Expression of a functional receptor for IL-17E on structural cells of the airways

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Abstract

Asthma is a common inflammatory airway disease affecting over 2.5-3 million Canadians. This disease involves a Th2-driven immune response. Th2-type cytokines, (interleukin (IL)-4, IL-5, and IL-13) are upregulated in human asthmatics and in animals models of allergic airway inflammation. A recently characterized cytokine, IL-17E (IL-25), expressed *in vitro* by Th2 lymphocytes, macrophages and mast cells, generates a Th2 phenotype in mice. Overexpression of IL-17E promotes asthma-like features in animal models, namely, elevated levels of IL-4, IL-5 and IL-13, increased airway hyperreactivity, eosinophilia, increased IgE, airway epithelial cell hypertrophy/hyperplasia, and airway mucus hypersecretion. IL-17E binds the interleukin-17B receptor (IL-17BR), which is expressed in a variety of tissues and is upregulated under inflammatory conditions. We have investigated the expression of this receptor on structural cells of the airways in addition to assessing the effect of IL-17E on these cells. We hypothesized that airway smooth muscle cells (ASMC), lung epithelial cells and lung fibroblasts express IL-17BR and that proinflammatory cytokines could modulate its expression. The three cell types constitutively express IL-17BR mRNA and protein, and its expression was consistently upregulated by the proinflammatory cytokine TNFα. We also observed that the Th1 type cytokine IFNγ decreased IL-17BR expression in ASMC, we observed a similar decrease by TGFβ in fibroblasts. IL-17E appears to have profibrotic properties, as it upregulates the expression of pro-collagen α1 mRNA in ASMC, this could suggest that it could increase

basement membrane thickening *in vivo*, a characteristic feature of airway remodelling. Pro-inflammatory and pro-eosinophilic cytokines such as RANTES, eotaxin, IL-8 and GM-CSF are increased in lung fibroblasts treated with IL-17E. In A549 lung epithelial cells, IL-17E also acts synergistically with TNFα to induce GROα mRNA and this effect can be inhibited by recombinant IL-17BR. In addition, IL-17E promoted the proliferation of bronchial epithelial cells isolated from biopsies taken from control and mild asthmatic donors. Collectively, our results show that the IL-17E receptor is consistutively present in structural cells of the airways and increases in the presence of TNFα. Our results also suggests a potential pro-remodelling and pro-inflammatory role for IL-17E on these cells.

Résumé

L'asthme est une maladie inflammatoire des voies respiratoires qui affecte au-delà de 2.5 à 3 millions de Canadiens. Cette condition est associée à une immunité de type Th2, le niveau de cytokines dominant sont de type Th2 (interleukin (IL)-4, IL-5, et IL-13), elles sont augmentées chez les individus asthmatiques, ainsi que chez les modèles animaux d'inflammation des voies respiratoires. IL-17E (IL-25) est une cytokine nouvellement identifiée, qui lorsque surexprimée chez des souris, favorise l'apparition d'un phénotype similaire à l'asthme humain, tels que l'augmentation de l'expression de l'IL-4, l'IL-5 et l'IL-13, une hyperreactivitée bronchique, une augmentation des IgE, une éosininophilie, une hypertrophie/hyperplasie de l'épithélium, ainsi qu'une sécretion excessive de mucus. L'IL-17E se lie au récepteur à l'IL-17B (IL-17BR), avec une affinité plus grande que l'IL-17B. Ce récepteur est exprimé par plusieurs type de tissus humains, notamment la trachée et les poumons, de plus, il est surrégulé dans un milieu inflammatoire. Nous avons investigué l'expression de ce récepteur par des cellules structurales des voies aériennes et des poumons, ainsi que l'effet de l'IL-17E sur ces cellules. L'expression de l'IL-17BR a été testée sur des cellules musculaires lisses des voies aériennes (ASMC), et sur des cellules épithéliales et fibroblastes pulmonaires. Nous avons aussi évalué l'effet de cytokines proinflamamtoires sur son niveau d'expression. Des trois types cellulaires testés, tous expriment constitutivement le transcript et la protéine de ce récepteur. De plus, son niveau est augmenté par la cytokine proinflammatoire TNFα. Au contraire, la cytokine de type Th1,

IFNγ, sousrégule IL-17BR chez les ASMC, et le TGFβ fait de même chez les fibroblastes. Nous avons noté que l'IL-17E avait des propriétés inflammatoires et de remodelage. Chez les ASMC, elle induit l'expression du collagène de type I au niveau de l'ARNm, une composante importante de la matrice extracellulaire. L'IL-17E surrégule les ARNm codant pour des cytokines pro-inflammatoires et pro-éosinophiliques tels l'éotaxine, RANTES, l'IL-8 et le GM-CSF dans des fibroblastes pulmonaires. Chez ces mêmes cellules, l'IL-17E crée une synergie en combinaison avec le TNFα, causant ainsi une forte augmentation de la relâche de GM-CSF. Nous notons aussi une synergie avec le TNFα dans l'expression de la chimiokine pro-neutrophilique GROα dans une lignée cellulaire épithéliale des poumons (A549). De plus, l'IL-17E semble promouvoir la prolifération de cellules épithéliales bronchiques isolées à patir de biopsies obtenues de donneurs contrôles et d'asthmatiques légers. Dans leur ensemble, nos résultats démontrent donc l'expression constitutive d'un récepteur pour la cytokine de type Th2, IL-17E, dans trois types de cellules structurales des poumons. Le niveau d'expression de ce récepteur est aussi augmenté par le TNFα. Nos résultats suggèrent aussi un rôle pro-inflammatoire et de remodelage potentiel pour l'IL-17E.

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List of abbreviations

aa:	amino acid
AHR:	airway hyperresponsiveness
ASMC:	airway smooth muscle cell
BAL:	bronchoalveolar lavage
BrdU:	bromodeoxyuridine
bp:	base pair
CCR:	CC chemokine receptor
cDNA:	complementary DNA
Ci:	Curie
DMSO:	dimethyl sulfoxide
DPBS:	Dulbecco's phosphate-buffered saline
ECL:	enhanced chemiluminescence
ECM:	extracellular matrix
ERK:	extracellular regulated kinase
Evi27:	viral integration site # 27
FEV1:	forced expiratory volume in 1 second
G-CSF:	granulocyte-colony stimulating factor
GM-CSF:	granulocyte/monocyte-colony stimulating factor
GROα:	growth-related oncogene-α
IFNγ:	interferon-γ
IL:	interleukin
IL-17BR:	interleukin-17B receptor
IL-17Rh1:	interleukin-17 receptor homologue 1
JNK:	c-JUN terminal kinase
kb:	kilobase
kDa:	kilodalton
MAPK:	mitogen activated protein kinase
MBP:	major basic protein
NF-κB:	nuclear factor-κB

MCP:	monocyte chemotactic protein
PC_{20}:	provocative concentration of a bronchoconstrictor that causes a 20% drop in FEV1
PDGF-bb:	platelet-derived growth factor-bb
PBMC:	peripheral blood mononuclear cells
PMA:	phorbol myristate acetate
RANTES:	regulated upon activation, normal T cells expressed and secreted
rh:	recombinant human
RT:	room teperature
TGFβ:	transforming growth factor-β
Th:	T helper
TNFα:	tumor necrosis factor-α
TRAF:	TNF receptor associated factor
3'-UTR:	3' unstranslated region
5'-UTR:	5'-unstranslated region

Chapter 1 - Introduction - Literature Review

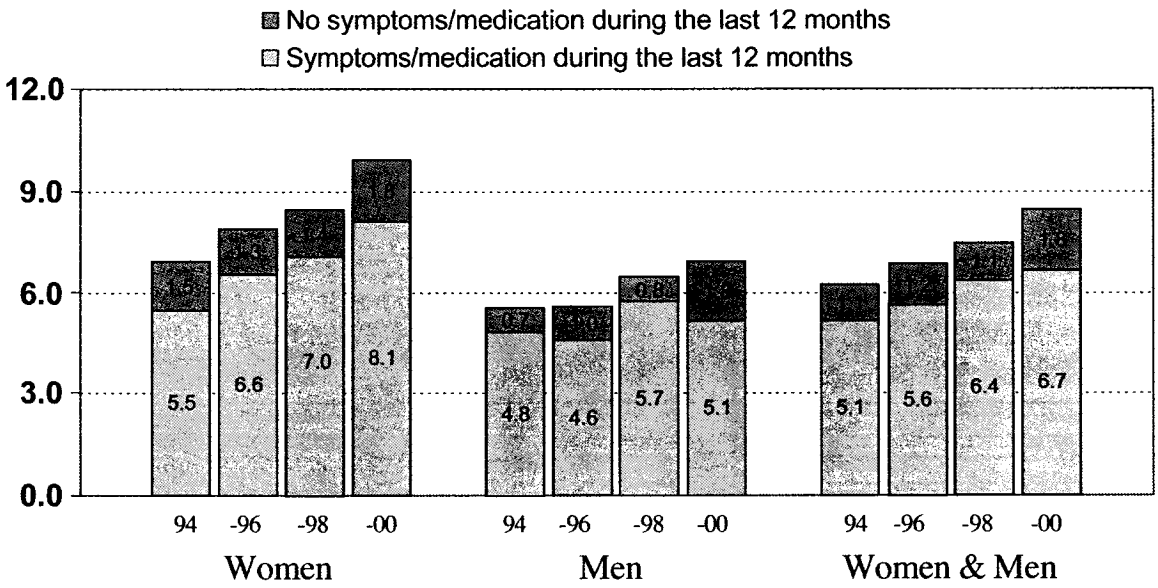
1.1 Asthma history

The first description of asthma goes back to Greek antiquity. Asthma is derived from the Greek verb *aazein*, which means to exhale with an open mouth, to pant, to gasp [1]. The first written account of this word was found in the Iliad written by Homer 2700 years ago. It is likely that it was then used to describe breathing symptoms rather than a disease in itself. British physician and asthma sufferer Sir John Floyer (1649-1734) also provided a detailed account of asthma symptoms, treatment and prevention in his *Treatise of Asthma* of 1698. He had also noted that there were several factors exacerbating asthma symptoms such as cold air, exercise and stress. It was only during the 19th century that the British physician Henry Hide Salter (1823-1871) described this condition in *On Asthma: its Pathology and Treatment* published in 1868. He was the first to give a definition of asthma to the medical community, describing it as a: "*paroxysmal dyspnoea of a peculiar character, generally periodic with intervals of healthy respiration between the attacks*" [2]. Interestingly, he discovered that strong black coffee could alleviate asthma symptoms, which later on lead to the discovery of theophylline, a molecule with bronchodilating activity and a similar chemical structure to caffeine.

1.2 Asthma prevalence

Despite our increasing understanding of this disease and efficacious treatment options, the number of reported cases of asthma in the last two to three decades has increased worldwide, especially in children. Currently about 2.5-3 million Canadians have a

diagnosis of asthma (8% in adults and 12% in children) and its prevalence it increasing (Fig 1). The general trend is of increased deaths and hospitalizations from the disease in all the industrialized countries of the world. Although genetic risk factors have been identified [3,4] and asthma certainly has a hereditary component [5-9], mutations and modifications of human genes that would increase susceptibility are likely to take more than 20-30 years to occur. Life style and environmental factors are now thought to be very important in the recent increased prevalence of asthma; indeed urbanization and pollution have been linked to increases in asthma cases worldwide [10,11]. The Hygiene hypothesis has been proposed as an explanation for the rising cases of asthma in developed countries. This hypothesis suggests that the increased prevalence is caused by use of antibiotics, vaccination and improved hygiene [7,12].



Source : Centre de prévention et de contrôle des maladies chroniques, Santé Canada, d'après les données de l'Enquête nationale sur la santé de la population, Statistique Canada.

Fig 1. Prevalence of asthma in adults (12 years old +) from 1994 to 2000 in Canada (http://www.phac-aspc.gc.ca/ccdpc-cpcmc/crd-mrc/facts_asthma_f.html).

1.3 Asthma definition

Asthma is a complex disease because of its mutlifactorial etiology . Allergic (atopic) and non-allergic (non-atopic) asthma have been described [13]. Atopic asthma, also refered to as extrinsic asthma, is characterized by elevated levels of circulating IgE and positive skin tests to common allergens such as pollen, dander, house dust mite, cockroach antigen, etc. In non-atopic asthma, also referred to as intrinsic asthma, individuals show a negative skin test and have normal levels of IgE. Non-atopic asthma usually develops later in life with more severe symptoms.

On the whole, asthma is a chronic disorder of the airways characterized by reversible airflow obstruction, airway inflammation and airway hyperresponsiveness (AHR). The combination of airway inflammation and tightening of the airway smooth muscle (bronchoconstriction) results in narrowing of the bronchi leading to the classical symptoms of expiratory wheeze, chest tightness, shortness of breath and cough. These symptoms are usually reversible either spontaneously or with treatment. A variety of environmental risk factors as well genetic predispositions combine to promote the development of the disease [14]. Several single nucleotide polymorphisms (SNPs) have been associated with asthma or asthma-associated traits such as hyperresponsiveness or atopy [15]. Asthma affects approximately 8% of the adult population and up to 20% of children in North America, Europe and Australia [16]. The severity of the disease is highly variable among asthmatics ranging from mild disease with infrequent symptoms to severe debilitating disease with daily symptoms and daily requirements for oral corticosteroid therapy. Severe asthmatics comprise 10% or less of the asthmatic population, but are responsible for a disproportionately high fraction of the health care budget. The impact of

severe disease or poorly controlled disease on the individual's quality of life is enormous with high rates of absenteeism from school or work. Asthma can also be fatal and Canadian statistics show that approximately 20 children and 500 adults die each year in this country from asthma (10 deaths per week). However, with adequate treatment most of these deaths can be prevented.

1.3.1 Reversible airflow obstruction

Airflow limitation in asthma is initially completely reversible. Airflow obstruction is responsible for recurrent episodes of wheeze, breathlessness, chest tightness and coughing. Spirometry (spirometry is a pulmonary function test that measures the volume of air inspired or expired as a function of time) provides an objective assessment of airflow limitation and is important in staging asthma severity. Classic spirometry measures the maximal expired volume during a forced expiration. During asthma exacerbation or once chronic airflow limitation is insalled, the forced expiratory volume in 1 second (FEV1) is usually decreased, FEV measures the amount of air a person can exhale during a forced breath. The forced vital capacity (FVC), which is the total amount of air exhaled during the FEV test, is generally normal and therefore the FEV1/FVC ratio is decreased, consistent with airflow obstruction. FEV1 is considered a central measure for classifying asthma severity and correlates well with airway diameter. In fact, it gives more information about disease status than physical examination or symptoms reported by the patient. The elevated expression of certain genes in asthmatics also correlate with lower FEV1 values, such IL-5 [17] and its receptor [18], a disintegrin and metalloproteinase domain (ADAM) 33 or the GM-CSF receptor [19].

Reversible airflow obstruction is demonstrated by a reduced FEV1 value (relative to the predicted value for the subject's height, age, weight and gender), which reverses following use the administration of an inhaled β2 agonist (bronchodilator). An increase in FEV1 of ≥12% following the administration of an inhaled β2 agonist is required to demonstrate reversibility. However in some individuals with asthma, airflow obstruction is only partially reversible and indeed with further disease progression, may become irreversible and is associated with permanent structural changes in the airways known as airway remodelling. Patients with severe airflow obstruction may need a short course of oral steroid therapy before they can demonstrate reversibility. In some cases a diagnosis of asthma may still be suspected even when spirometry results are normal as the nature of airflow limitation in patients is often periodic and variable, and FEV1 values can thus be normal at the time it is measured. In such cases, the diagnosis may be established by peak flow measurements or by bronchial provocation. Peak expiratory flow rates (PEFRs) are generally measured and recorded by patients themselves as an indication of asthma control or to demonstrate reversibility of airflow obstruction.

1.3.2 Airway hyperresponsiveness (AHR)

Asthmatic subjects have hyperresponsive airways, meaning that their bronchi react too quickly and too forcefully to a given stimulus. Bronchoprovocation is a diagnostic test used to assess hyperreactivity of the airways. A bronchoconstrictor (histamine or methacholine) is aerosolized into the airways at incremental concentrations. A diagnosis of asthma is made if the FEV1 drops by at least 20% from baseline following an inhaled

dose of bronchoconstrictor. The dose required to cause a 20% or more fall in FEV1 is known as the PC_{20} (provocative concentration 20%).

The exercise challenge test is another common bronchoprovocation test, which is used to establish a diagnosis in subjects whose asthma, is predominantly triggered by exercise but who have normal resting spirometry. The patient performs spirometry and then exercises, usually on a treadmill or exercise cycle. After the patient exercises, spirometry is repeated. This may be done several times, immediately after exercise and periodically, until there is a drop in the FEV1 greater than 20% or until 30 minutes have elapsed.

1.3.3 Inflammation

Airway inflammation correlates with the development of airway hyperesponsiveness (AHR) and airflow limitation. T cells and eosinophils play a primordial role in the onset and maintenance of airway inflammation. Eosinophilic inflammation has long been considered a hallmark of asthma [20,21]. However, infiltration of neutrophils into the airways has recently been considered to be a potential contributor to the inflammation present in the disease. Indeed neutrophilic inflammation in the airway has been demonstrated in several studies during exacerbations of asthma and in *status asthmaticus* [22-26]. *Status asthmaticus* is an intense and prolonged asthma attack that is unresponsive to normal bronchodilating treatment and often requires hospitalization. It has been suggested that the phenotype of 'severe asthma' comprises at least two different and distinct pathologic subtypes based on the level of eosinophils in the bronchoalveolar lavage (BAL) fluid. The subtype with eosinophil predominance is associated with increased basement membrane thickening compared to the other subtype that has little to no eosinophils compared to mild and moderate asthmatics[25]. Physiologically, however,

the two subtypes of severe asthmatics behave similarly, although the eosinophil negative group showed a slightly lower FEV1 [25]. Increased numbers of neutrophils have also been reported in the BAL, endobronchial and transbronchial biopsy specimens of subjects with refractory asthma [25,26] compared with those with milder disease, indicating that there is ongoing inflammation in the airways of severe asthmatics with poor airway function despite very high doses of oral and inhaled steroids [27]. Neutrophil numbers are similar in the two subgroups of severe asthma [25].

1.4 Asthma pathogenesis

Asthma can be described in macroscopic and microscopic terms. Morphologically, the lungs of individuals that have died of asthma are hyperinflated due to blockage of the small airways, formation of mucus plugs and thickening of the airway wall. At the cellular or microscopic level, the most notable changes are epithelial desquamation, enlargement of the smooth muscle mass area, thickening of the subepithelial layer due to enhanced matrix deposition, increased numbers of fibroblasts, and infiltration of inflammatory cells, notably T lymphocytes and eosinophils [28].

Clinically, the pathogenesis of asthma is characterized with increased airway hyperresponsiveness (AHR) and airflow obstruction, this is manifested through symptoms of wheezing, breathlessness and coughing. Individuals with asthma have hyperresponsive airways that narrow or constrict too much in response to a nonspecific stimulus. It is thought that several factors are involved in the development of AHR, although it is still unclear as to what causes this, though it has been shown that the degree of airway inflammation correlates with AHR [29]. However, there is some debate about

that, some studies show that the degree of airway inflammation (number of inflammatory cells, especially eosinophils) does not match the severity of the disease as assessed by FEV_1 and AHR measurements [30]. Furthermore, an anti-IL-5 monoclonal antibody (mAb) treatment of asthmatic patients reduced blood and sputum eosinophil numbers without affecting their AHR [31]. Interestingly, patients with eosinophilic bronchitis develop an eosinophil-dominated airway inflammation, yet show no evidence of asthma symptoms (AHR) [32]. Genetic factors might also explain some differences between control and asthmatic airways, where the asthmatic smooth muscle (ASM) has a different intrinsic phenotype, with increased contractility and force generated compared to non-asthmatic ASM. Indeed, single smooth muscle cells taken from asthmatic biopsies were shown individually to contract more quickly and contain a greater amount myosin light chain kinase, a protein associated with the contractile apparatus [33], but this is not always the case [34,35]. Moreover, smooth muscle from asthmatics have increased force generation more force per cross-sectional area compared to from non-asthmatics, in addition to greater shortening [36].

The inflammatory nature of asthma has been recognized for almost a century [37]. Tissues obtained from autopsies as well as bronchocospic biopsies consistently demonstrated airway mucosal inflammation in asthmatics. The existing view is that inflammation leads to alteration of the structural components of the airways [38,39]. On the other hand, there is the possibility that inflammation and structural changes are events that occur in parallel rather than sequentially [30,38]. In lung transplantation, asthma and AHR can be transferred to a normal recipient receiving the lungs of a donor that had mild asthma, however, asthmatic recipients of non-asthmatic lungs do not develop the disease

[40]. Disease developed in recipients of "asthmatic lungs", despite being treated with the potent immunosuppressant cyclosporin and oral corticosteroids.

Another pathological observation in asthma is the apparent evidence of epithelial damage and fragility [41] this may explain the increased shedding of epithelial cells found in sputum and BAL samples [42,43]. Although epithelial fragility and abnormal expression of proliferation markers might be authentic observations [44,45], epithelial desquamation has been debated as an artifact of tissue manipulation [46,47]. Mucus hypersecretion is characteristic of asthma pathology (Fig. 2) and is produced by goblet cells that are part of the airway epithelium as well as by submucosal glands. Excessive mucus secretions that completely plug the airways have been found post-mortem in individuals who died of asthma. In asthma of varying severity, the number mucus secreting cells are increased, goblet cells undergo hyperplasia and submucosal mucous glands show signs of hypertrophy [46,48-50].

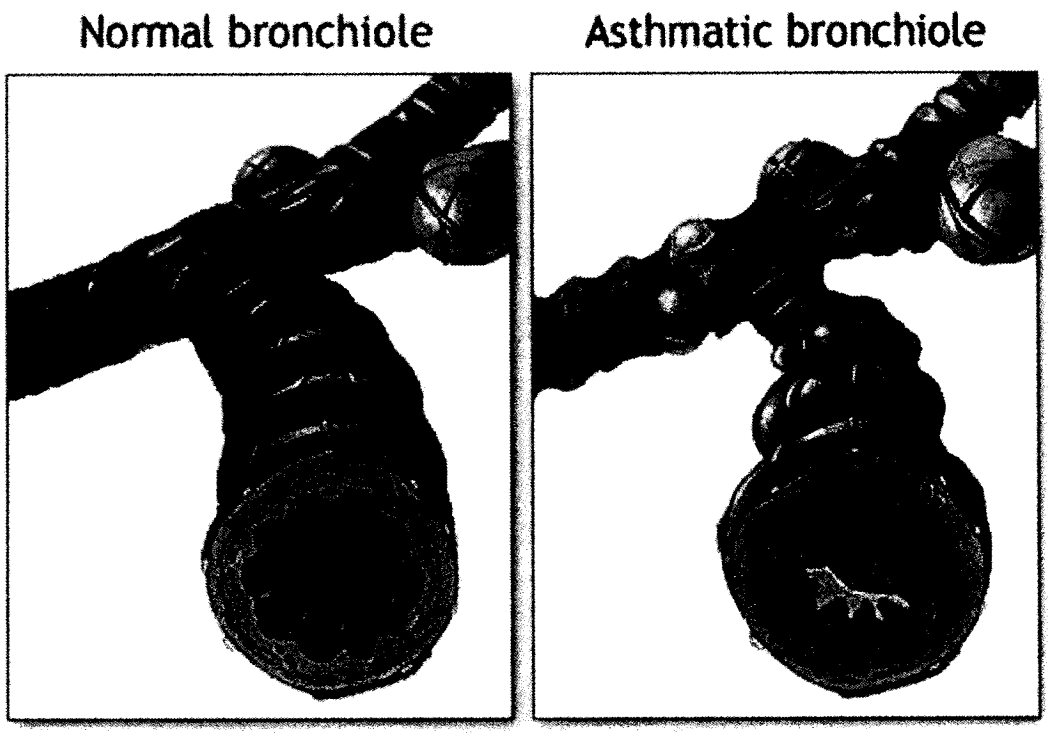


Fig 2. Schematic representation of a normal versus an asthmatic bronchiole. The airway smooth muscle excessively contracts, thus contracting the asthmatic bronchiole. The thickened airway wall and excessive mucus, contribute to narrowing of the lumen and airflow limitation (www.nlm.nih.gov/medlineplus/ency/imagepages/19346.htm).

As mentioned earlier, it is thought that asthmatic airways that are subjected to chronic and prolonged inflammation can develop structural changes in the airway wall that can eventually become with time, irreversible [51]. Theses changes are jointly referred to as airway remodelling [51]. Remodelling is thought to arise from the interaction of infiltrated inflammatory cells with structural cells, principally through the action of cytokines. Possibly, the remodelling of tissues in the airways is an attempt by structural cells to repair damage caused by the local inflammation [52 39]. In addition to what happens to the

epithelium, the structural changes that take place involve the thickening of the airway wall. This is due to an increase in smooth muscle mass, increased number of fibroblasts/myofibroblasts, deposition of extracellular matrix in the basement membrane and increased vascularization [39]. This thickening, in addition to increased mucus, leads to a narrowing of the lumen diameter and causes restricted airflow. Severe asthmatics that suffer from persistent airflow limitation show greater airway wall thickening than those that have no airflow obstruction [53].

1.5 Inflammatory cells in asthma

It is well known that there is an influx of inflammatory cells in asthmatics airways; this is observed in biopsy samples from humans and also in mice models of allergic airway inflammation. These cells infiltrate the mucosa and come into contact with all the structural cells that compose the airways. Several different leukocyte populations infiltrate the airway tissue; indeed, depending on disease severity and individuals, mast cells, macrophages and neutrophils can infiltrate the airway wall. However, CD4+ T helper 2 (Th2) lymphocytes, eosinophils and mast cells are the predominant cell types found in asthmatic airways. They are considered the central effector cells of this disease. These cells produce many different inflammatory cytokines, and express adhesion molecules, which initiate and maintain local airway inflammation in the airways.

1.5.1 CD4+ T cells

CD4+ T helper cells coordinate inflammation by producing several type of mediators. The concept of a T helper 1 (Th1) or T helper 2 (Th2) immune response originates from findings published two decades ago in a landmark paper that identified two subtypes of

mouse T helper cells [54]. Th1 or Th2 cells are identified by the profile of cytokine they secrete, moreover, these T cell subsets promote different types of immune responses. Th1 cells produce IFNγ, IL-2, IL-12 and IL-18; these are necessary for cell-mediated immunity and protection against intracellular pathogens. A Th2 response is necessary for antibody-mediated immunity against extracellular pathogens such as parasites. Th2 cells secrete preferentially IL-4, IL-5 and IL-13 [55]. These cytokines are known to promote mucus production IgE class switch and eosinophilia.

The importance of the Th2 lymphocyte for the development of airway hyperresponsiveness, mucus production and eosinophilia was demonstrated using animal models of allergic airway disease [56,57]. Mucus hypersecretion and eosinophilia is observed after allergen challenge, in mice receiving ovalbumin-specific Th2 but not Th1 cells [58]. In fact, Th1 cells inhibit these events [59]. Mucus secretion is still inducible by IL-4 -/- and IL-5-/- Th2 cells to the same extent as regular Th2 cells, but absolutely requires the presence of IL-4Rα [60]. IL-13 necessitates IL-4Rα for its signaling and has been shown to be critical for mucus production [61]. While mucus hypersecretion and eosinophilia are triggered only by Th2 cells transfer, both Th1 and Th2 can drive airway inflammation and AHR [62].

1.5.2 Eosinophils

Eosinophils, first named by Paul Erlich in 1879 because of intense eosin staining, are bilobar-nucleated cells that contain distinctive granules in their cytoplasm. The content of these granules are released upon cell activation or during cell necrosis. These granules are packed with highly basic and cytotoxic proteins, and also contain enzymes destined to

inflict oxidative damage to their targets. Eosinophils have long been attributed a central function in the pathogenesis of asthma because of their role in airway inflammation and remodelling. The presence of these in asthmatics was established early on, in 1860 the British physician Henry Salter identified binucleated cells in the sputum of his asthmatic patients, these were later identified to be eosinophils. Eighty-two years later, Huber and Kossler reported that individuals who had died of asthma had eosinophilic infiltration [37]. The relevance of this cell in allergic airway disease has been assessed by two groups that generated mice devoid of eosinophils, however their findings diverge [63,64]. In one type of eosinophil-deficient mice, Humbles *et al.* showed that allergen challenge still induced mucus hypersecretion and airway hyperesponsiveness, however these mice exhibited lesser changes associated with airway remodelling [63]. Notably, after allergen challenge they showed less subepithelial thickening, collagen deposition, fewer proliferative ASM cells and a lower total ASM cell count than wild-type mice [63]. Interestingly, eosinophil-negative severe asthmatics showed much less subbasement membrane thickness than eosinohpil-positive asthmatics [25]. These findings by Humbles *et al.* also concord with observations made in both mice and men where IL-5 was targeted. IL-5 is a crucial cytokine needed for eosinophil growth and differentiation. Mice which were either IL-5 deficient or treated with an anti-IL-5 antibody to deplete eosinophils [65,66], and asthmatics subjects were intravenously infused with a humanized anti-IL-5 monoclonal antibody [31,67]. The mice showed a reduction in subepithelial fibrosis [65,66] and smooth muscle mass thickness [66]. The asthmatic patients showed a decrease in the deposition of certain ECM proteins in the subepithelial membrane [67] but no improvement in airway hyperesponsiveness or FEV1 [31]. It is important to mention that in these patients, there

was a significant number of eosinophils remaining in the bronchial tissue even after 3 months of treatment with the anti-IL-5 mAb [31]. In contrast to these findings, the second type of eosinophil-deficient mice developed significantly less mucus production/goblet cell metaplasia and AHR after allergen challenge [64]. Comparable results were obtained in other animal models targeting IL-5, in that they did not develop AHR following challenge [68,69] and were protected against airway remodelling [68]. In asthma, the eosinophil could also serve as an antigen-presenting cell [70] as it can directly interact with Th2 lymphocytes to promote their expansion and the release of IL-4, IL-5 and IL-13 through the costimulatory molecules CD80 (B7.1) and CD86 (B7.2) [71,72].

1.5.3 Neutrophils

Neutrophils are also known as polymorphonucleated leukocytes because of their segmented nucleus that contains 3-5 connected lobules. They are the most abundant of circulating white blood cells, and they are the first responders in the early phases of an inflammatory response. They have a granule-filled cytoplasm and these granules do not stain for hematoxylin or eosin, thus differentiating them from basophils and eosinophils. The neutrophils granules contain degradative enzymes (lysozyme, elastase and collagenase). These cells are equipped to destroy pathogens, but can also damage surrounding host tissue. Their role in asthma is not clearly understood, though as was discussed earlier in section 1.3.3, it is suggested that there are two subsets of asthma, one having a predominance of eosinophils, and the other neutrophils [73]. Although there is a preponderance of literature exposing the presence of eosinophils in asthmatic bronchial tissue, several studies have shown the presence of increased neutrophils in the airway

mucosa, BAL and sputum of severe asthmatics or those undergoing asthma exacerbations [22,24,25,74]. Neutrophils are also particularly increased in biopsies from chronic obstructive pulmonary disease (COPD) patients [75,76].

1.5.4 Mast cells

Also discovered by Paul Erlich, mast cells have unsegmented oval-shaped nuclei and contain several cytoplasmic granules. They also show characteristic thin protuberances of their plasma membrane. Mast cell precursors migrate from the bone marrow to various tissues such as the skin and airways. They participate in the innate and adaptive immune responses. Two types of mast cells exist, those that produce tryptase only (MC_T) and those that make tryptase, chymase, carboxylpeptidase and cathepsin G (MC_{TC}). Mast cells express the high affinity immunoglobulin E receptor (FceRI), cross-linking of bound IgE by antigen induces a series of complex intracellular signaling events that activatess the mastocyte, and are thought to promote the release of granule content (eg.: histamine), cytokine gene expression (eg.: TNFα, IL-6, IL-13, IL-17E) and the production of lipid mediators (eg.: prostaglandin D_2, leukotriene C_4) [77-80].

