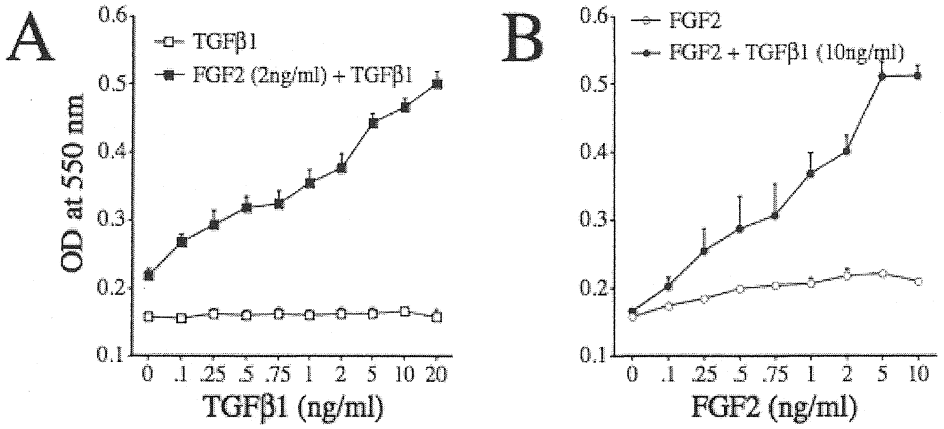
A
TGFβ1
FGF2 (2ng/ml) + TGFβ1
OD at 550 nm
0.6
0.5
0.4
0.3
0.2
0.1
0 .1 .25 .5 .75 1 2 5 10 20
TGFβ1 (ng/ml)
B
FGF2
FGF2 + TGFβ1 (10ng/ml)
0.6
0.5
0.4
0.3
0.2
0.1
0 .1 .25 .5 .75 1 2 5 10
FGF2 (ng/ml)

Figure 1

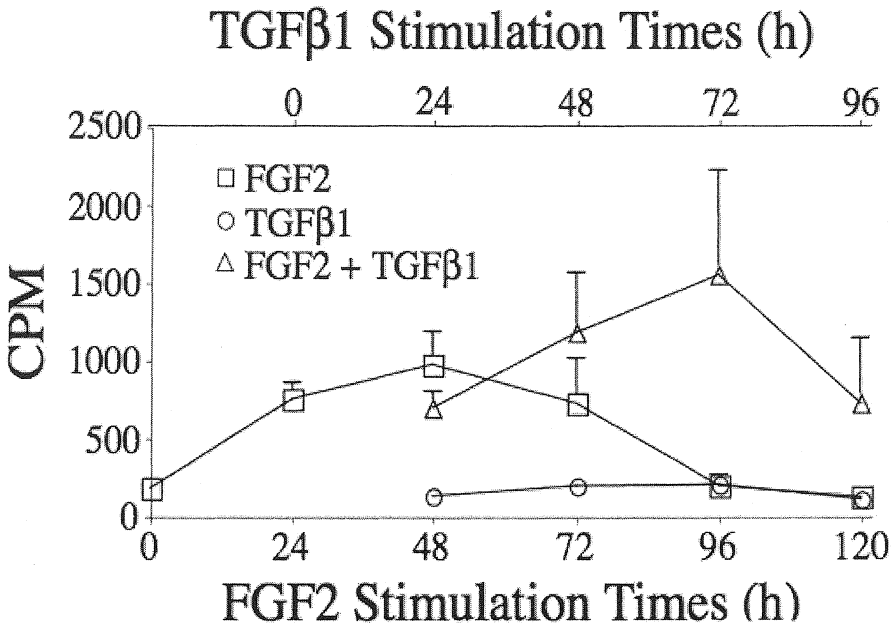
TGFβ1 Stimulation Times (h)
0
24
48
72
96
2500
2000
1500
1000
500
0
CPM
FGF2
TGFβ1
FGF2 + TGFβ1
0
24
48
72
96
120
FGF2 Stimulation Times (h)

Figure 2

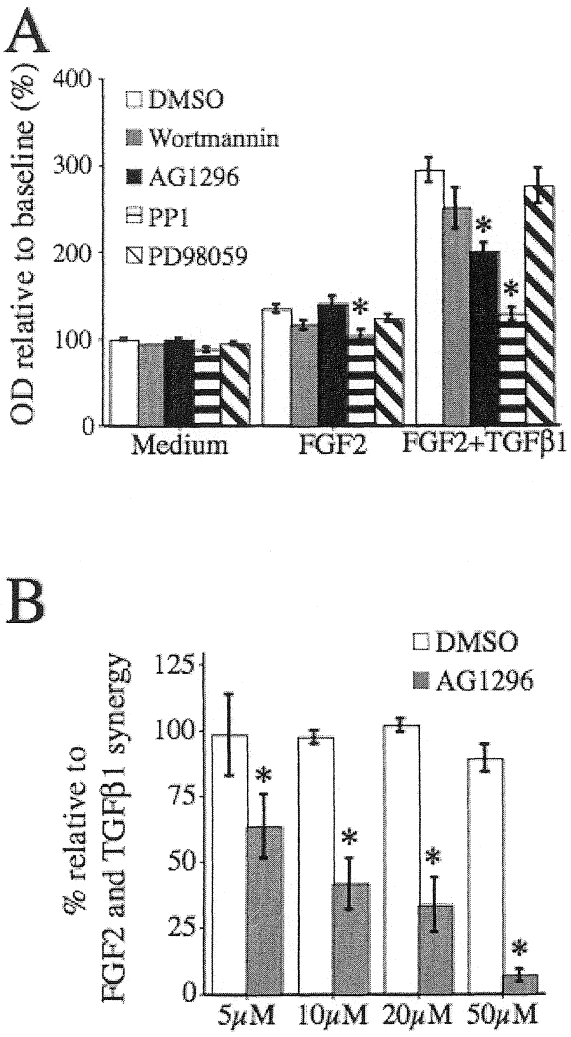
A
OD relative to baseline (%)
400
300
200
100
0
DMSO
Wortmannin
AG1296
PP1
PD98059
Medium
FGF2
FGF2+TGFβ1
B
% relative to FGF2 and TGFβ1 synergy
125
100
75
50
25
0
DMSO
AG1296
5μM
10μM
20μM
50μM

Figure 3

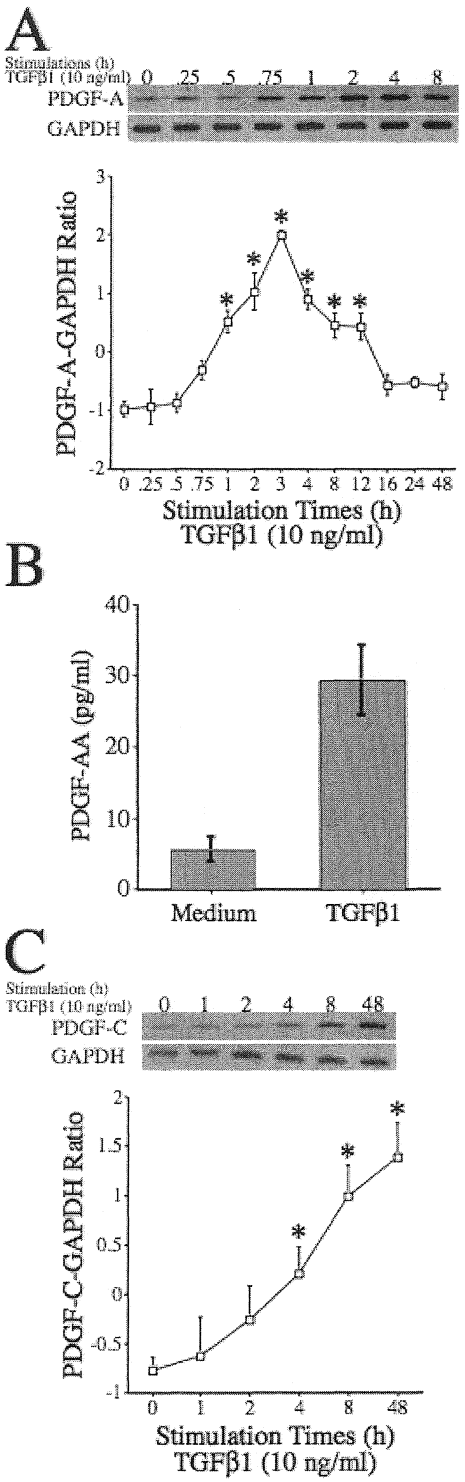
A
Stimulations (h)
TGFβ1 (10 ng/ml) 0 .25 .5 .75 1 2 4 8
PDGF-A
GAPDH
PDGF-A-GAPDH Ratio
3
2
1
0
-1
-2
0 .25 .5 .75 1 2 3 4 8 12 16 24 48
Stimulation Times (h)
TGFβ1 (10 ng/ml)
B
PDGF-AA (pg/ml)
40
30
20
10
0
Medium
TGFβ1
C
Stimulation (h)
TGFβ1 (10 ng/ml) 0 1 2 4 8 48
PDGF-C
GAPDH
PDGF-C-GAPDH Ratio
2
1.5
1
0.5
0
-0.5
-1
0 1 2 4 8 48
Stimulation Times (h)
TGFβ1 (10 ng/ml)

Figure 4

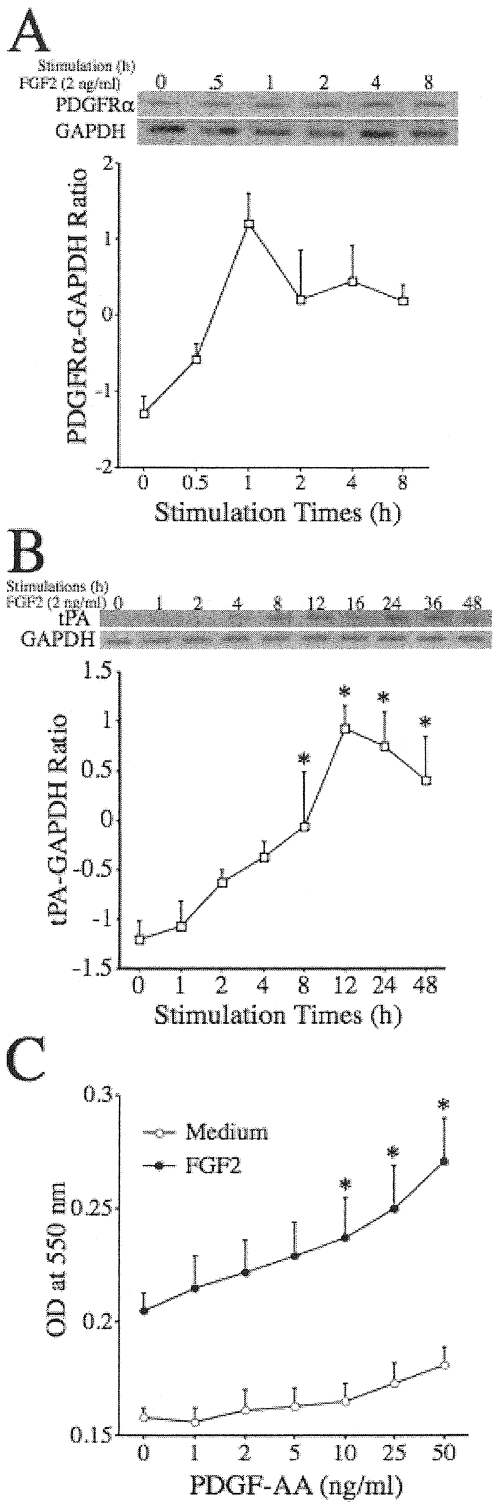
A
Stimulation (h)
FGF2 (2 ng/ml)
0 .5 1 2 4 8
PDGFRα
GAPDH
PDGFRα-GAPDH Ratio
2
1
0
-1
-2
0 0.5 1 2 4 8
Stimulation Times (h)
B
Stimulations (h)
FGF2 (2 ng/ml) 0 1 2 4 8 12 16 24 36 48
tPA
GAPDH
tPA-GAPDH Ratio
1.5
1
0.5
0
-0.5
-1
-1.5
0 1 2 4 8 12 24 48
Stimulation Times (h)
C
OD at 550 nm
0.3
0.25
0.2
0.15
Medium
FGF2
0 1 2 5 10 25 50
PDGF-AA (ng/ml)

Figure 5

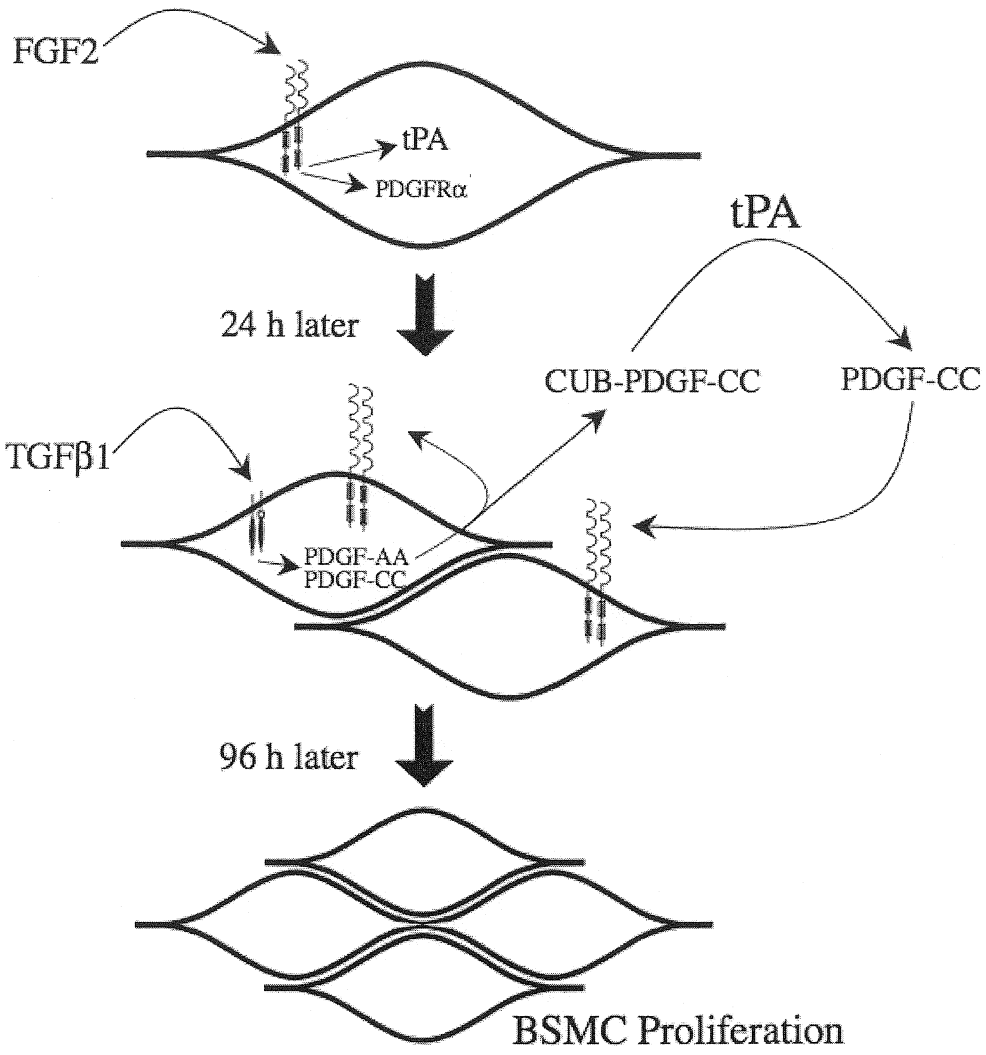
FGF2
tPA
PDGFRα
tPA
24 h later
CUB-PDGF-CC
PDGF-CC
TGFβ1
PDGF-AA
PDGF-CC
96 h later
BSMC Proliferation

Figure 6

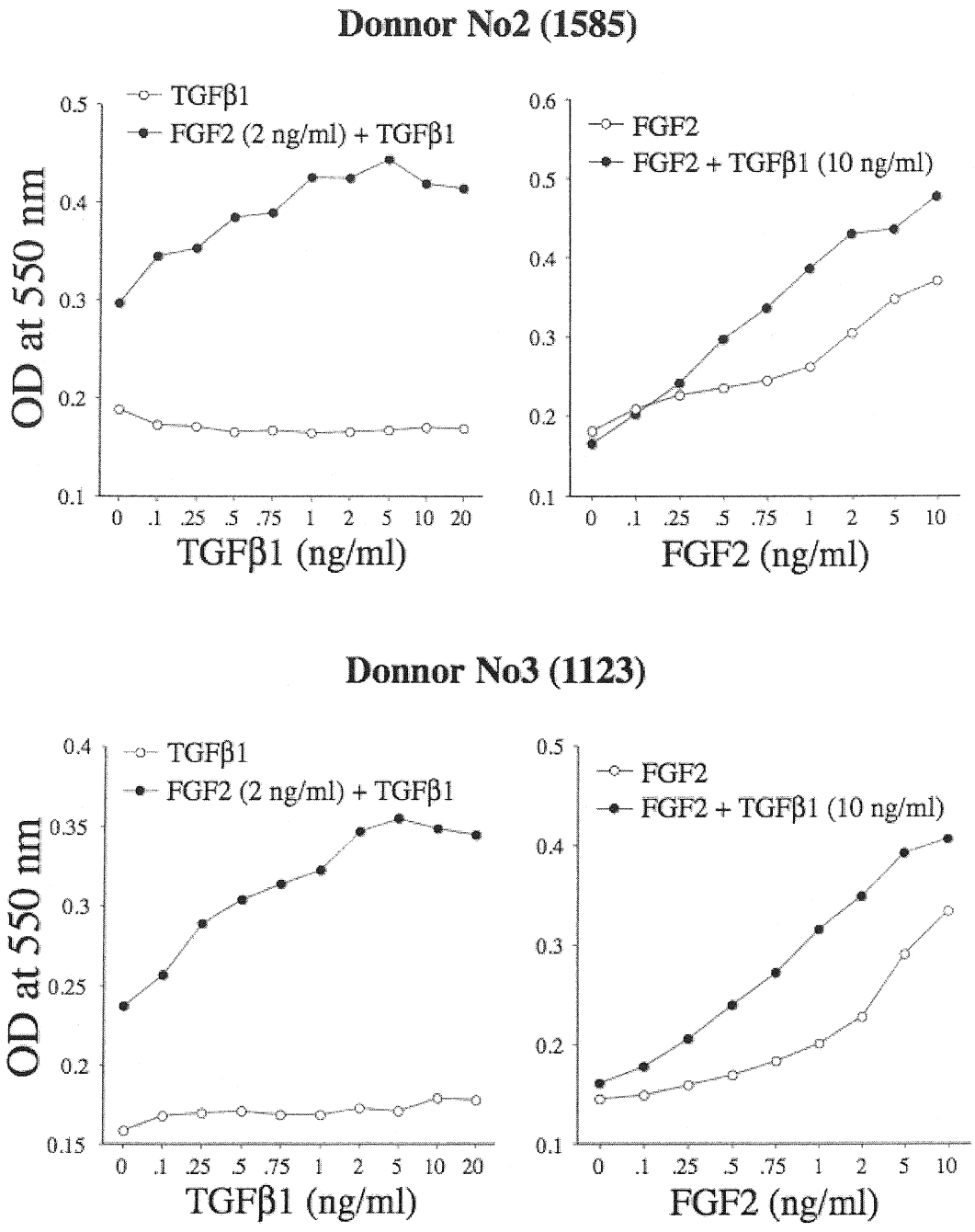

Figure 1 (Supplementary)

The T_H2 Cytokines, IL-4 and IL-13, Enhance Human Bronchial Smooth Muscle Cell Proliferation

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Number of words in the text: 3529

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ABSTRACT

Background: T_H2 inflammation and bronchial smooth muscle cell (BSMC) hyperplasia are characteristic features of asthma, but whether these phenomena are linked remains to be clarified.

Objectives: To define the effect of the T_H2 cytokines IL-4 and IL-13 on human BSMC proliferation when administered alone or in combination with the FGF2 growth factor. To test the additional effects of pro-inflammatory mediators such as TNFα and IL-1β and the involvement of members of the well-known family of platelet-derived growth factor (PDGF) mitogens.

Methods: BSMC proliferation was measured by crystal violet staining and PDGF mRNA and PDGF receptor (PDGFR) expression were determined by RT-PCR and immunocytochemistry, respectively.

Results: Neither IL-4 nor IL-13 (0.1 to 20 ng/ml) alone induced BSMC proliferation, despite being both potent inducers of PDGF-A and PDGF-C mRNA. However, following a pre-treatment with FGF2 (2 ng/ml), which increased PDGF receptor α chain expression, both IL-4 and IL-13 increased FGF2-induced BSMC proliferation in a time and concentration-dependent manner (70% and 40% increase compared to FGF2 alone, respectively.). On the other hand, TNFα and IL-1β did not affect basal or FGF2-induced BSMC proliferation, but both pro-inflammatory

mediators enhanced the proliferative synergism between FGF2 and the T_H2 cytokines.

Conclusions: The typical T_H2 cytokines, IL-4 and IL-13, potently induce BSMC proliferation when combined with FGF2 and this proliferative synergism is amplified by pro-inflammatory cytokines.