In asthma, a greater number of mast cells are detected within the airway smooth muscle, in bronchial epithelial brushings and in BAL fluid [81-83]. However some have failed to see a difference in mast cells numbers in bronchial biopsies between asthmatics and controls [84,85].

1.5.5 Basophils

Erlich first described this cell type as one that presented with cell structures stained by basic dyes such as hematoxylin. Like mast cells, basophils carry the FceRI and release histamine, however unlike mastocytes, which are found in tissue, they are almost solely present in peripheral blood and account for about 1% of circulating leukocytes. Basophils can infiltrate tissue at sites of inflammation. They are found in higher number in asthmatic airway mucosal tissue than in non-asthmatics, and increase after allergen challenge [86-88]. Moreover, these tissue-inflitrated basophils express IL-4 mRNA [87].

1.5.6 Monocytes/Macrophages

Macrophages and their blood precursors, monocytes, serve critical roles in host defense. They are an integral component of the innate immunity and are active during adaptive immunity. There are several types of lung tissue resident macrophages present in health; including peritoneal, pleural and alveolar macrophages, while, monocytes can be recruited to sites of inflammation. Some have reported an increase in macrophage infiltration in the bronchial mucosa of asthmatics compared to controls [89,90]. Macrophages in asthma are apparently more activated and show less apoptosis [91,92]. This cell type is particularly associated with other airway diseases such as COPD [75,76].

1.6 Cytokines in asthma

1.6.1 Th2 cytokines

Certain diseases are characterized as being either Th1- or Th2-dominant based on the type of cytokines observed in the tissues and/or biological fluids. Asthma is considered a Th2-type inflammatory disease because the levels of the Th2 cytokines IL-4, IL-5 and IL-

13 in the bronchial mucosa, blood, bronchoalveolar lavage (BAL) fluid and sputum of asthmatic patients are often increased [18,93-104]. Interestingly, these interleukins (eg.: IL-4, IL-5, IL-13) along with other genes (eg.: GM-CSF) coding for cytokines that may be important in asthma pathogenesis are located on chromosome 5q. CD4+ Th2 lymphocytes are thought to be the principal source of these cytokines in asthma [96,100].

Cytokine	Main function	Status in asthma
Th2 type		
IL-4	Th2 cell differentiation, IgE synthesis	increased
IL-5	eosinophil maturation	increased
IL-13	eosinophil and mast cell activation, IgE synthesis	increased
Th1 type		
IFNγ	inhibits IgE synthesis and IgE receptor expression	decreased, neutral or increased
Proinflammatory		
TNFα	induces expression of cytokines, and adhesion molecules	increased
IL-1β	induces expression of cytokines, and adhesion molecules	increased
Chemokines		
IL-8	chemoattractant for neutrophils	increased
RANTES	chemoattractant for eosinophils	increased
Eotaxin	chemoattractant for eosinophils	increased
Others		
IL-11	pro-fibrotic	increased
GM-CSF	supports eosinophil survival	increased
TGFβ	pro-fibrotic	increased
IL-6	B and T cell activation, enhance Th2 proliferation	increased

Table 1. List of some cytokines involved in asthma pathogenesis

IL-4

Most IL-4 comes from CD4+ as well as CD8+ T cells, but is also found in eosinophils [105,106], mast cells [106,107] and basophils. Principally, it targets T cells to promote Th2 differentiation and B cells to induce IgE antibody production. Although T cells are

though to be the principal source of IL-4 in asthma [98], it also found in smooth muscle-infiltrated mast cells [79]. Inhalation of IL-4 by patients with mild asthma results in an increase in AHR and eosinophil influx [108].

IL-5

Much like IL-4, CD4+, CD8+ T cells, eosinophils and mast cells secrete IL-5 [106];, airway epithelial cells can also be a source of this cytokine [109]. IL-5 is thought to be one of the most important mediators responsible for the eosinophilia observed in asthmatics [110,111]. Its level correlates with the number of eosinophils in the bronchial mucosa of asthmatics [94]. Human trials using an anti-IL-5 mAb show a marked decrease in blood and sputum eosinophils [31].

IL-13

IL-13 comes in large part from CD4+ Th2 lymphocytes but has been detected in mast cells, basophils and eosinophils. It targets B cells as well as endothelial cells to induce vascular cell adhesion molecule (VCAM)-1 expression, and epithelial cells and fibroblasts to promote mucus secretion and eotaxin release, respectively. Akin to what was mentioned about IL-4, while IL-13 is largely detected in T cells in asthma [98], it also found in smooth muscle-infiltrated mast cells [79].

1.7.2 Th1 cytokines

While the majority of studies find elevated Th2-type cytokines in asthmatics, findings are not as consistent regarding levels of Th1 type cytokines in asthma. Some suggest that

asthma is driven by a mixed Th1/Th2 response as the levels of typical Th1 cytokines can even be increased in asthmatics and have deleterious effects on asthma-like symptoms in murine models [112]. Nevertheless, several studies point to the inhibitory potential of Th1 cytokines on asthma-like features in mice models of allergic airway inflammation [113,114] and find them downregulated in asthmatic airways [99,115].

IFNγ

IFNγ is primarily secreted by Th1 cells and natural killer (NK) cells. IFNγ inhibits IgE isotype switching and antagonizes IL-4-mediated expression of low affinity IgE receptors. It also increases the production of IL-12, another key Th1 cytokine. Increased IFNγ in asthmatic airways might come from a viral infection, which not only exacerbate asthma, but also enhance IFNγ production [116]. On the other hand, decreased IFNγ is possibly the result of preferential apoptosis of IFNγ-producing T cells compared to IL-4-producing ones [117]. The role of IFNγ in asthma pathogenesis is likely to be a complex one; as reflected by the observation that in asthmatics, the levels of this prototypical Th1 cytokine can be either unchanged [103,118], upregulated [103,119,120] or downregulated [102,121 115]. It might, however, be beneficial to asthma pathogenesis in humans; a 3-week treatment of mild asthmatics with nebulized recombinant IFNγ reduced the amount of airway eosinophils in the BAL (in 4 out of 6 patients) without promoting the influx of other inflammatory cells [122]. In mice models of acute airway inflammation, mixed results have been observed. A number of studies show that IFNγ-producing T cells can inhibit allergen-induced eosinophilia, mucus production and AHR [59,123,124] or conversely, enhance airway inflammation and AHR [121,125,126].

1.6.3 Proinflammatory cytokines

TNFα and IL-1β

As asthma is an inflammatory disease, it is also characterized by increased levels of proinflammatory cytokines like TNFα and IL-1β [127-129] that correlate with disease severity[130]. The primary source of TNFα are macrophages, but mast cells, eosinophils, neutrophils, T cells and structural cells also secrete it. TNFα is upregulated in the BAL of asthmatics who are challenged with allergen and correlates with an increase in their bronchial hyperresponsiveness [131,132]. TNFα acts by inducing the production of many cytokines, chemokines and adhesion molecules that serve to attract, activate and enhance the survival of leukocytes. Indeed, healthy and mild asthmatics that inhaled recombinant TNFα showed increased bronchial hyperresponsiveness and increased eosinophils and neutrophils in the sputum [133,134]. Treating severe asthmatic patients with a recombinant soluble TNF receptor (etanercept) significantly improved their symptoms, FEV1 and PC_{20} values [130,135].

1.6.4 Chemokines

Chemokines are a subgroup of the larger cytokine family. They are small (8-12 kDa) secreted proteins with chemotactic properties primarily for leukocytes. Hematopoietic cells are the source for most chemokines, although they are now being identified in structural cells both *in vivo* and *in vitro*. The 40+ chemokines identified so far fall into four main groups based on the position of conserved cysteine residues. However, the majority belong to the CC or CXC class of chemokines. The CC chemokines have two

adjacent cysteines and are involved in the chemotaxis of eosinophils and monocytes. The CXC chemokines share two cysteines separated by a single amino acid (X), and this group contains members that attract neutrophils and lymphocytes. Chemokines serve other roles besides chemoattraction: they have been shown to have angiogenic or angiostatic effects, can promote degranulation of leukocytes and mediator release.

IL-8 (CXCL8)

IL-8 is a member of the CXC chemokine family and is an important chemotattractant for neutrophils. It is produced by macrophages, eosinophils, lymphocytes as well as neutrophils themselves [136,137]. In addition, IL-8 is also detected in macrophages, epithelial and airway smooth muscle cells from asthmatic bronchial biopsies [138,139]. IL-8 is though to be a marker of asthma severity, as it is especially elevated in severe asthmatics and not in mild asthmatics [138]. In patients with acute asthma exacerbation, neutrophils are predominantly detected and this parallels high IL-8 levels in sputum [22].

RANTES (CCL5)

Regulated upon activation, normal T cells expressed and secreted (RANTES), a member of the CC chemokine family, is chemotactic for eosinophils, T cells as well as monocytes [140-142]. Originally identified in T cells [143], fibroblasts, bronchial epithelial cells and smooth muscle cells express this chemokine in bronchial biopsies [138,144,145]. Several reports show that in asthmatics, RANTES protein is increased in BAL [146] as well as RANTES mRNA in bronchial mucosa [145,147]. However, one report notes no difference in RANTES mRNA in the BAL or RANTES protein expression in bronchial biopsies from

non-asthmatic and asthmatic subjects, but shows an increase in RANTES mRNA in bronchial biopsies from asthmatics [144].

Eotaxin (CCL11)

Eotaxin is a CC chemokine involved in the recruitment of eosinophils from the circulation into the tissues [148]. Its expression is increased in the asthmatic bronchial mucosa [145] and BAL and correlates with disease severity [138]. It is expressed by the bronchial epithelium as well as by airway smooth muscle and infiltrated macrophages [138,145,149].

1.6.5 Other cytokines

IL-11

IL-11 is a member of the IL-6-like family and predominantly secreted by stromal cells. It is overexpressed in asthma [150,151] and is found in the epithelium and subepithelium as well as in eosinophils [151]. IL-11 increases with disease severity and correlates inversely with FEV1 [151]. IL-11 is also associated with collagen I and III deposition in asthmatics [150] and promotes subepithelial fibrosis when overexpressed in murine airways [152-154].

TGFβ

There are more than 30 members in the TGF superfamily. Within this superfamily is the TGFβ family. Three isoforms have been identified in humans, β1, β2, and β3 [155], though several studies only refer to TFGβ without specifying the isoform. TGFβ is

released from inflammatory cells (T cells, eosinophils) and bronchial epithelial cells, fibroblasts and smooth muscle cells. TGFβ is involved in the repair response through its action on matrix deposition and remodelling. Moreover, TGFβ-producing T cells are classified as a distinct subset called Th3 or Treg (T regulatory) because they have anti-inflammatory properties. Indeed, T cells engineered to overexpress TGFβ abolish airway eosinophilia and AHR in mice models [156].

The biological activity of TGFβ1 *in vitro* includes myofibroblasts differentiation and increased production of collagen molecules and other extracellular matrix proteins and therefore could participate in the subepithelial fibrosis and in the increase in the number of fibroblasts/myofibroblasts seen in asthma. TGFβ is upregulated in the bronchial submucosa [150,157-159] and BAL [160] of asthmatic patients, and eosinophils appear to be the main source of this cytokine in the airways [157-159].

GM-CSF

In the context of asthma, granulocyte/monocyte-colony stimulating factor (GM-CSF) is the most studied of the three colony-stimulating factors (GM-CSF, Granulocyte-CSF and Monocyte-CSF). It comes mainly from T lymphocytes and macrophages, though it has been shown that endothelial and epithelial cells, fibroblasts and airway smooth muscle cells can synthesize this growth factor *in vitro* and *in vivo* [129,161-165]. One of its functions is to promote the survival of eosinophils and neutrophils [166,167]. It is increased in the BAL [96] and bronchial mucosa [147] from asthmatic airways and is higher in symptomatic versus non-symptomatic asthmatics [118].

IL-6

IL-6 is produced by monocytes, T and B lymphocytes as well as by airway structural cells. IL-6 is viewed as a pleiotropic cytokine because of its many effects. It promotes B cell maturation, T cell activation and proliferation, has anti-tumor effects and can activate neutrophils [168-170]. Moreover, it can promote Th2 polarization by inducing the initial production of IL-4 in CD4+ T cells [171]. IL-6 also induces several acute-phase proteins (C-reactive protein, fibrinogen, etc) synthesis by hepatocytes and contributes to the systemic effects of inflammation. Its synthesis is strongly induced by IL-1β and TNFα. Its overexpression in airway epithelial cells in mice promotes lymphocytic cell influx but reduces AHR [172]. Its expression is increased in the bronchial epithelium of asthmatics and in the BAL of symptomatic asthmatic individuals [128,173].

1.7 Airway remodelling

As mentioned previously, airway remodelling is a concept that encompasses several structural changes within the asthmatic airways. These changes occur mostly in the large airways, although the small airways are also affected in more severe cases of asthma. Microscopically, these structural changes manifest themselves through the altered behavior of the structural cell types that compose the airways. In the remodeled asthmatic airway, the airway smooth muscle cells, fibroblasts, endothelial cells and the epithelium undergo noticeable changes due to intrinsic and/or extrinsic factors. Asthmatics have a thickened airway wall, and, patients with severe asthma show a greater thickening than those with mild asthma [174,175] and thicker airway walls (Fig. 2) enhance airway narrowing during bronchoprovocation.

The bronchial epithelium is subject to structural changes in asthma. Several studies have shown that the epithelium demonstrates signs of damage, inflammation (increased cytokine production) and a deregulated proliferative process [44,55,176,177]. The phenomenon of epithelial shedding in asthma has been the subject of some dispute recently [47,49]. There is also a noticeable increase in the number of mucus-producing goblet cells in the epithelium of asthmatics [178]. Mucous glands are more prominent and enlarged in individuals with fatal asthma [179]. The synthetic ability of epithelial cells in asthma is increased, the bronchial epithelium has been shown to generate more cytokines with proinflammatory and/or profibrotic properties as well as chemokines that attract T lymphocytes, eosinophils and neutrophils to the airway mucosa [144,180].

Increased interstitial deposition of extracellular matrix proteins (ECM) such as collagen type II and V and fibronectin promotes the thickening of the basement membrane [181,182]. Activated fibroblasts are likely the source of the ECM, and their number is increased in the subepithelial layer in asthmatic airways [183]. Because the thickening occurs below the epithelium and involves increased production of fibrotic material, it is also referred to as subepithelial fibrosis. This phenomenon is often observed in cases of fatal asthma [184-186], but also in asthmatics with mild disease [182]. Increase in smooth muscle cell mass and thickness is a typical structural change associated with remodelling [36,187] (Fig. 3). This fact was first reported in postmortem examination of patients that had died of asthma [37,179]: their smooth muscle area was greatly enlarged in both the large and small airways. The increased mass is thought to occur primarily through hyperplasia and/or hypertrophy of the smooth muscle cells [34,188,189].

The increase in smooth muscle also appears to correlate with disease severity [138,188] and a decrease in FEV1 [138]. The chronic inflammation associated with asthma is thought to be in part responsible for the initiation and perpetuation of airway remodelling or at least happens concomitantly to it [190]. However, manifestations of tissue remodelling can happen independently of the presence of markers of allergic inflammation (ie: without concurrent eosinophilic infiltration) [191]. For a long time, airways inflammation was primarily linked with infiltrated leukocytes, but we now know that structural cells are not solely passive responders to the inflammatory stimuli. Importantly, the synthetic ability (eg.: cytokine and chemokine production) of airway smooth muscle cells could make them a significant source of cytokines and chemokines in asthmatic airways [192]. *In vitro*, airway smooth muscle cells have a strong synthetic and secretory potential, as they can produce large amounts of cytokines involved in inflammation and remodelling. While, most of the findings in this area have been done using cultured cells from airway biopsies the majority being from control, non-asthmatic individuals, they expose a new dimension to the functions of these cells *in vivo*. The details of the synthetic ability of ASMC, lung fibroblasts and epithelium will be discussed in later sections.

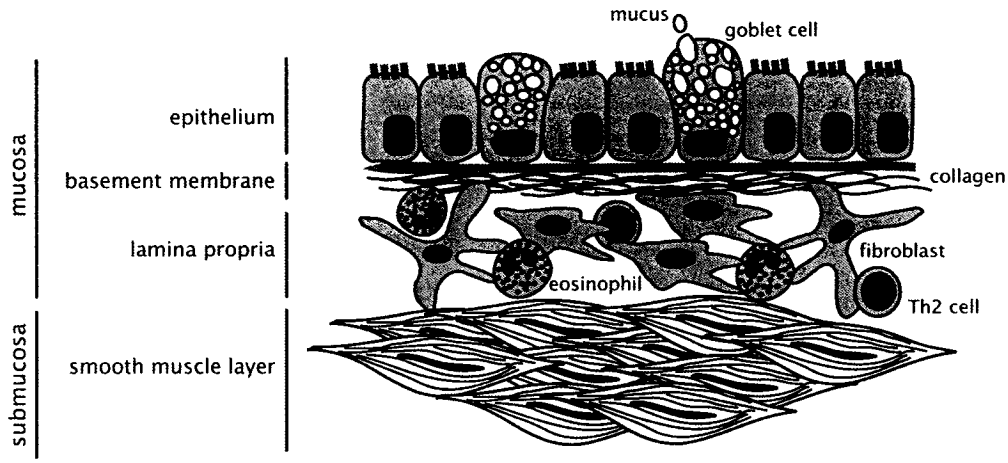


Fig.3. Asthmatic airways show evidence of inflammation and tissue remodelling. Characteristic changes are: increased number of mucus producing-goblet cells, increased deposition of collagen that thickens the basememt membrane, augmentation of fibroblasts numbers, the presence of infiltrated inflammatory cells such as eosinophils, mast cells and T cells as well as an increase in airways smooth muscle mass.

1.8 The Airway Smooth Muscle Cell

1.8.1 Structure of the ASMC

The airway smooth muscle (ASM) is an elongated cell and its cytoplasm filled with myofilaments. These filaments are composed of actin and myosin and are the primary components of the contractile apparatus [193]. Cells are grouped in bundles and are physically attached to the extracellular matrix. ASMC are connected to each other via desmosomes (intermediate junctions) and by transmembrane glycoproteins (cadherins) that attach to the cytoskeleton. The smooth muscle bundles are innervated with parasympathetic nerve fibers. Immune cells such as mastocytes can be found within the smooth muscle layer [79,81]. In the bronchus wall, the ASM encircles the airways as a broken circle, expect in the trachea where it is only present in the posterior wall and referred to as the trachealis muscle.

1.8.2 Function of the ASMC

The airway smooth muscel cell is responsible for contracting the bronchi, this narrows the lumen and restricts airflow. In a treatise published in 1868, the British physician Henry Hyde Salter described many symptoms of asthma including bronchospams [2]. The pathological role of the ASM in asthma is evident given its role in bronchoconstriction. In normal airways, ASM contraction and relaxation regulates the diameter of the airways and thus the passage of air. Recently ASMC have been shown to secrete a wide range of cytokines and chemokines as well as extracellular matrix components [194].

1.8.3 Basis of ASM contraction

Smooth muscle cell contraction is regulated by intracellular calcium. An increase in intracellular calcium by a contractile agonist activates the contractile machinery. This increase can either come from intracellular stores or from outside the cell, brought inside by calcium channels. Elevation of free calcium within the cell activates the calcium/calmodulin-sensitive myosin light chain kinase (MLCK); this leads to phosphorylation of the regulatory 20 kDa light chain of smooth muscle myosin (MLC_{20}) located on the myosin head. This promotes the ATPase activity of the myosin heavy chain head, which can now form cross-bridges with actin filaments and induce cell contraction. Relaxation is induced by the MLC phosphatase that dephosphorylates the MLC_{20} and terminates cross-bridge cycling. Certain cytokines can affect the contractile response of smooth muscle cells and/or decrease its relaxation. TNFα can affect the contractility of ASM in isolated bronchial strips by enhancing the response to the spasmogen acetylcholine (ACh) [195]. While it cannot modify calcium concentration on its own, TNFα increases the calcium concentration values of known contractile agonists such as bradikynin and carbachol [196]. The Th2 cytokines IL-5 and IL-13 increase the contractile response to Ach and carbachol in isolated tracheas [197,198]. On the other hand, they decrease the relaxation induced by isoprotenerol [197-200]. This effect on smooth muscle relaxation is also seen with TNFα in isolated tracheas [201,202], but not in cultured human ASMC [203].

1.8.4 Basis of ASM proliferation

Hyperplasia of smooth muscle in asthmatic airways is one of the means by which the ASM content is thought to increase in asthma. An increase in cell number implies for the

most part an increase in cell proliferation and/or a decrease in apoptosis. In animal models of airway inflammation, the ASM has been shown to be more proliferative and show a decrease in apoptosis [204-206]. The observation of *in vivo* ASM proliferation in bronchial biopsies of asthmatics is not as clear [34,187,207].

Most of the studies looking at the mechanisms behind human ASM proliferation were performed *in vitro*. Several growth factors, contractile agonists and lipid mediators directly induce an increase in ASMC division. These include growth factors such as platelet-derived growth factor (PDGF) isoforms AB and BB, epidermal growth factor (EGF), insulin-like growth factor (IGF) and basic fibroblast growth factor (bFGF) [208]. Other molecules induce ASMC proliferation, such as thrombin, tryptase, histamine, endothelin-1 and serotonin [208-213]. The lipid mediators thromboxane A_2, and leukotriene D_4 promote proliferation [214], while prostaglandin E_2 (PGE_2) inhibits growth; this effect is associated with an increase in cyclooxygenase-2 (COX-2) expression [215,216]. Cytokines also act on ASMC division. For example, the profibrotic cytokine transforming growth factor (TGF)-β1 is known to induce ASMC proliferation [217,218]. The effect of TNFα on ASMC proliferation is considered modest, though several report none to little effect [219,220]. TNFα has been shown to promote some ASMC proliferation [221] at low doses [220] but has no effect at higher concentrations [220]. TNFα also inhibits the effect of several mitogens on cell growth [220], possibly because TNFα can also induce COX-2 expression as well as PGE_2 [222-224]. IL-1β, which also a positive inducer of COX-2 and PGE_2 [222-224], has a negative effect on ASM proliferation [225]. Interestingly, the Th1-type cytokine IFNγ [226] and the Th2-type cytokine IL-4 [227] have both been shown to inhibit mitogen-induced

proliferation of human ASMC. Conversely, IL-4 and IL-13 induced rat aortic smooth muscle cells proliferation, but this was inhibited by IFNγ [228].

1.8.5 The ASMC in the pathogenesis of asthma

A key finding in remodeled asthmatic airways is that the ASM content is increased and correlates with disease severity. Indeed, Huber and Koessler first reported increase in ASM mass in 1922 [36,37,229]. They had shown airway cross-sections where the muscle mass was greater in cases of fatal asthma when compared to individuals that had died of other causes. In more recent times, Ebina *et al.* [189] performed a 3D morphometric study of airway smooth muscle in airways of fatal asthma cases and showed ASM hyperplasia. In nonfatal asthmatics, Woodruff *et al.* [34] and Benayoun *et al.* [187,188] measured increased ASM content. While Woodruff *et al.* [34] reported that hyperplasia and not hypertrophy was responsible for the increased ASM mass, Benayoun *et al.* [188] noted that ASMC in asthmatic biopsies were hypertrophied, an observation that was particularly salient in severe patients, but show no evidence of proliferation. The pathological effects of increased ASM mass could be attributed to augmenting normal muscle contraction, or induction of excessive and abnormal shortening in addition to taking up more space in the airways. The mechanisms that lead to increased ASM mass in asthmatics airways is not fully understood. Hyperplasia due to increased cell proliferation and/or decreased apoptosis is likely, but actually, only a few reports show this *in vivo*. Importantly, Roth *et al.* have shown that cultured airway smooth muscle cells from asthmatics proliferate more than cells from non-asthmatics [230]. Recently, *in vitro* studies using human ASMC have shown that they can migrate when exposed to a chemotactic stimulus [208,231-233].

Additionally, it has been shown in a murine model that fibrocytes can infiltrate the airway mucosa after allergen challenge [234]. These fibrocytes are CD34 and collagen I positive: CD34 is a marker of hematopoeitic cells, while collagen I is seen in mesenchymal-type cells, such as fibroblasts or smooth muscle cells. Thus cell migration and fibrocyte influx have also been raised as other possible mechanisms that could participate in increased ASM mass [234]. In recent times, the ASMC has been studied beyond its capacity as purely a contractile cell, indeed, ASMC can secrete several types of inflammatory mediators that are likely to participate in asthma pathogenesis [235].

1.8.6 The ASMC as a proinflammatory cell

For a long time the airway smooth muscle (ASM) was considered a purely contractile tissue responding to various substances inducing contraction, however, our perception of this cell is rapidly changing [235]. In the last decade new experimental evidence, particularly *in vitro*, has shown that this cell is capable of synthesizing a wide range of cytokines, chemokines and prostanoids as well as expressing receptors for many of them [192]. Airway inflammation has typically been associated with airway infiltrated T lymphocytes, eosinophils, neutrophils, mast cells and macrophages and the inflammatory mediators they release. However, many of the same mediators of inflammation can also be synthesized to varying degrees by ASMC, though the relative importance of ASMC-derived cytokines *in vivo* is not fully known. Furthermore, in human airway biopsies, ASM have been shown to express certain cytokines and chemokines.

1.8.7 Cytokine and chemokine production by ASMC

In vivo

Reports of cultured ASMC producing chemokines and cytokines are plentiful. This phenomenon does not appear to be due to the effect of cell culturing, indeed, some papers have demonstrated that the smooth muscle in biopsies from human bronchial mucosa stain positively for several cytokines and chemokines. For example, i*n vivo* the ASM express RANTES [138,144] eotaxin [138,149], IL-8 [138], monocyte chemotactic family (MCP)-1 [236], as well as TGFβ1 [237].

In vitro

Cultured ASMC express many cytokines at baseline and their expression can be increased or decreased by a diverse array of stimuli. Hakonarson *et al.* [198] stimulated ASMC with human serum from non-atopic, non-asthmatic subjects and showed that expression for IL-2, GM-CSF, IFNγ, IL-5 and IL-10 was detectable, although at very low level. However when ASMC were treated with serum from atopic asthmatics, cytokine mRNA levels were strongly increased for IL-2, IL-5, IL-10, IL-12, GM-CSF and IFNγ. IL-5 and IFNγ protein were also increased by atopic asthmatic serum. IL-5 protein could also be released by stimulation with IL-1β, IL-10, IL-13 or IgE [197,198]. IL-4 mRNA is undetected in ASMC in several experimental conditions [197,198], although it detected the supernatant after IgE treatment [238]. Human ASMC also synthesize IL-13, which is expressed at baseline and readily upregulated by IgE [197,238].

Proinflammatory cytokines such as TNFα and IL-1β are known to promote the synthesis of many cytokines/chemokines. For example, they stimulate the release of IL-6,

IL-8, GM-CSF and RANTES in ASMC. Moreover, IL-17A induces IL-6 and IL-8 and is synergistic when added to TNFα [164,239]. IL-1β and TGFβ1 can increase IL-11 production, while combining TNFα+IL-1β also induces IL-1β and IL-6 mRNA. ASMC also secrete TGFβ that can act in an autocrine fashion on collagen I synthesis [240].

ASMC release several types of chemokines. In asthma, some chemokines are increased, such as eotaxin, thymus- and activation-regulated chemokines (TARC), regulated upon activation T cell expressed and secreted (RANTES), IL-8, and the monocyte chemotactic protein (MCP) family [144]. These chemokines are also produced by cultured ASMC [149,225,241-244]. Eotaxin, a ligand for CCR3, has received particular attention because it is one of the most important eosinophil chemoattractant. In ASMC, production of eotaxin is increased by oncostatin M (OSM) [243], IL-4 and IL-13 [245], by TNFα, IL-1β [149] and IgE [238]. IL-9 has a positive effect on eotaxin release: Gounni *et al.* reported that IL-9 alone was enough to induce eotaxin release [246], while others have shown that IL-9 had no effect on its own but could enhance IL-13-mediated eotaxin secretion [247]. RANTES, another eosinophil chemoattractant, is also increased in ASMC by TNFα and IL-1β, but not by IFNγ. However the combination of TNFα and IFNγ provided a synergistic effect on RANTES secretion from ASMC [241,248]. ASMC also produce significant amounts of TARC, it selectively promotes Th2 lymphocyte migration by binding to CCR4, which is primarily expressed by Th2 cells and not found on Th1 cells. Although little TARC is present at baseline and further stimulation with IL-4, IL-13, TNFα or IFNγ does not stimulate its release from ASMC, it is grealy induced in cells treated with TNFα in combination with either IL-4 or IL-13[244]. IL-8 is probably the most potent chemotactic mediator for neutrophils and can act on eosinophil chemotaxix

through the endothelium and epithelium [249]. IL-1β and TNFα are potent inducers of IL-8 release, on the other hand, IL-4, IL-10 and IL-13 inhibit its synthesis [250]. TGFβ can also stimulate IL-8 production in ASMC [251].

1.8.8 Cytokine and chemokine receptors expressed by ASMC

The action of cytokines is totally dependent on the presence of their cognate receptors on responding cells. ASMC can respond to several Th1, Th2 and inflammatory cytokines. In one of the first papers on the subject, Hakonarson *et al.* showed the presence of that certain cytokines receptors on ASMC. Some were constitutively expressed at baseline, while others were undetected, but increased after appropriate cell stimulation [198]. The IL-2R, IL-5R, GM-CSFR and IFNγR mRNA were upregulated by atopic asthmatic serum. IL-2R, IL-4R, IL-5R, GM-CSFR, IFNγR protein were also increased. IL-12R was undetected at the mRNA or protein levels. ASMC constitutively express the mRNA for IL-4Rα, as well as IL-13Rα1 and none to very little of the common γ chain [198,252]. This γ chain is an essential component of the receptors for several type of cytokines (eg.: IL-2, IL-4, IL-9, IL-13, IL-15). Ligation of these by IL-4 or IL-13 causes signal transducer and activator of transcription (STAT)1, STAT6 and ERK1/2 phosphorylation [198,252]. Smooth muscle cells also respond to IL-9 by phosphorylation of ERK [247] and release of eotaxin [246,247] through the constitutive expression of IL-9Rα [246].

ASMC also express receptors for profibrotic cytokines. They carry the three TGFβ receptors (TGFβRI, TGFβRII, and TGFβRIII) [253] as well as receptors for the IL-6 family of cytokines: there is baseline expression of IL-6R, gp130, IL-11R and OSM receptor mRNA [254]. Function of these receptors was verified by stimulation with their

ligands and assessment of downstream signaling molecule activation. STAT3 was phosphorylated in response to IL-6, IL-11 and OSM [254]. As mentioned earlier, ASMC respond very well to TNFα, these cells carry both TNFα receptors, TNFR1 and TNFR2. TNFR1 seems more abundant and is necessary for TNFα-induced activation of NF-κB and expression of IL-6 and RANTES [255]. TNFα also induces the activation of the ERK and p38 pathways through TNFR1 [221]. Recently, a functional IL-17R has been characterized on ASMC [256], IL-17A binds this receptor and promotes the phosphorylation of MAPK and the release of several chemokines, including IL-8 [239,256,257].

Recently, CCR3, a receptor for eotaxin was found in cultured ASMC as well as by human smooth muscle *in vivo*. CCR3 mediates an eotaxin-dependent chemotaxis of ASMC in a Boyden chamber assay [231].

1.9 Bronchial epithelial cells

1.9.1 Structure of the bronchial epithelium

The epithelium covers the airway mucosa and within the epithelium, several cell types are found. There are ciliated cells, secretory cells and basal cells. In the upper airways, almost 80% of the epithelium is comprised of ciliated cells with a columnar shape. They are covered with microvilli on their apical surface. Interspaced through the epithelium are secretory cells, which are are much less abundant than their ciliated counterparts. Among them we find goblet cells, which are metabolically active and contain large granules filled with mucins [49]. Throughout the large airways there are submucosal glands that are lined by mucous and serous cells. Basal cells are smaller in size and are found between the basolateral surface of columnar cells and the basement membrane [258] and serve to anchor the epithelium to the basement membrane.

Different types of adhesion complexes hold epithelial cells together [258]. Tight junctions, at the apical surface, seal adjacent columnar cells and prevent the passage of molecules or ions between them. Adherens junctions hold the epithelium together; these are made from cadherins and catenins. Desmosomes, also composed of cadherins, serve to bind every type of epithelial cells tightly together, while hemidesmosomes connect basal cells to the extracellular matrix of the basement membrane [258].

1.9.2 Function of the bronchial epithelium

The airway epithelium serves several physiological functions and its constituent comprised of several cell types, each having particular functions. The epithelium forms a protective barrier between the external environment and the internal components of the

bronchi and lungs. A protective layer of mucus that humidifies the inhaled air and prevents desiccation covers the airways epithelium [48,259]. The ciliated epithelial cells are responsible for mucocilliary clearance of pathogens and debris trapped in the mucus [48,259]. The goblet cells compose 10% of the normal epithelium, but can become much more abundant in chronically irritated airways and lead to increased mucus secretions [49,50]. Mucus secretions are made up of 90% water and 10% of proteins, carbohydrate and lipids. The proteins in mucus include mucins, a group of heavily glycosylated proteins, that are 50%-90% carbohydrate by weight [259]. Mucins are exceptionally large molecules ranging from 3 to 23 million daltons. So far, 19 different mucin genes (MUC) have been identified in humans [48]. In the airway lumen, mucus is mixed with inflammatory cells and sloughed epithelial cells and can enhance airway narrowing and thus airflow limitation [48].

The airway epithelium is far from being only a structural entity; epithelial cells have very potent secretory abilities. They express cytokines, chemokines, lipid mediators, adhesion molecules, ECM proteins and gases [260]. Some of these are constitutively expressed by the airway epithelium, while others need the action of a specific stimulus to be detected [260].

1.9.3 The bronchial epithelial cell in the pathogenesis of asthma

In asthma, the bronchial epithelium is subject to both structural and functional changes. Indeed, bronchial epithelial damage and denudation have been shown in early post-mortem studies of asthmatics, and later using biopsy samples of the airways analyzed by light and electron microscopy [261]. Epithelial damage and desquamation in

asthmatic airways has been long-held as an important pathological feature of this disease. This phenomenon may be associated with disease severity: some authors have observed more epithelial disruption in more severe cases of asthma [262]. Amin *et al.* found that there was no difference in epithelial integrity between control subjects and non-atopic asthmatics, whereas atopic asthmatics showed much greater epithelial damage [55]. Another group showed epithelial damage in both atopic and non-atopic asthmatics compared to controls [261]. Also degree of epithelial integrity might also not be associated with asthma severity, mild asthmatic showed less epithelium than individuals with severe disease [188]. However, this could be attributed to the effect of corticosteroids treatment on increased epithelial survival and proliferation [176]. Certain researchers have attributed the occurrence of epithelial desquamation as being an artifact caused by tissue handling and/or processing [46,47,49].

Interestingly, a greater number of activated eosinophils and T lymphocytes are found in areas of epithelial damage in asthmatic bronchial biopsies [55]. Activated eosinophils release potent cytotoxic proteins such as major basic protein (MBP) or eosinophil cationic protein (ECP), which have been shown to cause direct damage to bronchial epithelial cells *in vitro* [263].

Activated T cells release TNFα and IFNγ. TNFα, can induce pro-apoptotic processes in epithelial cells [264]. Also, IFNγ is known to increase TNFα responsiveness by upregulating the TNF receptor [265]. Cultured bronchial epithelial cells exposed to T cells and eosinophils undergo significant apoptosis, and this is effectively inhibited using blocking antibodies to TNFα and IFNγ [263]. This finding was correlated with biopsy data showing increased apoptosis as assessed by TUNEL (Terminal deoxynucleotidyl

Transferase Biotin-dUTP Nick End Labeling) staining in the bronchial epithelial cells of mild asthmatics. Other experimental studies have shown that the amount of desmosomes is decreased in bronchial epithelial cell cultures exposed to TNFα and IFNγ [266]. Desmosomes are cellular structures critical for cell-to-cell adhesion [266], and are decreased in asthmatic bronchial tissue [261]. This is thought to facilitate epithelial cell detachment. The asthmatic epithelium is subject to other structural changes associated with remodelling, such as an increase in goblet cell numbers [261]. This occurs through hyperplasia [178,267] and also metaplasia [268,269] of columnar epithelial cells into mucus-secreting cells [178,267].

Deregulated expression of proliferative and apoptotic markers is a well-documented aspect of bronchial epithelial cell biology in asthma [45,176]. Vignola *et al.* did not detect differences in epithelial apoptosis between control and asthmatic subjects [44]. However, they have shown that the epithelium of asthmatics expresses more of the anti-apoptotic protein Bcl-2 (B-cell lymphoma 2) than normal epithelium, thus possibly enhancing its survival. Staining for proliferating cell nuclear antigen (PCNA) showed similar scores for the epithelium in control and untreated asthmatics. However, markers of epithelial survival (Bcl-2) and proliferation (PCNA) of corticosteroid-treated asthmatics were significantly higher than in the epithelium from control or untreated asthmatics. Proliferation of the bronchial epithelium is increased in allergen-challenged asthmatics (mild atopic) and correlates with the number of activated eosinophils [270].

1.9.4 Cytokine and chemokine production by epithelial cells

<u>In vivo</u>

Many different cytokines are detected in the bronchial epithelium, and their expression can be increased or decreased in asthma. The epithelium of asthmatic airways shows increased expression of the CD4+ T cell chemotactic cytokine IL-16, the profibrotic cytokines IL-6, IL-11 and TGFβ1, the chemokines eotaxin, RANTES, stem cell factor (SCF), TARC, monocyte chemotactic protein (MCP)- 3 and -4 and IL-8, and the growth factor GM-CSF [55,145,151,271-275], but shows decreased expression of IL-18 [276]. Certain cytokine and chemokine receptors are increased in asthma in the bronchial epithelium. This is the case for the IL-4Rα which is increased in atopic asthmatics [277], the stem cell factor (SCF) receptor, c-kit, and IL-9Rα are also increased in asthmatic epithelium [101,275,278].

In vitro

Cultured bronchial epithelial cells express a large range of cytokines and chemokines. They release eosinophil chemotactic proteins such as RANTES and eotaxin and the neutrophil chemoattractants GROα and IL-8. Epithelial cells are also a source of cytokines such as IL-11 and TGF-β1 and colony-stimulating factors like G-CSF and GM-CSF. Cytokine production in several cell types including epithelial cells is potently inhibited by dexamethasone, a very potent synthetic corticosteroid often used in *in vitro* experiments.

Like ASMC, epithelial cells are an important source of eotaxin; its expression can be induced by TNFα, IL-4 and IL-13 [279,280] as well as by *in vitro* rhinovirus infection [281]. They also synthesize RANTES which is increased by TNFα, IL-1β and IFNγ [282,283]. Moreover infection of epithelial cultures with rhinovirus, influenza A or the respiratory syncytial virus (RSV), a common cause of respiratory infection in infants and children,

causes RANTES synthesis [284-286]. RANTES is also detected in the sputum of children undergoing a viral asthma exacerbation [287]. This suggests an active role for RANTES in viral defense as it is chemotactic for leukocytes and promotes their recruitment to sites of inlammation. MCP-4 is upregulated in epithelial cells after treatment with IL-4, TNFα, and IL-1β [279]. Epithelial cells also appear to have a basal expression of TARC which, unlike in ASMC, is upregulated by IL-4 and IL-13, though much greater levels are obtained when these cytokines are combined with either TNFα or IFNγ [272].

GM-CSF is thought to contribute to the survival of eosinophils and neutrophils within the mucosa [165-167,288]. Indeed, GM-CSF produced in the supernatant of bronchial epithelial cells prolongs the survival of cultured eosinophils and neutrophils [167]. Cultured epithelial cells produce constitutive GM-CSF [161] and its expression is upregulated by proinflammatory cytokines and respiratory viruses such as RSV, but this upregulation is corticosteroid sensitive. TNFα, IL-4 and dust mite allergen (*Der p*, from the house dust mite *Dermatophagoides pteronyssinus*) induce GM-CSF release [289]. IL-1β induces the gene transcription and release of GM-CSF in A549 pulmonary epithelial cells and primary bronchial epithelial cells. However, IL-4 and IL-13 inhibit its expression in A549 cells through a transcriptional mechanism [290].

Epithelial cells can also be a source of G-CSF, interestingly, G-CSF can enhance the survival and activation of neutrophils *in vivo* [167] and TNFα-induced G-CSF production by the immortalized human bronchial epithelial cell line BEAS-2B is not inhibited by dexamethasone [291]. Notably, corticosteroid treatment does not affect neutrophil numbers *in vivo* as it does with eosinophils.

Bronchial epithelial cells are a very important source of neutrophil attracting chemokines. While they produce constitutive levels of IL-8 [161,292], several types of inflammatory stimuli are potent at increasing its synthesis and release such as IL-4, IL-13, IFNγ, IL-17A, TNFα, viruses, bacteria and allergen [289 292 293 281]. Pathogens rapidly induce IL-8 synthesis in the host, and because of this, neutrophils are usually the first responders on a site of inflammation. Indeed, RSV quickly increases IL-8 mRNA synthesis by BEAS-2B [294]. Similarly, rhinovirus infection of A549 epithelial cells yields a potent upregulation of IL-8 message in as little as an hour, although in these cells, IL-8 protein levels continue to rise for as many as four days [295]. Epithelial cells produce other cytokines of the same family, namely GROα and epithelial-derived neutrophilic peptide (ENA-78), that are upregulated proinflammatory by cytokines [293] and serve to chemoattract neutrophils.

Bronchial epithelial cells constitutively express IL-6, but it is strongly upregulated by TNFα and IL-1β, but also by IL-17A and *in vitro* viral infections. IL-11, which shares several properties with IL-6 [296] is increased in epithelial cells by viruses, IL-17A, IL-1β and TGFβ [296-298]. Bronchial epithelial cells are themselves capable of expressing TGFβ [299,300] and its release is inhibited by IFNγ [300]. Cultured bronchial epithelial cells can also produce another member of the TGF family, TGFα, which is present at baseline in cells from normal and asthmatic donors. It is increased by TNFα, allergen, IL-4 and IL-13 but only in asthmatic cells [301]. TGFα binds the epithelial growth factor receptor (EGFR) and induces fibroblast proliferation as well as goblet cell differentiation.

1.9.5 Cytokine and chemokine receptors expressed by bronchial epithelial cells

Although bronchial epithelial cells are known to respond to various cytokines, direct demonstration and characterization of their putative receptors has not been systematically performed. Bronchial epithelial cells express receptors for the IL-4, IL-9, IL-13 [289,302], IL-6 [302] and IL-10 [303]. Like in ASMC, chemokine receptor characterization in bronchial epithelial cells is a recent endeavor. They express CXCR1 and CXCR2 [304] which are receptors for both IL-8 and GROα, CCR3 [305] as well as CXCR3 that binds CXCL11 (interferon-gamma inducible T cell alpha chemoattractant or I-TAC), a chemoattractant for activated T cells [306].

1.10 Lung fibroblasts

1.10.1 Structure of the lung fibroblast

Lung fibroblasts are cells of mesenchymal origin. In the airways, they are present in the connective tissue underlying the epithelial layer and are an integral structural cell. These cells are thin and elongated and form a sheath around the basement membrane [307].

1.10.2 Function of the lung fibroblast

The primary function of fibroblasts is to maintain integrity of supporting tissues by a constant slow turnover of components of the extracellular matrix; they are also associated with wound repair [307]. Indeed, active fibroblasts secrete factors that are involved in wound healing, including deposition of extracellular matrix proteins (ECM). Under physiological conditions, the ECM serves several functions: it provides structural support by acting as a scaffold, and separates the epithelium and endothelium from other

structures [308]. The ECM also affects cellular function, such as adhesion, spreading, growth and gene expression.

1.10.3 Lung fibroblasts in the pathogenesis of asthma

In asthmatic airways, ECM such as type I, III and V collagen and fibronectin derived from subepithelial fibroblasts contribute to the thickening of the subepithelial reticular layer. Increased deposition of ECM is thought to happen partly because there are more fibroblasts. Indeed the increased number of subepithelial fibroblasts in asthmatics is one of the characteristics of airway remodelling. In fact, this increase correlates with the thickness of the reticular layer [309]. However, it was shown that cultured non-asthmatic fibroblasts survived longer and proliferated more than asthmatic fibroblasts [310] . In asthma, bronchial epithelial cells are thought to interact with the underlying fibroblasts and promote their proliferation and ECM synthetic ability. Conditioned media of bovine bronchial epithelial cells enhances bronchial fibroblast proliferation and co-culturing these two cell types also increases fibroblast proliferation [311].

Certain cytokines can affect the behavior of fibroblasts by either promoting their proliferation, or their ECM synthetic ability and thus contributes to features associated with airway remodelling. Bronchial epithelial cells are a source of the potent profibrotic cytokine TGFβ1 [312]. This cytokine plays a role in the *in vitro* proliferation of human fibroblasts and the production of collagen I and III as well as fibronectin [313]. TGFβ1, 2 and 3 have been reported to be proliferative for lung fibroblasts at low concentrations but is inhibitory at higher concentrations [314], yet others have reported no effect on proliferation [311]. Furthermore, TGFβ1 enhances fibroblast growth factor (FGF)-

induced proliferation of fibroblasts by upregulating FGF receptors [315]. TGFβ1 and TGFβ3 also inhibit the action of metalloprotease-2 (MMP-2), a collagen degrading enzyme, and increase the expression of tissue inhibitor of metalloprotease (TIMP)-1 [313]. Importantly, TIMP-1 happens to be increased in the BAL of asthmatics [316], and TGFβ1 is upregulated in human asthma as well as in animal models of airway inflammation [157,160]. The proinflammatory cytokines TNFα and IL-1β, which are increased in the airways of asthmatics [128,317,318], affect the proliferation of fibroblasts and the synthesis of certain ECM. Several reports show that TNFα and IL-1β inhibit collagen I synthesis in several cell types [319-322], but can increase the production of other ECM [323]. Data from human fibroblasts show that TNFα and IL-1β can also inhibit their proliferation, however, others have found that TNFα promotes proliferation and collagen I production in mouse intestinal myofibroblasts [324]. A possible reason for this discrepancy might be in the use of human lung [323] versus murine intestinal fibroblasts [324]. Inflammatory cells are thought to interact directly with structural cells in the airways and enhance the inflammatory/remodelling response of these cells. Indeed, human lung fibroblasts co-cultured with eosinophils will synthesize more fibronectin and TIMP-1 mRNA [325] which can contribute to subepithelial fibrosis. IL-4 and IL-13 promote the proliferation of lung fibroblasts [326-328], some report that these cytokines have no profibrotic effect [327], however, IL-4 has been shown to increase collagen I in primary lung fibroblasts [329]. OSM, a member of the IL-6 family, has been shown to decrease apoptosis and increase proliferation of lung fibroblasts in addition to enhancing collagen I synthesis [330].

1.10.4 Cytokine and chemokine production by lung fibroblasts

In vivo

Most *in vivo* studies that stain human biopsy tissues to investigate expression of cytokines will assess expression in easily recognizable structural tissues such as the epithelium, smooth muscle and endothelium, although in several studies the submucosal layer is also positively stained and is the location for airway fibroblasts [236,271]. Using antibodies to both eotaxin and a fibroblast marker (Ab-1), Wenzel *et al.* demonstrated that bronchial biopsies from severe asthmatics show more eotaxin-positive fibroblasts than normal [331].

In vitro

Fibroblasts have the potential to directly participate in airway inflammation and fibrosis because they can release several types of immune mediators. Much like ASMC and bronchial epithelial cells, the majority of this type of data is derived from *in vitro* experiments. Nevertheless, this points to an active inflammatory role for these cells, which eventually could lead to the initiation and/or support of existing airway remodelling. Microarray analysis of normal human lung fibroblasts treated with the Th2 cytokine IL-13 revealed that genes like MCP-1 and IL-6 were among the most upregulated [250]. IL-6 has been shown, in a transgenic mice model, to promote subepithelial fibrosis [172]. The chemokine MCP-1, a ligand for CCR2, has been implicated in lung fibrosis, also, CCR2 -/- mice are protected against pulmonary fibrosis [332]. This phenomenon might be in part explained by the fact that CCR2 -/- airway epithelial cells suppress fibroblast proliferation to a greater extent than CCR2+/+ airway epithelial cells [333].

In response to several types of cytokines, fibroblasts can generate large amounts of chemokines like IL-8, eotaxin and RANTES that are responsible for attracting inflammatory cells to the bronchial mucosa, as well as, factors like GM-CSF [162] that support their survival within mucosal tissue. These chemokines could contribute to initiating and/or maintaining chronic inflammation in asthmatic airways. Lung fibroblasts secrete IL-8 in response to IL-17A, TNFα, and TGFβ1 [331,334,335]. In contrast, IL-13 inhibits IL-8 release in fibroblasts [331], though it enhances its secretion in normal and asthmatic bronchial epithelial cells [289]. IL-17A and TNFα have also been shown to induce IL-6 [334]. In addition, lung fibroblasts, like other airway structural cells, are important sources of eotaxin. Fibroblasts stimulated with TNFα, IL-4 and IL-13 produce eotaxin [331,336]. TNFα in combination with either IL-4 or IL-13 creates a synergistic effect on eotaxin release [336]. IFNγ has no effect alone, but suppresses TNFα-induced eotaxin [336]. Also, TGFβ1 and IL-13 together show a strong synergy on eotaxin production [331]. Collectively, it appears that inflammatory conditions in association with a Th2 milieu (IL-4, IL-13) favors the synthesis of eotaxin and thus eosinophil recruitment.

RANTES is detected at constitutive levels in cultured fibroblasts, and like most chemokines, it is strongly induced by TNFα. Stimulation with IFNγ or IL-4 has no effect on RANTES. Unlike what is seen with eotaxin, the combination of TNFα plus IL-4 does not further enhance the expression of RANTES. Instead of the inhibitory effect on TNFα-induced eotaxin, IFNγ produces a synergistic effect when combined with TNFα on RANTES synthesis and release [336]. In an unstimulated state, supernatants from human lung fibroblast have monocyte chemokinetic activity, mediated through GM-SCF, TGFβ,

MCP-1 and leukotriene B_4, but not by RANTES [337]. Though MCP-1 is produced by fibroblasts, MCP-3 or MCP-4 are undetected in the supernatant [336].

1.10.5 Cytokine and chemokine receptors expressed by lung fibroblasts

Little data is available in the literature on actual expression of particular cytokine receptors by human lung fibroblasts. Both isoforms of the TNFα receptor (TNFRI and TNFRII) are expressed on the surface of fibroblasts [338,339] and their expression is lower in fibroblasts obtained from fibrotic lungs as compared to control lungs [339]. IL-4Rα as well as both the signaling (IL-13Rα1) and the decoy IL-13 receptor (IL-13Rα2), are expressed by human nasal and lung fibroblasts [340], though only IL-13Rα2 is increased by both TNFα and IL-4 [340]. IL-17R is also present on these cells [338].

1.11 The interleukin-17 family

Interleukin-17, now called IL-17A, is the founding member of a relatively novel family of cytokines. Since its discovery, five other members of this family have been identified: IL-17B, IL-17C, IL-17D, IL-17E (IL-25) and IL-17F. Members of the IL-17 family are synthesized as monomers, and secreted as glycosylated homodimers held together by disulfide bonds (Fig. 4). All IL-17's share four conserved cysteines (Fig. 5A), necessary for the formation of a cysteine knot held together by disulfide linkages. These cysteines are thus essential for the 3D structure of the protein (Fig. 4). Interestingly, the growth factor superfamily also has a cysteine-knot conformation held by six cysteine residues, and resembles the one found in IL-17 members [341,342].

All IL-17's are located on different chromosomes (Table 2) with the exception of IL-17A and IL-17F, which are both found in tandem on human chromosome 6p12. Among the IL-17's, IL-17F shares the highest level of homology with IL-17A (55%), then IL-17B (29%), IL-17D (25%), IL-17C (23%) and IL-17E with the least similarity (17%). Some of the IL-17's might even play a role in the pathogenesis of airway and lung diseases such as nasal polyps, asthma and cystic fibrosis, in which levels of IL-17A and/or IL-17F are increased [150,297,343,344].

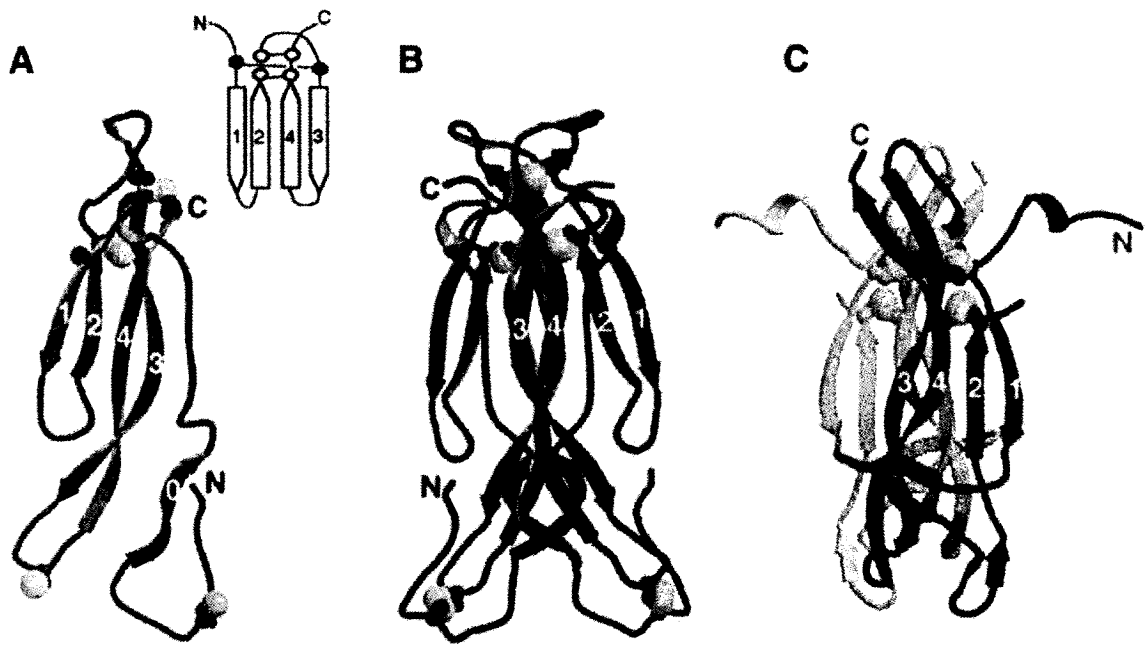


Fig.4 All members of the IL-17 family adopt a similar 3-dimensional structure because of the disulfide bonds that are created through their 4 conserved cysteine residues. (A) Structure of the IL-17F monomer. (B) Structure of the IL-17F homodimer. (C) Structure of nerve growth factor has similarities to IL-17F.

A

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IL-17A          ----------------MTPGKTSLVSLLLL--LSLEAIVKAGIT-IPRNPGCPNSEDKNF 41
IL-17B          MDWPHNLLFLLTISIFLGLGQPRSPKSKRKG-QGRPGPLAPGPHQVPLDLVSRMKPYARM 59
IL-17C          MTLLPGLLFLTWLHTCLAHHDPSLRGHPHS--HGTPHCYSAEELPLGQAPPHLLARGAKW 58
IL-17D          ---MLVAGFLLALPPSWAAGAPRAGRRPARPRGCADRPEELLEQLYGRLAAGVLSAFHHT 57
IL-17E          ------MRERPRLGEDSSLISLFLQVVAFLAMVMGTHTYSHWPSCCPSKGQDTSEELLRW 54
IL-17F          ------------MTVKTLHGPAMVKYLLLSILGLAFLSEAAARKIPKVGHTFFQKPESC 47

IL-17A          PRT--VMVNLNIHNRNTNTNPKRS----------------SDYYNRSTSPWNLHRNEDPE 83
IL-17B          EEYERNIEEMVAQLRNSSELAQRKCEVN---------LQLWMSNKRSLSPWGYSINHDPS 110
IL-17C          GQALPVALVSSLEAASHRGRHERPSATTQCPVLRPEEVLEADTHQRSISPWRYRVDTDED 118
IL-17D          LQLGPREQARNASCPAGGRPADRR--------------FRPPTNLRSVSPWAYRISYDPA 103
IL-17E          STVPVPPLEPARPNRHPESCRASE---------------DGPLNSRAISPWRYELDRDLN 99
IL-17F          PPVPGGSMKLDIGIINENQRVSMS----------------RNIESRSTSPWNYTVTWDPN 91

IL-17A          RYPSVIWEAKCRHLGCINAD--GNVDYHMNSVPIQQEILVLRRE---------PPHCPNS 132
IL-17B          RIPVDLPEARCLCLGCVNPFT-MQEDRSMVSVPVFSQVPVRRRLCPP-----PPRTGPCR 164
IL-17C          RYPQKLAFAECLCRGCIDART-GRETAALNSVRLLQSLLVLRRRPCSRDGSGLPTPGAFA 177
IL-17D          RYPRYLPEAYCLCRGCLTGLF-GEEDVRFRSAPVYMPTVVLRRTP-------ACAGGRSV 155
IL-17E          RLPQDLYHARCLCPHCVSLQTGSHMDPRGNSELLYHNQTVFYRRPCHGE---KGTHKGYC 156
IL-17F          RYPSEVVQAQCRNLGCINAQ--GKEDISMNSVPIQQETLVVRRK---------HQGCSVS 140

IL-17A          FRLEKILVSVDCTCVTPIVHHVA------------------------ 155
IL-17B          QRAVMETIAVGCTCIF------------------------------- 180
IL-17C          FHTEFIHVPVGCTCVLPRSV--------------------------- 197
IL-17D          YTEAYVTIPVGCTCVPEPEKDADSINSSIDKQGAKLLLGPNDAPAGP 202
IL-17E          LERRLYRVSLACVCVRPRVMG-------------------------- 177
IL-17F          FQLEKVLVTVGCTCVTPVIHHVQ------------------------ 163
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B

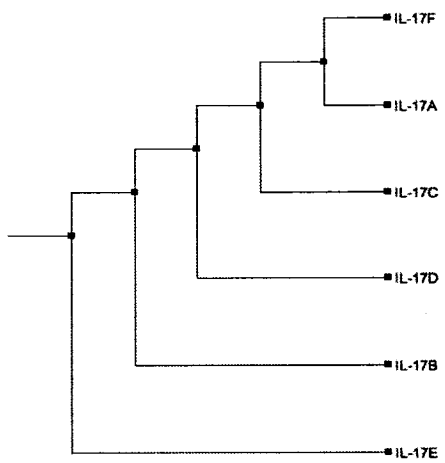

Fig. 5: Alignement of the amino acid (aa) sequences of the IL-17 family members. The four cysteine residues that form the disulide bonds are conserved in all IL-17's, they are highlighted in bold (in rectangles). Sequences were aligned using ClustalW (A). Phylogenetetic analysis of the aa sequence reveal their relatedness Tree construction was performed using the online SATCHMO method (B).

1.11.1 Interleukin-17A

This interleukin was first identified in 1993 by subtractive cDNA library screening. This library was generated using cDNA from mouse T cell of various sources. The new gene was termed cytotoxic T lymphocyte associated antigen 8 (CTLA8) as it was specifically expressed in cytotoxic T cells activated with PMA and ionomycin. Rouvier *et al.* found that the amino acid sequence of CTLA8 was 56.8% identical to the 13th open reading frame (ORF) of the T cell trophic herpervirus, *Herpesvirus saimiri*, also called HSV13 [345]. Both recombinant HSV13 and CTLA8 induce the activation of NF-kB and the secretion of IL-6 in fibroblasts. In 1995 Yao *et al.* also described IL-17A in activated CD4+ memory T cells from peripheral blood and proposed to name CTLA8 as IL-17A and HSV13 as viral IL-17 (vIL-17). The newly identified cognate receptor for IL-17A was hence referred to as IL-17R. Later, data from mice and human stimulated memory CD8+ T lymphocytes demonstrated production of IL-17A.

IL-17A is on the whole a product of activated T cells as it is noticeably absent from resting T lymphocytes. T lymphocytes are the primary source of IL-17A although it has been detected in eosinophils and neutrophils [297,346]. The biological relevance of eosinophils and neutrophils as a source of IL-17A is uncertain since the release of IL-17A in the supernatant of either has not been measured [297,346]. IL-23, a cytokine primarily secreted by dendritic cells, is a strong stimulus for IL-17A production in cultured CD4+ T lymphocytes. T cells from IL-23-deficient mice can still make IL-17A, although to a lesser extent [347]. In line with this observation, T cells stimulated *in vitro* only through the TCR with an anti-CD3 antibody secrete IL-17A without the need for exogenous IL-23 or presence of dendritic cells, although addition of IL-23 enhances IL-17A production [348].

IL-17A does not appear to be particularly associated with the Th1 or Th2 subsets of T cells as it has been found in both lineages [349-352]. Recently, two groups identified a distinct subset of CD4+ T cells that produce IL-17A [353,354]. They showed that IL-23 was critically important in the development of a newly identified T cell lineage called Th_{IL-17} (or T_H-17). Interestingly, the prototypical Th1 and Th2 cytokines (IFNγ and IL-4) inhibit the development of the Th_{IL-17} lineage.

Cytokine	Length (aa)	Size (kDa)	Chromosome	Homology to mouse	Receptor
IL-17A (CTLA8)	155	35	6p12	62	IL-17R
IL-17B	180	41	5q32-34	88	IL-17BR
IL-17C	197	40	16q24	83	unidentified
IL-17D	202	52	13q12.11	78	unidentified
IL-17E (IL-25)	161	34	14q11.2	81	IL-17BR
IL-17F (ML1)	153	44	6p12	77	IL-17R?

Table 2: IL-17 family members

1.11.2 Potential role of IL-17A in asthma

Under physiological conditions, IL-17A is present at low levels in the airways [355] but increases during inflammation or in airway diseases such as asthma [150,297], though its role in normal and asthmatic airways is still unclear. In asthma, it is likely to participate in maintaining or enhancing airway inflammation and promoting fibrosis. IL-17A is increased in asthmatic bronchial mucosa, sputum and in BAL [297,356]. In addition, blood eosinophils from asthmatics express more IL-17A protein than control subjects [297,357] and this correlates with increased airway hyperreactivity [357]. IL-17A is considered to have profibrotic properties because it can induce the release of IL-6 and IL-11 from airway fibroblasts [297] and epithelial cells [334].