Clinical Implication: Increased expression of FGF2 observed in asthmatic airways following allergen challenge may prime BSMC to proliferate when subsequently exposed to T_H2-type inflammation, which, in turn, contributes to airway hyperresponsiveness.

CAPSULE SUMMARY

Treating T_H2 inflammation with current anti-inflammatory therapies may prevent some remodeling features of asthma. This article reports that IL-4 and IL-13 induce bronchial smooth muscle cell hyperplasia when combined with the FGF2 growth factor.

KEYWORDS

Human, stromal cells, cytokines, lung, allergy

ABBREVIATIONS

AHR, airway hyperresponsiveness; BALF, bronchoalveolar lavage fluid; BSMC, bronchial smooth muscle cell; FGF2, fibroblast growth factor 2; PDGF, platelet-derived growth factor; PDGFR, PDGF receptor.

INTRODUCTION

Allergic asthma is an acute and recurent T_H2-mediated disease initiated by allergen encounters and characterized by airway inflammation and reversible airway hyperresponsiveness (AHR). It is also accepted that exaggerated or uncontrolled healing processes following each asthma exacerbation lead to cumulative structural changes in the airways over time translating into more chronic and irreversible airway dysfunction [1]. Among the remodeling phenomena observed in asthmatic airways, BSMC hyperplasia is thought to be the main contributor to the asthma diathesis [2 3].

A decade ago, increased expression of the T_H2 cytokines IL-4 and IL-13 in the lungs of asthmatic patients has been recognised [4 5 6]. Since then, IL-4 and IL-13 have both been shown to contribute extensively to the pathology of asthma [7]. *In vivo*, repetitive intratracheal instillation or intranasal administration of recombinant forms of IL-13 or IL-4 to naive mice recapitulates most of the features of allergic asthma [8 9], including enlargement of airway smooth mucle (ASM) mass [10]. Transient or constitutive overexpression of IL-13 transgene in the airway epithelium of mice also mimics many pathological features of asthma [11 12]. On the other hand, IL-13 and IL-4 knock-out mice are protected against allergen-induced AHR [13 14] and neutralization of IL-4 with monoclonal antibodies during the sensitization period prevents the development of AHR in a mouse model of allergic inflammation [15]. Furthermore, deficiency in IL-4Rα or in STAT6, the main signaling transducer

and activator of transcription (STAT) activated by IL-4 or IL-13 after binding to their cognate receptors, also renders mice resistant to experimentally-induced allergic asthma [9 16 17].

Based on recombination-activating gene 1 (Rag1)-deficient mice, it was suggested that IL-13 acts directly on the bronchial tissue through non-T cell and non-B cell mechanisms to induce the asthma phenotype [9]. Moreover, pre-treatment with vinblastin, a granulocyte-depleting agent, was ineffective in blocking IL-13-induced AHR [18], thus discarding the possible involvement of neutrophils, eosinophils and basophils in the asthma phenotype induce by IL-13. Correspondingly, IL-5 and eotaxin double knock-out mice, which are severely impaired in their ability to recruit eosinophils into the airways, still demonstrated AHR upon IL-13 exposure [19]. In addition, a single dose of IL-13 or IL-4 was sufficient to induce AHR at an early time point (6 hours), when leucocyte infiltration in the airways was still comparable to that of mice treated with saline alone [20]. Intranasal administration of conditioned medium derived from T_H2-activated cells also induced AHR in wild-type mice. This effect was blunted in IL-4Rα- or STAT6-deficient mice, but was still present in mast cell-deficient W/W^v mice [20], discarding the possible involvement of immune tissue residential mast cells in the asthma phenotype induced by IL-4 or IL-13. Finally, an elegant complementary study has confirmed all these observations by demonstrating with adoptive transfer experiments that IL-4Rα chain in the irradiated recipient, but not in the bone marrow donors, is a prerequisite for asthma development upon antigen exposure [21]. Hence, ample evidence now suggests that

T_H2 cytokines, at least in mice, exerts their effector functions by acting primarily through airway structural cells. Using gene microarray analysis, it was also established that among structural cells present in the airways, BSMC were the most responsive to IL-13 stimulation [22].

Most of the information gained in the aforementioned studies regarding the role of T_H2 cytokines in the pathology of asthma has been obtained using acute models of pulmonary inflammation [7]. Presently, different models of chronic asthma are established and some investigators have started to delineate the contribution of IL-4 and IL-13 in AHR, goblet-cell metaplasia and peribronchial inflammation in such models [23 24]. However, the potential role of these cytokines in BSMC hyperplasia is still unknown. This study aims at defining the effect of the T_H2 cytokines, IL-4 and IL-13, in primary human BSMC proliferation. The results show that administration of either IL-4 or IL-13 alone has no effect on BSMC proliferation, but both cytokines can synergize in a time- and concentration-dependent manner when administered following exposure of the cells to the growth factor FGF2.

MATERIALS AND METHODS

Cell culture

Human primary BSMC (BioWhittaker, Inc. Walkersville, MD) were used for all experiments. BSMC were derived from a 5 week-old black male and a 1 year-old and a 21 year-old caucasian females. All donors had negative history of smoking and were free of pre-existing lung disease. The identity and purity of the cells have been confirmed with α-SMA positive and with von Willebrand Factor negative staining. Upon reception, cryopreserved cells were cultured in T-75 flasks in Smooth muscle Growth Medium (SmGM) (SmGM-2 Bulletkit) provided by the manufacturer and consisting of Smooth muscle Basal Medium (SmBM), 5% fetal bovine serum (FBS) and a mixture of growth factors, including fibroblast growth factor 2 (FGF2) (2 ng/ml), epidermal growth factor (EGF) (0.5 ng/ml) and insulin (5 μg/ml), as well as a mixture of antibiotics, including Gentamicin (100 ng/ml) and Amphotericin B (0.1 ng/ml). Thawing, subculturing and harvesting procedures were performed according to manufacturer's instructions. Experiments were performed with cells at the 4^{th} passage.

Cell proliferation: Crystal violet staining

Cells were subcultured into 96-well plates at 3000 cells/well in a starvation medium, consisting of SmBM + 1% FBS, with or without FGF2 (2 ng/ml) as previously described [25]. Cells were maintained in these conditions for 24 h before IL-4 (PeproTech Canada, Inc. Ottawa, ON) or IL-13 (R&D, Minneapolis, MN)

stimulations (10 ng/ml; unless otherwise specified). BSMC proliferation was measured at different time intervals after cytokine administration using the DNA staining property of crystal violet (Sigma, Oakville, ON) as previously described [25].The validity of crystal violet staining as a surrogate of cell proliferation in the BSMC cell line has been determined elsewhere [25].

To investigate the involvement of PDGF receptors in BSMC proliferation induced by sequential FGF2 and T_H2 cytokines stimulation, a pharmacological inhibitor of PDGF receptor tyrosine kinase activity, tyrphostin AG1296 (10 μM; unless otherwise specified) (Biomol. Plymouth Meeting, PA) was added 1 h before cytokine treatments. DMSO (0.1%) was used as the control vehicle.

Immunocytochemistry

BSMC were allowed to reach confluency in cover slip-containing 6-well plates before being serum-starved for 24 h in SmBM 1% FBS and stimulated with FGF2 (2 ng/ml) in fresh medium for different time intervals. The cells were then washed in HBSS and sequentially fixed and permeabilized in paraformaldehyde (2%) and saponine (0.1%) PBS solutions, respectively. Non-specific binding sites were blocked with 5% (w:v) milk in PBS. A mAb against human PDGFRα (R&D, Minneapolis, MN) at 15 μg/ml final concentration was then applied as primary Ab followed by fluorescein (FITC)-conjugated goat anti-mouse IgG Ab (1:250) (Jackson ImmunoResearch Laboratories, Inc. West Groove, PA) as secondary Ab. Finally, cells were counterstained with Hoechst solution to identify cell nuclei. Appropriate washing with PBS BSA (2%) solution was performed after each step.

Following staining procedures, cover slips were transferred onto slides and examined under microscope, using a LEICA DMIRE2 fluorescent microscope (Leica microsystems. Wetzlar, Germany).

RT-PCR

Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) was used to measure mRNA expression, as previously described [25]. After reaching confluency in 6-well plates, cells were starved for 24 h in SmBM 1% FBS prior to IL-13 or IL-4 (10 ng/ml) stimulations in fresh medium. For all experiments, GAPDH mRNA was used as an internal housekeeping gene control. The PCR primers used were: PDGF-A forward, 5′-gaccaggacggtcatttacg-3′; PDGF-A reverse, 3′-cctcacatccgtgtcctctt-5′; PDGF-C forward, 5′-ttcagcaacaaggaacagaac-3′; PDGF-C reverse, 3′-ctgaaggggtagctctgaa-3′; GAPDH forward, 5′-gatgacatcaagaaggtggtgaa-3′; GAPDH reverse, 3′-gtcttactccttggaggccatgt-5′.

Statistical analysis

Results illustrated in the figures, as well as their statistical analysis, are compiled data obtained using BSMC derived from the 3 donors. To appreciate the independent effect of the T_H2 cytokines (IL-4 or IL-13) and the time of stimulation as well as the interaction between these two variables, the effect of FGF2 alone and in combination with either of the T_H2 cytokines was compared by using two-way ANOVA (Fig. 1). Similarly, in the proliferation experiments with increasing or fixed concentrations of either FGF2 or the T_H2 cytokines (Fig. 2), two-way

ANOVA was performed to determine the effect of both factors individually and their interactions. In experiments with the pharmacological inhibitor AG1296 (Fig. 6), unpaired Student's t test was used to compare the FGF2-IL-4/IL-13 proliferative synergism between AG1296- and DMSO-treated cells (analysis was performed on raw data before conversion to percentage). Finally, an ANOVA followed by Tukey's test *a posteriori* for comparison of all pairs of conditions was performed to determine the effect of the pro-inflammatory cytokines TNFα and IL-1β on the synergism between FGF2 and IL-4 or IL-13 (Fig. 7). All statistical analysis were performed using Prism 4 (GraphPad Software, San Diego CA) and a $p \leq 0.05$ was arbitrarily considered sufficient to reject the null hypothesis.

RESULTS

IL-4 and IL-13 induce BSMC proliferation

The proliferative effect of IL-4 and IL-13, alone or 24 hours after FGF2 stimulation, was examined in primary human BSMC. Kinetic responses were first determined with fixed concentrations of the T_H2 cytokines (10 ng/ml) and FGF2 (2 ng/ml). As shown in Fig. 1, neither IL-4 nor IL-13 alone induced BSMC proliferation compared to the starvation medium alone (SmBM + 1% FBS). However, when BSMC were primed with FGF2, both IL-4 and IL-13 potentiated the proliferation induced by FGF2 in a time-dependent manner. Based on two-way ANOVA analysis comparing the effect of FGF2 alone with the effect of FGF2 in combination with either of the T_H2 cytokines, both IL-4 and IL-13 significantly increased FGF2-induced BSMC proliferation independently of time. Since no apparent effect could be appreciated on the first two days following IL-4 or IL-13 administration (Fig. 1), the independent effect of both T_H2 cytokines are obviously the result of large differences observed at the 3 and 4-day time points. In fact, the interaction between the conditions tested (FGF2 alone or in combination with either of the T_H2 cytokines) and the time of stimulation was significant for IL-4 ($p = 0.02$) and tended toward significance for IL-13 ($p = 0.1$), meaning that the proliferative behavior of BSMC to FGF2 in function of time depends on whether or not they were treated with the T_H2 cytokines. Considering the significant increase in BSMC proliferation over that with FGF2 alone 4 days following T_H2 cytokines administration, this time point was chosen to measure BSMC proliferation for the remaining experiments.

Concentration-response studies were then performed for both the T_H2 cytokines and the FGF2 growth factor. As shown in Fig. 2A and B, FGF2 alone induced BSMC proliferation in a concentration-dependent manner ($p < 0.0001$). Consistent with the findings in Fig. 1, a fixed concentration of IL-4 or IL-13 (10 ng/ml) alone did not lead to BSMC proliferation. However, both cytokines used at that concentration induced significant BSMC proliferation when combined with increasing concentrations of FGF2 ($p < 0.0001$ for IL-4 as well as IL-13) and their effects were proportional to the concentration of FGF2 used (interaction : $p = .001$ and .05 for IL-4 and IL-13, respectively). On the other hand, Fig. 2C and D show that none of the concentrations of T_H2 cytokines tested were able to support BSMC proliferation when administered alone. Once again, the proliferative effect of a fixed concentration of FGF2 (2 ng/ml) could be appreciated ($p < 0.0001$ for both Fig. 2C and D) and its effect was gradually augmented by increasing concentrations of either IL-4 or IL-13 (interaction : $p = 0.002$ and not significant, respectively). Interestingly, administration of IL-4 and IL-13 together (both at 10 ng/ml) did not lead to an additive effect on the proliferative synergism with FGF2 (data not shown), which was consistent with both cytokines acting on the same receptor in structural cells.

PDGFRα is upregulated by FGF2

We [25] and others [26] have previously demonstrated that PDGFRα mRNA and protein expression are upregulated following FGF2 treatment. In the present study, these

results were confirmed by immunocytochemistry using an anti-human PDGFRα monoclonal antibody. As observed in Fig. 3, the major increase occured in the 8 hour gap between 16 and 24 hours post-FGF2 stimulation and was maintained for at least 48 hours. [26]

T_H2 cytokines increase PDGF-A and PDGF-C expression in a time-dependent manner

To determine whether IL-4 and IL-13 synergized with FGF2 by increasing the expression of agonists for PDGFRα, steady state mRNA expression levels of PDGF-A and PDGF-C were measured by semi-quantitative RT-PCR. As shown in Fig. 4A, PDGF-A, and most importantly PDGF-C, were upregulated in a time-dependent fashion following IL-4 or IL-13 stimulation. However, the kinetics of expression of PDGF-A and PDGF-C mRNA following T_H2 cytokine stimulation were different. Whereas PDGF-A was upregulated transiently (between 4 and 16 h post-stimulation), the increased expression of PDGF-C was maintained for at least 48 h. PDGF-B, on the other hand, was not detected in BSMC (data not shown).

Intrinsic tyrosine kinase activity of PDGFR is required for optimal proliferative synergism between FGF2 and the T_H2 cytokines

To be of any significance in the proliferative synergism observed between FGF2 and the T_H2 cytokines, PDGF production must lead to the activation of PDGFR. AG1296, a selective pharmacological inhibitor of PDGFR tyrosine kinase activity, was used to determine the role of PDGFR activation in FGF2 and IL-4 or IL-13

proliferative synergism. As shown in Fig. 5, the synergism between FGF2 and either IL-4 or IL-13 was significantly reduced (~ 50 %) by AG1296 compared to equivalent concentrations of the vehicle (DMSO). Neither DMSO, nor AG1296 affected FGF2-induced BSMC proliferation (data not shown). This result suggests that PDGFR activation is required for optimal proliferative synergism to occur and that induction of PDGFRα-binding ligands by IL-4 or IL-13 may act as potent mitogens in FGF2-primed BSMC.

Pro-inflammatory cytokines TNFα and IL-1β enhance the proliferative synergism between FGF2 and the T_H2 cytokines.

To determine the influence of pro-inflammatory mediators on the observed proliferative synergism between FGF2 and the T_H2 cytokines, TNFα (10 ng/ml) and IL-1β (10 ng/ml) were co-administered with the T_H2 cytokines (10 ng/ml) with or without a 24 hours pre-treatment with FGF2 (2 ng/ml). As demonstrated in Fig. 6A, IL-1β did not influence BSMC proliferation alone or in combination with FGF2, but increased by 79% the synergism between FGF2 and IL-4 ($p < 0.05$). Similarly, TNFα did not affect BSMC proliferation when administered alone, and even if it reduced the proliferative effect of FGF2, it increased by 111% the synergism obtained by FGF2+IL-4 ($p < 0.05$). The same trends were observed for the synergism between FGF2 and IL-13 (115 and 70% increases for TNFα and IL-1β respectively compared to FGF2+IL-13 synergism alone) (Fig. 6B), but the effects were not statistically significant.

DISCUSSION

The mitogenic effects of FGF2 on BSMC have been documented in several *in vitro* studies. FGF2 is a member of the IL-1 family of pro-inflammatory cytokines, which now comprises the newly identified interleukin with a T_H2 biology, IL-33 [27]. Histological examination of lung sections have shown that FGF2 expression is increased in human asthmatics as well as in non-human primate models of asthma [28] [29] [30] [31]. FGF2 expression is also higher in BALF and induced sputum samples of asthmatics compared to healthy subjects [32] [33]. Moreover, BALF concentrations of FGF2 are further increased following segmental allergen challenge in asthmatics [32], an effect occuring rapidly (10 min) and mediated by FGF2 desequestration from ECM constituents, mast cell degranulation or epithelial cell damage.

On the other hand, IL-4 and IL-13 are predominantly produced by T lymphocytes, mast cells and eosinophils [7]. Consequently, their peak expression following allergen challenge occurs during the late asthmatic response, where lymphocytic and eosinophilic inflammation is prominent. Hence, in order to mimic the sequence of upregulated factors in the airways following allergic challenge, BSMC proliferation was measured, in our study, after sequential co-stimulation with FGF2 and IL-4 or IL-13. Our results demonstrate for the first time that both IL-4 and IL-13 synergize with FGF2 to induce human BSMC proliferation.

Even if they share a common signaling chain (IL-4Rα) in cells of hematopoietic lineage and both IL-4 and IL-13 bind to the same IL-4 type II receptor in structural cells of the airways, they demonstrate divergent functions in the development of asthma. The currently accepted paradigm is that IL-4 is primarily involved in the sensitization process, whereas IL-13 seems to be the main effector cytokine in inducing the asthma phenotype [7]. However, Batra and coworkers [6] have recently demonstrated that both IL-4 and IL-13 are increased in BALF of asthmatic patients 24 h following segmental allergen challenge, but only IL-4 was still elevated after 1 week. Interestingly, the investigators have shown in the same study that only IL-4 was capable of inducing human lung fibroblast differentiation into myofibroblast and stimulating collagen III secretion *in vitro*. A murine model of allergic pulmonary inflammation also suggests that IL-4 may be more involved than IL-13 in the remodeling processes occuring in the chronic phase of the disease [34]. Hence, even if IL-13 is the main effector cytokine in the acute phase responses, the sustained IL-4 upregulation in asthmatic airways likely participates in the long term remodeling processes observed in asthma, such as BSMC hyperplasia. Herein, we report a similar effect of IL-4 and IL-13 on BSMC proliferation, supporting a potential role for both cytokines in airway remodeling. Moreover, since addition of both cytokines simultaneously did not further potentiate the synergism with FGF2, our results indicate that IL-4 and IL-13 may have redundant effects on BSMC proliferation.

Despite being defined as a T_H2-dominated immune response, the allergic airway inflammation in asthma is also characterized by elevated expression of other common pro-inflammatory mediators found in both T_H1 and T_H2 type inflammatory responses. Among these, TNFα and IL-1β were repeatedly reported to be up-regulated in the airways of asthmatics and were shown here to enhance the synergism between FGF2 and the T_H2 cytokines. It is thus anticipated that other aspects of the ongoing inflammation, which occurs in the airways of sensitized individuals following allergen challenge, would amplify the proliferation of BSMC induced by the sequential stimulation with FGF2 and IL-4 or IL-13. These results also suggest that not one, but multiple mediators are likely responsible for the BSMC hyperplasia observed in remodeled asthmatic airways.

In contrast to our results, Hawker and coworkers [35] have demonstrated that IL-4 reduced the mitogenic effect of several BSMC growth factors, including FGF2. Multiple issues may underlie the apparently conflicting results, such as the concentration of IL-4 and FGF2 used (30 vs 2 ng/ml for FGF2 and 50 vs 10 ng/ml for IL-4 in Hawker's and our studies, respectively), the cell density employed (confluent vs underconfluent) and the simultaneous vs sequential administration of the two factors. However, in our opinion, the method employed to measure cellular proliferation was the main issue explaining the discrepancy between the two studies. Hawker's group did not measure BSMC proliferation, but instead measured DNA synthesis by pulsing the cells with radiolabeled-thymidine in the last 5 h of a 24 h stimulation. DNA synthesis precedes cell division and likely reflects cellular

proliferation, but only if the entire DNA synthesis induced by the stimulation is measured, or, alternatively, if several pulses of thymidine are performed after cell stimulation to follow the kinetics of DNA synthesis over time. Evidence in the literature shows that the synergism between two factors might not be the result of an increased DNA synthesis at a particular time point, but rather the result of an extended period of DNA synthesis [25]. This extended DNA synthesis might not be appreciated by a single pulse of radiolabeled thymidine. Based on our kinetics study, the synergism between FGF2 and IL-4 occurs at later time points and the exclusive measurement of DNA synthesis between 19 and 24 h post-stimulation may not represent the right window where the synergism between FGF2 and IL-4 will be apparent. Moreover, since the synergism is partially dependent on a PDGF loop, it is more likely that the synergistic mitogenesis induced by sequential FGF2 and IL-4 will be the result of an extended, rather than a particular time-point increase in DNA synthesis.

The results presented here also suggest that the mitogenic effect of the T_H2 cytokines are indirect and mediated, at least partially, by the induction of PDGFRα-acting ligands, PDGF-A and PDGF-C. Under basal conditions, BSMC are unresponsive to the mitogenic effects of IL-4- or IL-13-induced PDGF-A and PDGF-C due to the lack of sufficient PDGFRα chain expression. However, following PDGFRα upregulation by FGF2, PDGFRα-binding ligands induced by T_H2 cytokines would trigger PDGFR chain dimerization and activation, which would be subsequently translated into extended BSMC proliferation. In support of

this contention, the PDGFR tyrosine kinase inhibitor AG1296 effectively reduced the proliferative synergism. Collectively, this study suggests that autocrine loops of PDGF are likely operational in the observed proliferative synergism between FGF2 and the T_H2 cytokines IL-4 and IL-13. Of note, AG1296 was unable to completely block this synergism, indicating that other mechanisms are also operative and remain to be identified.

Finally, we have recently demonstrated that TGFβ1 also synergized with FGF2 in BSMC proliferation and that this synergism similarly involves an autocrine loop of PDGF [25]. Collectively, these studies suggest that several mediators, with elevated levels in the airways following allergic challenge, likely cooperate to orchestrate the enlargement of airway smooth muscle tissue observed in asthmatic individuals. For the first time in this paper, the two classical T_H2 cytokines IL-4 and IL-13 are highlighted as such potent mediators potentially involved in BSMC hyperplasia.

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FIGURE LEGENDS

FIG. 1. IL-4 and IL-13 increase FGF2-induced BSMC proliferation in a time-dependent fashion. BSMC were sequentially stimulated with FGF2 (2 ng/ml) and IL-4 or IL-13 (10 ng/ml) at 24 h intervals and BSMC proliferation was investigated for 4 consecutive days following T_H2 cytokine administration. A two-way ANOVA was performed to compare the effect of FGF2 alone with the effect of FGF2 in combination with either of the T_H2 cytokines in function of time. P values are presented in the insets for each analysis. Symbols are means ± SEM of quadruplicate measurements of four independent experiments.

FIG. 2. FGF2 and the T_H2 cytokines IL-4 and IL-13 synergize in a concentration-dependent manner to induce BSMC proliferation. BSMC proliferation was measured 4 days following T_H2 cytokine admnistration. P values in the insets correspond to a two-way ANOVA evaluating the two main effects and the interaction effect of the following factors: (a) an increasing dose of FGF2 with a fixed dose of IL-4, (b) an increasing dose of FGF2 with a fixed dose of IL-13, (c) an increasing dose of IL-4 with a fixed dose of FGF2, and (d) an increasing dose of IL-13 with a fixed dose of FGF2. Symbols are means ± SEM of compiled data obtained in three different cell lines in A and B ($n = 6$) and in two different cell lines in C and D ($n = 5$).

FIG. 3. FGF2 increases PDGFRα chain expression. BSMC were stimulated with 2 ng/ml of FGF2 and PDGFRα expression (shown in green) was investigated at different time intervals by immunocytochemistry. Represented in blue is the Hoechst staining to reveal cell nuclei. Figures presented are from a single experiment representative of three.

FIG. 4. IL-4 and IL-13 increase mRNA expression of PDGFRα-acting agonists in a time-dependent manner. BSMC were stimulated with 10 ng/ml of IL-4 (A and C) or IL-13 (B and D) and kinetics of PDGF-A (A and B) and PDGF-C (C and D) mRNA expression were investigated up to 48 hours thereafter by RT-PCR. Shown is one representative experiment out of three.

FIG. 5. Tyrphostin AG1296, a pharmacological inhibitor of PDGFR tyrosine kinase activity, reduced the proliferative synergism between FGF2 and the T_H2 cytokines. Experiments were performed as described in Fig. 2, but AG1296 (10μM) was administered 1 hour before IL-4 (A) or IL-13 (B) administration. Results are expressed as percentage of the synergism, 100% being defined as the difference between the proliferation induced by FGF2 + IL-4 (A) or FGF2 + IL-13 (B) with DMSO and the proliferation induced by FGF2 alone similarly treated with DMSO (A and B).* indicates statistically significant differences with respective DMSO-treated controls ($p \leq 0.05$). Bars represent means ± SEM of five independent experiments performed in quadruplicates.

FIG. 6. Pro-inflammatory cytokines TNFα and IL-1β increased the proliferative synergism between FGF2 and IL-4 or IL-13. Experiments were performed as described in Fig. 2, but TNFα (10 ng/ml) or IL-1β (10 ng/ml) was administered simultaneously with IL-4 (A) or IL-13 (B). Synergism is indicated by subtracting from raw data either baseline OD (-) or OD obtained with FGF2 alone (+). * indicates statistically significant differences based on ANOVA and Tukey *a posteriori* test ($p \leq 0.05$). Bars represent means ± SEM of five independent experiments performed in quadruplicates.

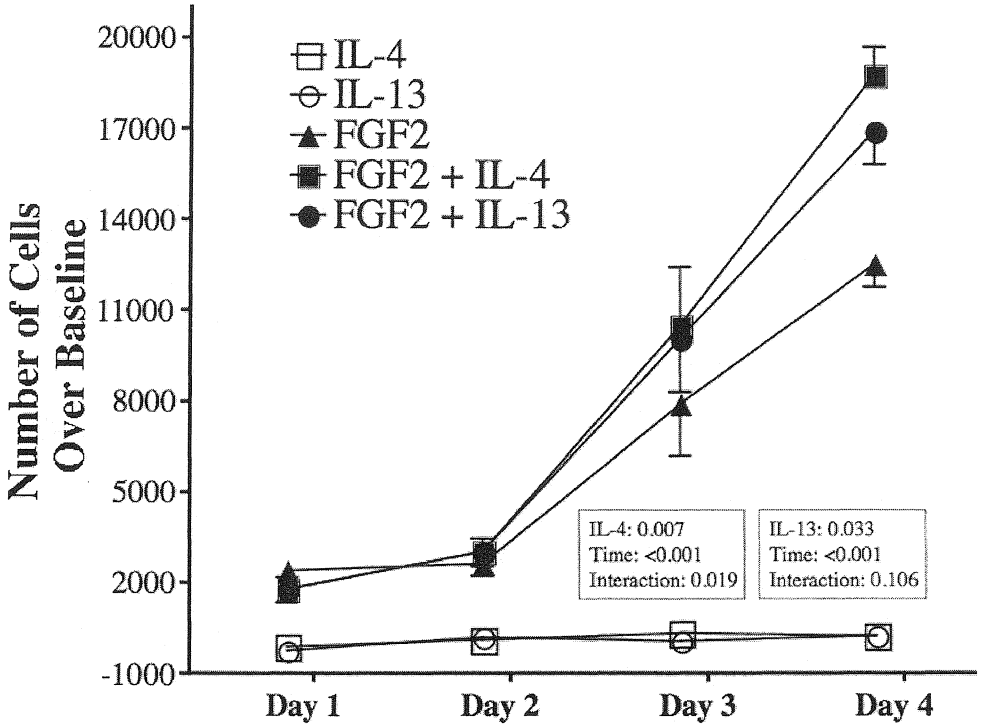
IL-4
IL-13
FGF2
FGF2 + IL-4
FGF2 + IL-13
Number of Cells Over Baseline
20000
17000
14000
11000
8000
5000
2000
-1000
Day 1
Day 2
Day 3
Day 4
IL-4: 0.007
Time: <0.001
Interaction: 0.019
IL-13: 0.033
Time: <0.001
Interaction: 0.106

Figure 1

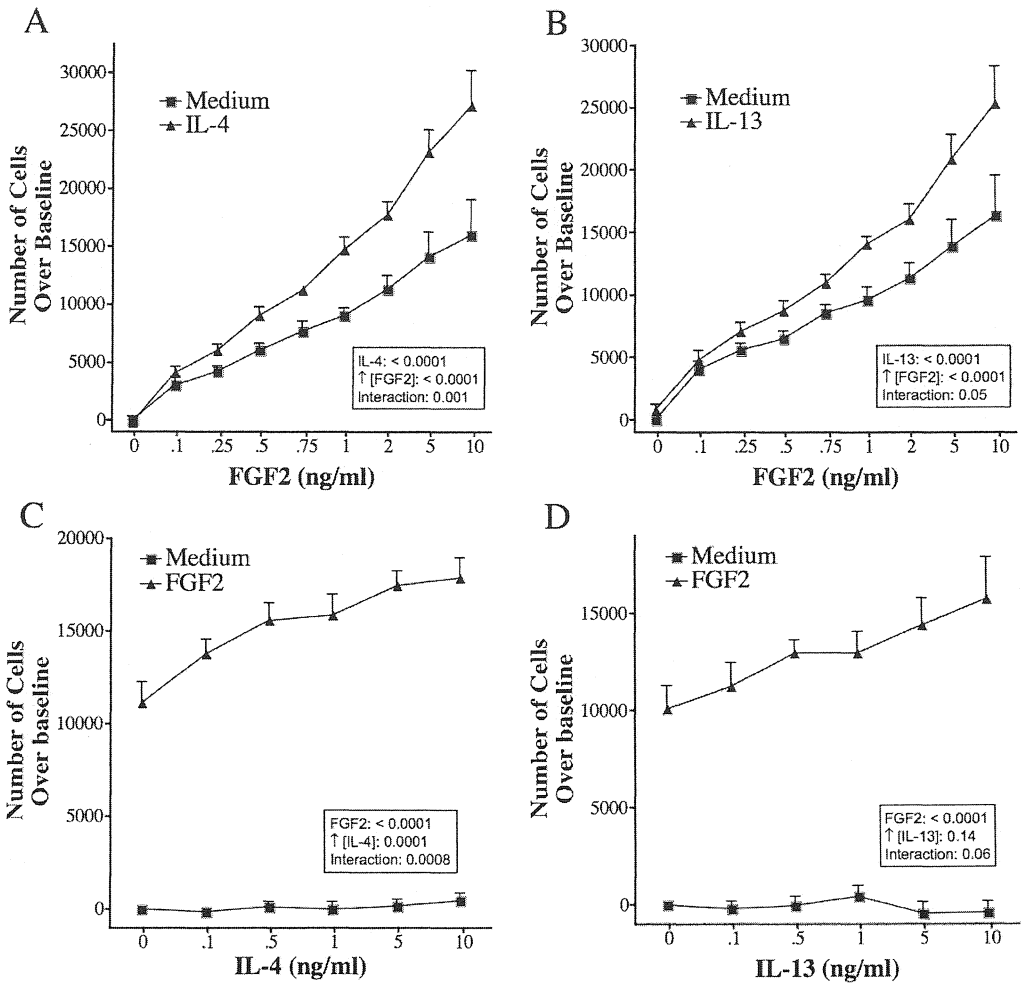
A
Medium
IL-4
Number of Cells Over Baseline
30000
25000
20000
15000
10000
5000
0
IL-4: < 0.0001
↑ [FGF2]: < 0.0001
Interaction: 0.001
0 .1 .25 .5 .75 1 2 5 10
FGF2 (ng/ml)
B
Medium
IL-13
Number of Cells Over Baseline
IL-13: < 0.0001
↑ [FGF2]: < 0.0001
Interaction: 0.05
FGF2 (ng/ml)
C
Medium
FGF2
Number of Cells Over baseline
20000
15000
10000
5000
0
FGF2: < 0.0001
↑ [IL-4]: 0.0001
Interaction: 0.0008
0 .1 .5 1 5 10
IL-4 (ng/ml)
D
Medium
FGF2
Number of Cells Over baseline
FGF2: < 0.0001
↑ [IL-13]: 0.14
Interaction: 0.06
IL-13 (ng/ml)

Figure 2

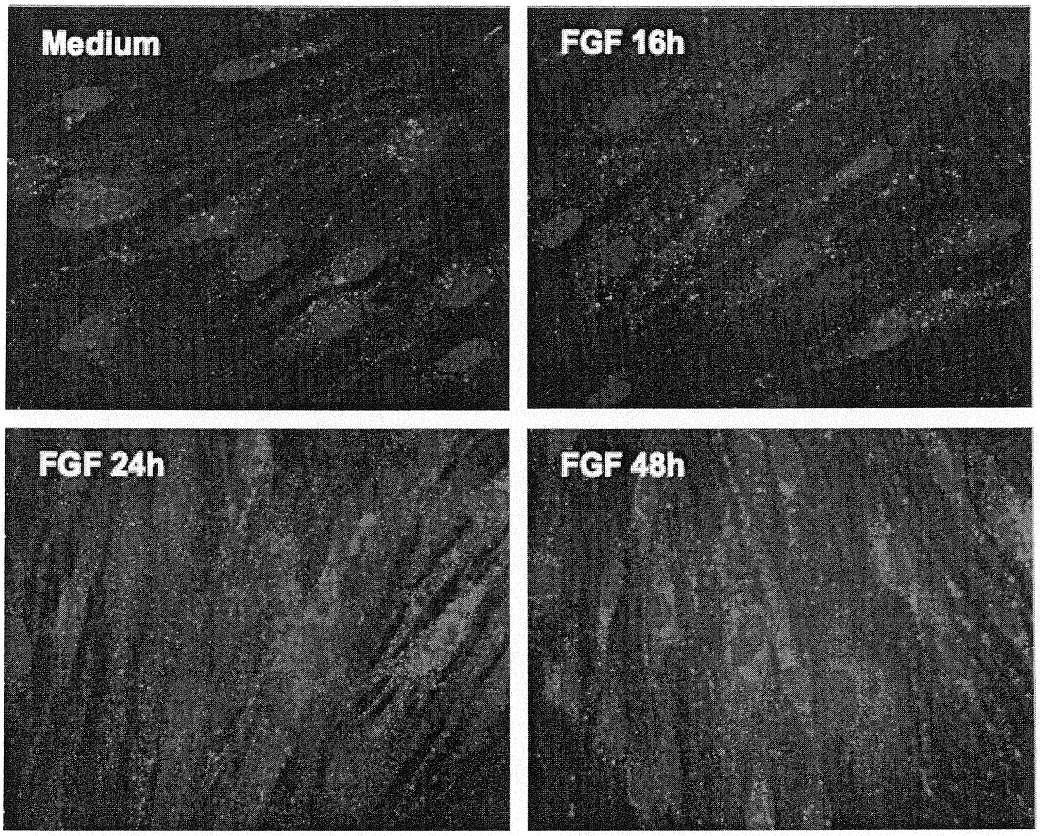

Figure 3

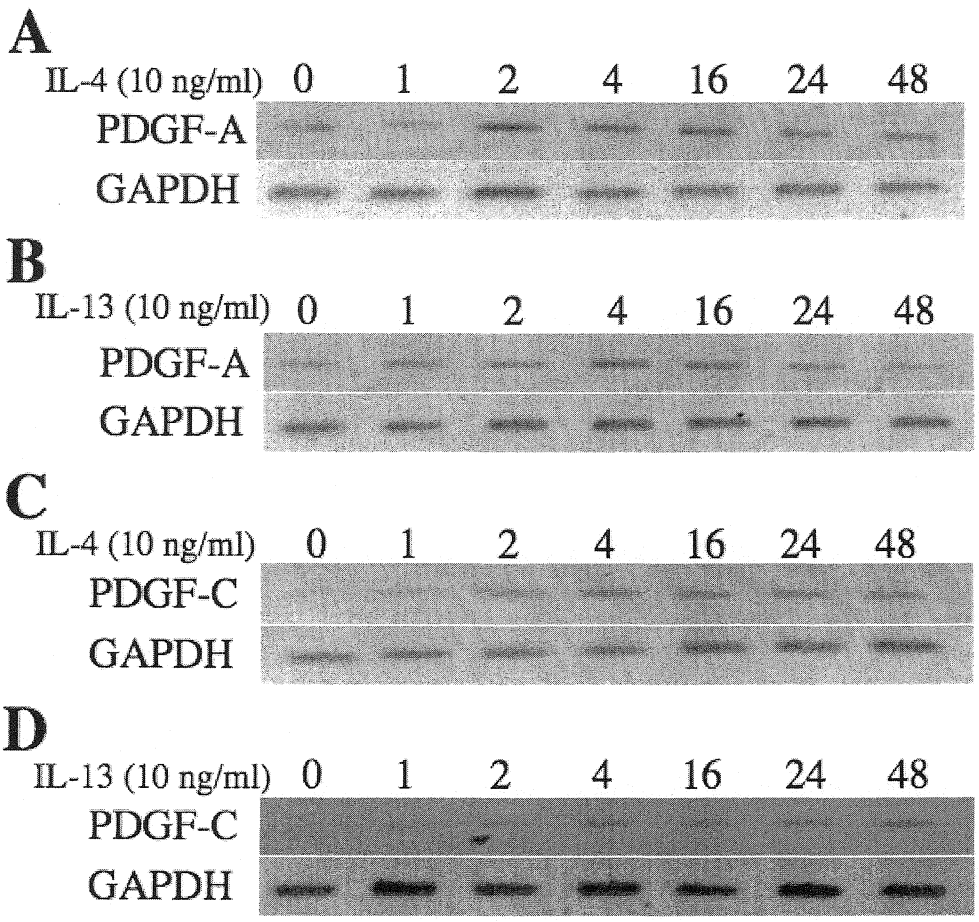
A
IL-4 (10 ng/ml) 0 1 2 4 16 24 48
PDGF-A
GAPDH
B
IL-13 (10 ng/ml) 0 1 2 4 16 24 48
PDGF-A
GAPDH
C
IL-4 (10 ng/ml) 0 1 2 4 16 24 48
PDGF-C
GAPDH
D
IL-13 (10 ng/ml) 0 1 2 4 16 24 48
PDGF-C
GAPDH

Figure 4

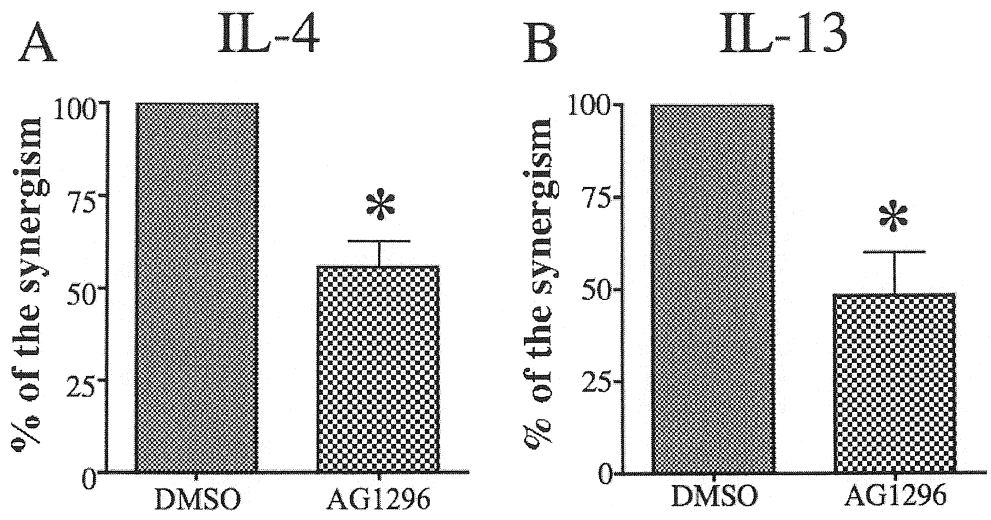
A
IL-4
% of the synergism
100
75
50
25
0
*
DMSO
AG1296
B
IL-13
% of the synergism
100
75
50
25
0
*
DMSO
AG1296