Overexpression of IL-6 in murine epithelial cells of the airways causes subepithelial fibrosis and increased collagen deposition [172]. IL-11 transgenic animals share a similar

phenotype to the IL-6 transgenic mice, both develop fibrosis below the basement membrane and show increased deposition of collagen I and III. In asthmatics airways, both IL-11 and IL-17A are upregulated and their expression correlates with disease severity [150]. Their expression in control and mild asthmatic subjects are similar, whereas it is much higher in moderate-to-severe asthmatics [150]. This suggests that IL-17A is involved in a more severe form of the disease, which is characterized by neutrophilic infiltration. The presence of higher IL-11 and IL-17A levels in the airways of these patients is also associated with an increased deposition of collagen I and III [150]. Notably, a 2-week course of oral corticosteroids decreased IL-17A levels, but not collagen I or III [150]. IL-17A as well as IL-6 also appear involved in mucin production by bronchial epithelial cells *in vitro* as both can induce an increase in MUC5AC and MUC5B mRNA [358]. In these cells, IL-17A increased the MUC5B gene partly through IL-6. Interestingly, the Th2 cytokines IL-4, IL-5, IL-9 and IL-13 did not increase either mucin gene. Although there are 19 mucin genes characterized so far, MUC5AC and MUC5B are the major gel-forming mucins [267]. Moreover, there was significant increase in mucus production in the aiways of IL-17A transgenic mice [353]. Collectively these results suggest that IL-17A could participate in asthma pathogenesis through increased neutrophilic infiltration, fibrosis and mucus production.

1.11.3 Biological effect of IL-17A

IL-17A is known to induce the activation of several proinflammatory transcription factors and cytokines [359]. IL-17A activates NF-kB and AP1 as well as members of the MAPK and Jak/STAT pathways in many types of cells [360-364]. The induction of IL-6 and

IL-8 production were the first biological effects associated with IL-17A [359,365,366]. Later, IL-17A was shown to promote the expression of other neutrophil chemoattractants of the CXC chemokine family besides IL-8, such as growth related oncogene (GRO)-α and granulocyte chemotactic protein (GCP)-2 [367,368]. In human bronchial epithelial cells IL-17A induces IL-6 and IL-8 through p38 and ERK [363]. Similarly, GROα and GCP-2 release is dependent on p38 but inhibition of the ERK or the calcineurin pathways has no effect [368].

Considering the ability of IL-17A to increase neutrophil activating and chemotactic cytokines, it is no surprise that it causes a potent neutrophilia *in vivo* [367,369] and their migration *in vitro* [369], but this effect can be effectively blocked using antibodies against IL-8 or KC (murine analogue of human GROα) [367,369]. IL-17A on its own had no chemotactic abilities for neutrophils *in vitro* [369]. IL-17A also has proangiogenic properties *in vivo* and *in vitro*. *In vitro* it induces vascular endothelial growth factor (VEGF) in mouse lung fibroblasts, and chemotaxis of endothelial cells [370,371]. IL-17A promotes angiogenesis in rat cornea and enhances tumor survival through increased blood vessel formation [370,372,373]. IL-17A also promotes the release of the growth factors granulocyte-colony stimulating factor (G-CSF) and granulocyte/monocyte-colony stimulating factor (GM-CSF) [163,359,374] and can mediate granulopoeisis in mice [375].

1.11.4 Cooperation of IL-17A with other cytokines

An important aspect of IL-17A biology is its ability to synergize with other inflammatory mediators and it is suggested that the primary function of this cytokine is to amplify or enhance an existing inflammatory reaction [376,377].

The combination of IL-17A with the CD40 ligand (CD40L) [378] or with the proinflammatory cytokines TNFα, IL-1β or IFNγ can induce a potent synergistic effect with respect to the release of several cytokines, chemokines and growth factors such as IL-8, IL-6, GROα, G-CSF and GM-CSF [164,293,334,344,364,379-381], complement pathway proteins C3 and factor B [382] or expression of the adhesion molecule (ICAM)-1 [383]. Many reports attribute this synergism to increased mRNA stability of transcripts like IL-6, IL-8 and GROα [367]. In ASMC, IL-17A on its own fails to induce IL-6, however it increases the level of TNFα-induced IL-6 but not of IL-1β. In this report, the synergistic mechanism does not occur through increased transcription of the IL-6 gene, as assessed by a promoter activity assay, but through enhanced stabilization of the IL-6 mRNA transcript by IL-17A [164]. Cooperation between IL-17A and TNFα does not only occur at the post-transcriptional level, as observed with mRNA stabilization: there is evidence of enhanced gene transcription as well [380]. IL-17A can also synergize with IFNγ. Although there is less data the mechanism behind this particular synergistic effect, overall, the data shows similar results than with TNFα. Combination of IL-17A+IFNγ enhances the production of IL-6, IL-8, GROα and GM-CSF as well as ICAM-1 in bronchial epithelial cells and keratinocytes [351,352,384].

While there is plentiful evidence that IL-17A combines well with TNFα to create a potent synergistic end result, some have reported that IL-17A can antagonize the action of TNFα. IL-17A can suppress TNFα-induced CCL27 expression [385] as well as VCAM-1 surface expression [338]. Other reports show that IL-17A is effective at blocking TNFα- or IFNγ-induced RANTES expression in a variety of cell types [338,379,383,386].

1.11.5 The IL-17A receptor

The interleukin-17A receptor (IL-17R) is structurally unrelated to any other cytokine receptor. IL-17R is a type I transmembrane receptor (single pass through the cell membrane) that mediates the signaling of IL-17A and possibly IL-17F [342,387]. It was first cloned in 1995 by using HSV13 as bait for screening by flow cytometry a cDNA library expressed by a murine T cell line (EL4) [365]. Later the human IL-17R was cloned using a probe based on the murine IL-17R sequence [388]. Only very recently has the configuration of the IL-17R been studied and identified [387]. The majority of cytokine receptors belong to one of four families.

An important aspect of cytokine receptor biology is the status of the receptor molecules before ligand binding. Some receptor subunits come preassembled, while in other cases, the receptor dimerizes upon ligand binding. Recent data shows that the functional IL-17R contains at least two identical subunits preassembled in the cell membrane before IL-17A binding [387]. Affinity binding experiments show that recombinant IL-17A has a low affinity for its receptor [342,388], yet low concentrations (0.1-10 ng/ml) of IL-17A can elicit a biological response suggesting that there are maybe other subunits to the IL-17R. Knowledge of the signal transduction events that follow IL-17A binding is for the most part limited to downstream events such as the activation of NF-kB or the phoshorylation p38, JNK and ERK [239].

Work performed by Schwandner and colleagues revealed the importance of the TNF receptor-associated factor (TRAF) 6 in promulgating the IL-17A signal to NF-kB and JNK [389]. Using murine embryonic fibroblasts (MEF) deficient in TRAF6, they showed that, both IL-1β and IL-17A, but not TNFα, were unable to initiate IKK or NF-kB

activity and JNK phosphorylation. However, IL-1β and IL-17A initiated activation of NF-kB and JNK in TRAF2 -/- cells, similar to wild-type. TNFα-induced activation of NF-kB and JNK was reduced in TRAF2-/- cells. The presence TRAF6 is also required for IL-17A-mediated IL-6 production, ICAM-1 expression [389] and activation of the cytokine-induced neutrophil chemoattractant (CINC) promoter [362].

IL-17R most likely mediates the destructive effect of IL-17A in rheumatoid arthritis, a chronic inflammatory disease of the joints. IL-17A is increased in the synovial fluid of patients with rheumatoid arthritis [390-392], and promotes destruction of human articular cartilage explants [342,393]. Moreover, increased IL-17R expression is associated with this condition in humans, as cells isolated from the synovium of arthritic patients show higher levels of this receptor. To further support this, mice lacking the IL-17R show no cartilage destruction, fewer infiltrating cells and a decrease in proinflammatory cytokines [394]. The increased IL-17A levels observed in asthma and cystic fibrosis [150,297,344] could act via the IL-17R expressed in the human airways by ASM bundles [256] and the bronchial epithelium [344]. Furthermore, the airway epithelium expresses IL-17R primarily on its basolateral surface [344], possibly as the source of IL-17A comes from tissues and cells below the epithelium, such as infiltrating T cells. McAllister *et al.* cultured human bronchial epithelial cells in an air-liquid interface and treated them with IL-17A on either their apical or basolateral surface. After 24 h, the basolateral media was tested for GROα or G-CSF, and only cells treated with IL-17A on the basolateral surface showed any upregulation of these cytokines [344].

While IL-17A and its receptor are deleterious in a disease such as arthritis, they are a critical component of innate immunity. The necessity of IL-17R in host defense against

various types of pathogens is evident in IL-17R knockout animals. The lack of IL-17R has a dramatic effect on the survival of mice infected with *Klebsiella pneumoniae*, *Candida albicans* or *Toxoplasma gondii* [395-397]. Because IL-17A is a potent inducer of several neutrophil chemoattractants such as IL-8, GROα, CINC and MIP-2, IL-17R-/- animals have greatly impaired neutrophils influx in response to infection.

1.11.6 IL-17A receptor regulation

Little data is available on the *in vitro* regulation of IL-17R expression. IL-17R expression is upregulated by treatment with TNFα in colonic fibroblasts but not in human chondrocytes or synovial fibroblasts [398]. IL-17R is unaffected by IFNγ in bronchial epithelial cells or keratinocytes [351,384]. IL-17A and IL-4 also have no effect on IL-17R expression in keratinocytes [351].

1.11.7 Interleukin-17B

IL-17B has been mostly detected at the mRNA level in the pancreas, stomach and small intestines. Unlike IL-17A, IL-17E, and IL-17F, it is undetected in either resting or stimulated CD4+ T cells. IL-17B cannot induce IL-6 in human foreskin fibroblasts but promotes TNFα as well as IL-1β release from the monocytic cell line THP-1 [399].

1.11.8 Interleukin -17C

IL-17C is one of the least studied IL-17's. Similar to IL-17B, it is undetected in either resting or stimulated CD4+ T cells. Itcannot induce IL-6 in human foreskin fibroblasts

but induces both TNFa and IL-1β release from THP-1 cells [399]. Also, a human IL-17C adenoviral construct promotes neutrophilia in mice [400].

1.11.9 Interleukin-17D

Besides IL-17A, IL-17E and IL-17F, little has been published on the expression, regulation or function of the other IL-17's. IL-17D is present in several tissues, but is most strongly expressed in skeletal muscle, brain, heart and lung. Cellularly, IL-17D mRNA is detected in resting CD4+ and CD19+ cells [401]. Much like IL-17A or IL-17E, IL-17D induces the release of IL-6, IL-8 and GM-CSF by human umbilical cord endothelial cells (HUVEC) and activates NF-kB in 293 epithelial cells [401]. This suggests that IL-17A, D and E share similar intracellular signaling events. So far, a unique feature of this cytokine is that it inhibits division of myeloid progenitors *in vitro*. Granulocyte-monocyte, erythroid and multipotential progenitor cells derived from human bone marrow and treated with IL-17D showed a reduction in the formation of colony forming units (CFU) [401].

1.11.10 Interleukin-17F

IL-17F is the last IL-17 to have been identified, it also the most closely related to IL-17A in terms of amino acid sequence and biological activity [402]. Moreover both genes are located in tandem on human chromosome 6p12. IL-17F mRNA is observed in unstimulated human mast cells and in activated in Th0, Th1, Th2, PMBC and basophils [402]. It is undetected in monocytes and unstimulated CD8+ T cells, but its expression is highly induced by PMA+ionomycin in CD4+ T lymphocytes [342,402]. As with IL-17A, IL-17F promotes the secretion of IL-6 and IL-8 by human fibroblasts [342], induces IL-6, IL-8,

G-CSF, GM-CSF, GROα and MCP-1 in human primary bronchial epithelial cells [344,402,403] and can synergize with TNFα to induce greater amounts of certain of these cytokines [344]. The synergy of IL-17F with TNFα is mediated by both TNFRI and TNFRII [344]. Similar to IL-17A in the case of TNFα-induced RANTES, IL-17F can also have an inhibitory action on G-CSF and GM-CSF release induced by TNFα by lung endothelial cells [163,404]. IL-17F also increases ICAM-1 surface expression in epithelial cells [402]. The receptor for IL-17F has not been formally identified; it appears however that it can signal through IL-17R. A blocking antibody against IL-17R prevents the upregulation of G-CSF, GROα and MCP-1 [344]. While the majority of the data recorded for this cytokines have been from *in vitro* experiments, it has been shown that IL-17F is increased in certain diseases such as asthma and cystic fibrosis [344,402], thus implicating this cytokine in their pathogenesis.

1.11.11 Interleukin-17E

IL-17E, also known as IL-25 [78,400,405-408], is structurally related to the other members of the IL-17 family, but shares the least sequence homology. This could account for its distinct biological effects *in vivo*. In fact, phylogenetic analysis of all six IL-17's shows IL-17E to be the most divergent (Fig. 5B). Several regions of IL-17E, ncluding the four characteristic cysteine residues, are conserved across mammalian orthologs, which suggests their importance in proper function of this cytokine (Fig. 6B). The gene for IL-17E spans 3.6 kb and is located in humans on chromosome 14q11.2; this chromosomal region is linked to skin prick reactivity [409]. 14q11.2 also contains the gene for mast cell chymase and a certain restriction site polymorphism associated with eczema [410].

The IL-17E gene contains 2 exons that yield a 1.36 kb message and the open reading frame encodes a 177 amino acid (aa) protein. The mature protein contains 145 residues after the 32 aa-signal peptide is cleaved. So far, nothing is known of the transcriptional regulation of this cytokine, though, IL-17E is likely to be regulated at the post-transcriptional level as the 3'-untranslated (UTR) region of its mRNA molecule contains several instability sequences [411] (Fig. 6A). This sequence motif is 5'-AUUUA-3' and was first identified in the 3'-UTR of the human GM-CSF mRNA by Shaw and Kamen [411]. These pentamers are also known as Shaw-Kamen sequences. Their presence induces mRNA instability and accelerates its decay. Shaw-Kamen sequences are often found in messages coding for cytokines, cytokine receptors, growth factors and oncogenes.

Constitutive tissue expression of IL-17E is limited, the highest levels being observed in testes, while levels of expression are low to moderate in the lungs and trachea [412]. *In vitro*, IL-17E is expressed by human Th2-polarized T lymphocytes [408], murine bone marrow-derived mast cells stimulated with IgE [78] and by mouse alveolar macrophages [406]. The first evidence of IL-17E *in vitro* production shows that it is undetected in naïve CD4+ T cells and only Th2 polarized lymphocytes can express IL-17E mRNA [408]. IL-17E message is observed in unstimulated murine bone marrow-derived mast cells but its expression increases after IgE crosslinking [78]. Interestingly, in addition to Th2 lymphocytes making IL-17E [78,408], unpolarized Th0 cells express it at baseline; expression is higher in both lineages when stimulated with anti-CD3 [78]. The BAL fluid of rats treated with titanium oxide particles show elevated IL-13 and IL-17E; both cytokines are also upregulated in cultured alveolar macrophages from these animals [406].

A

CTCAAGTCACTCCCTAAAAAGACAGTGGAAATAAATTTGAATAAACAAAACAGGCTTGCTGAAAATAAAA
TCAGGACTCCTAACCTGCTCCAGTCAGCCTGCTTCCACGAGGCCTGTCAGTCAGTGCCCCACTTGTGACT
GAGTGTGCAGTGCCCAGC**ATG**TACCAGGTCAGTGCAGAGGGCTGCCTGAGGGCTGTGCTGAGAGGGAGAG
GAGCAGAGATGCTGCTGAGGGTGGAGGGAGGCCAAGCTGCCAGGTTTGGGGCTGGGGGCCAAGTGGAGTG
AGAAACTGGGATCCCAGGGGGAGGGTGCAGATGAGGGAGCGACCCAGATTAGGTGAGGACAGTTCTCTCA
TTAGCCTTTTCCTACAGGTGGTTGCATTCTTGGCAATGGTCATGGGAACCCACACCTACAGCCACTGGCC
CAGCTGCTGCCCCAGCAAAGGGCAGGACACCTCTGAGGAGCTGCTGAGGTGGAGCACTGTGCCTGTGCCT
CCCCTAGAGCCTGC**TAG**GCCCAACCGCCACCCAGAGTCCTGTAGGGCCAGTGAAGATGGACCCCTCAACA
GCAGGGCCATCTCCCCCTGGAGATATGAGTTGGACAGAGACTTGAACCGGCTCCCCCAGGACCTGTACCA
CGCCCGTTGCCTGTGCCCGCACTGCGTCAGCCTACAGACAGGCTCCCACATGGACCCCCGGGGCAACTCG
GAGCTGCTCTACCACAACCAGACTGTCTTCTACCGGCGGCCATGCCATGGCGAGAAGGGCACCCACAAGG
GCTACTGCCTGGAGCGCAGGCTGTACCGTGTTTCCTTAGCTTGTGTGTGTGTGCGGCCCCGTGTGATGGG
CTAGCCGGACCTGCTGGAGGCTGGTCCCTTTTTGGGAAACCTGGAGCCAGGTGTACAACCACTTGCCATG
AAGGGCCAGGATGCCCAGATGCTTGGCCCCTGTGAAGTGCTGTCTGGAGCAGCAGGATCCCGGGACAGGA
TGGGGGGCTTTGGGGAAAGCCTGCACTTCTGCACATTTTGAAAAGAGCAGCTGCTGCTTAGGGCCGCCGG
AAGCTGGTGTCCTGTCATTTTCTCTCAGGAAAGGTTTTCAAAGTTCTGCCCATTTCTGGAGGCCACCACT
CCTGTCTCTTCCTCTTTTCCCATCCCCTGCTACCCTGGCCCAGCACAGGCACTTTCTAGATATTTCCCCC
TTGCTGGAGAAGAAAGAGCCCCTGGTTTTATTTGTTTGTTTACTCATCACTCAGTGAGCATCTACTTTGG
GTGCATTCTAGTGTAGTTACTAGTCTTTTGACATGGATGATTCTGAGGAGGAAGCTGTTATTGAATGTAT
AGAG<u>ATTTA</u>TCCAAATAAATATCTTT<u>ATTTA</u>AAAATGAAAAAAAAAAAAAAAAAAAA

B

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Mice            ------------------AVAFLAMIVGTHTVSLRIQEGCSHLPSCCPSKEQEPPEEWL 41
Rat             ----------------MYQAVAFLAMVVGTHTVSLRIQEDCSHLPRCCPSKQQEFPEEWL 44
Human           MRERPRLGEDSSLISLFLQVVAFLAMVMGTHTY--------SHWPSCCPSKGQDTSEELL 52
Chimp           MRERPRLGEDSSLISLFLQVVAFLAMVMGTHTY--------SHWPSCCPSKGQDTSEELL 52
Rhesus          MRGQPRLGENSSLISLFLQVIAFLAMVMGTHTY--------SHWPSCCPSKG-YPSEELL 51
Dog             --------------------IVFLVMAMGTHTLNFRIRKDCTHWPNCCPSKRQDPTHEWL 40
Opposum         KRERETLPRMDSKISEFLQLTGILVVALGTQALNLRLRRDYNHHHRGCCHQGQGFSEEWL 60
                      :     :  :     :*.: :**::          .*    *  :    ..* *

Mice            KWSSASVSPPEPLS-------HTHHAESCRASKDGPLNSRAISPWSYELDRDLNRVPQDL 94
Rat             KWNPAPVSPPEPLR-------HTHHPESCRASKDGPLNSRAISPWSYELDRDLNRVPQDL 97
Human           RWSTVPVPPLEPAR-------PNRHPESCRASEDGPLNSRAISPWRYELDRDLNRLPQDL 105
Chimp           RWSTVPVPPLEPAR-------LDRHPESCRASEDGPLNSRAISPWRYELDRDLNRLPQDL 105
Rhesus          RWSTVPVPPLKPAS-------LDFHSVSCRASEDGPLNSRAISPWRYELDRDLNRLPQDL 104
Dog             KRDTVLKFPGETTS-------LTHHPESCKASEDGPLNSRSISPWKYELDRDLNRLPQDL 93
Opposum         QRNSVPSTLLQVSSNLQVPNIEVSKPFKCQASRDGPLNSRAISPWKYVLDRDENRLPQDL 120
                : ...    :             :. .*:**.*******:**** * **** **:****

Mice            YHARCLCPHCVSLQTGSHMDPLGNSVPLYHNQTVFYRRPCH--GEEGTHRRYCLERRLYR 152
Rat             YHARCLCPHCVSLQTGSHMDPMGNSVPLYHNQTVFYRRPCH--GEQGAHGRYCLERRLYR 155
Human           YHARCLCPHCVSLQTGSHMDPRGNSELLYHNQTVFYRRPCH--GEKGTHKGYCLERRLYR 163
Chimp           YHARCLCPHCVSLQTGSHMDPRGNSELLYHNQTVFYRRPCH--GKKGTHKGYCLERRLYR 163
Rhesus          YHARCLCPHCVSLQTGSHMDPRGNSELLYHNQTVFYRRPCH--GKKGNHKGYCLERRLYR 162
Dog             YHARCLCPHCVSLQTGSHMDPLGNSELLYHNQTVFYRRPCP--GEQGAPDGYCLEQRLYR 151
Opposum         YHARCLCPHCINLRSGHDIDYRGNSELLWHNQTVFYRRPCHGHGEQQGSDCYYLEPRTYL 180
                ******* **:.*::* .:*  ***  *:***********   *::     * ** * *

Mice            VSLACVCVRPRVMA 166
Rat             VSLACVCVRPRMMA 169
Human           VSLACVCVRPRVMG 177
Chimp           VSLACVCVRPRVMG 177
Rhesus          VSLACVCVRPRVMG 176
Dog             VSLACVCVRPRVMA 165
Opposum         VSFACICVLPRIMA 194
                **:** :** **:*.
```

Fig. 6: (A) Human IL-17E cDNA sequence. Start and stop codons are highked bold; Shaw-Kamen sequences (ATTTA) are underlined (B).Comparison of the different mammalian homologues of IL-17E. The four cysteine residues (in rectangles) that form the disulide bonds are conserved accross all species aligned. Identicalresidues are marked with a star; residues that share the same side chain type are marked by two dots; residues that have different side chain type are marked by a single dot. Numbers appearing at right refer to the last amino acid on that line. Sequences were aligned using ClustalW.

1.11.12 Biological effect of IL-17E

IL-17E has distinct biological properties not reported for the other IL-17's, as IL-17E induces elements of allergic asthma and a Th2-type phenotype in mice [400,408,413,414]. Using diverse strategies of IL-17E overexpression, three independent groups showed similar findings [400,408,413,414]. Their primary results show that the mice develop eosinophilia, increased serum IgE and increased Th2-type cytokines (IL-4, -5, and -13). In addition, mice receiving intranasal recombinant IL-17E show production of mucus (positive periodic acid shiff staining) by the airway epithelium. Detailed accounts of the main findings are summarized in Table 3. The increase in Th2 cytokines appears specific to IL-17E, as it is not observed in animals overexpressing either IL-17A [353] or IL-17F [415]. Using various knockout mice or blocking antibodies, Fort *et al.* and Hurst *et al.* showed the necessity of IL-4, IL-4Rα, IL-5 or IL-13 in IL-17E-mediated eosinophilia, gene expression, histological changes and serum antibody production [400,408]. While IL-4 -/- and IL-4Rα -/- mice given IL-17E still developed eosinophilia [400,408], such mice did not produce elevated IgA, IgE and IgG_1 levels [408]. IL-13-/- animals administered IL-17E had no detectable BAL fluid eosinophils [400] and less blood eosinophils than wild-type [408]. IL-13 is also necessary for much of the IL-17E-mediated increase in IL-4 and IL-5. Lack of IL-4 does not affect elevated circulating eosinophils in mice infused with IL-17E. In contrast, eosinophilia is totally dependent on IL-5, and an IL-5 blocking antibody effectively prevents eosinophilia in IL-17E-treated mice [400,408]. Interestingly, mice lacking IL-4Rα or IL-13 were protected against the tissue changes caused by IL-17E, though IL-4Rα KO mice still developed eosinophilia and had higher levels of IL-4, IL-5 and IL-13 than WT [400,408]. Interestingly, histological changes are maintained in IL-17E-

treated RAG (recombination-activating gene) KO mice which lack mature B and T cells, and while lymphocyte deficiency totally prevents IL-4 production, IL-5 and IL-13 levels are still increased in these mice, but to a somewhat lesser extent than in WT [408].

Although eosinophils are the predominant inflammatory cell observed in blood and tissue in all mice models overexpressing IL-17E, some have reported a rise in neutrophils. Mice given recombinant IL-17E only show BAL fluid eosinophilia and no neutrophilia, while mice receiving an adenoviral construct of IL-17E showed both. As neutrophil recruitment is not observed in mice receiving the control adenoviral construct (Ad-GFP), the authors suggest that IL-17E promotes this effect because of adenoviral infection [400]. In addition to eosinophilia, mice expressing either a human or mouse IL-17E transgene also report increased circulating neutrophils [413,414]. Moreover, *in vitro*, IL-17E induces IL-8 production, a potent neutrophil chemoattractant. Collectively, the evidence also supports a role for IL-17E in neutrophil recruitment. The cell types responsible for producing the elevated levels of IL-4, IL-5 and IL-13 and thus mediating eosinophilia in the IL-17E mice models have yet to be identified. RAG KO mice could not produce IL-4 when given recombinant IL-17E, suggesting that cells of a lymphocytic lineage make the IL-4 [408]. Although, *in vitro* stimulation of αβTCR+CD4+ T cells or CD19+ B cells with IL-17E failed to induce IL-4, authors further suggested the role of γδ TCR+CD4+ T cells and NK-T cells as a source for this cytokine [408]. Nevertheless, IL-17E can still promote eosinophilia in mice without lymphocytes, NK or mast cells [400] and induces IL-5 and IL-13 from cells of a non-lymphocyte, non-NK and non-granulocyte origin [400,408]. While T cells, basophils and mast cells are primarily associated with IL-5 and IL-13 secretion, other cell types have been shown to produce these cytokines [109,238].

Together, these results show that IL-4 production is lymphocyte-dependent, but not the expression of IL-5 and IL-13. The pathologies observed in the tissues occur without lymphocytes and require IL-13 and IL-4Rα, while eosinophilia is dependent on IL-5. IL-17E is also very important for developing the proper Th2 immunity against parasitic infections. Recently, using IL-17E knockout mice, this cytokine was shown to be of critical importance in eradicating helminth infection [416,417]

IL-17E induces the release of several cytokines and chemokines from various structural cells as well as activating the MAPK and NF-kB pathways. The biological action of IL-17A and IL-17E overlap as both promote the production of IL-6 and IL-8, NF-kB activation and phosphorylation of ERK1/2, p38 and JNK. Also, both these IL-17's necessitate the presence of TRAF6 for proper signaling [389,405].

Method of overexpression	Pathologies and histological changes	Inflammatory cells	Increased cytokines						
			Lung	Liver	Kidney	Spleen	Stomach	S.I.	Blood
Purified IL17E protein (i.p.) (Fort et al.)	Splenomegaly, hyperplasia/hypertrophy/ mucus production of lung and GI tract epithelium. Increased IgE levels	eosinophils (blood, spleen lungs)	n.d.	IL-5 IL-13	IL-5 IL-13	IL-13	IL-4 IL-13	IL-13	n.d.
Purified IL17E protein (i.n.) (Hurst et al.)	Hyperplasia/mucus of lung epithelium.	eosinophils (blood, spleen lungs)	IL-5 IL-13 IFNγ Eotaxin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Adenoviral IL-17E construct (s.c.) (Hurst et al.)		eosinophils/ neutrophils (lungs)	IL-4 IL-13 Eotaxin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
hIL-17E transgene (Kim et al.)	Splenomegaly, lymphadenopathy, bone marrow myeloid hyperplasia. B cell hyperplasia (CD19+). Increased IgE levels	eosinophils/ neutrophils/ lymphocytes (blood)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	IL-2 IL-4 IL-5 G-CSF Eotaxin IFNγ
mIL-17E transgene (Pan et al.)	Jaundice, growth retardation. **Liver**: fibrosis,severe periportal inflammation, adenomatous hyperplasia of bilde ducts, hypertrophy of bile duct epithelium. **Lung:** inflammation and hyperplasia of bronchiole epithelium. Increased IgE levels	eosinophils/ neutrophils/ (blood)	IL-4 GROα IL-10 IFNγ	IL-4 TNFα G-CSF IFNγ ICAM1	IL-4 GROα G-CSF IL-10	n.d.	n.d.	n.d.	IL-5 IL-13 TNFα G-CSF

n.d.: not determined; i.p.: intraperitonneal; i.n.: intranasal: s.c.: subcutaneous; S.I.: small intestines. Cytokines: IL: interleukin; IFNγ: interferon gamma; G-CSF: granulocyte-colony stimulating factor; GROα: growth related oncogene alpha; ICAM1: intercellular adhesion molecule 1.

Table 3. Overview of the biological changes observed in mice treated with or overexpressing IL-17E.

1.11.13 The IL-17E receptor (IL-17BR)

IL-17BR is the second receptor of the IL-17 family to be identified [412,418,419]. Identification of this novel receptor was made by screening expressed sequence tags (EST) databases for IL-17R homologs. In human it is mapped to chromosome 3p21, a region found to harbor several deletions in breast, lung and renal carcinomas [420-422] and is the most frequently deleted region in chronic myeloid leukemia [423,424]. As with IL-17R, it is a single transmembrane receptor that shares similar position and amino acid sequence of its transmembrane domain [419]. Structurally, IL-17BR has a much shorter cytoplasmic tail than the unusually long one of IL-17R. Also, IL-17BR is thought to encode a soluble form, a feature not known to exist with IL-17R. The full-length IL-17BR mRNA is encoded from 11 exons spanning approximately 20 kb, it contains the coding region for the 22 amino acid (aa) transmembrane region on exon 10 (IL-17R has a 21 aa transmembrane domain). The soluble form of the receptor is though to arise from retention of intron 8 during transcription, yielding a longer IL-17BR message. The retained intron contains an in-frame stop codon that halts translation and produces a truncated version of the receptor lacking the transmembrane domain. Another notable difference between the two receptors is the affinity for their ligands, the binding kinetics, of IL-17A for IL-17R shows a dissociation constant value (K_d) of 74 nM, whereas it is only of 1.1 nM for IL-17E for IL-17BR [342]. This indicates that an almost 10-fold greater concentration of IL-17A is necessary to bind IL-17R than is necessary for IL-17E to bind IL-17BR.

IL-17BR is expressed in a wide variety of human tissues, the highest mRNA content being found in the kidneys [412,418,419]. The receptor also shows constitutive expression in the lungs and trachea [412]. At the cellular level, IL-17BR is present in various murine and human leukocytic cell lines as well as in some human epithelial cancer cell lines [412,419]. IL-17BR binds IL-17B and IL-17E, but shows a greater affinity for IL-17E [342,412]. IL-17BR is also known as IL-17Rh1 [412] and Evi27 [419]. The murine ortholog of this receptor, initially identified as *Evi27*, is a common integration site for retroviral integration in BHX2 murine myeloid leukemias [425]. Only one report is available on IL-17BR regulation. Gratchev *et al.* showed that human monocytes and monocyte-derived dendritic cells express IL-17BR [426]. In this study, the baseline measurement of the receptor is unclear, but the combination of IL-4 and TGFβ for monocytes and IL-10 for dendritic cells are strong stimuli in increasing IL-17BR mRNA. Similar to findings in ASMC [427], IFNγ appears to decrease both IL-17BR message and protein in monocytes [426]. TNFα is an effective inducer of IL-17BR expression in both ASMC and lung fibroblasts [427,428].

While data in the literature concerning the signaling components associated with IL-17BR is very limited, IL-17E is known to activate NF-kB in several cell type [405,407,412]. It was suggested that IL-17E might signal through one of the TNF receptor-associated factor (TRAF) molecules because IL-17A is dependent on TRAF6 for NF-kB and JNK activation [389]. Indeed, TRAF6 is also necessary for NF-kB activation by IL-17E as assessed using TRAF6 -/- mouse embryonic fibroblasts (MEF) [405]. IL-17BR associates specifically with TRAF6, not TRAF2 or TRAF5, to convey the signal to the NF-kB pathway. In addition, IL-17E activates ERK, p38 and JNK phosphorylation

independently of TRAF6. IL-17E also activates p38 and JNK in human eosinophils [407]. In MEF, TRAF6 is necessary for the induction of IL-6, TGFβ, G-CSF and TARC mRNA after IL-17BR ligation [405]. In contradiction with the Th2 nature of IL-17E and the necessity of TRAF6 for some of its signaling, TRAF6 knockout mice develop a Th2-type inflammation [429]. Anti-CD3 and anti-CD28-stimulated CD4+ T cells from TRAF6-/- mice secrete similar amounts of IFNγ as wild-type, but inordinately higher quantities of IL-4, IL-10 and TGFβ [429]. However, levels of IL-5 or IL-13 were not assessed in these mice. In addition, lack of TRAF6/NF-kB signaling might not adversely affect IL-17E-induced Th2 cytokines, as IL-4, IL-5 and IL-13 secretion can be mediated by other signaling pathways [430]. Since TNFα is also increased in the transgenic animals [414], and as TNFα upregulates IL-17BR in ASMC and lung fibroblasts [427,428], it is possible that it is responsible for the elevated IL-17E receptor expression. Also, i*n vitro* data from eosinophils suggests that IL-17E itself may also increase expression of IL-17BR [407]. Interestingly, IL-17BR expression is upregulated in IL-17E transgenic mice [413,414].

1.12 Rationale

IL-17E promotes a Th2-type inflammatory response in mice and has been shown *in vitro* to be produced by Th2 lymphocytes as well as mast cells, two cells types that are known to infiltrate the bronchial mucosa of asthmatics. Little is known about the biology of IL-17E on human cells because most data generated so far have been in mice models. IL-17E is an important target of study in the context of human asthma since it induces asthma-like features in mice. The importance of airway structural cells in the participation of asthma has been well identified. However their exact contribution to airway inflammation remains to be elucidated. Thus, understanding their interaction with cytokines important in asthma pathogenesis is crucial.

1.13 Hypothesis

We hypothesized that structural cells of the airways would express the IL-17E receptor, and that the levels of expression could be modulated by Th1, Th2 or proinflammatory cytokines. Secondly, we hypothesized these structural cells would respond to IL-17E via IL-17BR by generating mediators and effects important to to asthma pathogenesis, such as cytokines and chemokines that support airway inflammation, deposition of components of the extracellular matrix and promoting cellular proliferation.

1.14 Aims

1.14.1 General Aims

The general aim of this thesis was to clarify the link between IL-17E and structural cells of the airways. We wanted to assess the presence of the Il-17E receptor (IL-17BR) in cultured airway structural cells as well as in human bronchial biopsies. In addition, we examied the biological effect of IL-17E on these cells *in vitro*.

1.14.2 Specific Aims

1. To assess the presence of IL-17BR on airway smooth muscle in sections of biopsies taken from human bronchial tissue. Then examine its expression in cultured airway smooth muscle cells as well as its modulation by various types of cytokines. If the receptor was to be present, evaluate the effect of IL-17E on the production of components of the extracellular matrix.

2. To investigate the presence of the IL-17E receptor on lung fibroblasts and evaluate the effect of this cytokine on the production of mediators that promote allergic airway inflammation.

3. To examine the presence of IL-17BR on the epithelium in sections of biopsies taken from human bronchial tissue. Following that, to investigate its expression in cultured lung epithelial cells as well as its modulation by various types of cytokines. To compare levels of IL-17BR between bronchial epithelial cells isolated from asthmatics vs. cells from control donors. If the receptor was to be present, evaluate the effect of IL-17E on the production of inflammatory cytokines and chemokines as well as it role in epithelial proliferation.

Chapter 2 – Materials and Methods

2.0 Summary of experimental design

To address the questions put forward in our hypothesis, three different studies were performed. We cultured three different types of airway structural cells (airway smooth muscle cells, fibroblasts and epithelial cells) for this purpose of studying the presence of the IL-17E receptor as well as the effect of IL-17E on these cells *in vitro*. Initially we used primary airway smooth muscle cells from non-asthmatic donors; these were purified from lung resected tissue and characterized as smooth muscle cells. We also used airway sections from biopsies to assess the *in vivo* presence of IL-17BR on airway smooth muscle cells. For *in vitro* experiments, we used recombinant human cytokines to stimulate the cells in culture and assess expression of IL-17BR by experiments involving real-time PCR, western blot, laser capture microdissection, immunohistochemistry and immunofluorescence.

Secondly, primary lung fibroblasts were commercially obtained and were tested for the presence of IL-17BR on by immunofluorescence, western blot and real-time PCR. We then treated the fibroblasts with recombinant human IL-17E and measured by real-time PCR and enzyme-linked immunosorbent assay (ELISA) chemokine production.

Finally, we used lung epithelial cells in which we assessed the presence of IL-17BR by western blot and real-time PCR. We then treated the epithelial cells with recombinant human IL-17E and measured their proliferative response by radioactive thymidine uptake and production of chemokines by real-time PCR.

2.1 Materials

This section mentions the commercial and non-commercial sources for the materials used in the experiments performed in this thesis. Smooth muscle Growth medium was purchased from Cambrex (East Rutherford, NJ, USA). LabTek chamber slides were bought from Nalgene Nunc (Naperville, IL, USA). Human recombinant cytokines, rhIL-17BR:Fc, mouse monoclonal anti-IL-17BR and isotype antibodies were obtained from R&D Systems (Minneapolis, MN, USA). QuantiTect SYBR Green PCR kit, RNeasy mini and micro kit extraction columns, HiSpeed Maxi plasmid kit, the QIAquick PCR purification kit and the Endo-free Maxiprep kit were purchased from Qiagen (Mississauga, ON, Canada). CXCL8 and GM-CSF ELISA kits were obtained from Biosource International (Camarillo, CA, USA). Trypan Blue dye, Superscript II, Taq Platinum polymerase, PCR primers, 100 bp DNA ladder and restriction enzymes were obtained from Invitrogen (Burlington, ON, Canada). Bradford assay reagent and Western blot precision plus molecular weight standards were purchased from Bio-Rad (Mississauga, ON, Canada). Donkey anti-mouse HRP-conjugated secondary antibody, oligo(dT)12-18 primer, RNAguard, Hybond-P polyvinylidene fluoride (PVDF) membrane, ECL Plus, and X-ray films were purchased from Amersham Pharmacia Biotech (Baie d'Urfé, QC, Canada). The LightCycler real-time PCR, the minicomplete protease inhibitor cocktail tablets were bought from Roche Diagnostics (Laval, QC, Canada). pGEM-T, pGL3 and pRL-TK plasmids and the Dual luciferase assay were purchased from Promega (Madison, WI, USA). Pharmacological inhibitors (U0126, SB203580, and SP600125, resuspended in dimethyl sulfoxide - DMSO), dexamethasone 21-phosphate disodium salt (resuspended in water) and Fast Red were purchased from

Sigma-Aldrich (Oakville, ON, Canada). The specific IKK2 inhibitor, AS602868, was a generous gift from Dr. Ian M. Adcock (National Heart and Lung Institute, Imperial College London). Alexa Fluor 555 labelled goat antimouse IgG was obtained from Molecular Probes (Eugene, OR). Universal Blocking Solution and mouse alkaline phosphatase anti-alkaline phosphatase (APAAP) were bought from Dako, (Mississauga, ON, Canada), Gill modified hematoxylin was from Surgipath (Winnipeg, MB, Canada). The BX51 Olympus epifluorescence microscope attached to a CoolSNAP-Pro color digital camera was purchased from Carsen Group (Markham, ON, Canada). The Cap-Sure Macro LCM Caps and the PixCell II System were obtained from Arcturus (Mountain View, CA, USA). The GeneAmp high fidelity PCR enzyme mix was obtained from Applied Biosystems (Foster City, CA, USA). The antibody array was obtained from RayBiotech (Norcross, GA, USA). All sequencing reactions were performed at McGill's University Genome Centre.

2.2 General techniques

Several of the techniques used in this thesis were performed across all three main projects and will be included in this section.

2.2.1 RNA extraction

Total RNA was isolated from (i) cultured ASMC and (ii) ASMC bundles from human airway biopsies (see *2.2.9* section) using RNeasy mini and micro kit extraction columns, respectively. The columns are composed of a specially engineered silica membrane that captures and holds the RNA. The frozen samples in RLT buffer are thawed out and an

equal volume (350 ul) of 70% ethanol is added to each tube to promote selective binding of RNA to the RNeasy column. This serves to acidify the solution and helps the RNA to bind the silica membrane. Buffers are used to wash away residual proteins and other impurities. RNA is eluted from the extraction columns using 35 ul RNAse-free water and then quantified using spectrophotometry.

2.2.2 Complementary DNA (cDNA) preparation

cDNA is obtained by reverse transcribing RNA into DNA using a reverse transcriptase enzyme. We used 500 ng of total RNA from cultured airway smooth muscle to generate the cDNA. As messenger RNA (mRNA) is characterized by having a tail containing several non-coding adenosines (poly A tail), a synthetic primer composed of complementary thymidines (oligodT) that will initiate the reverse transcription reaction. This mix of RNA, and oligodT is heated at 65°C for 5 min in order to linearize the RNA molecules by undoing secondary structure formation. The mix is then quickly chilled on ice. 20 ul of a master mix composed of water, reverse transcription buffer (250 mM Tris-Cl, 375 mM KCl and 15 mM $MgCl_2$), 0.1 M dithiothreitol (DTT), dNTPs (dATP, dTTP, dGTP and dCTP), 40 units of ribonuclease inhibitor (RNasein), and 40 units of SuperScript II reverse transcriptase is then added to then RNA and the reverse transcription reaction was performed at 42C for 1 h. The enzyme is inactivated by heating the reaction for 15 min at 70°C. The cDNA is then stored at -20°C until used as a template for PCR.

2.2.3 Real-time quantitative PCR (QPCR)

Real-time QPCR was used to determine the expression of several genes of interest at the mRNA level. Real-time QPCR was done using the LightCycler system from Roche. The PCR reaction is done in a 20 µl volume containing 7 µl water, 1 µl cDNA, 0.3 µM of each primer, and 10 µl of 2X QuantiTect SYBR Green PCR Master Mix. The QuantiTect SYBR Green master mix contains HotStartTaq DNA polymerase, PCR buffer, dNTPs and the SYBR Green dye. The SYBR Green dye is used to quantify the generation of amplicons in the PCR reaction in real-time, it intercalates in the minor groove of double-stranded DNA, but cannot bind single-stranded DNA. Hence, the amount of double-stranded DNA in a PCR reaction correlates with the amount of SYBR Green dye bound, thus generating more fluorescence. The LightCycler uses a diode to excite the SYBR Green dye and a fluorescence detector to measure the resulting signal.

The PCR reaction is performed in a glass capillary that permits very rapid and efficient air-induced heating and cooling.

All primers used in this thesis were designed to span at least one intron and, in the case of IL-17BR, to include the region encoding the transmembrane domain. Designing primers that cover at least one intron avoids co-amplification of genomic DNA. Relative quantification of the genes was achieved by constructing standard curves using serial dilutions of PCR amplicons of the gene in question. PCR was employed to amplify the genes of interest from structural cell cDNA, All PCR amplicons were purified using the QIAquick PCR purification kit and specificity of the amplified products was assessed by gel electrophoresis, then quantified by spectrophotometry. A 10-fold dilution series ranging from 10 -1 to 10-10 ng/µl of each amplicon was then prepared in Tris-HCl pH 8.0. Relative quantification of gene expression was performed using the LightCycler

software. The software calculates a standard curve from the known concentrations in ng/ul vs. the Ct values (crossing point) and uses this curve to compute the concentrations by interpolating the Ct values of the experimental samples.

Gene name	Sequence	Amplicon size
S9	Sense: 5'- TGCTGACGCTTGATGAGAAG -3' Antisense: 5'- CGCAGAGAGAAGTCGATGTG-3'	307 bp