Figure 5

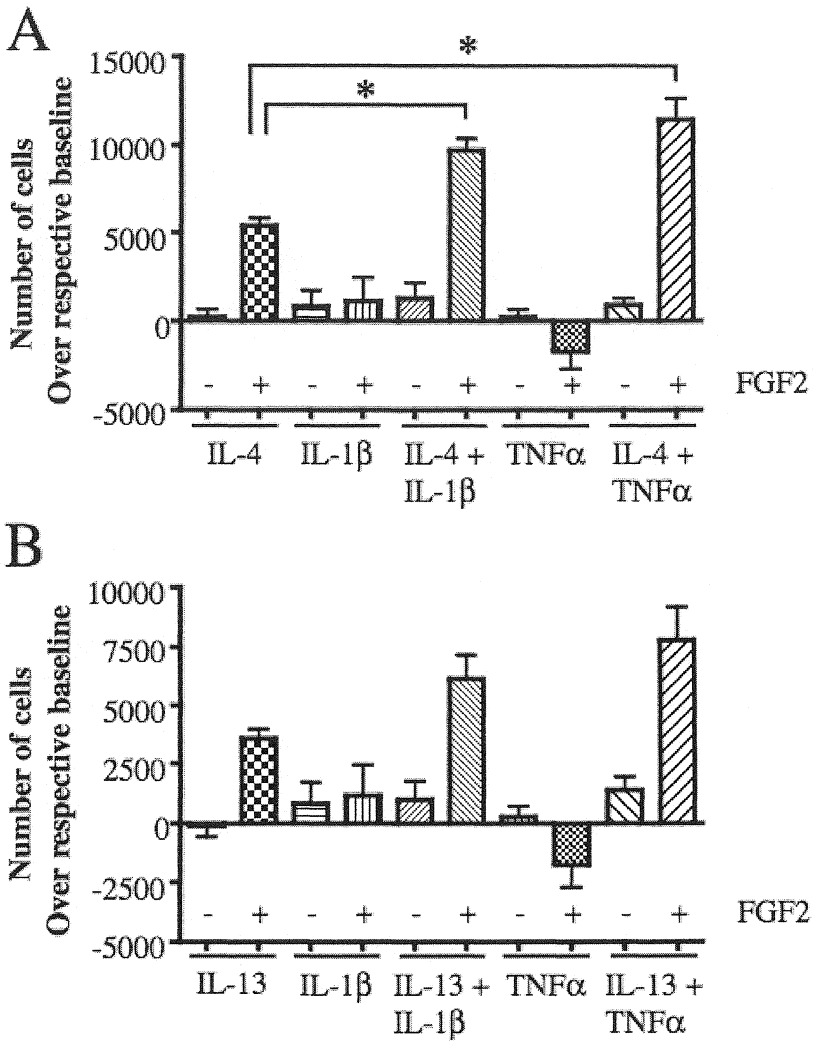
A
Number of cells
Over respective baseline
15000
10000
5000
0
-5000
*
*
- + - + - + - + - +
FGF2
IL-4
IL-1β
IL-4 + IL-1β
TNFα
IL-4 + TNFα
B
Number of cells
Over respective baseline
10000
7500
5000
2500
0
-2500
-5000
- + - + - + - + - +
FGF2
IL-13
IL-1β
IL-13 + IL-1β
TNFα
IL-13 + TNFα

Figure 6

CHAPTER 7

LEUKOTRIENES

7.1. Leukotrienes and receptors

Leukotrienes (LTs) are a subgroup of eicosanoids involved in several inflammatory processes (reviewed in (Capra, 2004)). They are produced by a series of enzymatic reactions begining after the cytosolic phospholipase (PL)A_2-mediated release of arachidonic acid (AA) from membrane glycerophospholipids by cleavage of the ester bond at the sn-2 position of the glycerol backbone (reviewed in (Peters-Golden and Brock, 2003; Shimizu et al., 2006)). The intitial step occurs in the vicinity of the nuclear envelope where the membrane-associated 5-lipoxygenase activating protein (FLAP) binds to AA and transfers endogenous AA to 5-lipoxygenase (5-LO). This enzyme then catalyses the conversion of AA by two successive reactions of oxygenation and dihydration to yield the 5-hydroperoxyeicosatetraenoic acid (5-HPETE) intermediate product and the epoxide metabolite LTA_4, respectively. LTA_4 is very unstable and is rapidly transformed by the action of either LTA_4 hydrolase or LTC_4 synthase (LTC_4S). The former hydrolyzes LTA_4 to form the double hydroxyl molecule LTB_4, and the latter conjugates the sulfhydryl group of gluthathione (GSH) to C6 of LTA_4 to form the first peptidoleukotriene LTC_4. Both of these lipid mediators are released in the extacellular space and act mainly as autacoids by binding on plasmalemma of adjacent cells to heptahelicoidal receptors coupled to cytoplasmic heterotrimeric G proteins (GPCR). LTB_4 transduces its biological effect by binding onto two different GPCRs. The high affinity receptor, BLT1, is specific for LTB_4 and is exclusively expressed in leukocytes (Okuno et al., 2005). The other receptor, BLT2,

is of lower affinity for LTB_4, binds to other eicosanoids and its expression is more widely distributed. Recent evidence in a mouse model of asthma suggests that the LTB_4-BLT1 axis mediates early T cells recruitment into the airways (Tager et al., 2003) and may thus be relevant in the understanding of asthma pathogenesis. However, for the sake of clarity and since its effect on ASM cell mitogenesis has never been reported, the LTB_4-BLT1 axis will not be discussed further here.

Peptidoleukotrienes are mainly produced by cells with constitutive expression of 5-LO, such as granulocytes and monocytes/macrophages. Neutrophils also contribute to the overall peptidoleukotriene synthesis by a transcellular metabolism. As such, neutrophil-derived LTA_4 can efficiently be taken up by platelets (reviewed in (Murphy et al., 1991)) and vascular endothelial cells (reviewed in (Sala and Folco, 2001)) and the laters possess the intracellular machinery to convert it to LTC_4. After its secretion, LTC_4 can be further processed extracellularly by gamma-glutamyl transpeptidase (γ-GT), which cleaves the glutamate aa of the GSH moiety of LTC_4 to form LTD_4, and then by a variety of dipetidases, which cleave the peptide of the remnant GSH moiety, consisting henceforth of a glycine and a cysteine residues, to form the single amino acid-bound leukotriene, LTE_4. Each of these cysteinyl-LTs (cys-LTs) bind to two GPCRs, called CysLT1 and CysLT2, with affinity in the following rank order of potency: $LTD_4 > LTC_4 > LTE_4$ for CysLT1 and $LTD_4 = LTC_4 > LTE_4$ for CysLT2 (Capra, 2004). CysLT1 is expressed predominantly in peripheral blood leukocytes and spleen with lower levels of expression found in placenta, small intestine and lung, including ASM tissue (Capra, 2004). On the other hand, CysLT2

expression demonstrates some overlaps with CysLT1, but it is peculiarly expressed in heart, adrenals, and brain (Capra, 2004).

7.2. Increased expression of cys-LTs and CysLT1 in asthma and experimental asthma

Release of cys-LTs has been observed in sensitized rats (Powell et al., 1995) and mice (Henderson et al., 1996; Lee et al., 2007) following allergic challenge. In human, cys-LTs have been consistently reported to increase in BALF (Lam et al., 1988; Wenzel et al., 1990), urine (Bellia et al., 1996; Christie et al., 1991; Daffern et al., 1999; Diamant et al., 1995; Green et al., 2004; Israel et al., 1993; Micheletto et al., 2006a; Micheletto et al., 2006b; Mita et al., 2004; Oosaki et al., 1997; Rasmussen et al., 1992a; Rasmussen et al., 1992b; Reiss et al., 1997; Sampson et al., 1995; Smith et al., 1992), bronchial biopsies (Cowburn et al., 1998; Volovitz et al., 1999), sputum (Macfarlane et al., 2000; Pavord et al., 1999), blood (Levy et al., 2005; Sampson et al., 1995), and exhaled breath condensate (EBC) (Baraldi et al., 2003; Csoma et al., 2002; Hanazawa et al., 2000; Lex et al., 2006) of asthmatic subjects both at baseline and following allergen exposure. Spontaneous, as well as C5a-induced release of cys-LTs, are also greater in blood leukocytes derived from asthmatics compared to normal individuals (Mewes et al., 1996).

Numbers of cells expressing CysLT1 receptor in the subepithelial zone are equally upregulated in stable asthmatics compared to non-asthmatic subjects and were further augmented in asthmatics hospitalized for acute exacerbation (Zhu et al., 2005). These

increased numbers of CysLT1 positive cells correlate with the increased numbers of CD45 positive cells, indicating an increase in leucocyte recruitment in the bronchial mucosa rather than an increased expression of this receptor on pre-existing mucosal cells. The cells positive for CysLT1 were identified as eosinophils, neutrophils, mast cells, macrophages, B lymphocytes and plasma cells, but not CD3 positive cells of either CD4 or CD8 positive phenotypes. However, structural cells, such as the vascular endothelial cells, also stained positive for CysLT1 in all groups of subjects. Moreover, CysLT1 expression was higher in the cytoplasm of epithelial cells derived from asthmatic patients suffering of acute exacerbation. This latter result suggests that the airway epithelium of asthmatics is likely to respond in an exaggerated manner to the increased expression of cys-LTs that occurs *in vivo* upon allergen challenge.

7.3. Cys-LTs in asthma pathogenesis

Cys-LTs has been recognised a long time ago as ASM cell spasmogens that were initially adopted as the slow reactive substances of anaphylaxis (SRS-A). It was thus shown that Cys-LTs are highly potent bronchial constrictors on isolated human bronchi (Dahlen et al., 1980). The spasmogenic effect of cys-LTs was equally demonstrated in monoculture of ASM cells *in vitro* (Amrani et al., 2001), as well as in humans *in vivo* (Weiss et al., 1982). In fact, LTC_4 was 600 to 9500 times more potent than histamine on a molar basis in producing an equivalent decrement in lung residual volume (Weiss et al., 1982). However, it is henceforth well established that cys-LTs' roles in asthma pathogenesis extended way above their initially described effects on ASM cell contraction. As has been reviewed by Hay and coworkers more then a

decade ago (Hay et al., 1995), cys-LTs were now suggested to: 1-increase vascular permeability, which leads to edema and concomitant increase in airway wall thickening; 2-induce mucus hypersecretion and ciliary dyskinesia, which alter mucus clearance; and 3-recruit inflammatory cells into the airways, such as eosinophils.

The exact cys-LTs mediating the influx of eosinophils into the airways of human subjects is, however, a matter of controversy. Gauvreau and coworkers have shown that inhalation of LTE_4, but not LTD_4, by atopic asthmatics causes eosinophil influx into the airway wall (Gauvreau et al., 2001). Laitinen and coworkers (Laitinen et al., 1993) had previously published similar observations, showing that LTE_4 increases the influx of eosinophils, and to lesser extent neutrophils, 4 h after inhalation. In contrast to Gauvreau and coworkers (Gauvreau et al., 2001), Diamant and coworkers (Diamant et al., 1997) have shown that LTD_4 inhalation also triggers an increased number of eosinophils in the sputum. Mobilisation of eosinophils into the airways in response to aerolized LTD_4 was also demonstrated in guinea pigs (Underwood et al., 1996). It is thus likely that several cys-LTs are involved in the inflammatory cell infiltration characterizing asthma.

The contribution of cys-LTs into the pathogenesis of allergic asthma was mainly elucidated in animal models of the disease using both knockout approaches and CysLT1 receptor antagonists (LTRA) or pharmacological inhibitors targeting key enzymes in their synthesis. Upon allergic challenge, sensitized 5-LO-deficient mice showed reduced AHR and pulmonary eosinophilia, as well as lower levels of total IgE

and antigen (Ag)-specific IgG in their serum, compared to wild type animals (Irvin et al., 1997). Since 5-LO is required for the synthesis of both LTB_4 and cys-LTs, reduced asthma features observed in mice containing the 5-$LO^{-/-}$ background (Irvin et al., 1997) did not discriminate which of the two pathways contributes to experimental asthma development. Recently, mice deficient for the terminal enzyme responsible for cys-LTs synthesis, LTC_4S, were studied in a model of allergic pulmonary inflammation (Kim et al., 2006a).

Kim and coworkers (Kim et al., 2006a) have shown that $LTC_4S^{-/-}$ mice were largely protected against experimentally-induced asthma. Among the features tested, eosinophil infiltration, goblet cell hyperplasia, mucus hypersecretion, mobilisation and activation of intraepithelial mast cells, AHR, and serum levels of Ag-specific IgE and IgG1, as well as lung mRNA expression of T_H2-type cytokines and chemokines such as IL-5, IL-13, IL-10 and CCL11, were significantly reduced in $LTC_4S^{-/-}$ animals. Moreover, higher numbers of cells were harvested from parabronchial lymph nodes of LTC_4S-null mice and these cells secreted less IL-4, IL-5, IL-13 and IFNγ on a per-cell basis when stimulated *ex vivo* with antigen compared to cells derived from parabronchial lymph nodes of wild type animals.

These results confirm the crucial role of cys-LTs in animal models of asthma that was previously demonstrated by the use of either 5-LO and FLAP inhibitors (Henderson et al., 1996; Salmon et al., 1999) or LTRA (Henderson et al., 2002; Lee et al., 2007; Muz et al., 2006; Salmon et al., 1999; Wang et al., 1993). It is thus believed that cys-

LTs orchestrate many features of asthma. In fact, intratracheal instillations of LTC_4, LTD_4 or LTE_4 in mice induced AHR, inflammatory cell recruitment, fibroblast growth and mucus production into the airways (Chavez et al., 2006; Vargaftig and Singer, 2003), indicating that any cys-LTs alone are sufficient to induce an asthma-like phenotype in mice. Similarly, mice deficient in γ-glutamyl leukotrienase ($GGL^{-/-}$), an enzyme that converts LTC_4 to LTD_4, showed higher amount of cys-LTs in their lungs and spontaneous AHR to acetylcholine (Chavez et al., 2006). Surprisingly, lung inflammatory cell infiltrates were not observed in $GGL^{-/-}$ mice, suggesting that cys-LTs act on airway resident cells to increase airway responsiveness to cholinergic agonists.

7.4. CysLT1 receptor transduces the asthma-like effects of cys-LTs.

Owing to the ability of LTRA to block several feature of asthma and their specificity for CysLT1 receptor, the effects of cys-LTs in asthma are thought to be mediate mainly by this particular receptor (Busse and Kraft, 2005). LTRA as been shown to: 1-attenuate eosinophilia in human asthma (Taylor et al., 1991), as well as in such different species models of asthma as mouse (Blain and Sirois, 2000), sheep (Abraham et al., 1993) and rat (Ihaku et al., 1999); 2- attenuate exercise-induced bronchospasm (Reiss et al., 1997); 3- prevent AMP- and neurokinin A-induced bronchoconstriction (Crimi et al., 2003; Rorke et al., 2002); 4- protect against early and late phases of bronchoconstriction induced by antigen challenge (Rasmussen et al., 1992a; Rasmussen et al., 1992c); 5- reduce airway reactivity to non-specific spasmogens such as methacholine (Rasmussen et al., 1992c); 6- improve pulmonary

functions and quality of life, and reduce asthma symptoms and the needs of rescue medication in aspirin-intolerant asthma (Dahlen et al., 2002); and 7- provide additive clinical benefit when used as initial treatment for preschool-aged children suffering from acute exacerbation relative to short-acting β2-agonist bronchodilators alone (Harmanci et al., 2006). *In vitro* studies also demonstrated that montelukast (a LTRA) prevented the increased stiffness of human ASM cells in response by LTD_4 (Amrani et al., 2001).

In addition, a recent genetic study have shown that polymorphisms in the CysLT1 receptor promoter are associated with aspirin-intolerant asthma in male Koreans (Kim et al., 2006b), furthering the contention that cys-LTs may required binding onto this receptor to induce asthma-like features. Interestingly, the same study showed that a luciferase construct containing the haplotype associated with an increased disease risk (T-C-G ; OR = 2.71) was shown to yield higher luciferase activity when transfected into Jurkat cells. This genetic association may thus be functionally related to increased CysLT1 expression in the carriers of the high risk haplotype (Kim et al., 2006b).

7.5. Effect of leukotrienes on ASM cell mitogenesis in vivo vs in vitro

The first evidence that cys-LTs can be mitogenic for ASM came from a study measuring the effect of thromboxane $(Tx)A_2$ on the proliferation of rabbit ASM cells (Noveral and Grunstein, 1992). It was demonstrated that the mitogenic effect of TxA_2 was inhibited by phospholipase A_2 and 5-LO inhibitors, as well as by the blockade of cys-LTs binding to its LTD_4 receptor (currently known as CysLT1). They pursued by

demonstrating that TxA_2 effectively enhances the release of endogenous cys-LTs and that administration of LTD_4 alone mimics the mitogenic effect of TxA_2 (Noveral and Grunstein, 1992). A year later, these results gained physiological significance when Wang and coworkers (Wang et al., 1993) demonstrated that LTRA (MK-571) was successful in preventing ASM cell enlargement in a rat model of allergic asthma.

It was then followed by a series of studies performed by the same group that demonstrated a LTD_4-dependent mitogenic effect of TxA_2 on rabbit ASM cells (Noveral and Grunstein, 1992). Using the cells from the same species, they contradicted their previous results and showed that LTD_4 alone was not sufficient to induce ASM cell proliferation, but was able to increase the proliferation induced by IGF-I (Cohen et al., 1995). They proposed an elegant mechanism to explain this proliferative synergism, whereby the key element was the induction of MMP-1 in response to LTD_4. The latter proteolytically degrades IGFBP-2, which normally sequesters IGF and prevents it from binding to its receptor and mediating its mitogenic action. Hence, it was concluded that LTD_4 potentiates the mitogenic effect of IGF-IGF receptor axis by fostering IGF-1 binding onto its cell surface receptor via a MMP-1-dependent degradation of IGFBP-2 (Cohen et al., 1995; Rajah et al., 1995; Rajah et al., 1996).

Elucidation of the LTD_4 effect on human ASM cell proliferation took longer and was not documented until 1998. At that moment, Panettieri's group (Panettieri et al., 1998) demonstrated that LTD_4 was not mitogenic on its own, but appeared to synergize with

EGF. The results were quite evident and concentration-dependent, with a perceptible effect starting at 10^{-7} M of LTD_4, when DNA synthesis was measured as the surrogate for cell proliferation. However, in terms of true cell proliferation, the growth-potentiating effect of LTD_4 on EGF-induced proliferation was less convincing and was only demonstrated at 10^{-5} M of LTD_4, which is a concentration much too high to be of any physiological significance.

Despite the weakness of these results, the contention that cys-LTs were mitogenic for ASM cells continues to reap momentum because of two other *in vivo* studies. Salmon and coworkers (Salmon et al., 1999) first showed that both an enzyme inhibitor of 5-LO (SB 210661) and a LTRA (pranlukast), but not a LTB_4 receptor antagonist (SB 205312), attenuate ASM cell DNA synthesis (measured by bromodeoxyuridine incorporation) in a rat model of allergic asthma. Henderson and coworkers (Henderson et al., 2002) pursued by confirming these results in a mouse model of allergic asthma, demonstrating this time the efficacy of montelukast (another LTRA) to reduce the development of ASM cell hyperplasia.

Since then, three studies have been conducted to measure the mitogenic potential of LTD_4 on human ASM cells. Among them, only Ravasi and coworkers (Ravasi et al., 2006) were able to show that LTD_4 alone is sufficient to stimulate DNA synthesis. However, they did not extend their experiments to measure true ASM cell proliferation and had to use two sequential stimulations, seperated by a 4 h interval, with 10^{-6} M of LTD_4 to observe the mitogenic effect. Once again, this concentration of

LTD_4 is unlikely to be encountered by ASM cells in *in vivo* condition and thus, this mitogenic effect may simply represent a non-physiologic cellular response.

Similar to Panettieri and coworkers (Panettieri et al., 1998), Ravasi (Ravasi et al., 2006) and Potter-Perigo's (Potter-Perigo et al., 2004) groups observed a mitogenic synergism between LTD_4 and EGF in term of DNA synthesis. Even though the former showed that this synergistic response required binding of LTD_4 to its cognate CysLT1 receptor by efficaciously blocking with pranlukast and zafirlukast, the latter showed that the response was insensitive to montelukast treatment, revealing another conflicting result on the matter. In addition, in both cases, they did not confirm their results by measuring true ASM cell proliferation.

Finally, we demonstrated, with a more physiological concentration (a single stimulation with 10^{-7} M), that LTD_4 was unable to support ASM cell proliferation when administered alone, but that following a 24 h pre-treatment with IL-13 or TGFβ1, which both increased CysLT1 expression, the same concentration of LTD_4 induced cellular proliferation (Espinosa et al., 2003). This raises the possibility that CysLT1 is not sufficiently expressed on ASM in baseline conditions to transduce the mitogenic signal of cys-LTs.

In support of this contention, *in situ* hybridization and immunohistochemical studies raise questions concerning the expression of CysLT1 on human ASM tissue (Zhu et al., 2005). The lack of significant CysLT1 expression in human ASM would readily

explain the weak, almost inexistence effect of LTD_4 on human ASM cell proliferation and would corroborate the results obtained with mouse airways, which did not contract in response to LTD_4 stimulation (Martin et al., 1988; Richter and Sirois, 2000).

Taken together, the presented evidence shows a conspicuous dichotomy between the *in vitro* and *in vivo* data documenting the effect of cys-LTs on ASM cell proliferation. Whereas all the *in vivo* data support a role for cys-LTs in ASM cell hyperplasia, *in vitro* data demonstrate that cys-LTs are not mitogenic when administered alone and their growth-potentiating effects with other mediators are marginal and only occur with supraphysiologic concentrations of cys-LTs (synthetized in Table 5). Otherwise, CysLT1 must be upregulated by specific stimuli before cellular proliferation could take place in response to cys-LTs, as we demonstrated with IL-13 and TGFβ1 pre-treatment (Espinosa et al., 2003). In this regard, airway responsiveness to cys-LTs was shown to be higher in asthmatic individuals (O'Hickey et al., 1988).

Altogether, these results may also imply that the mitogenic effect of cys-LTs on ASM cell proliferation *in vivo* may be indirect and depend on a paracrine loop involving the cys-LT-induced secretion of ASM cell mitogens by other airway cells. In this regard, the airway epithelium of asthmatics expresses CysLT1 (Zhu et al., 2005) and may release substances affecting the proliferative behaviour of the underlying ASM tissue following cys-LTs stimulation.