IL-17BR	Sense: 5'- AACAGGCGTCCCTTTCCCTCTGGA -3' Antisense: 5'- TTCTTGATCCTTTCGTGCCTCCAC -3'	141 bp
Pro-collagen αI	Sense: 5'- CTGGTCCCCAAGGCTTCCAAGG -3' Antisense: 5'- CTTCACCCTTAGCACCAACAGC -3'	478 bp
Versican	Sense: 5'- GAATTGGAGACCCAACCAGCC -3' Antisense: 5'- GGTATAGCCCATCTTCCATTTCC -3'	300 bp
Lumican	Sense: 5'- CCTGGTTGAGCTGGATCTGT -3' Antisense: 5'- TGGTTTCTGAGATGCGATTG -3'	194 bp
GROα	Sense: 5'- GAAAGCTTGCCTCAATCCTG -3' Antisense: 5'- CATTAGGCACAATCCAGGTG -3'	194 bp
IL-8 (CXCL8)	Sense: 5'- GCAGAGGGTTGTGGAGAAG-3' Antisense: 5'- TCTTGTATTGCATCTGGCAAC-3'	169 bp
IL-6	Sense: 5'- GGTACATCCTCGACGGCATC -3' Antisense: 5'- GCCTCTTTGCTGCTTTCACAC-3'	79 bp
Eotaxin (CCL11)	Sense: 5'- GGGCCAGCTTCTGTCCCAAC -3' Antisense: 5'- TTATGGCTTTGGAGTTGGAGATTT-3'	225 bp
GM-CSF	Sense: 5'- CCAGCCACTACAAGCAGCAC-3' Antisense: 5'- GGGATGACAAGCAGAAAGTCC -3'	112 bp
RANTES (CCL5)	Sense: 5'- TCCAACCCAGCAGTCGTC -3' Antisense: 5'- CAAGAGCAAGCAGAAACAGG -3'	162 bp

Table 4. List of primers used for real-time PCR

2.2.4 Western blotting

Western blot was used to detect IL-17BR protein after treatment of ASMC with TNFα and IFNγ. After cytokine stimulation, the media was removed and the cells were rinsed in ice-cold PBS and incubated in lysis buffer (150 mM NaCl; 10 mM Tris-HCl pH 7.4; 1 mM EDTA; 1 mM EGTA; 1% Triton X-100; 0.5% NP40, 100 mM sodium fluoride; 10 mM sodium pyrophosphate; 2 mM sodium orthovanadate) containing a mini-complete protease inhibitor cocktail tablet for 30 minutes on ice. Cell extracts were collected from the bottom of each wells and clarified at 14,000 x g at 4°C for 20 minutes to remove cell debris. Protein concentration was determined using the Bradford assay. 10 μg of protein was then resolved by one-dimensional 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to Hybond-P polyvinylidene difluoride (PVDF) membranes. Blocking of membranes was carried out using 5% bovine serum albumin (BSA) in Tris-buffered saline (TBS) containing Tween-20 (TBS-T) (0.05% Tween-20, 10 mM TBS, pH 7.5) for 2 h at room temperature. Blots were incubated overnight at 4°C with either 0.5 μg/ml of mouse monoclonal anti-IL-17BR antibody or with anti-IL-17BR preincubated with a 10 molar excess of rhIL-17BR:Fc chimera for 2 h at room temperature. After washing, membranes were treated with horseradish peroxidase (HRP)-conjugated anti-mouse secondary antibody for 1 h at room temperature. After washing, signal was developed using reagents from the ECL Plus kit that contains a substrate for the HRP enzyme that is converted to a chemiluminescent signal. Chemiluminescence was detected by exposing the membranes to an X-ray film.

2.2.5 Immunofluorescence

Immunofluorescence was used to visualize IL-17BR expression by cultured ASMC and lung fibroblasts. To do so, cells were grown to 50-60% confluence in LabTek chamber slides. These chamber slides offer 8 plastic chambers glued on top of a glass slide and permit 8 different conditions on the same slide. The slides were briefly rinsed in room temperature PBS, then fixed in a 4 % paraformaldehyde solution for 20 min at room temperature and washed again in PBS for 5 min before drying overnight. Fixed slides were stored at -80°C until use. When processed, the slides were first incubated with a solution of 50 μM NH_4Cl for 30 min, followed by blocking in 20% normal goat serum. Slides were then exposed overnight to a solution of mouse anti-human IL-17BR (5 μg/ml), or isotype matched control antibody. Slides were extensively washed and incubated with a 1/750 dilution of goat anti-mouse IgG labeled with the Alexa Fluor 555 dye. After 555 nm light excitation, the Alexa Fluor will emit fluorescence at 565 nm, visible as orange-red. Cell nuclei are visualized by staining with Hoechst 33342 (2 μg/ml); this dye stains nucleic acids and emits blue fluorescence (461 nm) after ultraviolet-light excitation. Slides were examined with a BX51 Olympus epifluorescence microscope attached to a CoolSNAP-Pro color digital camera.

2.2.6 Immunohistochemistry of sections from bronchial biopsies

Immunohistochemistry was performed on sections of major airways from 6 mild asthmatic subjects. Paraffin-embedded sections were deparaffinized and heat-induced antigen retrieval was performed using 10 mM citrate buffer. Slides were then extensively washed in PBS, blocked for 30 min in Universal Blocking Solution and incubated overnight at 4°C with 25 ug/ml murine anti-human IL-17BR, or IgG_{2B} isotype control,

antibodies. Sections were subsequently washed twice in TBS prior to 45 minute incubation at room temperature with rabbit anti-mouse Ig followed by mouse alkaline phosphatase anti-alkaline phosphatase (APAAP). The reaction was developed using Fast Red. Tissue was then counterstained with Gill modified hematoxylin and examined by light microscopy. Images were acquired with the BX51 microscope.

2.2.7 Statistical analysis

Data are expressed as means ± standard deviation. Analysis of Variance (ANOVA) followed by a Student's T test was performed on a minimum of 3 independent experiments whenever statistical analysis was used. In some cases the differences between groups were detected using the Kruskall-Wallis test. The Mann-Whitney U test was performed after the Kruskall-Wallis test when appropriate. A p value of <0.05 was considered significant.

2.3 Airway smooth muscle cell-specific experiments

2.3.1 Isolation and culture of human bronchial ASMC

Primary human airway smooth muscle cells (ASMC) were obtained from main bronchial airway segments (0.5–1.0 cm diameter) in pathologically uninvolved segments of resected lung specimens using isolation methods described previously (17, 46). Cells were then seeded at a density of 10^5 cells/cm^2 and grown at 37°C in Smooth Muscle Growth medium. At confluence, primary human ASMC exhibited spindle morphology and a hill-and-valley pattern characteristic of smooth muscle in culture. In cultures up to passage 5, over 90% of the cells at confluence retained smooth muscle-specific α-actin,

SM22, and calponin protein expression, and were able to mobilize intracellular Ca 2+ in response to acetylcholine. Growth rate (determined by cell number) of ASMC from all lung resection donors was similar to that reported previously for ASMC cultures from healthy human transplant donors. Morphologically, the ASMC from lung resection donors and from healthy human transplant donors were indistinguishable. Cell viability was always above 95% as assessed by Trypan Blue dye exclusion. ASMC were cultured in commercially available media containing 5 % fetal bovine serum (FBS), insulin, human fibroblast growth factor, human epidermal growth factor and gentamycin sulfate and amphotericin-B.

2.3.2 Cell stimulation

ASMC were grown in 6 or 12-well dishes. At confluency, cells were serum-starved for 24 h before stimulation to allow the cells to reach a quiescent state. Recombinant human cytokines (TNFα, IL-4, IFNγ or IL-17E) were added for various times and at different concentrations. We also used pharmacological inhibitors (table 5) that target the three main mitogen activated kinase (MAPK) pathways, namely extracellular-regulated kinase (ERK), p38 and c-Jun-N-terminal kinase (JNK) as well as the NF-kB pathway. These inhibitors help to distinguish the pathway(s) necessary by which a specific stimulus conducts its observed effect(s); in this case the stimuli are TNFα and IFNγ. NF-kB is a proinflammatory transcription factor that regulates several immune response genes. NF-kB activation is an important downstream effect of TNFα signaling. ASMC were therefore pre-treated with the pharmacological inhibitors 1 h prior to addition of TNFα or IFNγ. For RNA extraction, cells were lysed and homogenized directly from the

wells using 350 ul RLT buffer supplemented with β-mercaptoethanol. The RLT buffer is a guanidine isothiocyanate solution (RLT buffer) containing β-mercaptoethabol, which provides a denaturing and RNAse-free environment. Lysates were stored at -80°C until RNA extraction.

Pharmacological inhibitor	Function
U0126	Prevents phosphorylation of ERK1 and ERK2 by inhibiting MEK1 and MEK2 [431]
SB203580	Inhibits the phosphorylation of p38 [432]
SP600125	Inhibits the phosphorylation of c-Jun by inhibiting JNK [433]
AS602868	Inhibits IKK2 and prevents IkB phosphorylation and degradation, and therefore NF-kB activation.[434,435]

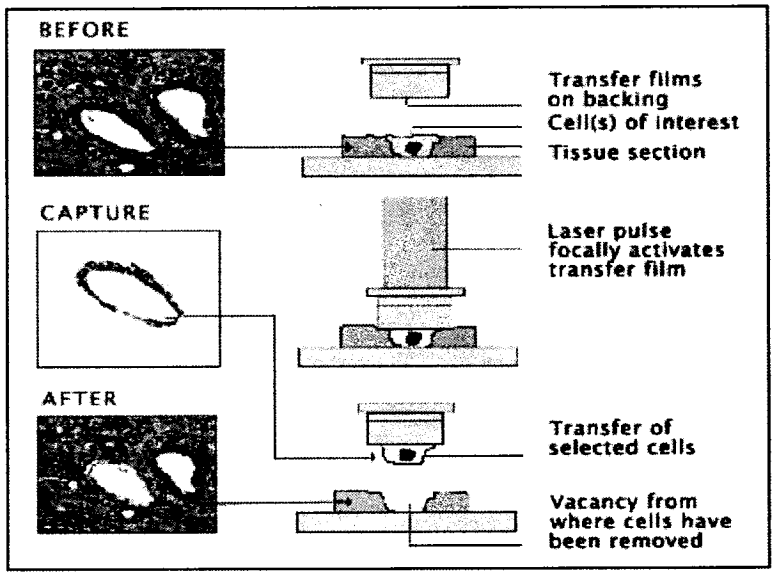
Table 5. List of pharmacological inhibitors used and their target.

2.3.3 Laser capture microdissection of ASMC bundle

Laser capture microdissection (LCM) is a technique that originated at the National Institutes of Health (NIH) [436,437] and allows for the removal of specific areas or cell-types from sectioned tissue preparations. The removed tissue can be used for RNA, DNA and even protein extraction and analysis. To assess the presence of IL-17BR mRNA in airway smooth muscle cells *in vivo*, LCM was performed on sections of the airways taken from biopsies of mild asthmatic patients in order to selectively remove smooth muscle bundles. Slides were completely air-dried and desiccated to prevent the activation of endogenous RNase in the tissues. LCM of smooth muscle bundles was performed using the PixCell II System with the following parameters: laser diameer, 15 μm; pulse duration, 1.5 milliseconds; and amplitudes, 80 to 90 milliWatts. The interval between staining of slides

and completion of microdissection was 1 to 2 hours. After samples were captured on Cap-Sure Macro LCM Caps, cells were lysed with RLT lysis buffer (Qiagen RNeasy kit), and samples were stored at -80°C until RNA extraction (Fig. 8).

A



B

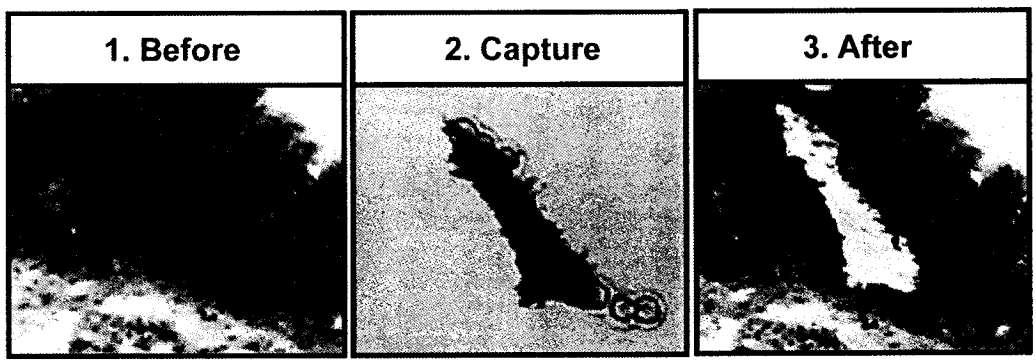


Fig. 8. Overview of laser capture microdissection (LCM). (A) A cap with a thermoplastic membrane is placed above the tissue of interested and pulses of low energy infrared laser activates the adhesiveness of the membrane and captures cells or cellular structure of interest which can then be lifted off the slide. The cells that were transferred to this membrane can then be processed for RNA/DNA extraction. (B) Actual example of a human airway biopsy stained with hematoxylin, where a smooth muscle bundle is captured and lifted off the slide.

2.3.4 Antibody array

Antibody arrays are small nitrocellulose membranes on which capture antibodies that recognize several types of target proteins (cytokine, chemokines, phosphorylated proteins, etc) are spotted. They allow for the comparison of supernatants, cell or tissue extracts, serum, etc, from different sources or treatments and gives information on possible increase or decrease of proteins represented on the membrane.

In our experiment, we used a commercial antibody array (RayBio) that contains 42 different cytokine and chemokine capture antibodies spotted in duplicate on membranes. ASMC were treated with either vehicle or IL-17E (30 ng/ml) for 24 h. Supernatants were collected and incubated with the antibody array overnight at 4°C to allow for the cytokines and chemokines in the supernatant to bind the capture antibodies on the array. Membranes were rinsed and incubated with a cocktail of biotin-labeled detecting antibodies (the cocktail contains antibodies against all 42 cytokines and chemokines represented on the array) overnight at 4°C. Membranes are then incubated with an HRP-conjugated streptavidin and incubated with ECL+. Chemiluminescence was detected using X-ray film. Dot intensity was quantified using the free ImageJ software from the National Institutes of Health (http://rsb.info.nih.gov/ij/).

2.4 Primary lung fibroblast-specific experiments

2.4.1 Growth of primary lung fibroblasts

Normal primary human lung fibroblasts (CCD-8Lu) were obtained from the American Type Culture Collection (ATCC). These fibroblasts were isolated from a single non-asthmatic 48 yr old male donor that deceased of cerebral thrombosis. Lung fibroblasts

were grown in Dulbecco's Modified Eagle Medium (DMEM) that was supplemented with the essential amino acid L-glutamine, penicillin/streptomycin and 10% FBS.

2.4.2 Cell stimulation

Lung fibroblasts were grown in 6-well dishes. At confluency, cells were serum-starved for 24 h before stimulation to allow the cells to reach a quiescent state. Various cytokines (TNFα, TGFβ1, IL-17E) were added for various times. Since IL-17E is often synergistic in combination with TNFα in the production of certain cytokines, we also combined IL-17E and TNFα. Supernatants from certain conditions were kept for ELISA analysis. For RNA extraction, cells were harvested directly from the wells using 350 ul RLT buffer supplemented with β-mercaptoethanol. Lysates were stored at -80°C until RNA extraction.

2.4.3 ELISA: measurement of GM-CSF and IL-8 (CXCL-8) release

Sandwich ELISA is a very sensitive method by which to measure the release of certain mediators such as cytokines or chemokines in supernatants of cultured cells. The sandwich term comes from the fact that the molecule being detected is held between the capture antibody and the detection antibody. This method was used to measure IL-8 and GM-CSF in the supernatants of lung fibroblasts treated with IL-17E, TNFα, IL-17E in combination with TNFα or in those receiving no stimulation. After 48 hours of stimulation, fibroblast supernatants were collected and centrifuged to remove residual cells. Samples were stored frozen at -80°C until quantification by using commercially

available ELISA kits specific for human GM-CSF and CXCL8 (IL-8). ELISA are performed in a 96-well plate format.

The capture antibody coats the bottom of the wells. The wells are blocked using a solution of BSA for 3 h at RT. Blocking solution is removed and wells are rinsed then 100 ul of supernatant is incubated in the wells for 1 h at RT. Wells are washed thoroughly and incubated with the detection antibody that is coupled to biotin molecules for 1 h at RT. After washing, a solution of streptavidin conjugated to HRP is added for another hour at RT. Wells are washed once more and then incubated with the HRP substrate, TMB blue for approximately 20-30 min at RT until the development of a clear blue color. This reaction is stopped by addition of 1N HCl, a plate reader reads the color intensity and calculates with the help of a standard curve the amount of cytokine of interest in pg/ml in each well. The detection limits were 3 pg/mL for GM-CSF and 5 pg/mL for CXCL8.

2.4 Lung epithelial cell-specific experiments

2.4.1 Growth of the lung epithelial cell line A549

The lung alveolar epithelial cell line A549 were obtained from the ATCC, these cells were isolated from lung carcinomatous tissue from a 58-year-old male. A549 cells are a widely used cell model in lung and respiratory research. Cells were maintained in a 1:1 mix of DMEM and F12 media that was supplemented with the essential amino acid L-glutamine, penicillin/streptomycin and 10% FBS.

2.4.2 Isolation and growth of primary bronchial epithelial cells from control and mild asthmatic donors

These epithelial cells were isolated from bronchial biopsies of mild asthmatic and non-asthmatic subjects as reported previously [438]. Volunteers aged between 20 to 35 years were recruited from the Asthma Clinic at the Laval Hospital (Quebec, QC, Canada). The ethics committee of Laval Hospital approved the study and all subjects gave written informed consent. The asthmatic subjects were diagnosed according to the American Thoracic Society criteria [439] (methacholine PC_{20}< 8 mg/ml) and used only inhaled β_2-agonists on demand. Their mean FEV_1 was 85 ± 3.1 % and mean PC_{20} was 4.0 ± 2.2 mg/ml. None used inhaled or systemic corticosteroids. All asthmatics were non-smokers atopics and had skin test positive for house dust mite. Control subjects (mean FEV_1 = 98.2 ± 5.1 and mean PC_{20}= 99.4 ± 1.2 mg/ml) were non-atopics and non-smokers with no history of asthma or systemic diseases. Bronchial biopsies were obtained by bronchoscopy from asthmatic and normal subjects and epithelial cells were isolated from bronchial biopsies as reported previously [440] and characterized by immunofluorescence and flow cytometry using anti-cytokeratin, anti-vimentin, and a mouse anti-human fibroblast antigen Ab-1 (CD90/Thy-1) antibody (Calbiochem) that showed no cross reactions with epithelial cells, endothelial cells, smooth muscle cells or many other cell types [441]. This characterization confirmed the purity of our bronchial cell culture. Primary epithelial cells from donors were grown in DMEM/F12 (3:1) supplemented with 10% FBS, 10 ng/ml epithelial growth factor (EGF), 5 µg/ml insulin, 5 µg/ml transferrin, $2x10^{-9}$ M T3, 0.4 µg/ml hydrocortisone, 10^{-10} M cholera toxin. Cells from control, non-

asthmatic individuals are labeled as BNE (bronchial normal epithelial) and cells isolated from mild asthmatics are labeled as BAE (bronchial asthmatic epithelium).

2.4.3 Cell stimulation

A549 cells were grown in 12-well dishes. At confluency, cells were serum-starved for 24 h before stimulation to allow the cells to reach a quiescent state. Various cytokines (TNFα, IL-17A, IL-17E) were added for various times and at different concentrations. Since IL-17E is often synergistic in combination with TNFα in the production of certain cytokines, we tried to block that effect by using the recombinant IL-17E receptor (IL-17BR). Accordingly, IL-17E (50 ng/ml) was pre-incubated with rhIL-17BR:Fc fusion protein for 2 h at RT prior to adding with TNFα to A549 cells. As control we also performed the same experiment using IL-17A. For RNA extraction, cells were harvested directly from the wells using 350 ul RLT buffer supplemented with β-mercaptoethanol. Lysates were stored at -80°C until RNA extraction.

Primary bronchial epithelial cells from donors were grown in 6-well dishes. At confluency, cells were serum-starved for 24 h before stimulation to allow the cells to reach a quiescent state. Cytokines (TNFα, IL-17E) were added for various times and doses. Stimulations were done for 4 or 24 h. Cells were detached and cell pellets were immediately lysed in 350 ul RLT buffer. All extracts were kept at -80°C until processing.

2.4.4 Assessment of A549 cell proliferation by radioactive thymidine incoporation

The effect of IL-17E on proliferation of A549 cells was measured by radioactive thymidine uptake. The initiation of DNA replication is a sign of cell division. To assess

it, radioactive thymidine is placed in the media where it enters the cells and is used by the DNA replication machinery in place of regular endogenous thymidine. An increase in radioactive signal emission correlates with increased incorporation of radioactive thymidine in the cell's DNA and an indicator of cell proliferation. For this experiment, A549 cells were plated in 96-well dishes at 3000 cells per well and allowed to adhere overnight in complete media (DMEM/F12 + 10% FBS). Cells were rinsed twice in starving media, (DMEM/F12 without FBS) and starved for 48 h. After starving, media is replaced with fresh starving media containing IL-17E (50 ng/ml) or 2% FBS (positive control) and stimulated for 24 h. Cells were pulsed with 0.5 μCi ^{3}H-thymidine/well for the last 5 hours of the 24 h stimulation period. After incubation, wells were rinsed once with 1x DPBS and incubated for 15 min with trypsin to detach the cells and stored at -80°C. Cells were harvested onto glass filter mats (Wallac, PerkinElmer Life Sciences, Boston, MA, USA) with a semiautomatic cell harvester (Micro96 hervester, Skatron Instruments, Sterling VA, USA). ^{3}H-thymidine incorporation was measured by liquid scintillation counting (1450 MicroBeta Trilux, Wallac) and expressed as counts per minute (CPM).

2.4.5 Assessment of BNE and BAE cell proliferation by bromodeoxyuridine incorporation

The effect of IL-17E on proliferation of BNE and BAE cells was assessed by uptake of the synthetic thymidine analog bromodeoxyuridine (BrdU). ELISA was used to measure total cellular BrdU incorporation. For this experiment, BNE and BAE cells were plated in 96-well dishes at 10 000 cells per well and allowed to adhere overnight. Cells were serum-starved for 24 h and then stimulated with either media or IL-17E (50 ng/ml)

for 48 h. Afterwards, 10 ul of BrdU labeling solution was added to each well and incubated for 2 h. Cells are rinsed and fixed and BrdU is detected by using an anti-BrdU antibody.

Chapter 3 - Results

3.1 Expression and function of the IL-17E receptor on human airway smooth muscle cells.

3.1.1 Introduction

Interest in the interleukin (IL)-17 family has been stimulated by the publication of data suggesting that its founding member, IL-17A, may contribute to the pathogenesis of conditions such as cancer, multiple sclerosis, arthritis, lupus and asthma [150,297,391,392,442,443]. The expression of this cytokine is significantly upregulated in the airways of subjects with moderate-to-severe asthma [150]. IL-17A is proinflammatory in nature and is produced primarily by activated memory CD4+ T lymphocytes, and does not appear to be particularly associated with either the Th1 or Th2 subset of these cells [349,351]. It has now been demonstrated that IL-17A is produced by a particular lineage of CD4+ T cells called Th_{17} or $Th_{IL\text{-}17}$ [353,354,444]. IL-17A is especially potent at synergizing with IL-1β or TNFα to induce production of cytokines such as IL-6, IL-8 and GM-CSF [163,164,334,344,364,380]. Among the six members of the IL-17 family, the homodimer IL-17E, also known as IL-25, is unique in that it is able to elicit protypical Th2 responses such as peripheral and lung eosinophilia, increased serum IgE, increased respiratory tract mucus production, as well as the induction of IL-4, IL-5 and IL-13 gene expression [400,408,413,414]. Recent *in vitro* experiments have shown that Th2 cells, mast cells and macrophages produce IL-17E [78,406,408]. This cytokine binds to the interleukin-17B receptor (IL-17BR) [418] also known as IL-17Rh1 [412] and Evi27 [419], and is expressed in a variety of human tissues including the lung [412,418]. IL-17E activates NF-κB and stimulates IL-8 production

on binding to IL-17BR in two human renal carcinoma cell lines, TK-10 and 293 [412]. It also induces p38 and JNK phosphorylation in eosinophils [407].

Airway smooth muscle cells (ASMC) are responsible for the airway narrowing of asthma. However, their role is not limited to this action, rather, they appear to play an active role in the pathogenesis of this condition through various mechanisms including airway remodelling. ASMC have been shown to express receptors for a variety of cytokines and chemokines, including the IL-17A receptor [256], and respond to their cognate ligands by releasing other proinflammatory mediators and extracellular matrix (ECM) proteins [192,308]. One of the characteristic features of remodelling is the increased deposition of ECM, in particular collagen I, III, V, and proteoglycans. We hypothesized that ASMC harboured IL-17BR and that proinflammatory cytokines could modulate its expression. We also assessed the potential of IL-17E to induce expression of ECM components. Here, we provide data showing the presence of the IL-17E receptor, IL-17BR, in ASMC *in vivo* and *in vitro*, as well as its modulation by TNFα and IFNγ. We also show that there was increased expression of pro-collagen α1 and lumican mRNA increased in these cells in response to IL-17E. Taken together, these data suggest the involvement of IL-17E and its receptor in the initiation and/or perpetuation of airway remodelling in asthma.

3.1.2 Results

3.1.2.1 IL-17BR is expressed by cultured primary human ASMC.

In order to determine whether human cultured ASMC constitutively expressed IL17BR at the mRNA and protein levels, we analysed unstimulated cells by RT-PCR, Western blot and immunofluorescence. The RT-PCR data illustrated that IL-17BR was constitutively expressed in all our human ASMC sources as shown in Fig. 1A. Western blot analysis from the same ASMC demonstrated basal expression of IL-17BR (Fig. 1A). To confirm the specificity of the anti-IL-17BR antibody, we incubated it with rhIL-17BR, when this was applied to the Western blot membrane, no signal was detected, thus confirming the specificity of the 55 kDa band observed (Fig. 3.1.4.1A). Immunofluorescence also revealed receptor protein expression by primary cultured ASMC (Fig. 3.1.4.1C and D). Compared to isotype control, specific fluorescent staining was observed on the cell membrane, as well as in the cytoplasm.

3.1.2.2 IL-17BR mRNA and protein expression are increased by TNFa and decreased by IFNγ.

We wanted to investigate whether IL-17BR expression could be regulated by TNFα and IFNγ. Real-time PCR and Western blot were used to evaluate receptor modulation by these cytokines. TNFα upregulated IL-17BR in ASMC (Fig 3.1.4.2A). A time course of the effect of TNFα shows a rapid peak expression for both mRNA and protein after 4 h (Fig. 3.1.4.2A). Furthermore, we observed a concentration-dependent effect of TNFα on IL-17BR message and protein levels after 4 h stimulation (Fig. 3.1.4.2B). Levels of IL-

17BR appeared to reach a plateau at 10 ng/ml TNFα at the mRNA level. In contrast, treatment of cells with IFNγ resulted in a decrease in the expression of IL-17BR mRNA and protein (Fig. 3.1.4.2B). IL-17BR mRNA showed an early tendency for downregulation by IFNγ, however, the maximal inhibitory effects for mRNA and protein were reached after 12 h. IFNγ also inhibited the receptor's expression at the mRNA and protein levels in a concentration-dependent manner (Fig. 3.1.4.2B) up until 10 ng/ml, the 100 ng/ml concentration did not further inhibit the receptor.

3.1.2.3 Th2 cytokines do not affect IL-17BR expression in ASMC

We wished to assess whether IL-17BR expression was sensitive to the prototypical Th2 cytokines IL-4 and IL-13. These cytokines had no noticeable effect on the receptor in ASMC after 4 or 24 h of stimulation (Fig. 3.2.4.3). In the same conditions the Th1 cytokine IFNγ induced a significant downregulation of IL-17BR mRNA.

3.1.2.4 Effect of pharmacological inhibitors on IL-17BR expression

As previously illustrated (Fig. 3.2.4.2 and 3.2.4.3), TNFα-induced a robust increase in IL-17BR mRNA, and although upregulation of IL-17BR mRNA by TNFα was strongest at 4 h, the receptor remained induced after 24 h. This time point was used to study the effects of TNFα plus, either IFNγ For dexamethasone. Addition of increasing concentrations of IFNγ to TNFα-treated cells abrogated IL-17BR expression in a concentration dependent fashion (Fig 3.1.4.4A). The addition of the synthetic corticosteroid dexamethasone to TNFα-stimulated ASMC caused an even greater reduction in expression of IL-17BR message (Fig 3.1.4.4B). Inhibition of the NF-kB

pathway with AS602868 produced a marked inhibition of IL-17BR expression with a similar potency to that of dexamethasone (Fig 3.1.4.4C). The JNK inhibitor SP600125 caused a partial decrease in IL-17BR message (Fig 3.1.4.4C). The inhibitor of ERK phosphorylation, U0126, appeared to produce some inhibition of IL-17BR expression, although this effect was only significant at the concentration of 1 μM. The p38 phosphorylation inhibitor SB203580 showed no effect (Fig 3.1.4.4C). Of note, the fold increase values observed with TNFα+DMSO compared to DMSO were less than those observed with TNFα alone as compared to medium (Fig 3.1.4.2A). This may be related to an effect of DMSO on ASMC. The inhibitory effect of IFNγ on IL-17BR expression was reversed by U0126 in a concentration-dependent manner (Fig. 3.1.4.4D). Treatment with SB203580 and SP600125, had no significant effect on this parameter (Fig. 3.1.4.4D).

3.1.2.5 IL-17E stimulates ECM mRNA expression in ASMC.

Cells were stimulated with either 100 ng/ml IL-17E or equivalent in vehicle (4 mM HCl/ 0.1 % BSA) for 4, 24 and 48 h and analyzed for their expression of ECM components such as pro-collagen αI, lumican and versican mRNA. Of the ECM tested, pro-collagen I was the most increased by IL-17E. Statistically significant data points showed that pro-collagen αI was on average 1.6 fold upregulated after 24 h and lumican was 1.2 fold increased after 4 h (Fig. 3.1.4.5) when compared to vehicle-treated cells at their respective time points. Pro-collagen αI appeared to remain elevated after 48 h of stimulation, but was not significant ($p = 0.054$).

3.1.2.6 IL-17BR is expressed by airway smooth muscle in vivo

We investigated IL-17BR expression *in vivo*, using immunohistochemistry on airway biopsies obtained from 6 different asthmatic donors. Positive staining was evident in the smooth muscle layers (Fig. 3.1.4.6B) demonstrating the relative abundance of this receptor in the airways. No staining was observed using isotype control antibody (Fig. 3.1.4.6A). We confirmed in vivo expression of IL-17BR by laser capture microdissection of ASMC bundles from suitable biopsies (n=3). cDNA obtained from these microdissected tissues were analyzed by real-time PCR and showed that the IL-17BR 141 bp amplicon was present in laser-captured ASMC bundles (lanes 1-3). Cultured ASMC were used as a positive control (Fig. 3.1.4.6C). Non-specific bands observed below 100 bp correspond to primer-dimers (lanes 1 and – ctrl).

3.1.2.7 IL-17E promotes cytokine release by ASMC

The supernatant of control- or IL-17E-treated ASMC was evaluated for cytokine production using an antibody array (Fig. 3.1.4.7A). There are 42 different cytokines represented in duplicate on the antibody array. The quantified signals showed that IL-6, IL-8, IL-10, grow-related oncogene (GRO)-α and GM-CSF were upregulated by IL-17E in ASMC (Fig. 3.1.4.7B).

3.1.3 Figures

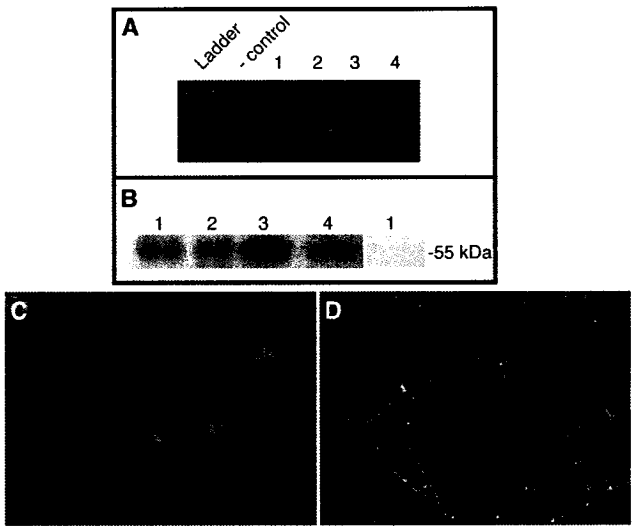


Figure 3.1.4.1. ***IL-17BR is expressed in cultured airway smooth muscle cells.*** IL-17BR mRNA (A) and protein (B) are constitutively expressed by cultured ASMC from different donors (n=4). Specificity of the 55 kDa band in the western blot was assessed using ASMC protein extract from donor 1 incubated with anti-IL-17BR preincubated with rhIL-17BR. Detection of IL-17BR in cultured ASMC was performed by immunofluorescence using an Alexa Fluor 555-labelled antibody. (C) 200X magnification of the staining obtained with the isotype control or (D) anti-IL17BR antibody.

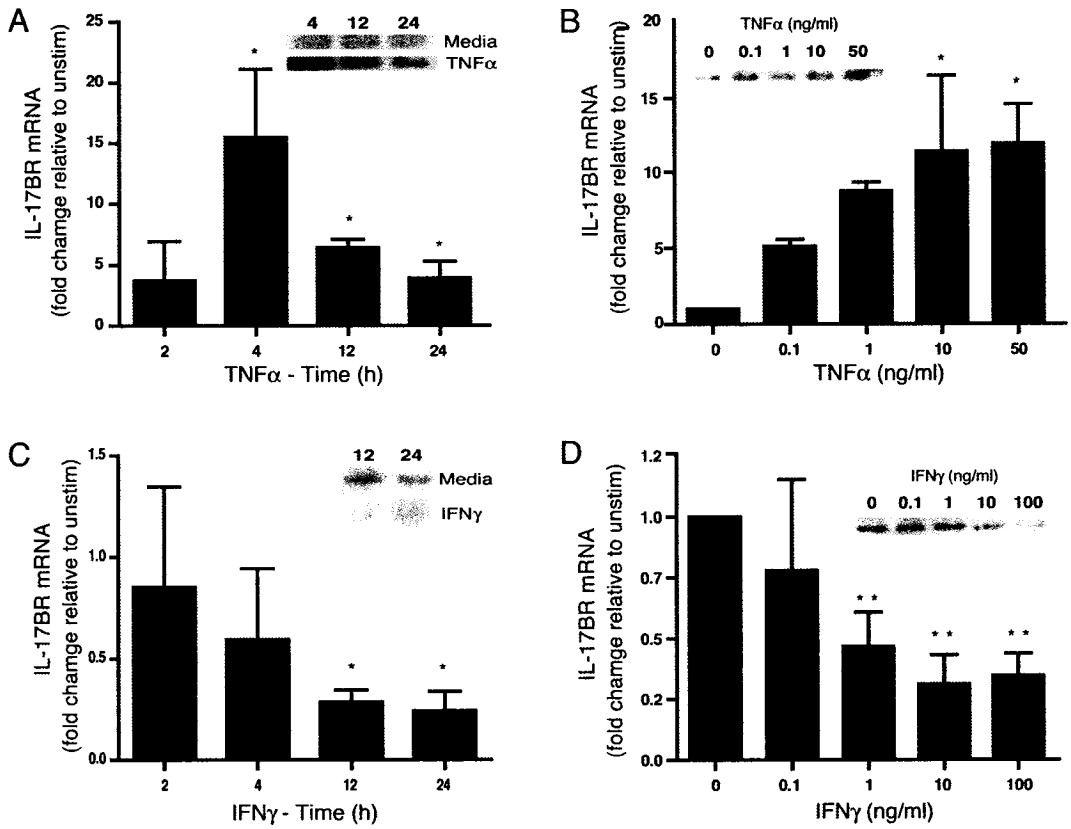


Figure 3.1.4.2. Time and concentration dependent effect of TNFα and IFNγ on IL-17BR mRNA and protein expression in ASMC. Cells were starved for 24 h before treatment with cytokines, cDNA and protein samples were analyzed by real-time PCR (bar graphs) and Western blot (insets). A) Time and B) concentration-dependent expression of IL-17BR mRNA and protein in ASMC in response to TNFα. C) Time and D) concentration-dependent, expression of IL-17BR mRNA and protein in ASMC after treatment with IFNγ. RNA was extracted at stated time and concentration and reverse-transcribed. cDNA was analyzed by real-time PCR, and data are represented as the mean of 3 to 5 independent experiments ± SD (*$p<0.05$). Western blots shown are representative of 3 independent experiments.

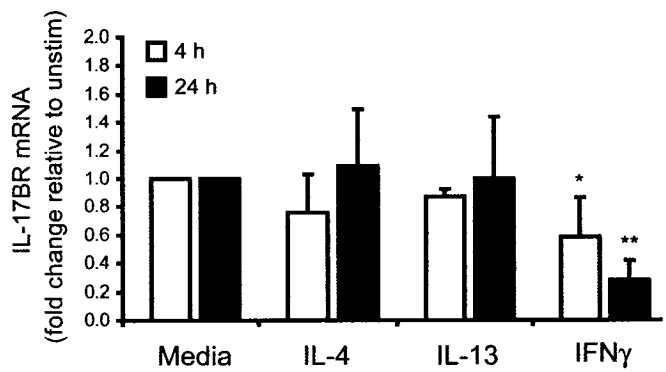


Figure 3.1.4.3. Effect of Th2-type cytokines on IL-17BR mRNA expression in ASMC. Cells were starved for 24 h and treated with 10 ng/ml IL-4, IL-13, or IFNγ. RNA was extracted at 4 and 24 h and reverse-transcribed. cDNA was analyzed for IL-17BR levels by real-time PCR. Data represent the mean of 3 to 6 independent experiments ± SD (*, $p<0.05$;** $p<0.01$).

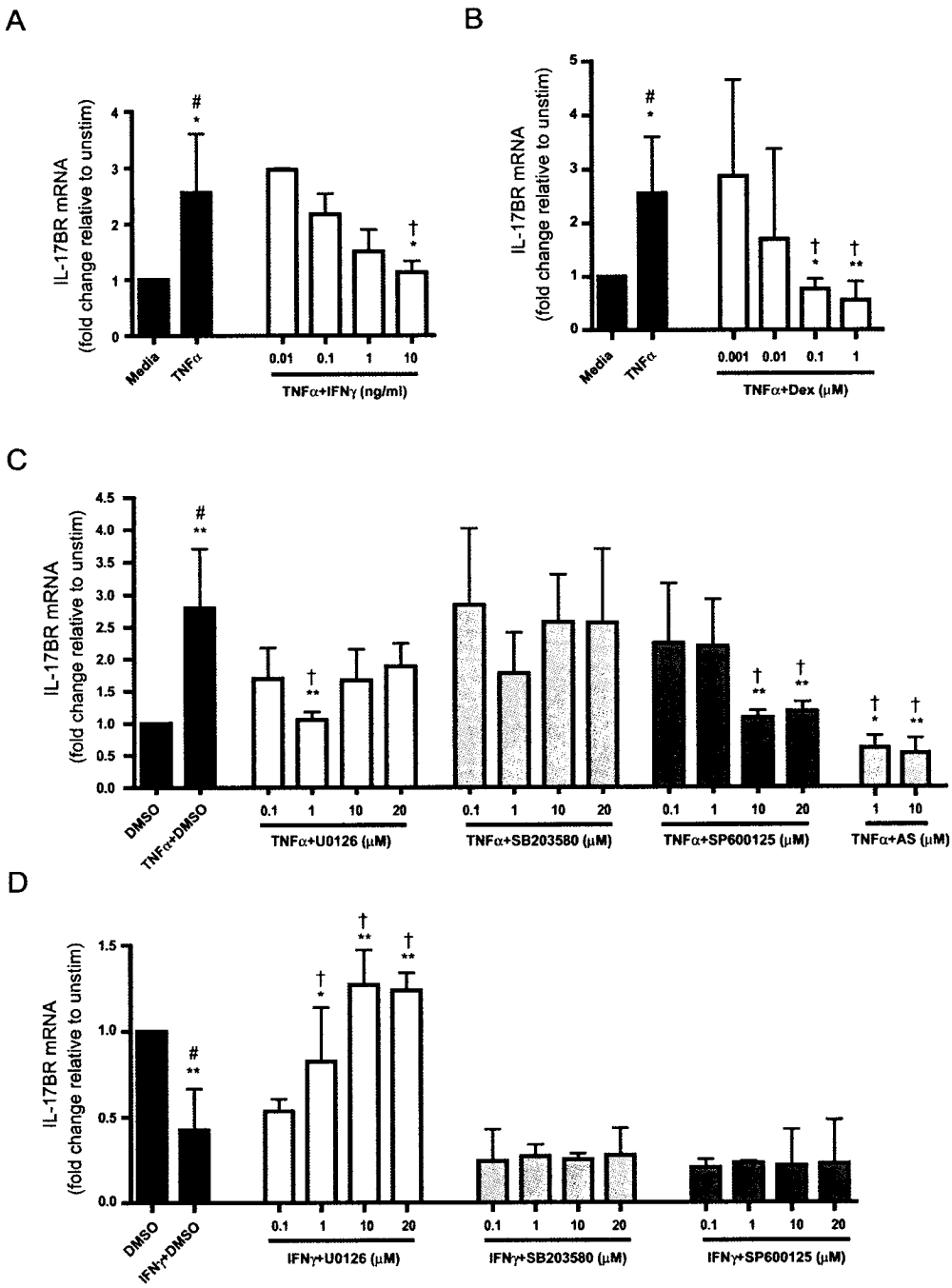


Figure 3.1.4.4. Effect of various inhibitors on IL-17BR expression. ASMC were starved for 24 h prior to stimulation and then treated with cytokines. Cells were stimulated 24 h with TNFα (10 ng/ml) and increasing concentrations of A) IFNγ or B) dexamethasone. C) ASMC were stimulated 4 h with TNFα (10 ng/ml) and increasing concentrations of MAPK (U0126, SB203580 and SP600125) or IKK2 (AS = AS602868)

inhibitors. D) ASMC were stimulated 24 h with IFNγ (10 ng/ml) and increasing concentrations of MAPK inhibitors. Effect of the solvent (DMSO, 0.2%) was tested for the equivalent volume used in the highest inhibitor concentration (20 μM). RNA was extracted after 4 or 24 h. RNA samples were reverse-transcribed and cDNA was analyzed for IL-17BR levels. Data are expressed as the mean of 3 to 4 independent experiments ± SD (*$p<0.05$; **$p<0.01$; # =significantly different from media or DMSO, †= significantly different from TNFα or IFNγ alone).

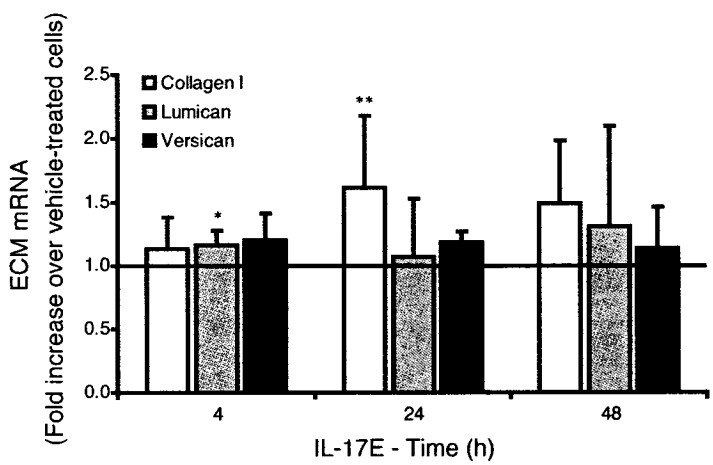


Figure 3.1.4.5. Effect of IL-17E on the expression of ECM mRNA in cultured ASMC. Cells were starved for 24 h and treated with 100 ng/ml IL-17E or its vehicle (4 mM HCl/0.1% BSA) and RNA was extracted at 4, 24 and 48 h. RNA samples were reverse-transcribed and cDNA was analyzed for pro-collagen αI, lumican and versican levels. Real time PCR data represents the mean of 4-8 independent experiments ± SD. (* $p < 0.05$; ** $p < 0.01$).

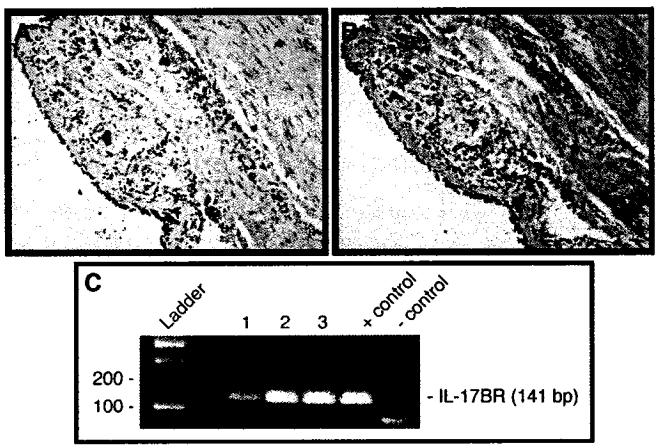
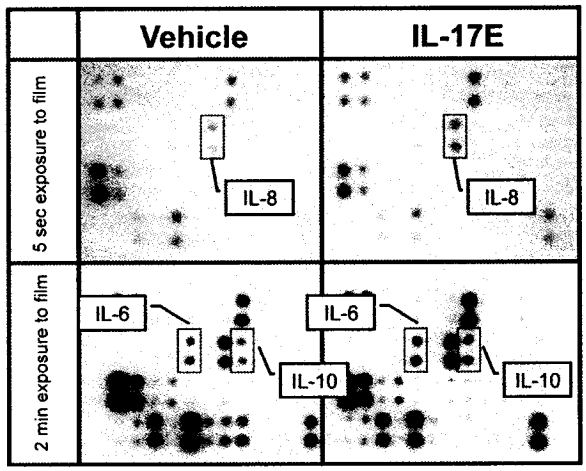


Figure 3.1.4.6. Expression of IL-17BR *in vivo*. IL-17BR is expressed at the protein level as assessed by immunohistochemistry. Representative staining of a human airway biopsy showing positive immunoreactivity in smooth muscle layers. 200X magnification of an airway biopsy treated with the isotype control (A) or the anti-IL17BR (B) antibody followed by APAAP detection. Expression of IL-17BR mRNA in ASMC bundles obtained by laser capture microdissection (lanes 1-3). Cultured ASMC were used as a positive control (C).

A



B

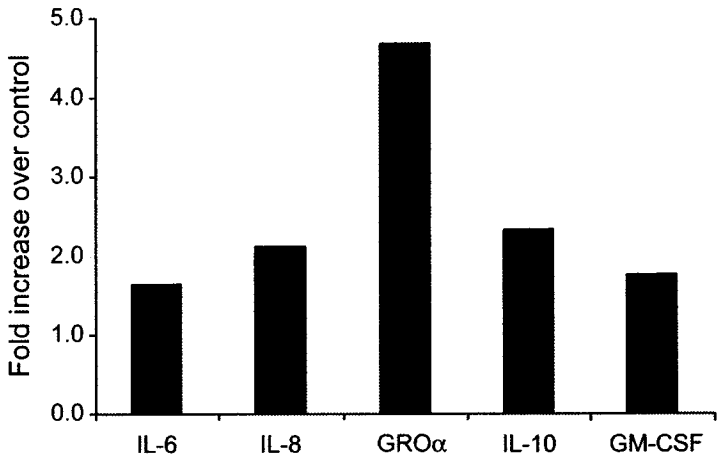


Figure 3.1.4.7. IL-17E induces the release of cytokines from ASMC. (A) ASMC were treated with vehicle or 30 ng/ml IL-17E for 24 h, supernatants were incubated with a cytokine antibody array. Signal was developed by X-ray autoradiography. (B) Cytokines increased by IL-17E as assessed by antibody array and expressed as the quantified dot intensity of IL-17E membrane over control membrane.

3.1.4 Discussion

We report for the first time the expression and modulation of the IL-17E receptor in a primary structural cell type. We show here that IL-17BR is expressed *in vitro* by primary ASMC and *in vivo* in airway biopsies with predominant staining in smooth muscle layers. Previous reports show that dendritic cells, leukocytes and some structural cell lines express this receptor [419,426]. Interestingly, a functional IL17R has recently been described on airway smooth muscle cells [256]. IL-17BR and IL-17R share partial amino acid homology but bind different IL-17 family members, IL-17BR binds IL-17E and to a lesser extent IL-17B but not IL-17, or IL-17C [412]. To date, there has been only one report describing the modulation of IL-17BR [426]. The authors found that IL-4, -10, and -13 upregulated IL-17BR mRNA in human dendritic cells, although the report of baseline levels of the receptor is unclear [426]. In ASMC, IL-4 and IL-13 did not have any effect on the constitutive expression of IL-17BR. However, our data shows that IL-17BR message and protein were readily upregulated by TNFα, an effect observed for IL-17R in human colonic myofibroblasts [364]. Preliminary bioinformatic analysis of the IL-17BR promoter region revealed several NF-κB binding sites, which could account for the TNFα-induced upregulation of the IL-17BR transcript we observed. TNFα is a well known inducer of NF-κB in ASMC. Consequently, we evaluated the effect of AS602868, a specific inhibitor of the IκB kinase 2 (IKK2), important for TNFα-mediated NF-κB activation. As expected, inhibition of NF-κB led to a marked reduction of TNFα-induced IL-17BR mRNA. IL-17BR expression is also corticosteroid sensitive, as demonstrated by the potent inhibition of TNFα-induced IL-17BR by dexamethasone. Besides activating the IκB kinase (IKK) pathway, TNFα can turn on the main MAPK signalling cascades

including, p42/44, p38 and c-Jun N-terminal kinase (JNK). The effect of inhibiting JNK was less drastic than with either dexamethasone or AS602868, SP600125 reduced TNFα-induced IL-17BR levels to baseline. Taken together, our data show NF-κB and to a lesser extent JNK play a role in the induction of IL-17BR by TNFα. Most likely, NF-κB causes a direct transactivation of the IL-17BR promoter.

IFNγ reduced the constitutive expression of IL-17BR, a finding also observed in dendritic cells. Furthermore, the increase in IL-17BR transcript induced by TNFα could be antagonized at the mRNA level by the addition of IFNγ. The mechanism by which IFNγ downregulates IL-17BR is unknown; IFNγ has been shown to target gene expression at different levels. IFNγ downregulates IL-4-induced IgE and IL-4R as well as collagen I expression by mechanisms involving transcriptional suppression, and posttranscriptional inhibition mediated by increased mRNA degradation. The IL-17BR promoter sequence displays two potential IFNγ response elements (IgRE) that bind Y-Box-1, previously shown to be responsible for IFNγ-mediated collagen I α2 downregulation. Furthermore, the 3'-untranslated region of the mouse and human IL-17BR transcript contain 3 and 2 Shaw-Kamen motifs (5'AUUUA-3'), respectively. This pentamer motif was previously shown to be present in several immediate early genes, such as cytokines, cytokine receptors, and oncogenes, and confers instability to mRNAs [411]. Indeed, we have preliminary data suggesting that IFNγ accelerates IL-17BR mRNA decay when added to actinomycin D treated ASMC. Collectively, these observations hint at the possible downregulation of IL-17BR by IFNγ via possible IgRE elements present in its promoter and/or through an increase in mRNA decay. Besides the traditional

Jak/STAT pathway involved in IFNγ signaling, this cytokine has been shown to activate ERK 1/2 and p38 in some cell types, such as cardiac myocytes, macrophages, glioma cells, human bronchial epithelial cells and human vascular smooth muscle cells. IFNγ can also induce ERK 1/2, and JNK phosphorylation in ASMC (data not shown). It has also been reported that IFNγ can induce, through JAK2, the activation of Raf-1, an upstream kinase involved in ERK phosphorylation. Moreover, the MEK inhibitor U0126 can abrogate IFNγ induced gene expression in the RAW murine macrophage cell line. Accordingly, preincubation of ASMC with the MAPK inhibitor U0126 totally reversed the IFNγ mediated IL-17BR downregulation. Inhibition of p38 or JNK phosphorylation had no significant effect. This suggests that IFNγ exerts its inhibition at least through the MEK-ERK pathway.

Shi *et al.* found that IL-17BR mRNA was significantly upregulated in a murine model of intestinal inflammation [418], a condition characterized by elevated TNFα levels in humans. In asthma, TNFα is increased in the airways and is linked to disease severity [128,135]. No real consensus exists in the literature about IFNγ expression in asthma since published data shows that levels of this cytokine are either increased, decreased or unchanged in asthma and upregulated in subjects treated with corticosteroids. Airways exposed to increased TNFα and decreased IFNγ concentrations could thus favor IL-17BR expression *in vivo* by smooth muscle. This phenomenon could modulate their sensitivity to IL-17E released by airway-infiltrated Th2 cells and/or mast cells. Accordingly, mice that were administered either recombinant IL-17E or an adenoviral IL-17E construct, developed a robust eosinophilic infiltration of the airways and stimulated the release of

the Th2 cytokines IL-4, IL-5, and IL-13 [400,408,413,414]. IFNγ inhibits Th2 cells and Th2 cytokine release as well as eosinophilia in sensitized mice after exposure to allergen and could therefore antagonize the effects of IL-17E. Indeed, one of the positive effects of increasing IFNγ in asthmatic patients, through allergen immunotherapy, could be the reduction of IL-17BR expression on ASMC and other cell types and the subsequent reduction of IL-17E bioactivity in vivo. One of the hallmarks of remodelling is the increased deposition of ECM proteins in the airways [308]. We wanted to investigate whether IL-17E was potentially involved in airway remodelling and for this, evaluated production of extracellular matrix (ECM) components by ASMC after stimulation with IL-17E. Reports have shown that cultured ASMC can produce matrix proteins such as collagen I, versican, and decorin [308]. In addition, IL-17A has been associated with an increased deposition of type I collagen in the airways of moderate to severe asthmatics [150], but it is thought that IL-17A could induce collagen I deposition indirectly through the induction of the profibrotic cytokines IL-6 and IL-11 which have been shown to enhance collagen and tissue inhibitor of metalloprotease (TIMP)-1 production [297,353]. Moreover, IL-17A transgenic mice, showed increased deposition of collagen in the subepithelium of bronchioles. IL-17E has also been shown to induce IL-6 production in eosinophils and human cartilage [393,407]. Interestingly, IL-17BR ligation promotes TGFβ1 mRNA production by murine embryonic fibroblasts, a known profibrotic cytokine [405].

Primary ASMC cultured in the presence of IL-17E showed increased mRNA expression for lumican, and especially collagen α1 (60% increase approximately). For comparison, TGF-β1, an inducer of collagen αI, produced a 150% increase in collagen αI

mRNA (data not shown). In contrast, IFNγ, a known inhibitor of collagen αI synthesis led to a 40-50% decrease in pro-collagen α1 mRNA (data not shown). Similar values were found in the literature regarding collagen αI mRNA modulation by TGF-β1 and IFNγ in fibroblasts and hepatic stellate cells. This initial finding implies that IL-17E could participate with other Th2 and profibrotic cytokines and contribute to airway remodelling observed in asthmatics.

Altogether, our results provide initial insight into the *in vitro* and *in vivo* expression as well as the *in vitro* regulation of the IL-17E receptor and a potential mechanism of action of this novel cytokine on ASMC. Further work is necessary to better understand the mechanisms that lead to the modulation of IL-17BR in ASMC by TNFα and IFNγ respectively. Moreover, it would be of considerable interest to assess if IL-17E and IL-17BR are overexpressed in asthma, an area of ongoing research in our laboratory.

3.2 Expression and function of the IL-17E receptor on human epithelial cells.

3.2.1 Introduction

Interest in the interleukin (IL)-17 family has been stimulated by the publication of data suggesting that its founding member, IL-17A, may contribute to the pathogenesis of conditions such as multiple sclerosis, arthritis and asthma [150,297,390,392,445]. Indeed, the expression of this cytokine is significantly upregulated in the airway mucosa, sputum and BAL fluid of asthmatics [150,297]. IL-17 is proinflammatory in nature and is produced primarily by activated memory CD4+ and CD8+ T lymphocytes. In fact, the main source of IL-17 comes from a newly identified CD4+ T cell lineage that is neither Th1 or Th2, it is termed Th-17 (Th_{IL-17}) [353,354]. IL-17 is especially potent at synergizing with IL-1β or TNFα to induce production of cytokines such as IL-6, IL-8 and GM-CSF. Among the six members of the IL-17 family, the homodimer IL-17E, also known as IL-25, is unique in that it is able to elicit protypical Th2 responses such as peripheral and lung eosinophilia, increased serum IgE, increased respiratory tract mucus production, as well as the induction of IL-4, IL-5 and IL-13 gene expression [400,408,413,414]. Recent *in vitro* experiments have shown that IL-17E is produced by Th2 cells [408], mast cells [78] and macrophages [406]. IL-17E binds to the interleukin-17B receptor (IL-17BR) also known as IL-17Rh1 [412] and Evi27 [419]. IL-17BR is expressed in a variety of human tissues including the lung and trachea [412] and *in vitro* by human monocytes, dendritic cells, primary lung fibroblasts and airway smooth muscle cells [426-428]. In cultured cells, IL-17E activates NF-κB [405,412], upregulates IL-6, IL-8, TARC, TGF-β1, G-CSF and GM-CSF production

[393,405,407,412,414,428]. It also induces ERK, p38 and JNK phosphorylation in eosinophils and in mouse embryonic fibroblasts [405,407].

Epithelial cells are an integral component of the airways and serve as a physiological barrier. Besides its structural nature, the epithelial cell is also a source of potent mediators of inflammation that may be involved in asthma pathogenesis and remodeling [446]. In has been shown that the bronchial epithelium of asthmatics express several cytokines and chemokines as well as cytokine receptors. In asthma, there is evdicence of epithelial damage and increased proliferation [44,446]. Moreover, cultured primary and transformed bronchial epithelial cells show evidence of increased proliferation in the presence of Th2 cytokines such as IL-4 and IL-13 [289,446].

This study focuses on IL-17E, a novel Th2 cytokine capable of inducing allergic asthma-like characteristics in mice [400,408,413,414]. Interestingly, these mice show evidence of bronchial epithelial hypertrophy and hyperplasia [408,414]. However this might not be unique feature of IL-17E, intratracheal IL-17F gene transfer into mice causes goblet cell hyperplasia in small and large airways [415] and IL-17A transgenic mice have hypertrophic epithelium in the bronchus and bronchioles [353] . Moreover, IL-17E induces several types of cytokines and chemokines other thanthe Th2 type *in vivo* [413,414]. So far, human lung epithelial cells have not been shown to carry a functional IL-17E receptor. In addition, it is not known wether IL-17E can act directly on epithelial cells to induce gene expression or proliferation. Here, we show for the first time that human epithelial cells constitutively express the IL-17E receptor. Similar to data obtained with human primary lung fibroblasts and airway smooth muscle cells [427,428], TNFα increases the basal expression of IL-17BR in pulmonary (A549) and primary bronchial epithelial cells. Additionally,

treating bronchial epithelial cells with IL-17E promotes their proliferation. We show also that IL-17E can synergize with TNFα to induce greater levels of the GROα chemokine, and that this effect is completely abrogated by incubation with the recombinant receptor to IL-17E (IL-17BR).

3.2.2 Results

3.2.2.1 IL-17BR is constitutively expressed by epithelial cells and is upregulated by TNFα

Real-time PCR was performed on cDNA obtained from A549, airway smooth muscle cells (ASMC), primary lung fibroblasts and T cells. All cell types show a basal level of IL-17BR expression at the mRNA level (Fig. 3.2.3.1A). To determine protein expression of IL-17BR, western blot was performed on cell lysates of A549, and human bronchial epithelial cells. The full length isoform at 55 kDa is present in these cells (Fig 3.2.3.1B). Moreover, IL-17BR mRNA is increased by TNFα in A549 pulmonary epithelial cells (10 ng/ml TNFα for 4 h).

3.2.2.2 Trend towards synergism between IL-17E and TNFα on GROα mRNA.

The expression of GROα and IL-6 was measured by real-time PCR in A549 cells that were treated with IL-17A, or IL-17E alone or in combination with TNFα (Fig. 4). IL-17E had little to no effect on GROα transcript level, by comparison, IL-17A induced a 3.3 fold increase and TNFα, induced a 4.6 fold increase in GROα. While not as potent as the combination of IL-17A and TNFα (~22 fold increase, data not shown), there was a noted synergistic effect between IL-17E and TNFα (~7.3 fold increase) on GROα levels. By