Table 5: Cys-LTs on ASM cell mitogenesis

Leukotrienes	Type of studies	Species	Effects	References
LTD_4	*in vitro*	Rabbit	Induced	(Noveral and Grunstein, 1992)
LTD_4	*in vivo*	BN rat	Induced	(Wang et al., 1993)
LTD_4	*in vitro*	Rabbit	⇑ mitogenesis induced by IGF-1	(Cohen et al., 1995)
LTD_4	*in vitro*	Rabbit	⇑ mitogenesis induced by IGF-1	(Rajah et al., 1995)
LTD_4	*in vitro*	Rabbit	⇑ mitogenesis induced by IGF-1	(Rajah et al., 1996)
LTD_4	*in vitro*	Human	⇑ mitogenesis induced by EGF	(Panettieri et al., 1998)
Cys-LTs	*in vivo*	BN rat	Induced	(Salmon et al., 1999)
Cys-LTs	*in vivo*	BALB/c mice	Induced	(Henderson et al., 2002)
LTD_4	*in vitro*	Human	Induced following IL-13 or TGFβ1 pre-treatment.	(Espinosa et al., 2003)
LTD_4	*in vitro*	Human	⇑ mitogenesis induced by EGF	(Potter-Perigo et al., 2004)
LTD_4	*in vitro*	Human	Induced alone and ⇑ mitogenesis induced by EGF	(Ravasi et al., 2006)

7.6. Induction of TGFβ1 in vitro and in vivo

The mitogenic effect of cys-LTs *in vivo* with the lack of effect when administered alone *in vitro* is very reminiscent to the effect of TGFβ1 on ASM cell proliferation. These results are also suggestive that the *in vivo* action of cys-LTs on ASM cell mitogenesis may not be direct, but rather related to their capacity to induce TGFβ1

expression. In this regard, two recent papers have demonstrated the capacity of cys-LTs to induce TGFβ1 expression in two different cell types *in vitro*. Kato and coworkers (Kato et al., 2005) first demonstrated that LTD_4 increases TGFβ1 mRNA expression in eosinophils. Secondly, Perng and coworkers (Perng et al., 2006) have shown that LTC_4 increases TGFβ1 expression in airway epithelial cells. Interestingly, the induction of TGFβ1 was p38 MAPK-dependent in this last paper. Since p38 is crucial for the transduction of FGF2-dependent mitogenic signals (Fernandes et al., 2004) and it is involved in cys-LTs-induced TGFβ1 upregulation in airway epithelial cells (Perng et al., 2006), its inhibition may interfere with the FGF2-TGFβ1 synergism and may prove beneficial to prevent ASM cell hyperplasia if this synergy really occurs *in vivo*. As aforementioned, SD282 (a selective p38 inhibitor) was used successfully (Nath et al., 2006) to prevent ASM cell hyperplasia in a mouse model of allergen sensitization/challenge. Unfortunately, this result is not a proof of concept since it may be due to other p38-dependent mechanisms unrelated to FGF2-TGFβ1 synergism.

The next manuscript presents the first evidence that endogenous production of TGFβ1 by airway epithelial cells in response to LTD_4 is able to support FGF2-primed ASM cell proliferation *in vitro*. Consequently, it suggests that a paracrine loop of TGFβ1 production by airway epithelium in response to LTD_4 is required for cys-LTs-induced ASM cell hyperplasia *in vivo*.

CHAPTER 8

MANUSCRIPT 3

Ynuk Bossé, Charles Thompson, Stéphanie McMahon, Claire M Dubois, Jana Stankova, Marek Rola-Pleszczynski. **Leukotriene D_4-Induced, Epithelial Cell-derived Transforming Growth Factor β1 in Human Bronchial Smooth Muscle Cell Proliferation.** Submitted in *Journal of Allergy and Clinical Immunology.*

Contributions of first author:

All the experiments excepted the Northern blot in Fig. 2 and the ELISA in Fig. 3 were performed by the first author.

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Dear Dr. Marek Rola-Pleszczynski:

The Journal has received your submission. Thank you.

Best regards,

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Leukotriene D_4-induced, epithelial cell-derived Transforming Growth Factor β1 in human bronchial smooth muscle cell proliferation

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ABSTRACT

Background: Cysteinyl-leukotrienes (cysLTs) orchestrate many pathognomonic features of asthma in animal models of allergic airway inflammation, including bronchial smooth muscle cells (BSMC) hyperplasia. However, since cysLTs alone do not induce mitogenesis in monocultures of human BSMC, the effect observed *in vivo* seemingly involves indirect mechanisms, which are still undefined.

Objectives: To investigate the regulatory role of leukotriene (LT) D_4 on transforming growth factor (TGF)β1 expression in airway epithelial cells and the consequence of this interplay on BSMC proliferation.

Methods: HEK293 cells stably transfected with cysLT receptor 1 (CysLT1) (293LT1) were stimulated with LTD_4 and TGFβ1 mRNA and protein expression was measured using Northern blot and ELISA, respectively. Conditioned medium (CM) harvested from LTD_4-treated cells was then assayed for its proliferative effect on primary human BSMC. TGFβ1 mRNA expression was also determined in tumoral type II pneumocytes A549 and in normal human bronchial epithelial cells (NHBE) following LTD_4 stimulation.

Results: LTD_4 induced TGFβ1 mRNA production in a time- and concentration-dependent manner in 293LT1. TGFβ1 secretion was also upregulated and CM from LTD_4-treated 293LT1 was shown to increase BSMC proliferation in a TGFβ1-

dependent manner. The increased expression of TGFβ1 mRNA by LTD_4 also occured in A549 and NHBE cells via a CysLT1-dependent mechanism.

Conclusions: Increased secretion of TGFβ1 by airway epithelial cells in response to LTD_4 may contribute to BSMC proliferation.

Clinical Implication: Elevated expression of cysLTs in asthmatic airways might contribute to BSMC hyperplasia and concomitant clinical features of asthma such as airway hyperresponsiveness via a paracrine loop involving TGFβ1 production by airway epithelial cells.

CAPSULE SUMMARY

Long term treatment with CysLT1 receptor antagonists, might prevent BSMC hyperplasia and ensuing airway hyperresponsiveness by decreasing LTD_4-enhanced TGFβ1 secretion by airway epithelial cells.

KEYWORDS

Cysteinyl-leukotrienes, asthma, airway smooth muscle, mitogenesis, FGF2

ABBREVIATIONS

AHR, airway hyperresponsiveness; BALF, bronchoalveolar lavage fluid; BSMC, bronchial smooth muscle cells; CysLT1, type 1 receptor for cysLTs; cysLTs, cysteinyl-leukotrienes; FGF2, Fibroblast Growth Factor 2; LTD_4, leukotriene D_4; NHBE; normal human bronchial epithelial cells; TGFβ1, Transforming Growth Factor beta 1

INTRODUCTION

Bronchial smooth muscle cell (BSMC) hyperplasia is a characteristic feature of asthma [1] and contributes extensively to airway hyperresponsiveness (AHR) [2]. Among the mediators capable of supporting BSMC proliferation *in vitro*, fibroblast growth factor (FGF)2 was shown to be upregulated in human asthmatic airways [3 4 5 6 7] and its expression is further increased rapidly (10 min) following allergen challenge [3]. Expression of the pleiotropic cytokine, transforming growth factor (TGF)β1, is also increased in human asthmatic airways [8 9 10 11 12 13 14 15 16]. But, despite evidence suggesting that TGFβ1 supports BSMC hyperplasia in murine models *in vivo* [17 18], its effect on human BSMC proliferation *in vitro* is controversial [19 20 21 22 23 24]. In this regard, we have recently shown that TGFβ1 alone failed to affect human BSMC proliferation, but strikingly increased mitogenesis when administered 24 hours following FGF2 treatment [23]. These results indicate that TGFβ1 could be a major player in BSMC hyperplasia in an allergic inflammatory context where BSMC were primed with FGF2. However, the cellular source of TGFβ1 and the mechanism by which TGFβ1 is upregulated in response to allergen challenge in asthmatic airways are still uncertain.

Inflammatory cells that infiltrate the airways following allergen exposure as well as resident structural cells in the lung have been shown to produce TGFβ1. However, airway epithelial cells are the main source of TGFβ1 in normal murine [25 26] and human [27 28] lungs. Morevover, numerous reports suggested that epithelial cell-derived TGFβ1

could be upregulated by phlogogenic challenges *in vitro* [26 29] and *in vivo* [30 31]. Hastie and coworkers [32] have also demonstrated that *ex vivo* cultures of airway epithelial cells derived from asthmatic subjects secreted higher amounts of TGFβ1 compared to those derived from non-asthmatic subjects [32].

Histological evidence suggests that type 1 cysteinyl-leukotriene (cysLT) receptor, CysLT1, is expressed in airway epithelium of asthmatic patients [33]. CysLTs orchestrate many pathognomonic features of asthma in animal models of the disease [34 35 36 37 38 39] and have consistently been reported to be upregulated in bronchoalveolar lavage fluid (BALF) [40], urine [41], bronchial biopsies [42], sputum [43], blood [44], and exhaled breath condensates [45] of asthmatic subjects both at baseline and following allergen exposure. Interestingly, CysLT1 antagonists significantly attenuated BSMC hyperplasia in murine [36 37 46] and rat [34 35] models of allergic airway inflammation, but the effect of cysLTs on human BSMC proliferation *in vitro* is modest and only occurs with high concentrations (2 sequential stimulations with 10^{-6}M of LTD_4) [47] or when combined with other growth factors [48 24 49]. These observations suggest that cysLTs support BSMC proliferation *in vivo* via indirect mechanisms. The current study aims to determine whether epithelial cells secrete TGFβ1 in response to cysLTs and test the hypothesis that cysLT-induced BSMC proliferation, as observed *in vivo*, is mediated through a paracrine loop involving TGFβ1 production by epithelial cells.

METHODS

Cells and culture conditions

The 293LT1 cell line was generated in our laboratory as previously described [50]. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with glucose (Life technologies. Burlington, ON, Canada) with 10% FBS and 100 μg/ml hygromycin B (Wisent. St-Bruno, Qc, Canada), until the last passage. Human primary BSMC (BioWhittaker, Inc. Walkersville, MD) were derived from a 5 week-old black male, free of pre-existing lung disease. Culture procedures have previously been described [23] and all experiments with BSMC were performed with cells at the 4th passage. Cells from the human tumoral type 2 pneumocytic cell line A549 (ATCC), derived from a 58 years-old caucasian male, were cultured in RPMI-1640 (Life technologies) with 5% FBS. Normal human bronchial epithelial cells (NHBE) were derived from a 13 year-old caucasian male, free of pre-existing lung disease, and cultured as recommended by the supplier (BioWhittaker, Inc.). All experiments with NHBE were performed with cells at the 4th passage. At the last passage, 293LT1, A549 or NHBE cells were subcultured into 6-well plates and were allowed to reach confluence in their respective growth culture medium. Cells were then starved for 24 hours (DMEM with 0.2% FBS for 293LT1 and RPMI and BEBM alone for A549 and NHBE, respectively) before starting the stimulations in fresh starving medium.

Northern blot analysis

Cells were stimulated for the indicated time with the indicated concentration before mRNA extraction with TRIzol® reagent (Invitrogen. Burlington, ON, Canada). In some experiments, cells were pre-treated for 30 min with montelukast (MK-476) (Merck Frosst. Pointe-Claire, Qc, Canada) before LTD_4 or ethanol administration. mRNA was then separated on agarose gel (1%) by electrophoresis and transferred onto Hybond-N^+ (Amersham Pharmacia Biotech. Baie d'Urfé, Qc, Canada) membrane. The ethidium bromide-stained 18S band on the membrane was used as a loading control. Membranes were subsequently hybridized overnight at 68°C with human TGFβ1 riboprobe, washed extensively and radioactive signals were detected with Hyperfilm MP (Amersham Pharmacia Biotech).

ELISA

Aliquots of conditionned medium (CM) from 293LT1 cells were collected 24 hours following addition of LTD_4 (10^{-8}M) or equivalent concentration of the vehicle (ethanol). CM aliquots were centrifuged to remove cellular debris and TGFβ1 protein levels were quantified using Quantikine® ELISA kit (R&D System. Minneapolis, MN) as described in the manufacturer's instruction manual.

BSMC proliferation

The experimental design employed to assay BSMC proliferation is schematically presented in Fig. 1. BSMC were subcultured into 96-well plates at 3000 cells/well in a starvation medium, consisting of Smooth muscle Basal Medium (SmBM)

(BioWhittaker, Inc.) with 1% FBS, with or without FGF2 (2 ng/ml). Cells were maintained in these conditions for 24 hours before incubating them with heat-activated (10 min at 80°C for activation of latent TGFβ1) CM derived from LTD_4 (10^{-8}M)- or ethanol-treated 293LT1. In some experiments, BSMC were pre-treated for 1 hour with SB431542 (10μM) (Sigma-Aldrich. Oakville, ON, Canada) or an equivalent concentration of the vehicle (DMSO) before incubation with 293LT1 CM. BSMC proliferation was measured 4 days after 293LT1 CM administration using the DNA staining property of crystal violet (Sigma-Aldrich). Briefly, cells were washed once with a HBSS solution containing 2 mM $CaCl_2$ and 10 mM Hepes, fixed 20 min with ethanol (70%) at –20°C, incubated 15 min in crystal violet dilution (1% w/v) at room temperature, washed 6 times under tap water and dissolved in acetic acid (33%). Optical density (OD) was spectophotometrically determined at 550 nm with an ELISA reader (Bio-Rad Laboratory, Inc. Hercules, CA). The validity of crystal violet staining as a surrogate of cell proliferation in the BSMC cell line has been determined elsewhere [23].

RT-PCR

Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) was used to measure CysLT1 mRNA expression. Starved, unstimulated cells were resuspended in TRIzol® reagent for mRNA extraction. To avoid DNA contamination and mRNA degradation, mRNA extracts were treated for 15 min with dexoxyribonuclease (DNAse 1 from Amersham Biosciences. Piscataway, NJ) and ribonuclease inhibitor (RNasin from Promega, Madison, WI) at 37°C. RT reactions

were performed using 1 μg of mRNA extract and M-MLV reverse transcriptase kit (BioCan Scientific, Inc. Mississauga, ON) and PCR was performed with a Taq polymerase kit (New England BioLabs, Ltd. Pickering, ON). Peripheral blood mononucleated cells (PBMC) and the monocyte-like cell line THP-1 (ATCC) were used as CysLT1 mRNA positive controls and human umbilical vein endothelial cells (HUVEC) were used as negative controls. For all experiments, GAPDH mRNA was used as an internal housekeeping gene control. The PCR primers and conditions used were: CysLT1 forward, 5′-CGGGATCCGATGAAACAGGAAATC-3′; CysLT1 reverse, 3′-CCGGAATTCAATGGGTTTAAACTATAC-5′; 32 cycles at 60°C annealing temperature; GAPDH forward, 5′-GATGACATCAAGAAGGTGGTGAA-3′; GAPDH reverse, 3′-GTCTTACTCCTTGGAGGCCATGT-5′; 28 cycles at 55°C annealing temperature.

Flow cytometry

Flow cytometry analysis was used to measure CysLT1 expression in A549 and NHBE cells. Starved, unstimulated cells were harvested, fixed (2% paraformaldehyde), permeabilised (0.1% saponine) and labeled sequentially with rabbit anti-human CysLT1 Ab (1:2000) (Cayman Chemical. Ann Arbour, MI) or isotypic control (1:2000) (Southern Biotechnology Associates. Birmingham, AL) and with FITC-conjugated goat anti-rabbit IgG Ab (1:1000) (Jackson Immuno Research Laboratories Inc. West Grove, PA). Mean fluorescence intensity (MFI) on labeled cells was then measured using a FACScan flow cytometer (Becton Dickinson, San Jose, CA) and analysed using BD CellQuest Pro (version 4.0.1) software.

Statistical analyses

In ELISA experiments (Fig. 3), unpaired Student's t test was used to compare the level of TGFβ1 expression between LTD_4- and ethanol-treated 293LT1 CM. ANOVA followed by Tukey's test *a posteriori* for comparison of all pairs of conditions was performed to compared the effect of 293LT1-derived LTD_4- and ethanol-treated CM on FGF2-pre-treated-BSMC proliferation (Fig. 4A and B). In Northern blot analysis with NHBE cells (Fig. 6B), ratios of TGFβ1 expression over 18S were standardized in Z scores within each experiment before the data obtained for every experiment were compiled and analysed by paired Student's t test. All statistical analysis were performed using StatView or Prism software and p values are indicated in the figures.

RESULTS

LTD_4 upregulated TGFβ1 expression at the mRNA and protein levels in 293LT1 cells

To determine whether cysLTs can affect TGFβ1 expression, the CysLT1-expressing epithelial cells (293LT1) were stimulated with LTD_4 or equivalent concentrations of the vehicle (ethanol). As shown in Fig. 2A, LTD_4 (10^{-8}M) increased TGFβ1 mRNA expression in a time-dependent fashion. However, the kinetics of TGFβ1 upregulation by LTD_4 were relatively slow, beginning at 8 hours and continuing to increase up to 24 hours post-stimulation. When increasing concentrations of LTD_4 were tested at the earliest time point where LTD_4 increased TGFβ1 mRNA expression (8 hours), the effect of LTD_4 was shown to be concentration-dependent, starting at 10^{-11}M and increasing up to 10^{-7}M (Fig. 2B). The amount of TGFβ1 secreted in the conditioned medium (CM) was then determined following 24 hours of stimulation. As shown in Fig. 3, the constitutive expression of TGFβ1 in CM of 293LT1 cells was approximately 3 ng/ml. However, LTD_4 (10^{-8}M) stimulation doubled this amount to attain more than 6 ng/ml.

CM from LTD_4-treated 293LT1 cells increased BSMC proliferation in a TGFβ1-dependent fashion.

We previously demonstrated that TGFβ1 alone did not affect human BSMC proliferation. However, when TGFβ1 was sequentially administered 24 hours following FGF2 treatment, it synergized in a concentration-dependent manner with

FGF2-induced mitogenesis [23]. Together, these results prompted us to investigate whether endogenous TGFβ1 production by 293LT1, at baseline or following LTD_4 stimulation, could support BSMC proliferation when the latter had been pre-treated with FGF2. Primary human BSMC pre-treated or not for 24 hours with FGF2 (2 ng/ml) were thus incubated with CM derived from LTD_4-treated or ethanol-treated 293LT1 cells. Recombinant TGFβ1 at 10 ng/ml was used as a positive control. As shown previously, TGFβ1 had no effect on its own, but synergised with FGF2 to induce BSMC proliferation (Fig. 4A). Similarly, CM from 293LT1 cells induced proliferation only when BSMC were pre-treated with FGF2, suggesting that TGFβ1 in the CM of 293LT1 cells was responsible for the mitogenic effect. Moreover, this proliferative synergism with FGF2 was amplified with CM derived from LTD_4-treated cells (Fig. 4A), which is consistent with the amount of TGFβ1 measured in CM of LTD_4-treated (~ 6 ng/ml) or ethanol-treated (~ 3 ng/ml) 293LT1 cells (Fig. 3).

Most of the biological effects mediated by TGFβ1 after binding to its cognate cell surface receptor depend on the Smad signal transduction pathway. Hence, to confirm the involvement of endogenous TGFβ1 in BSMC proliferation induced by 293LT1-derived CM, BSMC were pre-treated for 1 hour with SB431542, used at a concentration (10 μM) that completely inhibit Smad protein activation [51], or an equivalent concentration of the vehicle (DMSO) before incubation with 293LT1 CM. As shown in Fig. 4B, SB431542, but not DMSO, abrogated BSMC proliferation induced by LTD_4-treated or ethanol-treated 293LT1 CM.

CysLT1 is expressed in airway epithelial cells and is required for LTD_4-induced TGFβ1 mRNA upregulation

We subsequently tested whether LTD_4 can enhance TGFβ1 expression in airway epithelial cells, as observed in 293LT1 cells. However, since endogenous expression of CysLT1 has never been demonstrated in cultured airway epithelial cells, CysLT1 mRNA and protein expression was first determined in tumoral type 2 pneumocytes A549 and in NHBE cells. As determined by RT-PCR, CysLT1 mRNA was expressed in both cell lines, but the level of expression was higher in A549 cells (Fig. 5A). PBMC and THP-1 cells were used as positive controls and HUVEC as negative control. CysLT1 protein expression was then assayed by flow cytometry and, again, it was seen in both cell lines with a higher expression observed in A549 (Fig. 5B).

A549 or NHBE cells were then stimulated with 10^{-7}M LTD_4 or equivalent concentration of ethanol for 24 hours and TGFβ1 mRNA expression was determined by Northern blot analysis. As shown in Fig. 6A and B, LTD_4 stimulation increased TGFβ1 mRNA expression in both cell lines. To measure whether the increased expression of TGFβ1 mRNA induced by LTD_4 was mediated by the CysLT1 receptor, cells were pre-treated with the specific CysLT1 receptor antagonist MK-476 (montelukast) during 30 min prior to LTD_4 stimulation. As shown in Fig. 6A and B, MK-476 totally prevented the induction of TGFβ1 mRNA by LTD_4, demonstrating the requirement for LTD_4 activation of its high affinity receptor CysLT1 in order to upregulate TGFβ1 mRNA expression in airway epithelial cells.

DISCUSSION

LTD_4 and TGFβ1 are both regarded as orchestrating factors in the pathology of asthma [52 53]. Whereas LTD_4 is synthesized and released early following allergenic challenge and is best known as an inflammatory and spasmogenic stimulus, TGFβ1 is secreted later on and is primarily involved in immunoregulation and tissue remodeling processes. However, it is unknown whether the rapid release of LTD_4 affects the sequential upregulation of TGFβ1 expression. Here, we demonstrated that LTD_4 enhances TGFβ1 secretion in a cell line stably transfected with the high affinity receptor for LTD_4 (293LT1). In addition, CM from LTD_4-stimulated cells was mitogenic for human BSMC pre-treated with FGF2. The magnitude of this proliferative response was consistent with the amount of TGFβ1 measured in CM of LTD_4-treated (~ 6 ng/ml) or vehicle-treated 293LT1 cells (~ 3 ng/ml) (Fig. 3) and was in accordance with the concentration-dependent synergistic effect of TGFβ1 on FGF2-induced BSMC proliferation that we reported previously [23]. In addition, intact Smad signaling was required for this proliferative response to occur, supporting the concept that TGFβ1 is the growth factor responsible for the mitogenic effect of the CM.

The airway epithelial tissue, with its overlaying apical-associated mucus layer, represents a large host-environment interface targeted by allergens, pollutants, virus, bacteria and several mediators secreted by host cells infiltrating the airways. Experts in the field consider the airway epithelium as the protagonist tissue in asthma aetiology [52]. They propose that asthmatic epithelium shows increased susceptibility to

injurious agents, leading to chronic inflammation, subsequent activation of repair processes in the epithelial-mesenchymal trophic unit which ultimately lead to airway remodeling.

In this regard, TGFβ1 was shown to contribute extensively to airway remodeling in different animal models [17 18] and airway epithelial cells were shown to be an important cellular source of TGFβ1, both in normal [27 28 25 26] and in inflammatory conditions [26 29 30 31]. It was also determined that airway epithelial cells derived from asthmatic subjects secreted higher amounts of TGFβ1 compared to cells from healthy donors [32]. However, whether airway epithelial cells released TGFβ1 in response to LTD_4 stimulation and whether the remodeling effect of cysLTs observed *in vivo* [36 37 34 35] was mediated by TGFβ1 was still unexplored.

In the initial report characterizing the CysLT1 receptor, Lynch and coworkers [54] identified CysLT1 mRNA expression mainly in BSMC and in lung macrophages with minimal expression detected in airway epithelial cells. More recently, CysLT1 mRNA and protein expression has been questioned in human BSMC using *in situ* hybridization and immunohistochemical approaches [33]. However, its expression has been confirmed in airway epithelium of nonsmoking, nonatopic control subjects and was shown to be higher in epithelial cells of asthmatic subjects [33]. In the current study, CysLT1 expression at the mRNA and protein levels was confirmed in A549 and NHBE, two airway epithelial cell lines. Our data also demonstrate that TGFβ1 mRNA expression in airway epithelial cells increased in a CysLT1-dependent manner in

response to LTD_4 stimulation, confirming the findings in 293LT1 cells. Taken together, these results suggest that LTD_4-induced BSMC proliferation *in vivo* may involve a paracrine loop of TGFβ1 secretion by airway epithelial cells (Fig. 7).

In conclusion, our data present a new mechanism by which cysLTs may lead to BSMC hyperplasia *in vivo* [36 37 34 35], despite their marginal effects on proliferation of human BSMC cultured *ex vivo* [47 48 24 49]. In addition, our results suggest that the increased secretion of TGFβ1 by airway epithelial cells in response to LTD_4 may represent an important paracrine loop where CysLT1 antagonists could interfere in order to prevent BSMC hyperplasia. Supportingly, long term therapy with CysLT1 antagonists was shown beneficial to prevent key features of airway remodeling in animal models of allergic airway inflammation [36 37 34 35]. Hence, anti-leukotrienes drugs might be considered in the treatment of asthma to prevent TGFβ1 production and its ensuing effect on airway remodeling, including BSMC hyperplasia. Finally, the results of the current study also highlight the importance of studying the cell-cell interplay that inevitably occurs *in vivo* and likely participates in the development of a phenotype as complex as airway remodeling.

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FIGURE LEGENDS

FIG. 1. 5 day time-course experimental design used in this study to measure BSMC proliferation.

FIG. 2. Northern blot analysis of TGFβ1 mRNA expression in 293LT1 cells following different times of incubation with LTD_4 (10^{-8}M) (**A**) or different concentrations of LTD_4 for 8 hours (**B**). Presented are representative blots of two independent experiments.

FIG. 3. TGFβ1 protein level in CM of 293LT1 following 24 hours of treatment with LTD_4 (10^{-8}M) or equivalent concentration of ethanol was determined by ELISA. Bars are means ± SEM of duplicate measurements of 2 independent experiments.

FIG. 4. BSMC proliferation in response to CM from 293LT1 cells. (**A**) BSMC were pre-treated or not with FGF2 (2 ng/ml) and then incubated with CM harvested from LTD_4- or ethanol-treated 293LT1. TGFβ1 (10 ng/ml) was used as a positive control. Proliferation was measured using crystal violet uptake. (**B**) As in (A), except that BSMC were pre-treated with SB431542 (10μM) or equivalent concentration of DMSO 1 hour before the addition of 293LT1 CM. Each bar represents means ± SEM of quadruplicate measurements of 3 independent experiments and are expressed relative to their respective control, i.e. compared to medium alone (-) or FGF2 alone (+).

FIG. 5. CysLT1 mRNA (A) and protein (B) expression were measured in unstimulated lung epithelial A549 and NHBE cells by RT-PCR and flow cytometry, respectively. In (A), PBMC and THP-1 cells were used as positive controls and HUVEC were used as a negative control. In (B), the difference in mean fluorescence intensity (Δ MFI) between anti-CysLT1 and isotypic control Ab-labeled cells is indicated. Presented are representative results of three independent experiments.

FIG. 6. TGFβ1 mRNA expression was measured in A549 and NHBE cells following 24 hours stimulation with LTD_4 (10^{-7}M) or equivalent concentration of ethanol in the absence or presence of prior treatment with MK-476 (10^{-6}M). (A) Presented are representative results of three independent experiments. Numbers below each lane represent the ratio of densitometric assessment of TGFβ1 and 18S bands. (B) Means ± SEM of the TGFβ1/18S ratios obtained in NHBE cells, n = 3 per condition.

FIG. 7. Inflammatory cells infiltrating the airway lumen, the epithelium and the submucosal compartment produced cysLT. In response to LTD_4, airway epithelial cells produce TGFβ1, which, in turn, induces cellular proliferation in FGF2-preexposed BSMC.

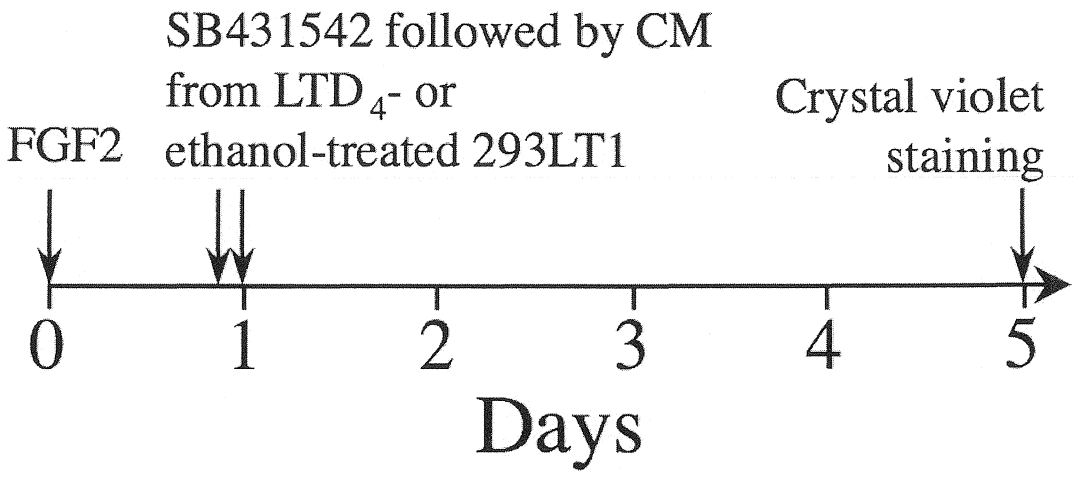

SB431542 followed by CM
from LTD4- or
ethanol-treated 293LT1
FGF2
Crystal violet
staining
0
1
2
3
4
5
Days

Figure 1

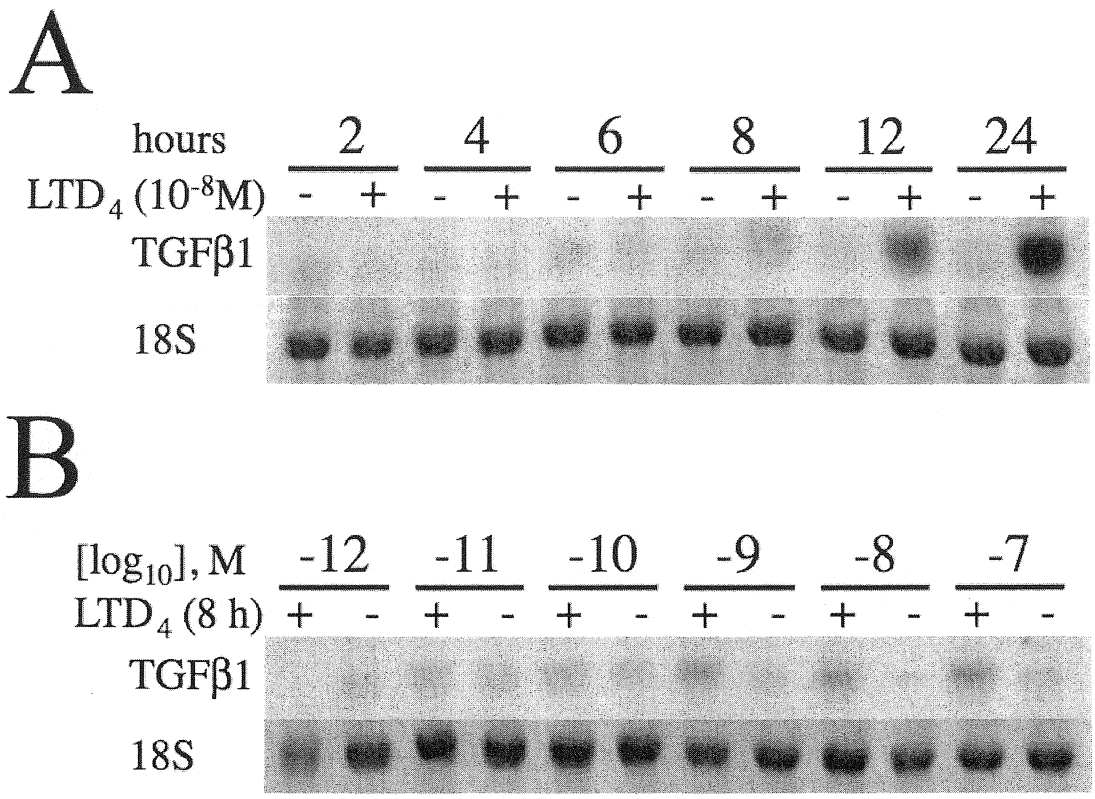

A
hours
2
4
6
8
12
24
LTD4 (10-8M)
- + - + - + - + - + - +
TGFβ1
18S
B
[log10], M
-12
-11
-10
-9
-8
-7
LTD4 (8 h)
+ - + - + - + - + - + -
TGFβ1
18S

Figure 2

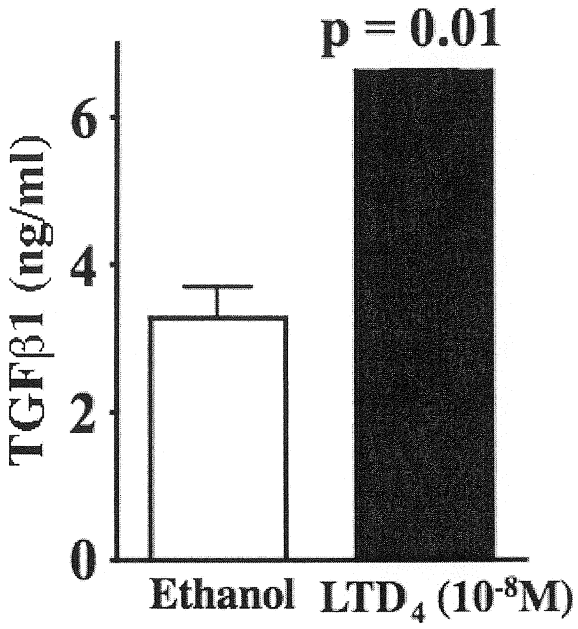


Figure 3

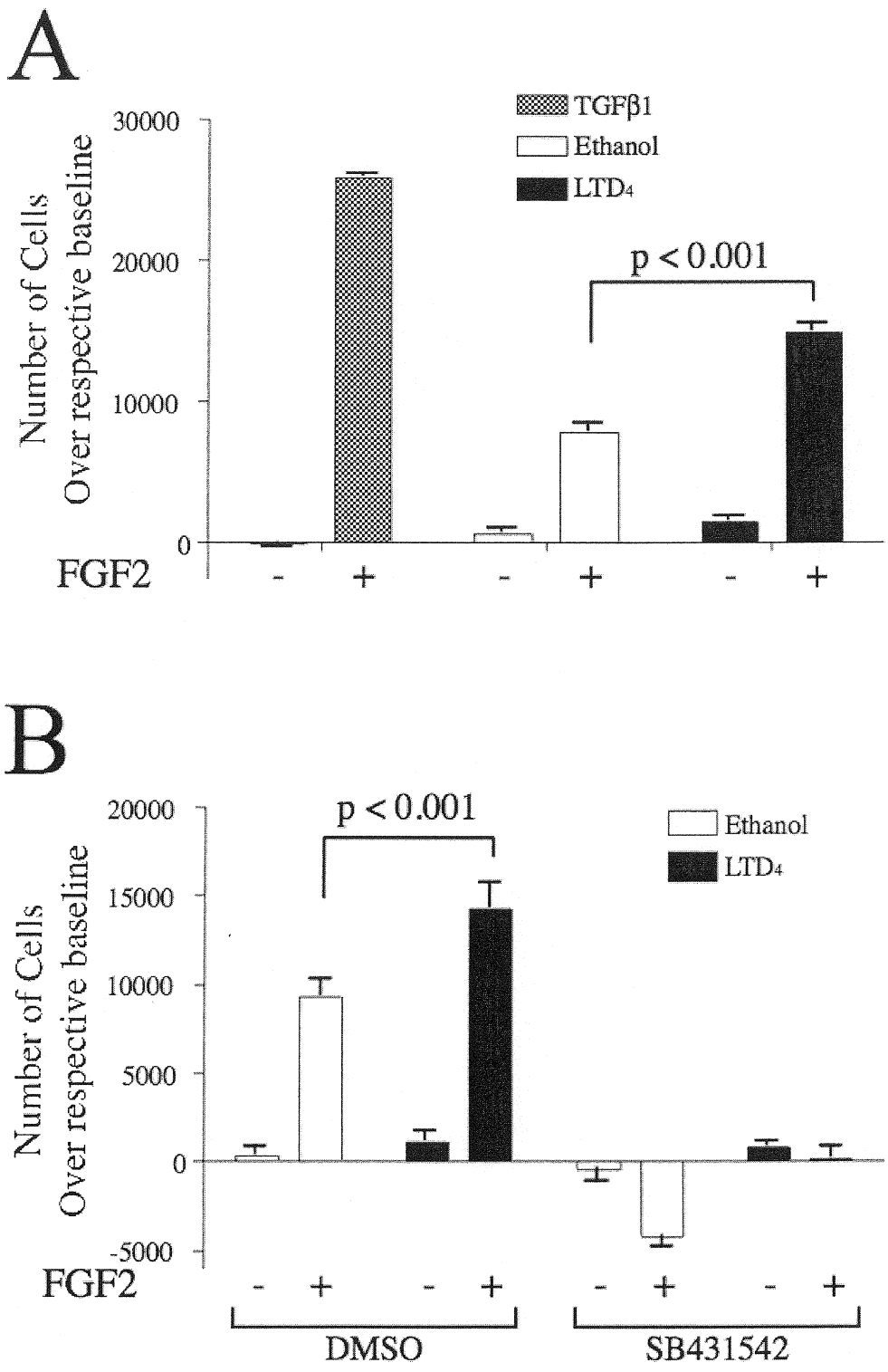

A
TGFβ1
Ethanol
LTD4
p < 0.001
Number of Cells
Over respective baseline
30000
20000
10000
0
FGF2
- + - + - +
B
p < 0.001
Ethanol
LTD4
Number of Cells
Over respective baseline
20000
15000
10000
5000
0
-5000
FGF2
- + - + - + - +
DMSO
SB431542

Figure 4

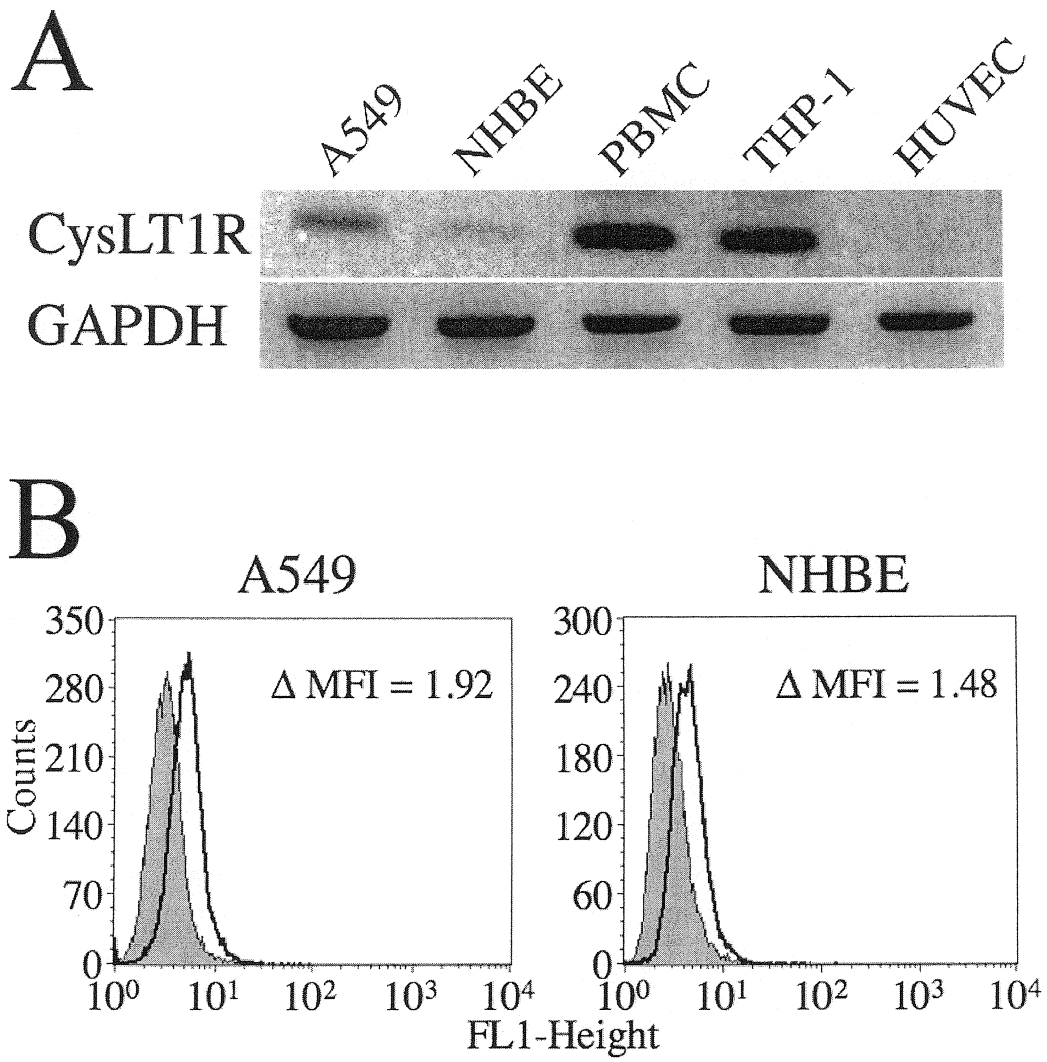

A
A549
NHBE
PBMC
THP-1
HUVEC
CysLT1R
GAPDH
B
A549
NHBE
Δ MFI = 1.92
Δ MFI = 1.48
Counts
350
280
210
140
70
0
300
240
180
120
60
0
10^0
10^1
10^2
10^3
10^4
FL1-Height

Figure 5

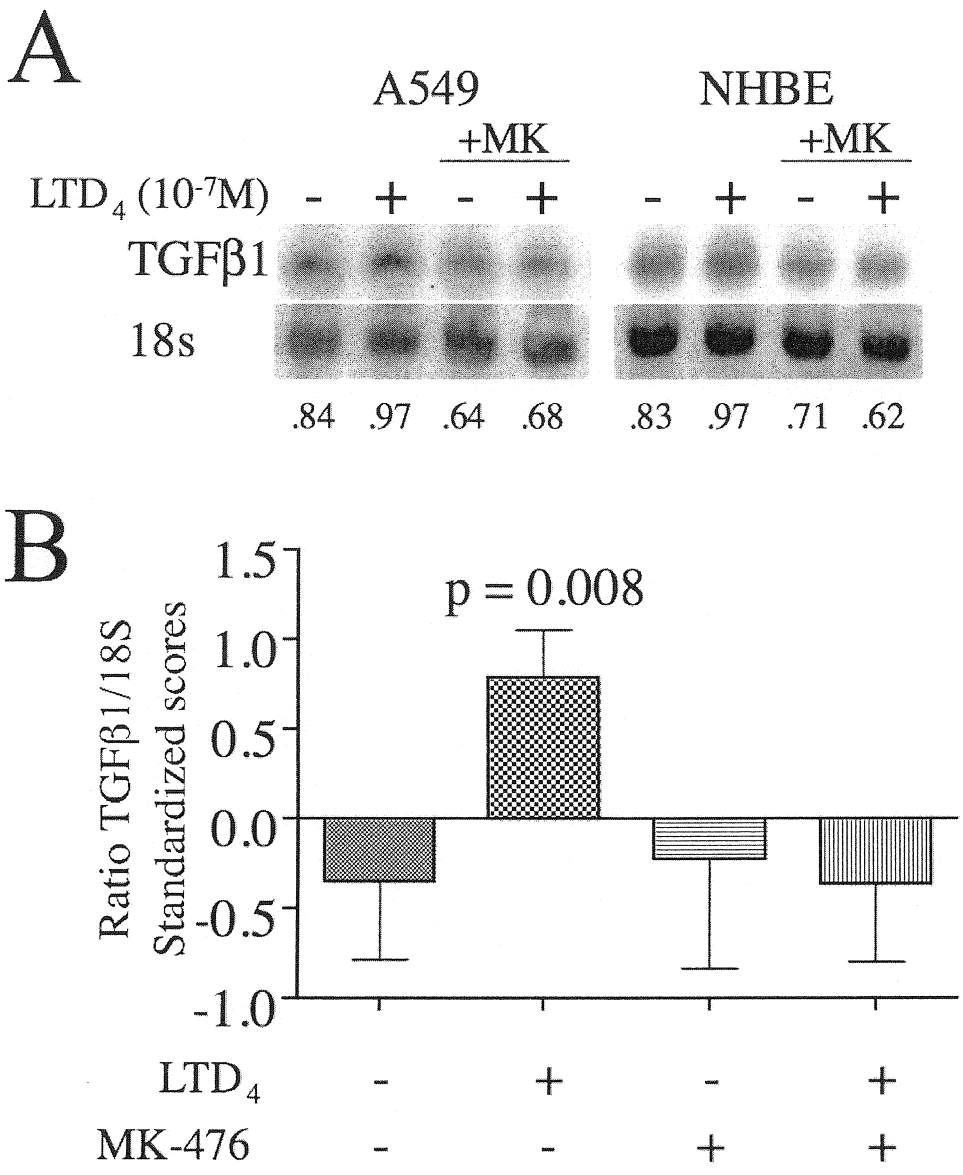

A
A549
NHBE
+MK
+MK
LTD4 (10-7M) - + - + - + - +
TGFβ1
18s
.84 .97 .64 .68 .83 .97 .71 .62
B
p = 0.008
Ratio TGFβ1/18S
Standardized scores
1.5
1.0
0.5
0.0
-0.5
-1.0
LTD4 - + - +
MK-476 - - + +

Figure 6

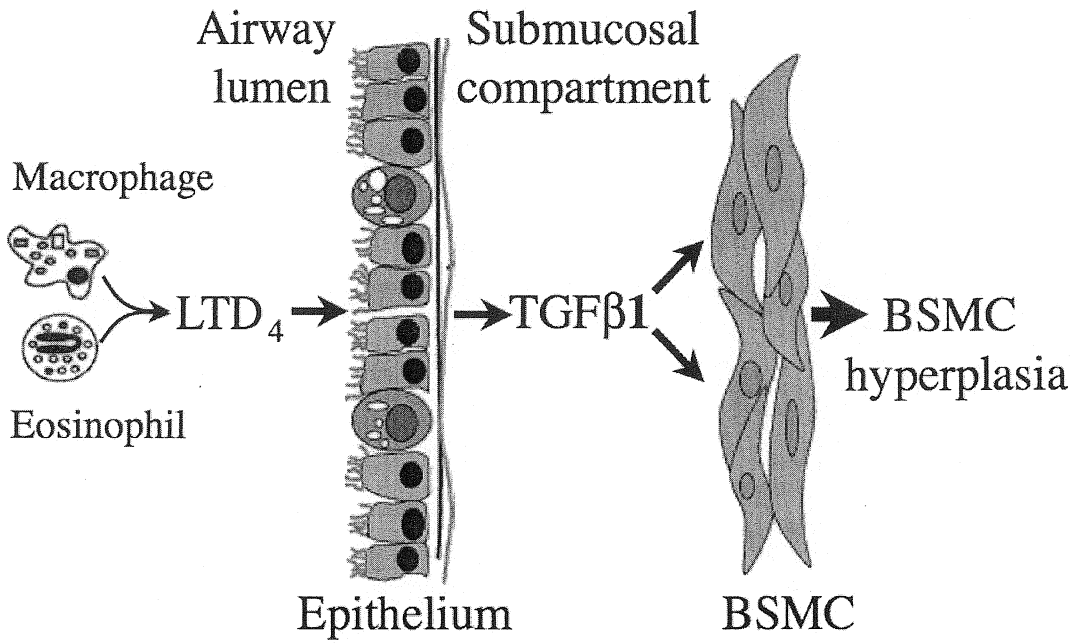

Airway
lumen
Submucosal
compartment
Macrophage
LTD 4
TGFβ1
BSMC
hyperplasia
Eosinophil
Epithelium
BSMC

Figure 7

CONCLUSION

The molecular mechanisms involved in ASM cell hyperplasia have been widely studied, but remain an open field of investigation since no clear picture of its etiology is yet defined. Numerous individual mediators have been shown to induce ASM cell proliferation *in vitro*, including growth factors, proteases, spamogens, reactive oxygen species (ROS) and cytokines (Hirst et al., 2004). Most of these mitogens are upregulated during asthma exacerbation and must act together, simultaneously or sequentially, to induce ASM cell hyperplasia. However, to date, interactions among these mediators and whether they can synergize or antagonize each other have not been explored extensively. Moreover, the order of production of all these mitogens in the airways after phlogogenic challenge is not clearly defined yet, but clues regarding the sequence of expression of certain growth factors have been documented. In this regard, Redington and coworkers (Redington et al., 2001) have reported a rapid FGF2 increase in BALF after a SAC, whereas TGFβ1 was upregulated only 24 h post-SAC (Redington et al., 1997). Similarly, increased expressions of IL-13 and IL-4 in the lungs of asthmatics are inducible, transient and with a slow onset (Batra et al., 2004; Tschopp et al., 2006). Their peaked expressions are thought to occur during the late asthmatic reaction (LAR), at the time that T_H2 lymphocytes and eosinophils are mobilised into the lungs (the former being the most important cellular source of these cytokines). Our results suggest that initial upregulation of FGF2 can render ASM cells responsive to the mitogenic effect of cytokines that are upregulated with slower kinetics following allergic challenge and which are otherwise non-mitogenic in the absence of FGF2 pre-treatment.

Even if asthma mediators are upregulated transiently, Phipps and coworkers (Phipps et al., 2004) have proposed that the effects of serial acute processes occurring as a result of multiple single allergenic insults culminate over time to cause airway remodeling. Hence, repeated allergic challenges with concomitant sequential increases of FGF2 and TGFβ1, IL-4 and IL-13 may lead to cumulative structural changes, such as ASM cell hyperplasia.

Evidence from *in vivo* studies suggests that TGFβ1 contributes to ASM cell hyperplasia. The results presented in this thesis confirm its mitogenic potential and shed light on the conundrum surrounding its mitogenic effect *in vitro*. In contrast, the *in vivo* data supporting a role of IL-4 or IL-13 in ASM cell proliferation are sparse. In this context, our results suggest that both IL-4 and IL-13 may be able to induce ASM cell hyperplasia in the context of allergic airway inflammation, i.e. when FGF2 has previously stimulated ASM cells (submitted article). Accordingly, we demonstrated that similar to TGFβ1, both IL-4 and IL-13 synergize with FGF2 to induce ASM cell proliferation *in vitro*. It is worthy of mention that the effects of both IL-4 and IL-13 were marginal compared to the effect observed with TGFβ1. However, it is likely that in *in vivo* conditions, IL-13 may induce ASM cell hyperplasia by either acting directly on its receptor expressed on ASM cells or indirectly via its ability to induce a paracrine loop of TGFβ1 (Lee et al., 2001a; Shim et al., 2006). In both cases, FGF2 priming is required for IL-13 and TGFβ1 to stimulate ASM cell proliferation *in vitro*. Our results also suggest that the mitogenic effect of cys-LTs observed *in vivo* may not

be a direct effect of cys-LTs on ASM cell behaviour, but instead a paracrine loop involving the secretion of TGFβ1 by other cells (submitted article).

Concerning leukotrienes, accumuling evidence now suggests that most of the asthma-like pathogenesis induced by IL-13 is dependent on leukotrienes-mediated mechanisms (Elias et al., 2003; Grunig, 2003; Vargaftig and Singer, 2003). In fact, leukotriene receptor antagonists (LTRAs) were shown to prevent AHR, inflammation and part of mucosal metaplasia and tissue injury induced by intratracheal instillation of IL-13 into the lungs of mice (Vargaftig and Singer, 2003). However, the conversion of LTC_4 into LTD_4 seems dispensable, since GGL-deficient mice still showed increased airway responsiveness after intranasal instillation of IL-13 (Chavez et al., 2006). The fact that IL-4Rα-deficient mice have increased AHR to LTC_4, but not to IL-13, is also suggestive that cys-LTs are downstream mediators of IL-13-induced AHR (Chavez et al., 2006). More confusing is that STAT6-deficient animals are protected against AHR development induced by intranasal instillation of either IL-13 or LTC_4 (Chavez et al., 2006). This result highlights the crucial role of STAT6 in the development AHR and suggests a yet unrecognized function of cys-LTs in the activation of this transcription factor.

Additionally, intranasal instillation of IL-13 augments the mRNA expression of the two cys-LTs receptors, CysLT1 and CysLT2, as well as the expression of 5-LO (Chavez et al., 2006). These results corroborate with our previous findings demonstrating an increased expression of CysLT1 in human ASM cells following IL-

13 stimulation (Espinosa et al., 2003). In Chavez and coworkers' study, IL-13 also increased the mRNA expression of one of its own receptor chain, the α1 chain (IL-13Rα1). On the other hand, intranasal instillation of LTC_4 increased pulmonary mRNA expression of its own CysLT1 receptor, as well as IL-13Rα1, highlighting the prominent *in vivo* cross-talk between IL-13 and cys-LTs (Chavez et al., 2006).

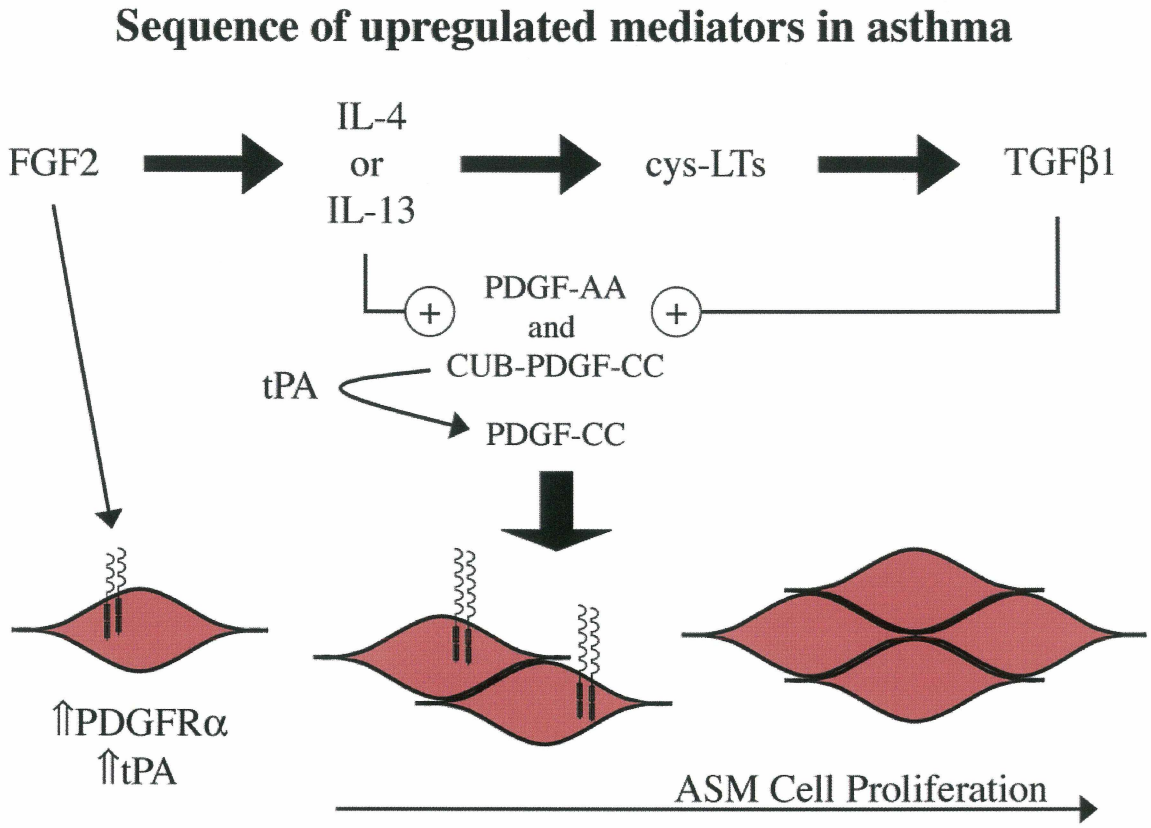
Additional studies support the potential interplay occurring between IL-13 and cys-LTs. For example, IL-13 increases the intracellular mobilisation of Ca^{2+} and the contractile response of cultured murine ASM cells to LTD_4 (Eum et al., 2005). Also of interest in that study, BALF levels of IL-13 were comparable between sham-challenged (111 pg/ml) and OVA-challenged (94 pg/ml) animals, indicating that IL-13 is not sufficient to induce AHR. Instead, IL-13 may require other active inflammatory mediators released during allergic challenge, such as cys-LTs, to induce ASM contraction.

In IL-13 transgenic mice, mRNA encoding cytosolic phospholipase A (cPLA)2, LTA_4 hydrolase, and 5-LO-activating protein (FLAP) were upregulated (Shim et al., 2006). However, in contrast to mice instilled via intranasal (Chavez et al., 2006) or intratracheal routes (Vargaftig and Singer, 2003) with IL-13, neither CysLT1 and CysLT2 nor 5-LO mRNA expressions were affected in IL-13 transgenic mice (Shim et al., 2006). To test the effect of 5-LO and its end products in the pathogenesis induced by IL-13 overexpression, crosses between IL-13 transgenic mice and 5-LO deficient mice have been generated (Shim et al., 2006). This study demonstrated that

5-LO, and by inference leukotrienes, play a critical role in the pathogenesis of IL-13-induced airway inflammation, fibrosis, and respiratory failure. As previously mentioned, TGFβ1 was induced and activated in this IL-13 transgenic model, and was shown to mediate most of the fibrotic effect of IL-13 transgene (Lee et al., 2001a). This was in concordance with the lack of active TGFβ1 upregulation in the epithelial and the submucosal zones of IL-13-deficient mice exposed to a prolonged challenge protocol of allergic asthma (Kumar et al., 2004). Interestingly, 5-LO was also required for optimal stimulation and activation of TGFβ1 in this IL-13 transgenic mouse (Shim et al., 2006). This *in vivo* result corroborates *in vitro* data demonstrating increased expression of TGFβ1 in both epithelial cells (Perng et al., 2006) (and submitted manuscript) and eosinophils (Kato et al., 2005) following cys-LTs stimulation. It also corroborates with new observations showing a substantial reduction of TGFβ1 expression in whole-lung lavages, as well as in lung tissues, in OVA sensitized and challenged mice treated with montelukast or pranlukast (Lee et al., 2007). However, the results presented in Shim and coworkers' study (Shim et al., 2006) represent the first *in vivo* demonstration that cys-LTs induced by IL-13 are required for TGFβ1 induction *in vivo*. Thus, if IL-13 is upstream of cys-LTs production and TGFβ1 is downstream of cys-LTs production, it is easy to imagine that this ordered sequence of upregulated mediators in the airways of asthmatics following allergic challenge may contribute to ASM cell hyperplasia (Figure 4).

In this sequence, every single upstream mediator is a stimulus to upregulate the expression of its downstream effectors and some intermediaries in the sequence (i.e.

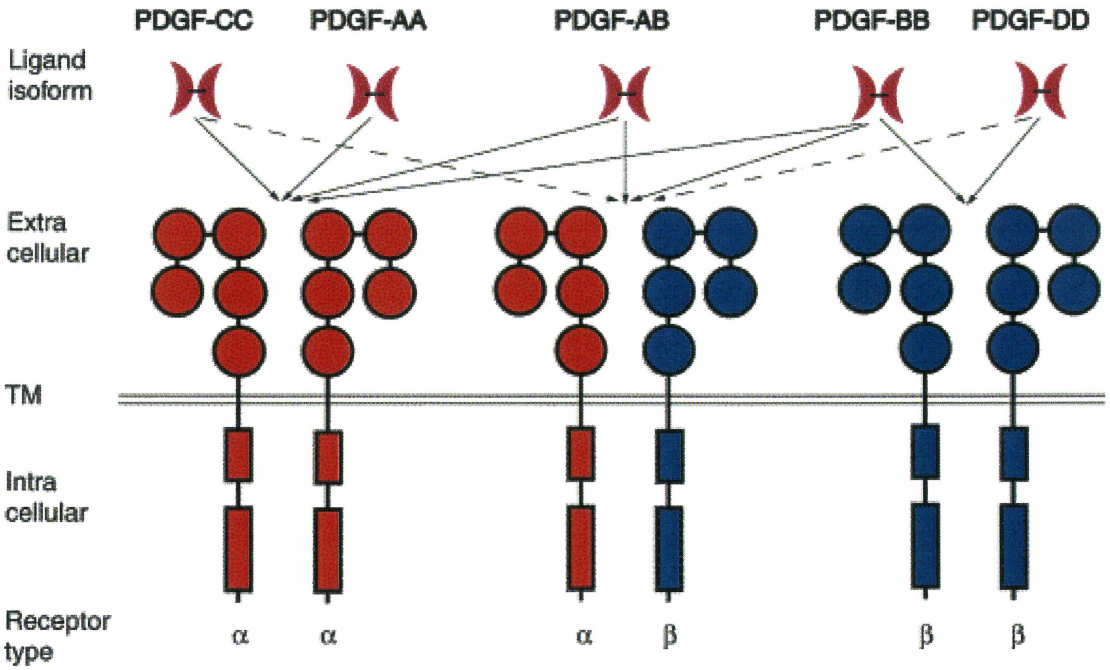
Figure 4: Sequence of upregulated mediators in asthma



onset of TGFβ1 upregulation occurring in the airways of asthmatics following SAC (Redington et al., 1997). As demonstrated in the results presented in this thesis, the key element in this pathway is FGF2, because none of the asthma mediators listed in the sequence is effective in inducing ASM cell proliferation in the absence of FGF2. Altogether, these results suggest that FGF2 may be a central regulator of ASM cell hyperplasia in asthma and must prone investigators in the field to turn their interests toward the understanding of this growth factor regulation in asthmatic airways and demystifying whether its growth-promoting effect with different asthma mediators occurs *in vivo*.

In turn, the operational mechanism involved in the synergistic proliferations occurring between FGF2 and different asthma mediators was partially elucidated in this thesis. It seems that an autocrine loop involving different members of the PDGF and PDGFR family of growth factors could account for approximately 50% of the synergism (Bosse et al., 2006) (and manuscript submitted). This family consists of four protein chains that yield 5 different isoforms (AA, BB, AB, CC and DD), where each binds and causes dimerization of PDGF receptor α and β chains with different specificity/promiscuity (Figure 5).

Figure 5: PDGF receptors



Li and Eriksson. Cytokine & Growth Factor Reviews 2003; 14(2): 91-8.

Even though we did not measure the protein expression PDGF-CC due to the lack of available Ab, we have many reasons to speculate that PDGF-CC might be the most important player in the part of the synergistic responses that were blocked by PDGFR tyrosine kinase inhibitors. The induction of PDGF-C mRNA by TGFβ1, IL-4 or IL-13 persisted much longer than the induction of PDGF-A mRNA. Moreover, PDGF-AA binds exclusively to the PDGFRα chain (Heldin and Westermark, 1990) (figure 5) and it was clearly established that homodimerization of this receptor induces a poor mitogenic signal in bronchial and vascular smooth muscle cells, as reported herein and by others (Bonner et al., 1996; Bosse et al., 2006; Stouffer and Owens, 1994). Like PDGF-AA, PDGF-CC binds and activates PDGFRα homodimers. However, PDGF-CC also engages PDGFRαβ heterodimers (Cao et al., 2002; Gilbertson et al., 2001) (figure 5), which have been demonstrated to be more efficient in inducing mitogenesis compared to PDGFRα homodimers (Seifert et al., 1993). Unlike PDGFRα chain, the PDGFRβ chain is constitutively expressed (Bonner et al., 1996). Taken together, the increased expression of PDGFRα chain by FGF2 may increase the binding of PDGF-AA and PDGF-CC on ASM cells, but may also increase the likelihood of αβ heterodimerization by PDGF-CC, which is more likely to be of major significance in ASM cell proliferation.

One can also discuss the fairly low amounts of PDGF-AA measured in the conditioned medium of TGFβ1-stimulated ASM cells. However, PDGF-AA, AB and BB are secreted in an active form. Consequently, in cells expressing both the receptors and the ligands, autocrine binding is likely to occur before externalisation.

Hence, low amounts are expected to be secreted by these cells since most of the PDGF content in the Golgi apparatus may already be bound to their cognate receptors prior to exocytosis. However, as soon as the complex reaches the plasmalemma, the activated receptor is likely to initiate signaling and cause the PDGF-mediated cellular effects. Certain factors subject to such an autocrine signaling (i.e. secreted by the cells that also express its receptor), are prevented from binding to its receptor prematurely by being secreted as an latent, inactive form. TGFβ1 for example, is associated non-covalently with its pro-peptide LAP before being secreted, which prevents its intracellular interaction with its receptors within the Golgi apparatus (Wakefield et al., 1987). Only cells that possess the extracellular machinery to intercept and to activate TGFβ1 are subjected to its biological effect. However, it is not the case for PDGF-AA, and this may explain the fairly low amount of PDGF-AA detected in the conditioned medium of TGFβ1-treated or non-treated ASM cells.

Similar to TGFβ1, PDGF-CC and the newly identified member of PDGF family, PDGF-DD, are secreted in a latent form, covalently associated with their extended N-terminal CUB domain (Bergsten et al., 2001; LaRochelle et al., 2001; Li et al., 2002). The CUB domain must be cleaved by tPA (Fredriksson et al., 2004) to permit binding of the growth-factor domain to its cognate PDGF receptor. In this regard, FGF2 has been shown to increase plasminogen activator (PA) activity in several *in vitro* systems (Cavallaro et al., 2001; Flaumenhaft et al., 1992). Our results suggest that tPA is increased following FGF2 stimulation and, based on the proliferative inhibition induced by leupeptin, its activity is required for optimal mitogenic effect of TGFβ1 on

FGF2-primed ASM cells. We thus speculate that the proteolytic activation of PDGF-CC might take place in this synergism and its autocrine binding may contribute to the overall mitogenic input of TGFβ1. It is also interesting to note that the protease inhibitor used (leupeptin) reduced FGF2-TGFβ1 synergism to an intermediate level compared to PDGFR tyrosine kinase inhibitors, suggesting that both PDGF-CC (the leupeptin blocking part) and PDGF-AA (the AG1296 blocking part not affected by leupeptin) are involved in FGF2-TGFβ1 proliferative synergism.

In contrast to other members of the PDGF family, PDGF-BB is a universal ligand for PDGF receptors (Heldin and Westermark, 1990). It can induce β and α homodimers as well as αβ heterodimers. By increasing PDGFRα expression, one might expect that FGF2 pre-treatment would enhance the proliferative effect of PDGF-BB. However, we did not observe a synergistic effect of FGF2 on PDGF-BB-induced proliferation in our study (data not shown), which was consistent with the results obtained by Bonner and coworkers (Bonner et al., 1996). In their study, FGF2 pre-treatment increased the mitogenic response of ASM cells to PDGF-AA and PDGF-AB, but did not potentiate the effect of PDGF-BB.

In conclusion, our data suggest that PDGF-AA and PDGF-CC are induced by TGFβ1, IL-4 and IL-13 and their mitogenic actions on ASM cells are potentiated by PDGFRα upregulation by FGF2. In normal development, a paracrine mode of action of PDGFs is well recognized. However, in exagerated growth pathologies such as cancer, PDGFs act largely in an autocrine fashion (reviewed in (Betsholtz, 2003)). After

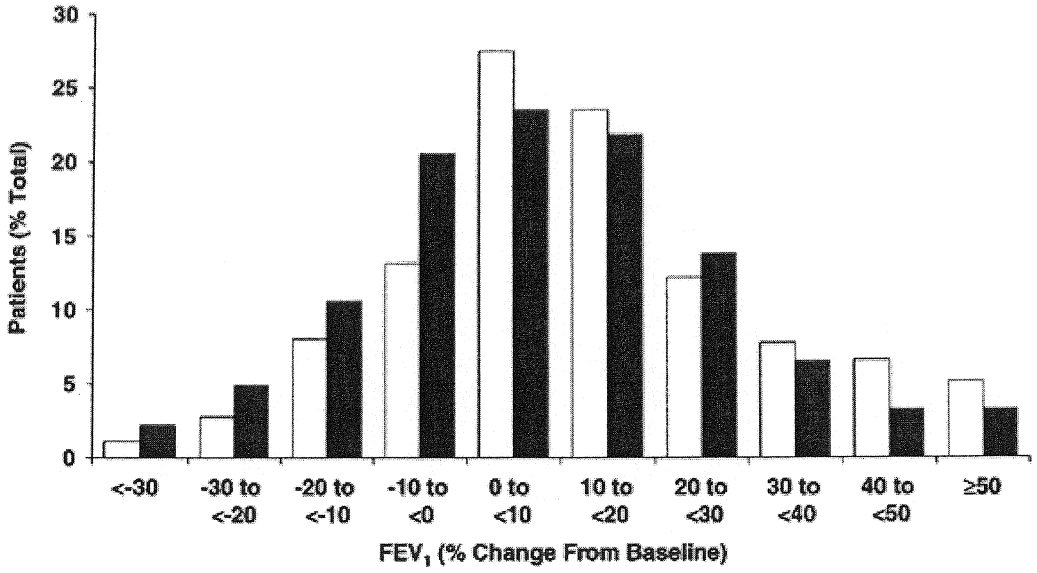
sequential FGF2 and TGFβ1/IL-4/IL-13 stimulations, which likely occur after allergic challenge *in vivo*, ASM cells will express α and β chains of PDGFR and secrete PDGF-AA and PDGF-CC ligands. Hence, unlike the normal paracrine mode of action of PDGFs, the PDGF signaling reported herein would act in an autocrine fashion and may lead to abnormal proliferation of ASM cells, as observed in asthmatic individuals.

Therapeutic options to alter these proliferative synergisms

Since its recognition in 1922 (Huber and Koessler, 1922), ASM cell hyperplasia has been regarded as the major structural alteration contributing to bronchial hyperresponsiveness (Lambert et al., 1993; Pare et al., 1997). In addtion to its active role in bronchial constriction, ASM hyperplasia increases airway wall thickening, which concomitantly decreases airway lumen diameter and increases susceptibility to airway closure even with limited bronchoconstriction (James et al., 1989; Wiggs et al., 1992). In addition, increased ASM mass around the airways participates in symptoms of asthma in many other ways. In particular, it has been recognized as an active participant in the inflammatory and fibrotic responses during asthma exacerbation by its ability to synthesize and secrete a diverse array of mediators involved in recruitment, activation and enhanced survival of inflammatory cells (Howarth et al., 2004; Joubert and Hamid, 2005; Panettieri, 2002). Considering all of these detrimental effects induced by ASM tissue enlargement, any strategy employed to reduce or reverse ASM cell hyperplasia is likely to improve asthma symptoms.

The results presented in this thesis demonstrate that FGF2 can confer a mitogenic potential to TGFβ1, IL-4 and IL-13, as well as to cys-LTs-induced TGFβ1, and suggest that all of these proliferative synergisms may work in concert *in vivo* to contribute to ASM cell hyperplasia. Hence, interfering with the function of any of these mediators may prevent the proliferative synergism to take place and thus, prevent ASM cell hyperplasia, together with the concomitant features of asthma associated with this altered phenotype. For instance, LTRAs, such as montelukast, pranlukast, zafirlukast and pobilukast are currently approved as asthma therapies in different countries and were shown to bring beneficial effects to appoximateley 40% of asthmatic patients (Figure 6).

Figure 6 : Variability in the therapeutic response of asthmatics to a leukotriene receptor antagonist



Peters, JACI 2003: 111(suppl. 1): S62-70. Distribution of treatment response to beclomethasone (white bars) and LTRA (black bars) for FEV_1. Adapted from Malmstrom *et al.*, Ann Intern Med 1999: 130: 487-95.

On the other hand, drugs targeting TGFβ1, IL-13 and IL-4 are not presently available, but many are under development. In the case of TGFβ1, several experimental and pharmacological tools currently exist to modulate its response (Schmidt-Weber and Blaser, 2004). Many of these effectively reduce TGFβ effects *in vivo* and were shown to have therapeutic effects on different animal models of fibrotic diseases. Among the strategies employed to block the biological effect of TGFβ *in vivo*, certain prevent TGFβ activation by treating the animals with the plasmin/serine protease inhibitor aprotinin (Lee et al., 2001a); others have blocked TGFβ binding to its receptor by gene transfer of decorin (Kolb et al., 2001b; Kolb et al., 2001c), neutralising Ab against TGFβ (Giri et al., 1993), soluble TβRII (Wang et al., 1999), TβRIII peptide (Ezquerro et al., 2003), soluble TβRIII (Liu et al., 2002) or by the use of a fusion protein consisting of soluble TβRII attached to the Fc fragment of human IgG (sTGFβ-Fc) (Lee et al., 2001a); and finally, others have opted for inhibiting the main signaling pathway initiated by TGFβ following its binding to cognate cell suface receptors, including gene transfer of Smad7 (Nakao et al., 1999), inhibitor of ALK5 (SD-208) (Bonniaud et al., 2005) or inhibitor of Smad3 phosphorylation (Halofuginone) (McGaha et al., 2002). Additional molecules inhibit TGFβ1 effects *in vitro* by blocking its interaction with the receptor, such as ursolic acid (Murakami et al., 2004), or by preventing ALK5 serine/threonine kinase activation, such as SB-431542 (Inman et al., 2002) and SB-505124 (DaCosta Byfield et al., 2004), but their *in vivo* effects are currently undefined.

As mentioned earlier, McMillan and coworkers (McMillan et al., 2005) have shown beneficial therapeutic effects of anti-TGFβ Ab on ASM cell hyperplasia and AHR in an animal model of allergic asthma. This Ab was also successful in preventing peribronchial ECM deposition and mucus production. This study implies that blocking TGFβ1 by neutralizing Ab may be an alternative and effective therapeutic option to cure asthma disease.

Drugs targeting other molecules with indirect influence on TGFβ1 expression could also be envisaged to alter TGFβ1-mediated effects. For example, mepolizumab, an anti-IL-5 antibody, was shown to decrease TGFβ1 expression in the airways of mild atopic asthmatics (Flood-Page et al., 2003). PPARγ agonists, such as rosiglitazone and pioglitazone, were also shown to reduce lung expression of TGFβ1 as well as several inflammatory and remodeling features of airway disease in a mouse model of occupational asthma (Lee et al., 2006a). The antifibrotic drug pirfenidone is also a likely canditate, since it was shown to reduce airway expression of TGFβ in a hamster model of bleomycin-induced pulmonary fibrosis (Iyer et al., 1999), and to potentially exert similar effect in a Brown Norway rat model of allergic asthma (Mansoor et al., 2006).

Another example would be the corticosteroid methylprednisolone, which had little effect on the secretion of TGFβ by alveolar macrophages *in vitro*, but reduced the total lung content of TGFβ by preventing the influx of alveolar macrophages into the lungs of rats in a model of bleomycin-induced pulmonary inflammation (Khalil et al.,

1993). However, others have shown that TGFβ1 expression in asthmatic subjects was unresponsive to corticosteroid treatments (Chakir et al., 2003) and one study even demonstrated that it increased TGFβ1 mRNA expression in T cells *in vitro* (AyanlarBatuman et al., 1991). Hence, the well-known efficacy of corticosteroids in the treatment of asthma may be related to mechanisms other than their capacity to alter TGFβ1 expression.

An early study with IL-4 antagonist altrakincept, which is a soluble recombinant IL-4 receptor, has shown therapeutic benefits as a steroid-replacing agent (Borish et al., 1999) and new drugs targeting one or both of IL-4 and IL-13 are now under development (reviewed in (Izuhara and Arima, 2004)). These drugs are soluble IL-13 receptor, chimera protein formed by linking the soluble forms of either IL-4Rα and IL-13Rα1 (IL-4/IL-13 Trap), mutant form of IL-4 able to bind to the type 1 receptor but unable to signal (IL-4 mutein) or Ab against IL-13Rα1.

Blocking IL-13 was shown to yield successful therapeutic results in animal models of asthma. For example, Ab against IL-13 prevents the progression of an established disease in a murine model of persistent asthma (Yang et al., 2005). In this study, mice were sensitized i.p. and challenged intranasaly at several occasions with OVA. It was shown that i.v. administration of anti-IL-13 Ab before every challenge following the establishment of the disease (after 2 challenges) prevents further progression of eosinophilia and mononuclear cell infiltrates as well as airway fibrosis. Some pathological features such as excessive mucus production and AHR were even

reversed to attain levels observed in non-challenged animals. This treatment regiment also reduced MMP-9 levels as well as the levels of IL-4, IL-5, TNFα, CCL11, CCL2 and KC expression in lung homogenates. The authors suggest that therapeutic doses of an anti-IL-13 Ab may be clinically beneficial to inhibit the progression of an established disease.

Since the bottom line of every synergies reported may involve an autocrine loop of PDGF, targeting PDGFR tyrosine kinase activity may also be an interesting strategy. In this regard, Gleevec (Imatinib) is an inhibitor of the tyrosine kinase activity of PDGF receptors and is already approved for treatment of different malignancies (Noble et al., 2004). In addition, it has been used with success for the treatment of leukaemias that are associated with translocation of the PDGFR gene (Apperley et al., 2002; Pietras et al., 2003). Unfortunately, Gleevec lacks selectivity as it also blocks the activity of other tyrosine kinases, such as ABL, ARG and the KIT receptor (Dibb et al., 2004).

LTRAs or drugs currently under development enumerated above may prove to be beneficial in asthma in part because they interfere with the synergistic proliferation of ASM cells induced by the combined action of FGF2 with one of the asthma mediators (TGFβ1, IL-4, IL-13 or cys-LTs). However, this speculation must be viewed with caution, because these drugs target mediators that orchestrate various aspects of the pathology of asthma, and consequently, their beneficial effects may be unrelated to their capacity to reduce ASM cell hyperplasia.

Final remarks and perspectives

Investigating the role of individual mediators in isolation is a convenient approach and an obligatory step undertaken in the last several years to understand the mechanisms leading to ASM cell hyperplasia. However, it is unlikely to reflect *in vivo* conditions, where several mediators act simultaneously or sequentially on ASM cells and could influence each other's function on ASM cell proliferation. By using a single mediator approach, we would have failed to reveal the tremendous growth factor-amplifying effect of TGFβ1 and the relatively weaker effects of IL-4 and IL-13. The results presented in this thesis also highlight the importance of kinetics. As such, not only the stimulus *per se*, but also the moment where the stimulus in added in a particular sequence of stimuli may alter cellular behaviour or the extent of the adopted behaviour. The sequence of stimulation used in these studies likely mimics the time sequence of triggers encountered by ASM tissue in *in vivo* conditions following allergic challenge. Consequently, the outcomes measured are more likely to reflect what is really happening *in vivo*. Finally, our results also shed light on some of the inconsistencies between *in vitro* and *in vivo* findings regarding the mitogenic effect of TGFβ1 and cys-LTs on ASM cells.

However, to gain further confidence in the role of these proliferative synergisms in ASM cell hyperplasia, several issues need to be clarified and an appreciable amount of research remains to be done. Firstly, the few data reporting an increased expression FGF2 must be considered only as the *stepping stone* for undertaking larger and more

controlled trials to confirm these early studies. Secondly, studies designed to harvest tissues at multiple-time points following allergic challenge would be very useful to understand the kinetics of cytokine upregulations. In the case of TGFβ1, its upregulation but also its kinetics of activation will require elucidation. Non-invasive techniques, such as exhaled breath condensate, could be helpful to perform this kind of experiments in humans. Otherwise, animal models will need to be considered. Finally, the effect of FGF2 and its direct or indirect synergisms with TGFβ1, IL-4, IL-13 and cys-LTs will need to be investigated *in vivo*, by the use of transgenic or knockout animals or with pharmacological inhibitors targeting each or several of these pathways. Determining whether other members of both FGF and TGFβ families could synergize with one another to induce ASM cell proliferation would also be interesting to document.

The interactions reported in this thesis also suggest that many other mediators are likely to influence the proliferative outcomes of ASM cells *in vivo*. The elucidation of such an intricate network of mediators will undoubtedly require high throughput techniques, such as genomic and proteomic platforms. Therefore, I think we've been walking in the right direction, but we'd better start running if we want to reach a complete understanding of ASM tissue enlargement before casting off this task to our progeny.

ACKNOWLEDGMENTS

Completing a PhD is like sailing a boat from one port to another with not much food in between to keep you alive. After swiming all the way to reach my Master's degree port, my reckless attitudes, together with my young and motivated (and maybe naïve) mind pushed me to travel even farther. I knew from the beginning that the water to be crossed over during a doctorate is covered by a thick layer of ice. However, the 3-year program boats in Immunology at the Université de Sherbrooke were quite appealing, and by jumping in one of them, I thought that it would prevent me from freezing by swimming all the way to reach my PhD degree port. I actually managed to stay unfrozen. Unfortunately, the cargo was heavier than I'd expected, and what about the distance to cover ? Ouf ! To be congruent with myself, it also seemed to me that the current was not alway on my side. Traveling this distance wasn't easy and it tooks more time then I'd envisaged, but I'm satisfied about the work accomplished.

With no surprise, reaching one's final destination is not a one-man effort. I've been fortunate to work with seasoned immunologists. First, I want to ackowledge my research supervisor, Dr Marek Rola-Pleszczynski. When I was busy propelling the boat with no clear picture of what was going on in front of me, Dr Pleszczynski was guiding me to avoid obstacles and unnecessary extra mileage. His patience and his positivity also helped me get through when the boat needed to be fixed up and when I was still unable the see the shore despite all the time and energy I'd invested in this project at these particular moments. I also want to address a special thanks to the program director Dr Jana Stankova, who has acted as a scientific adviser during my

doctorate studies. Her rigor toward well-done work have patched many holes in the boat to ensure a solid and trustworthy cruise.

Along my way, I also had the opportunity to meet other sailors and captains that were cruising by once in a while. I won't enumerate all of them, but I need to recognize Charles Thompson: By following his path, he acted as a co-pilote for me; and by being just a few knots in front of me, I profitted from his drag and his ice-breaking paths many times. For all the other people working in the Immunology Division, your name may not be cited in my thesis, but your whole person is engraved into my memory. I will be forever grateful, and I want to let you know that it has been a real pleasure to sail in your part of the sea.

Lastly, I want to express my gratitute to my family. Remember that I've grown-up beside Madawaska River in St-Jacques N.-B. This may sound funny, but it means everything to me. I learned to swim there. The education that I received from my parents and the attitude of being courageous, determined and tenacious that they implicitly transmitted to me during my childhood are the foundations for what I am right now. For these reasons, even in the harshest conditions, sinking during my doctorate degree has never been an option. My father, Réal, my mother, Corinne, my twin brother Yohan and my two sisters Anik and Heidi are the main reasons for me being myself right now. Everything was moving very fast in the last several years and my only regret in undertaking my Master's and Doctor's degrees is to have been distant from you. Yohan is also of great inspiration for me, not only as being an

astonishing scientist, but also as carrying at the same time the responsability with his wife Mélanie of being a new parent. The new family member, Francis, is growing pretty fast and I'm wondering if he is aware of his forthcoming sister. All of you, I want to let you know that thinking of my family is the only remedy which succesfully cures my blues when I'm feeling down and which gives me the strength and the motivation required to fulfil my task when I'm entirely beaten up.

By sailing my boat close to Moncton during the end of my Bachelor's degree, I met this 'living-by-the-seagirl', named Rachel. Since then, she has been beside me during my 7-year trip in Québec and Sherbrooke to attain my Master and Doctor's degrees. She is the one who has endured my sudden mood swings when things at work were deviating from the plan. She didn't help me push the boat, but she surely made this trip a lot more enjoyable. For all that, I will be eternally thankful and I hope she will stay in my boat for what we could henceforth call an unending trip, that will be pursued in Vancouver.

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