incubating IL-17E with its recombinant receptor (IL-17BR:Fc) we could abrogate the effect caused by IL-17E and return the GROα levels to those of TNFα alone (4.6 fold for TNFα and 4.8 fold for IL-17E+TNFα+IL-17BR:Fc). To confirm specificity of IL-17E for its receptor, IL-17BR:Fc was also incubated with IL-17A but had no consequence on GRO message (22 fold for IL-17A+TNFα and 21.9 fold for IL-17A+TNFα+IL-17BR:Fc, data not shown) (Fig. 4).

3.2.2.3 TNFα increases expression of IL-17BR mRNA in primary bronchial epithelial cells

Similarly to A549 cells, human bronchial epithelial cells isolated from non-asthmatic and mild asthmatic donors that were treated with TNFα (10 ng/ml) for 4 h showed an increased in their expression of the IL-17BR transcript. Baseline expression of IL-17BR mRNA in bronchial epithelial cells from mild asthmatics appear higher compared to control cells, but this difference is not statistically significant (p=0.13) (Fig. 3.2.3.3). Moreover, TNFα induced a much greater upregulation of IL-17BR message in control cells (~4.5-fold) than in asthmatics cells (~1.8-fold) when compared to media-treated cells.

3.2.2.4 IL-17E fails to enhance TNFa-induced GROa or IL-6 in primary bronchial epithelial cells.

Primary bronchial epithelial cells were treated with media alone, IL-17E, TNFα or a combination of IL-17E and TNFα. Levels of GROα were assessed by real-time PCR.

Message for either cytokine were not increased by IL-17E, but as expected, TNFα induced both in the bronchial epithelial cells. However, addition of IL-17E did not appear to further enhance the transcript levels for either GROα or IL-6. Unexpectedly, cells from asthmatic donors (BAE) were practically unresponsive to TNFα treatment, in a similar fashion to results obtained for IL-17BR in BAE (Fig. 3.2.3.3).

3.2.2.5 IL-17E induces bronchial epithelial cell proliferation.

IL-17E was tested for its ability to induce cellular proliferation on A549 and primary bronchial epithelial cells (Fig 3.2.3.6) 48 h-serum starved A549 cells were stimulated with media, IL-17E, or PDGF-bb (10 ng/ml) as positive control. Although IL-17E-treated cells show a trend towards increased proliferation, results did not reach statistical significance. However, the proliferative response of BNE and BAE after IL-17E treatment was more robust. We observed an increase in proliferation in both BNE and BAE cells, however the response to IL-17E as well as to the positive control (TNFa) was stronger in asthmatic-derived epithelial cells with an almost 50% increase in BrdU incorporation compared to media-treated cells.

3.2.2.6 IL-17BR is present on human airway epithelium.

Immunohistochemistry was performed on section of human airways to assess the presence of IL-17BR in bronchial epithelial cells (Fig. 3.2.3.7). Sections were treated with an anti-IL-17BR antibody or a matched isotype control antibody. There was a positive staining throughout the epithelium for IL-17BR. The signal was considered specific, as the isotype antibody did not stain the tissue. Constitutive expression of IL-

17BR mRNA was also verified by LCM and is expressed in the tested donors (Fig. 3.2.3.7C).

3.2.3 Figures

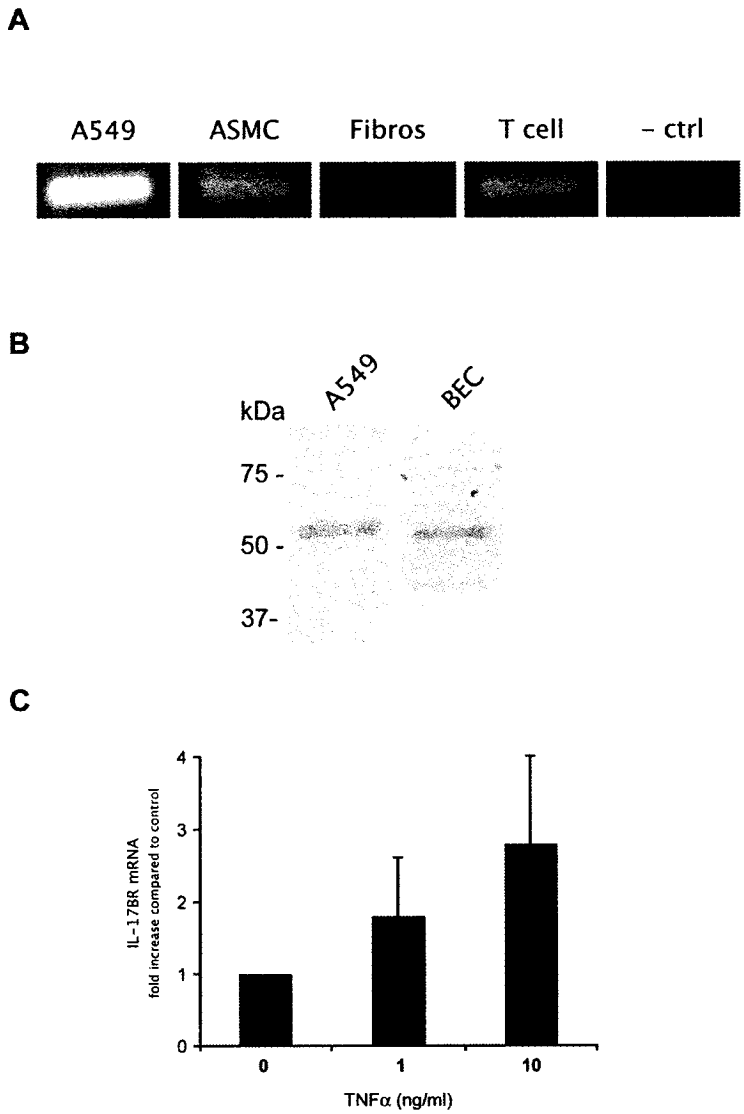


Figure 3.2.3.1. IL-17BR is constitutively expressed by several cell types, including epithelial cells and is upregulated by TNFα. (A) PCR for IL-17BR using cDNA from various cellular sources. (B) Western blot showing the IL-17BR 55 kDa band in A549 epithelial cells and in primary bronchial epithelial cells (BEC). (C) IL-17BR levels by real-time PCR in A549 cells treated for 4 h with 0, 1 or 10 ng/ml TNFα (n=2-3 ± SD).

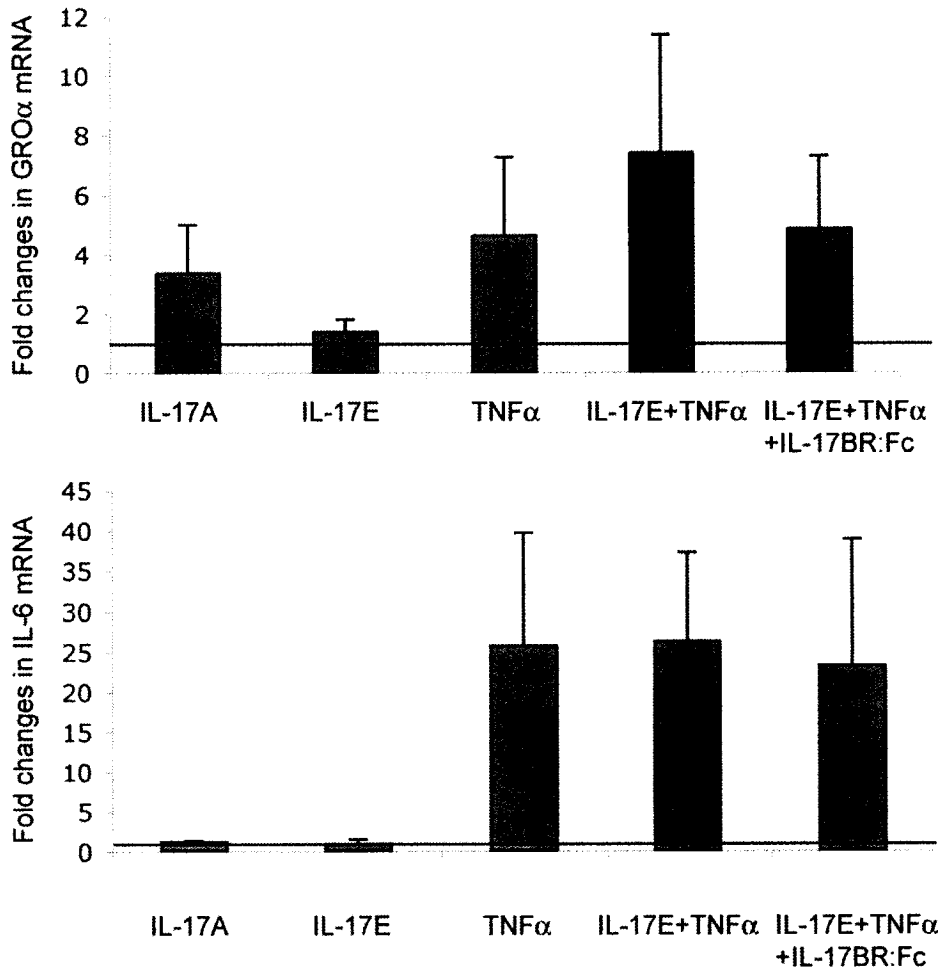


Figure 3.2.3.3. IL-17E synergizes with TNFα to induce GROα, but not IL-6. A549 cells were serum-starved for 24 h then stimulated with media, IL-17A (50 ng/ml), IL-17E (50 ng/ml), TNFα (1 ng/ml) or in combination for 24 h. The cytokine mixture (IL-17E+TNFα) was also preincubated with recombinant human IL-17BR:Fc (2 ug/ml) for 2 h prior to adding to the cells. RNA was extracted and reverse transcribed to cDNA. GROα and IL-6 expression was assessed by real-time PCR. Data is expressed as means± SD of 3-4 independent experiments.

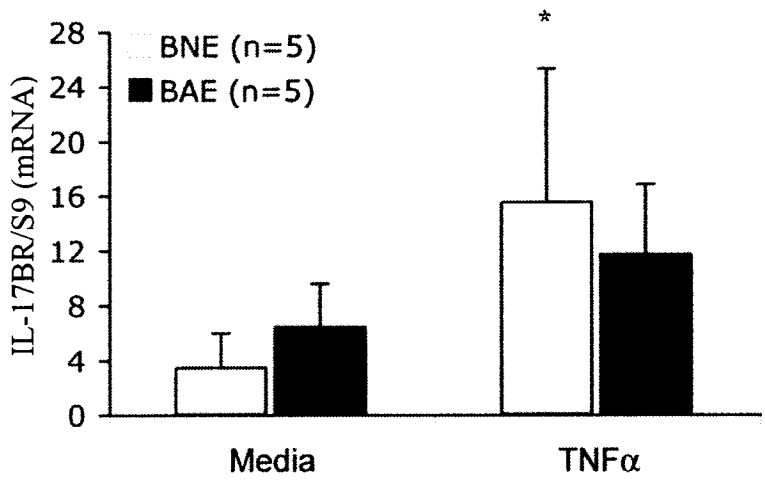


Figure 3.2.3.4. The proinflammatory cytokine TNFα increases expression of IL-17BR mRNA in bronchial epithelial cells. Primary bronchial epithelial cells from non-asthmatic (bronchial normal epithelial – BNE; n=5) and mild asthmatic donors (bronchial asthmatic epithelial – BAE; n=5) were cultured and treated with TNFα (10 ng/ml) for 4 h. RNA was extracted and reverse transcribed to cDNA. Level of IL-17BR was assessed by real-time PCR. Results are values of mean± SD. * $p<0.05$ for TNFα-treated BNE cells vs. media-treated BNE cells. Ribosomal protein S9 was used as the housekeeping gene (see Materials and Methods).

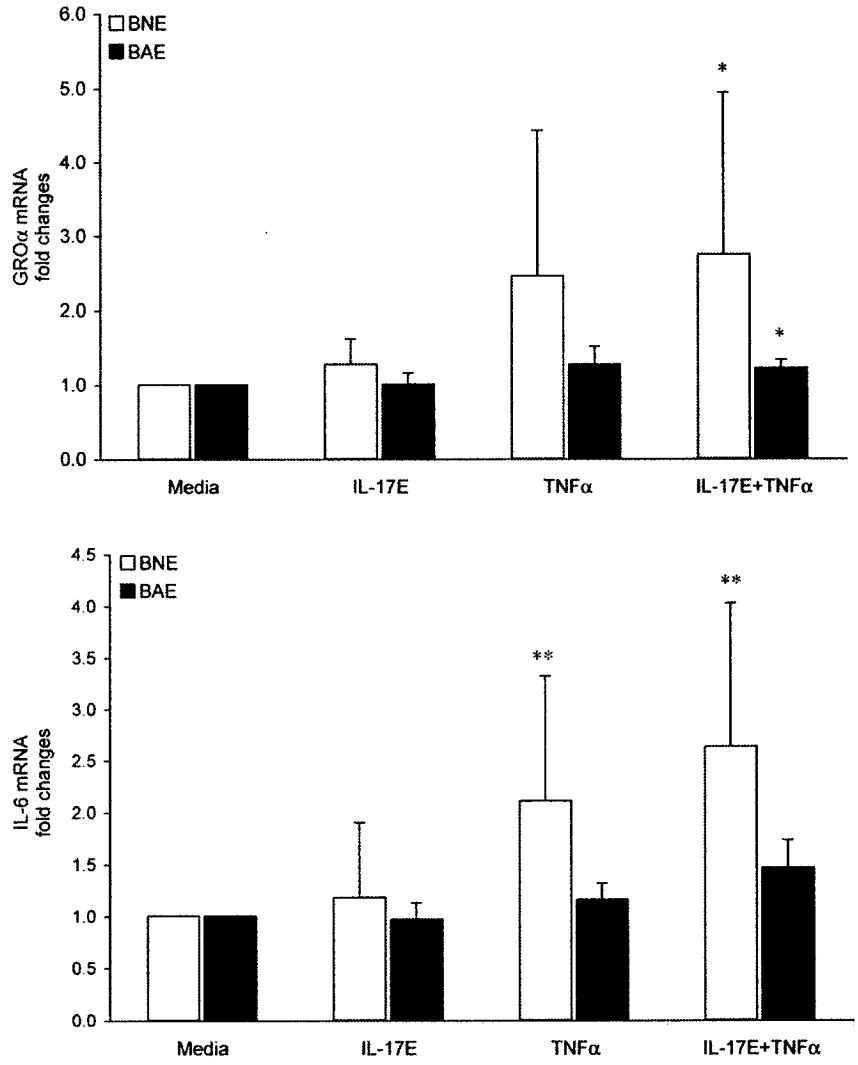


Figure 3.2.3.5. IL-17E fails to enhance TNFα-induced GROα or IL-6 in primary bronchial epithelial cells. Primary bronchial epithelial cells from non-asthmatic (bronchial normal epithelial – BNE; n=6) and mild asthmatic donors (bronchial asthmatic epithelial – BAE; n=5) were cultured and treated with media, IL-17E (50 ng/ml), TNFα (10 ng/ml) or in combination for 24 h. RNA was extracted at the indicated time and concentration and was reverse transcribed to cDNA. Level of GROα and IL-6 was assessed by real-time PCR. Results are values of mean± SD. * $p<0.05$; ** $p<0.01$ vs. media-treated cells.

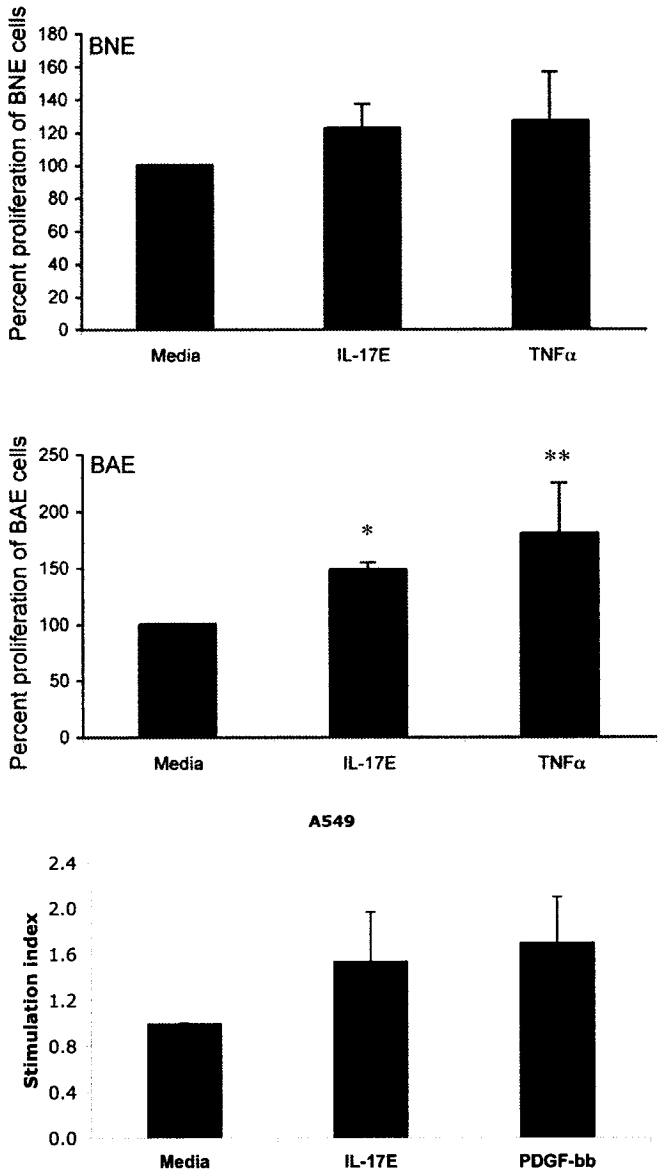


Figure 3.2.3.6. IL-17E has a positive effect on bronchial epithelial cell proliferation. BNE and BAE cells were serum starved for 24 h then treated with media, IL-17E (50 ng/ml) or TNFα (1 ng/ml) as positive control for 48 h. Proliferation of BNE and BAE cells was measured by BrdU incorporation and assessed by ELISA. A549 cells were serum-starved for 48 h and treated with media, IL-17E (50 ng/ml) or PDGF-bb (10 ng/ml) as a positive control. Cells were pulsed with 0.5 μCi ^{3}H-thymidine/well (25 μCi/ml) for the last 5 h of stimulation (n=3 ± SD). CPM values for media-treated A549 cells ranged from 6700 to 11900. Results of IL-17E in A549 cells did not reach statistical significance. * $p<0.05$; ** $p<0.01$ vs. media-treated cells.

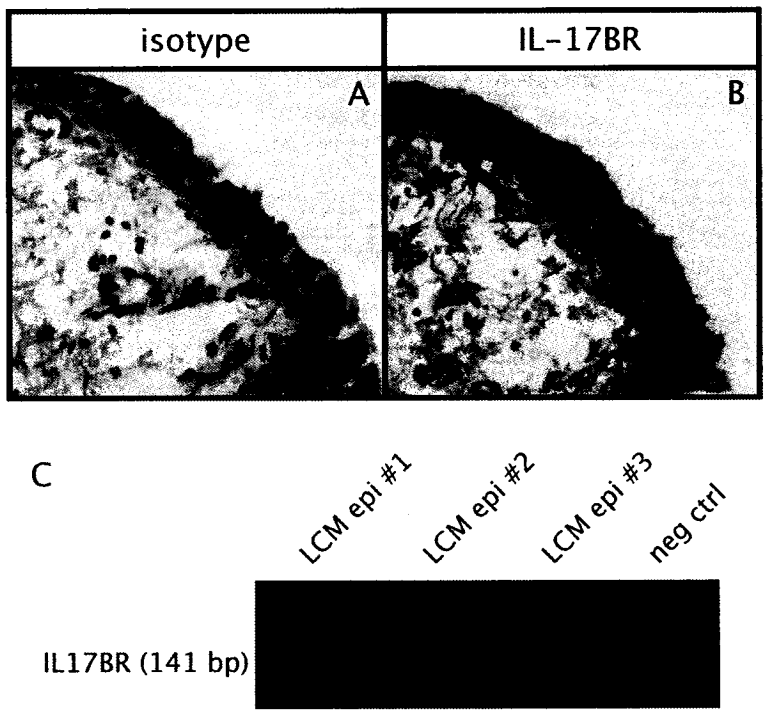


Figure 3.2.3.7. IL-17BR is expressed by human airway epithelium. IL-17BR is expressed at the protein level as assessed by immunohistochemistry. Representative staining of a human airway biopsy showing positive immunoreactivity in smooth muscle layers. 200X magnification of an airway biopsy treated with the isotype control (A) or the anti-IL-17BR (B) antibody followed by APAAP detection. Expression of IL-17BR mRNA in airway epithelium obtained by laser capture microdissection from biopsies (C).

3.2.4 Discussion

We have shown that IL-17E *in vitro*, in addition to inducing cytokines such as IL-6, IL-8, GM-CSF, and MCP-1; can promote the proliferation of bronchial epithelial cells. This finding is in agreement with epithelial and goblet cell hyperplasia observed in IL-17E transgenic mice or mice treated with IL-17E [408,414]. We also report that human epithelial cells carry a functional IL-17E receptor. Expression of this receptor (IL-17BR) is widely distributed among tissues, including the lung and trachea. In addition, cells of both a structural and immune nature express IL-17BR [419,426]. By comparison, IL-17R also shares a broad tissue and cellular distribution, and is expressed by cultured bronchial epithelial cells and human lung epithelium [344]. As with IL-17A, IL-17E is more restricted in its expression, as their production has only been mostly observed in immune-type cells [78,297,353,354,406,408]. *In vitro*, Th2 cells, alveolar macrophages and mast cells produce IL-17E [78,406,408]. Several groups have studied the effect of elevated levels of IL-17E in mice, either through an IL-17E transgene or by treating them with an adenoviral expression construct or intranasal/intraperitoneal administration of the recombinant cytokine. Collectively, their data shows that IL-17E promotes a Th2-type response in these animals [400,408,413,414]. The data presented in this study demonstrates the presence of the IL-17E receptor in cultured bronchial- and lung-derived epithelial cells as well as in the bronchial epithelium from human biopsies tissue; this suggests that epithelial cells can respond to IL-17E.

Of the six IL-17 family members, IL-17E shares the least amino acid homology with IL-17A, nevertheless, both share some biological activity: they activate NF-kB, induce

the profibrotic cytokine IL-6, [297,393,407,447] are associated with collagen I production [427] or deposition, [150] induce IL-8 [239,297,412] and GM-CSF [364,428]. The combination of IL-17A with TNFα provides a potent synergistic effect in the production of several growth factors, chemokines and cytokines, similarly, human lung fibroblasts treated with IL-17E in addition to TNFα had enhanced GM-CSF production [428]. As expected, the combination of IL-17A and TNFα induces a very strong effect on GROα and IL-6 mRNA production. IL-17E on its own is not as potent as IL-17A, it fails to upregulate IL-6, but lightly induces GROα. However, while not as potent as IL-17A+TNFα, IL-17E has a synergistic effect with TNFα on the induction of IL-6 and GROα transcripts. The synergistic effect between IL-17E and TNFα was partly abrogated by the recombinant IL-17E receptor (IL-17BR:Fc). Preincubating IL-17A with IL-17BR:Fc had not effect on GROα or IL-6 expression, confirming its specificity for IL-17E. The similarities between IL-17A and IL-17E biology might be explained by comparable intracellular events. Although there are differences in the size and amino acid composition of their respective receptors, both ligands can induce ERK and p38 phosphorylation [360,405,407,447] and require the presence of the adaptor protein TNF receptor-associated factor (TRAF) 6 for proper signaling to NF-kB and JNK [389,405].

Data regarding the effect of IL-17A on *in vitro* cellular proliferation is conflicting and could be due to differing cell types. Recently, IL-17A was shown to induce proliferation of tracheal epithelial cells [448], however, some show no effect on A549 cells [370] and can even be anti-proliferative on primary human intestinal epithelial cells [449]. IL-17A also appears to play a role in tumor cell growth and apoptosis [370,373,443,449]. IL-17A promotes the growth of different human non-small cell lung cancer (NSCLC) cell lines in SCID

mice. Interestingly, IL-17A had anti-apoptotic properties on the tumors generated by the NSCLC cell lines Sq19 and A549 [370]. In line with our observation that IL-17E promotes a proliferative phenotype, the murine B cell line Ba/F3 expressing the IL-17E receptor proliferated when cultured with supernatant from a mouse myeloma cell line (X63) engineered to produce IL-17E [405] and support the B cell hyperplasia noted in one of the IL-17E transgenic mice models [413].

3.3 Expression and function of the IL-17E receptor on human lung fibroblasts

3.3.1 Introduction

Asthma is a chronic inflammation of the airways and is characterized by the increase in expression of Th2 cytokines such as IL-4, IL-5 and IL-13, mucus hypersecretion, fibrosis eosinophil accumulation in airway tissues, and in allergic asthmatic increased IgE synthesis. IL-17E (IL-25), a newly characterized member of the IL-17 family, induces in mice several features typical of human asthma. Mice treated with or overexpressing IL-17E show increased expression of IL-4, IL-5 and IL-13, circulating IgE, mucus production, promotes a strong eosinophilia [400,408,413,414] and in some models, neutrophilia as well. Thus IL-17E is suggested to play an important role in allergic airway disease. Eosinophilia has long been considered a hallmark feature of asthma, the cytokines eotaxin, RANTES, IL-8 and GM-CSF are involved in regulation of eosinophil attraction and maintenance in the bronchial mucosa, all of which are upregulated in asthmatics. These tissue-infiltrated eosinophils are thought to play an important role in this disease because they are a source of proinflammatory and profibrotic cytokines, such as TNFα and TGFβ1.

In vitro, production of IL-17E has been shown in human Th2 lymphocytes, murine mast cells exposed to IgE and rat airway macrophages stimulated with titanium oxide particles. IL-17E is also constitutively found at the mRNA level in human lung and tracheal tissue. This cytokines is a ligand for the interleukin-17B receptor (IL-17BR) also known as IL-17Rh1 [412] and Evi27 [419]. IL-17B also binds IL-17BR but with less affinity

than IL-17E [342,412]. This receptor is expressed in a variety of human tissues including the trachea and lung [412]. *In vitro*, human macrophages and dendritic cells have been shown to express IL-17BR [426]. Like many proinflammatory cytokines such as IL-17A and TNFα, IL-17E activates NF-kB and the members of the mitogen-activated protein kinase (MAPK) pathway [405,407,412]. IL-17E can induce IL-6, G-CSF, IL-8 in murine fibroblasts, human articular cartilage, and a renal epithelial cell line [393,412,414]. It can also promote the release of IL-6, MIP-1α and IL-8 from cultured human eosinophils [407].

Because structural cells of the airways are a potent source of cytokines and chemokines both *in vivo* and *in vitro*, they are now regarded as important effector cells in the pathogenesis of airway diseases. Cultured primary pulmonary fibroblasts constitutively express IL-17BR and produce mediators involved in allergic airway disease in response to IL-17E. IL-17E induces synthesis of IL-8, GM-CSF, RANTES and eotaxin. Moreover, TNFα and TGFβ1 have the ability to modulate IL-17E bioactivity on human lung fibroblasts, maybe in part through increased or decreased IL-17BR expression.

3.3.2 Results

3.3.2.1 Human lung fibroblasts constitutively express IL-17BR: modulation by TNFα and TGFβ1.

Immunofluorescent staining of unstimulated fibroblasts shows constitutive expression of IL-17BR (Fig. 3.3.3.1A). Similarly, western blot analysis demonstrates that fibroblasts cell lysates contain de full-length protein at 56 kDa (Fig. 3.3.3.1B), as well as other previously demonstrated isoforms, thought to represent secreted and glycosylated variants [419]. The chimeric protein IL-17BR:Fc was used as a positive control and the expected band at 75 kDa was detected (Fig. 3.3.3.1B). Real-time PCR analysis showed that fibroblasts express IL-17BR mRNA at baseline. The IL-17BR transcript was strongly upregulated in cells stimulated with TNFα at 4 or 24 h, with the maximal effect observed after 4 h (Fig. 1C). On the other hand, TGFβ1-stimulated cells showed a decrease in their expression of the receptor's mRNA at both 4 and 24 h of stimulation, but reached greatest inhibitory effect after 24 h (Fig. 3.3.3.1C)

3.3.2.2 IL-17E increases mRNA for IL-8 (CXCL8), RANTES (CCL11), eotaxin (CCL5) and GM-CSF.

Fibroblasts were stimulated with either 50 ng/ml IL-17E or its vehicle (4 mM HCl+0.1% BSA) for 4 or 24 h. Real-time PCR was used to assess mRNA levels for IL-8 (CXCL8), RANTES (CCL11), eotaxin (CCL5) and GM-CSF. Although these cytokines were detected in vehicle treated cells, IL-17E upregulated their transcript levels (1.5- to 3-fold) after 4 and 24 h of stimulation. However, cytokine increase reached statistical significance only at the 24 h time point (Fig. 3.3.3.2). IL-8 (CXCL8) and GM-CSF

message were the most sensitive to IL-17E with an almost 3-fold increase over vehicle (Fig, 3.3.3.2C, D).

3.3.2.3 Trend towards synergism between IL-17E and TNFα on GM-CSF and CXCL8 mRNA production.

Levels of IL-8 (CXCL8), RANTES (CCL11), eotaxin (CCL5) and GM-CSF mRNA were measured on fibroblasts that were treated with either IL-17E, TNFα or in combination. TNFα strongly induced these cytokines, especially at 24 h (Fig. 3.3.3.3). The combination of IL-17E and TNFα had no noticeable effect on CCL5 and CCL11 transcript levels at either incubation times. IL-17E appeared to induce a synergistic effect on the amount of GM-CSF (2-fold for IL-17E and 118-fold for TNFα versus 176-fold in combination; Fig. 3.3.3.3C) and CXCL8 (464-fold for TNFα and 666-fold for IL-17E+TNFα; Fig. 3D), both at 24 h, however the differences between the TNFα and TNFα+IL-17E groups did not reach statistical significance.

3.3.3.4 TGFβ1 decreases production of CCL11 but increases CXCL8 production in IL-17E-treated cells.

Levels of IL-8 (CXCL8), RANTES (CCL11), eotaxin (CCL5) and GM-CSF mRNA were measured on fibroblasts that were treated with either IL-17E, TGFβ1 or in combination. In contrast to TNFα, TGFβ1 did not upregulate CCL5 or CCL11 mRNA (Fig. 3.3.3.4A, B). GM-CSF mRNA was modestly increased after 24 h with TGFβ1; however, it had a much stronger effect on CXCL8 expression at 4 and 24 h (Fig. 3.3.3.4C, D). The combination of IL-17E and TGFβ1 induced a transient (only at 4 h)

decrease in GM-CSF mRNA. Likewise, CCL11 mRNA was downregulated by IL-17E+TGFβ1 compared to IL-17E alone at 24 h (Fig. 3.3.3.4A). Conversely, IL-17E increased a TGFβ1-mediated upregulation of CXCL8 expression, especially at 24 h (Fig. 3.3.3.4D).

3.3.3.5 Trend towards synergism between IL-17E and TNFα on GM-CSF and CXCL8 release from lung fibroblasts.

ELISA was used to measure levels of GM-CSF and CXCL8 in the supernatants of cells treated with IL-17E alone or in combination with TNFα for 48 h. Low levels of GM-CSF and CXCL8 were detected in unstimulated fibroblasts. IL-17E induced a slight increase in the secretion of both cytokines, although it was not statistically significant. As expected, TNFα induced GM-CSF and CXCL8 release and this effect appeared to be accentuated by addition of IL-17E (Fig. 3.3.3.5). However, differences between TNFα alone and TNFα+IL-17E are not statistically significant.

3.3.3.6 IL-17E is present in human airways.

The expression of IL-17E in human asthmatic airways was done by immunohistochemistry of bronchial biopsy tissue. Mainly inflammatory cells, some with eosinophil-like morphology stained positive for IL-17E in the bronchial submucosa (Fig. 3.3.3.6). Moreover, the same tissue revealed eosinophil infiltration as assessed by positive MBP staining.

3.3.3 Figures

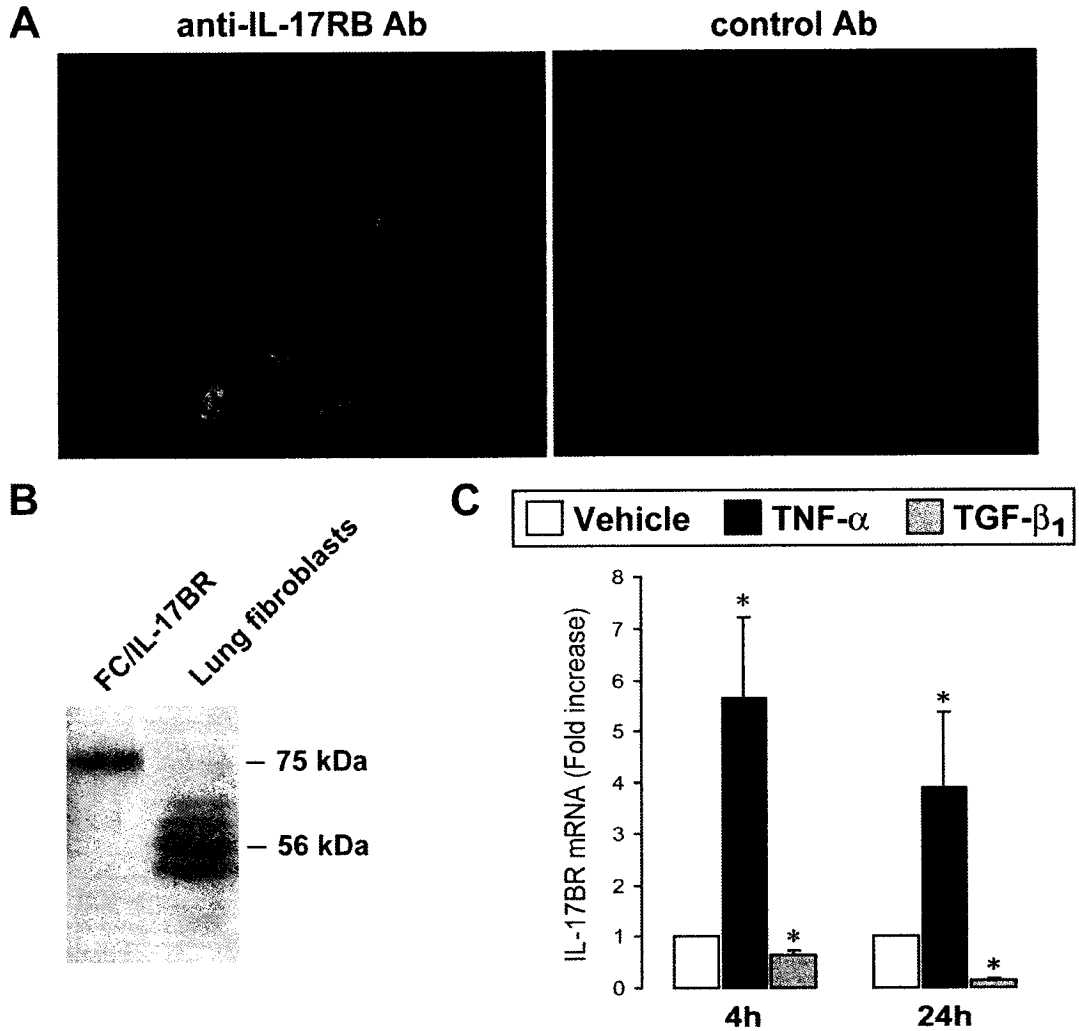


Figure 3.3.3.1. Expression of IL-17BR in human lung fibroblasts. (A) IL-17BR immunofluorescent staining in cultured lung fibroblasts (left panel). Negative control experiment using an isotype-matched control primary antibody is shown in right panel. Magnification: x200. (B) Detection of IL-17BR expression by Western blot in fibroblast lysates (lane 2) in parallel with the purified control fusion protein Fc/IL-17BR (lane 1). Results are representative of a minimum of 3 experiments. (C) Expression of IL-17BR mRNA, as quantified by real-time PCR in fibroblasts stimulated with vehicle, TNFa or TGFb1. IL-17BR levels were normalized over those of the housekeeping gene S9, and values are expressed as a fold increase compared with vehicle-treated cells. Values are means ± SEMs of 3 to 5 experiments. *P<0.05 compared with vehicle-treated fibroblasts at the same time point.

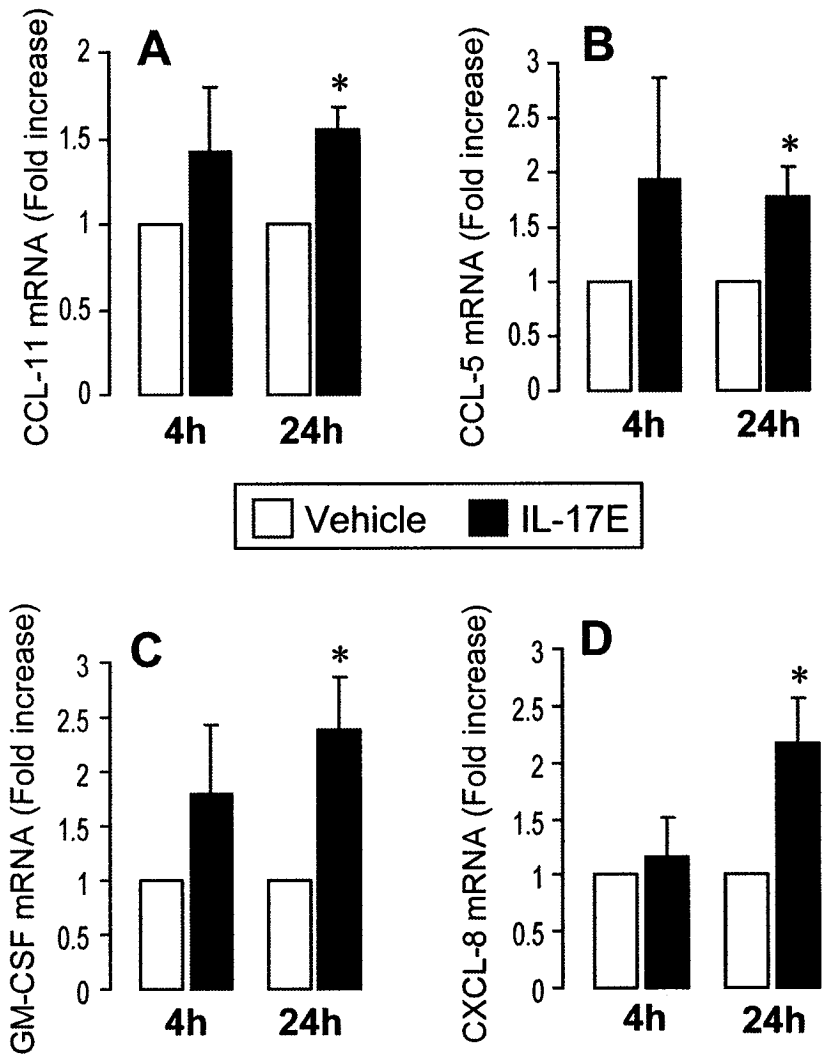


Figure 3.3.3.2. Effect of IL-17E on the expression of fibroblast-derived mediators. Serum-deprived lung fibroblasts were stimulated with IL-17E or its vehicle and (A) CCL11 (B), CCL5 (C), GM-CSF (D), and CXCL8 mRNA levels were evaluated. Results are presented as a fold increase compared with vehicle-treated cells at the same time point. Values are means ± SEMs of 6 experiments. *P<0.05 compared with vehicle-treated fibroblasts.

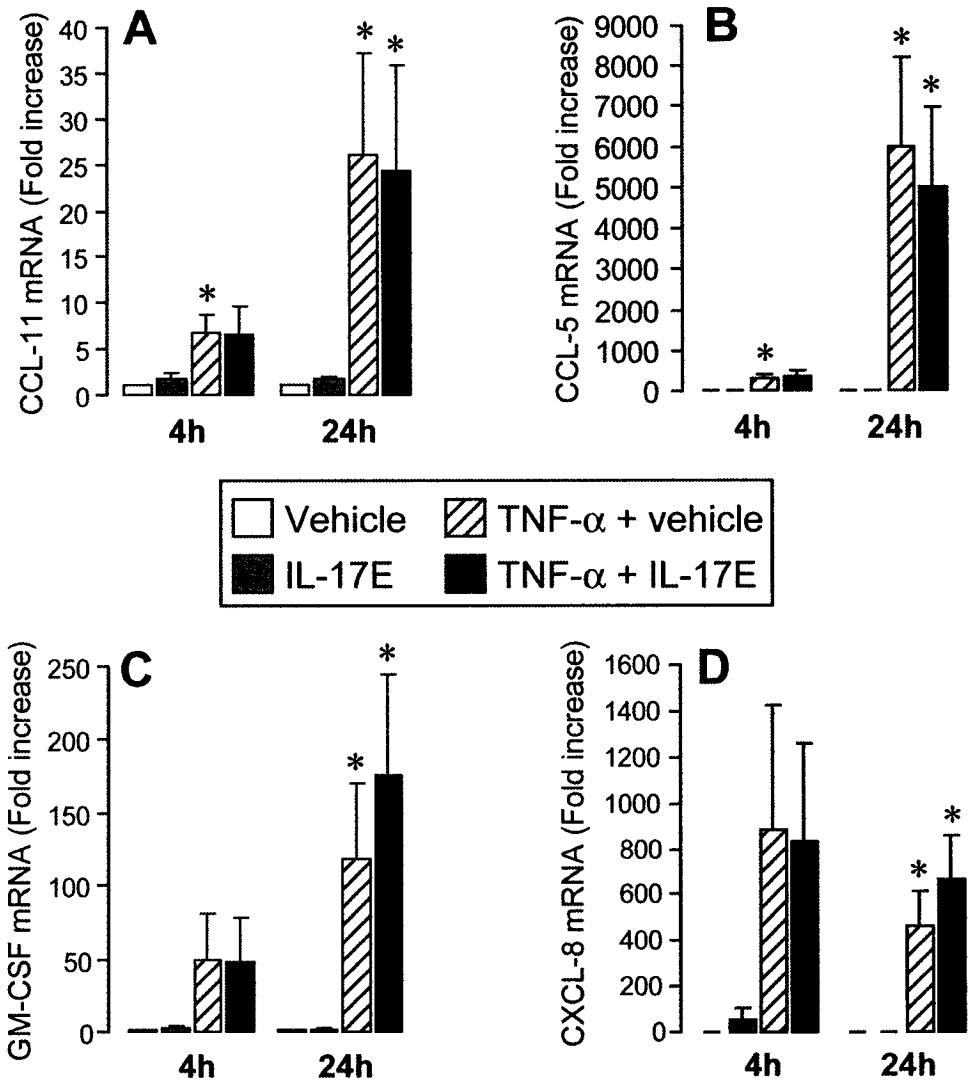


Figure 3.3.3.3. Effect of TNFα and IL-17E on the expression of cytokines in lung fibroblasts. Fibroblasts were stimulated with IL-17E or its vehicle, alone or in combination with TNFα, and (A) CCL11 (B), CCL5 (C), GM-CSF (D), and CXCL8 mRNA levels were quantified. Results are expressed as fold increase over values obtained with vehicle-treated cells at the same time point. Values are means ± SEMs of 3 to 7 experiments. *P<0.05 compared with IL-17E–treated cells at the same time point. Differences between TNFα alone and TNFα+IL-17E are not statistically significant.

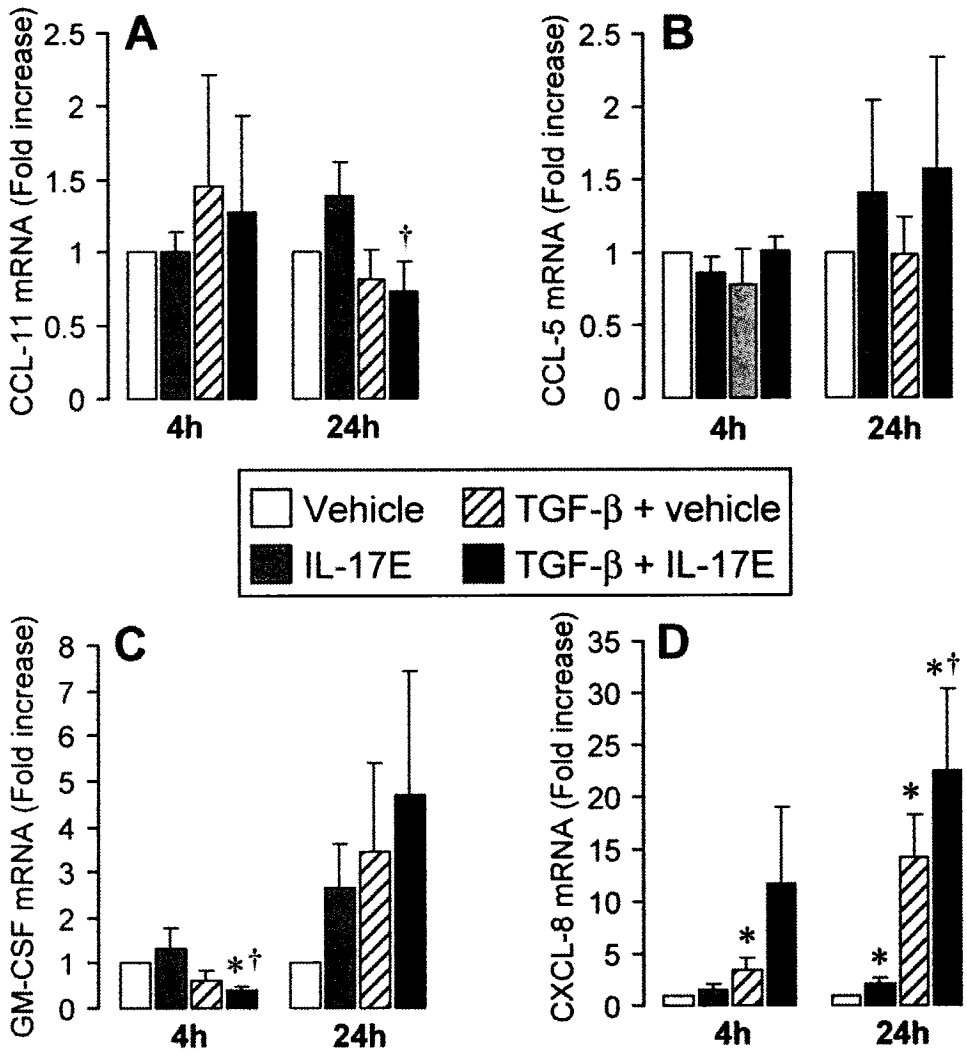


Figure 3.3.3.4. Effect of the combination of TGFβ1 and IL-17E on the expression of cytokine expression in fibroblasts. Fibroblasts were stimulated with IL-17E or its vehicle, alone or in combination with TGFβ1, and mRNA levels of (A) CCL11 (B), CCL5 (C), GM-CSF, and (D) CXCL8 were quantified. Results are expressed as a percentage of the value obtained in vehicle-treated cells at the same time point. Values are means ± SEMs of 3 to 6 experiments. *P<0.05 compared with IL-17E–stimulated cells and †P<0.05 compared with TGFβ1–treated cells at the same time point.

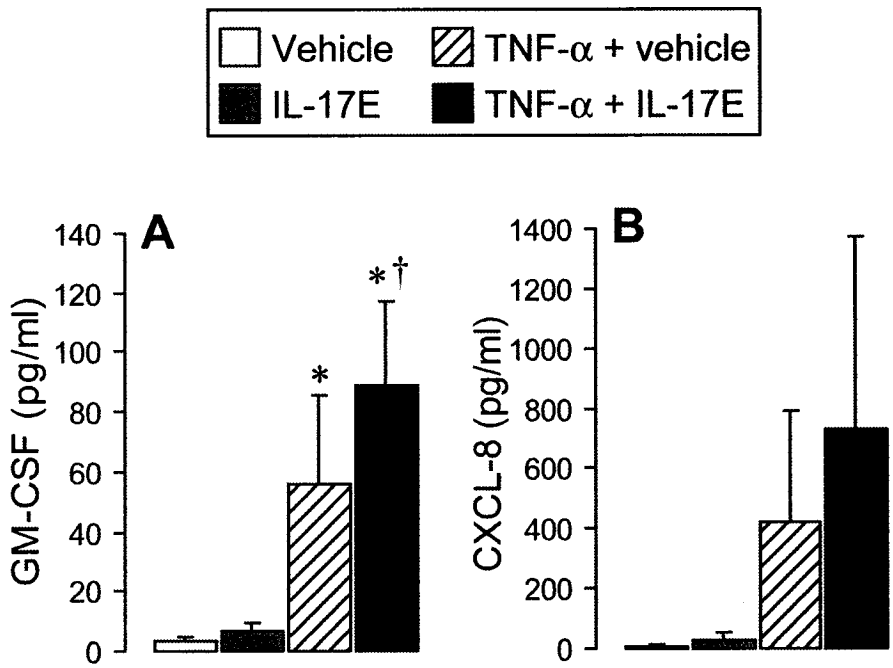


Figure 3.3.3.5. Effect of IL-17E and TNFα on cytokine release by fibroblasts. Fibroblasts were stimulated for 48 hours with IL-17E or its vehicle, with or without TNFα. (A) GM-CSF and (B) CXCL-8 were quantified in supernatants. Values are expressed as means ± SEMs of 3 to 6 experiments. *P<0.05 compared with vehicle and †P<0.05 compared with IL-17E–treated cells. Differences between TNFα alone and TNFα+IL-17E are not statistically significant.

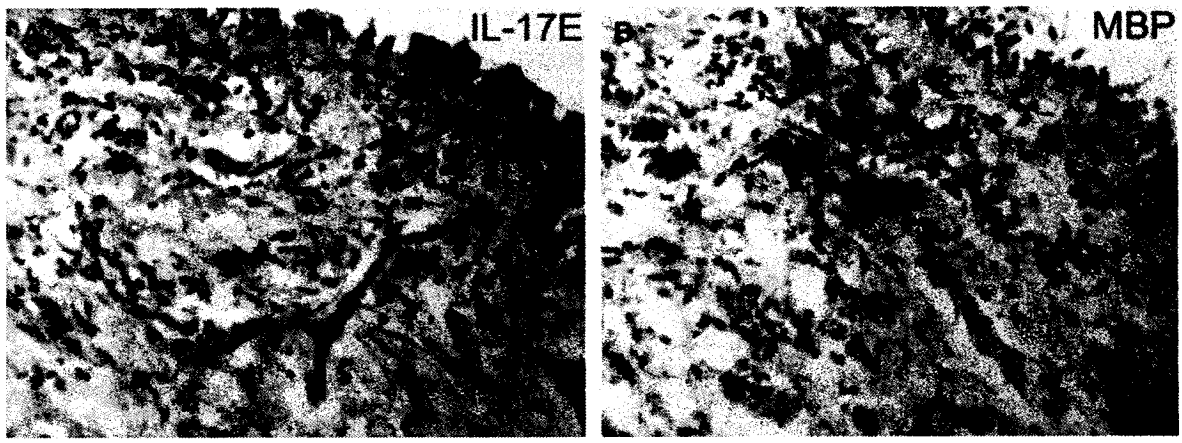


Figure 3.3.3.6. Expression of IL-17E in asthma. (A) Immunohistochemistry for IL-17E and (B) major basic protein (MBP) was performed on bronchial biopsies of subjects with asthma to identify eosinophils. Representative staining experiments are shown.

3.3.4 Discussion

Little is know about the biology of IL-17E in airway diseases such as asthma, however, its Th2 nature leads us to believe that it could indeed play a role in this disease. This report demonstrated for the first time the expression of IL-17BR in primary lung fibroblasts and that IL-17E promotes the production of pro-eosinophilic mediators in these cells (CCL11, CCL5, CXCL8 and GM-CSF).

Mice overexpressing an IL-17E transgene or treated with exogenous recombinant IL-17E show eosinophil infiltration in lung tissue, something not seen in transgenic IL-17A animals. Indeed, IL-8 is unpregulated in primary bronchial epithelial cells treated with IL-17A, but not eotaxin or RANTES [384]. The chemokines CCL11 (eotaxin) and CCL5 (RANTES) are selectively chemotactic for eosinophils and promote their infiltration from the peripheral blood to the airway mucosa. GM-CSF is a growth factor involved in the proliferation of granulocyte progenitors and promotes their differentiation into eosinophils. GM-CSF also supports eosinophil chemotaxis and activation. Reports using IL-17E transgenic mice also show an increase in neutrophils [413,414], this can be explained by the fact that IL-17E also induces CXCL8 (IL-8), an important neutrophil chemotattractant, in lung fibroblasts, but also in epithelial cells [412] and eosinophils [407]. Moreover, CXCL8 also induces chemotaxis of primed eosinophil and favors their adhesion to activated endothelium. Thus IL-17E may be involved in the initiation and maintenance of an eosinophilic infiltration through the upregulation of CCL11, CCl5, CXCL8 and GM-CSF.

Our data shows that the IL-17E receptor message is upregulated by the proinflammatory cytokine TNFα. Consistent with our observation, IL-17BR transcript is

elevated in the intestines a rat model of inflammatory bowel disease [418]. On the other hand, TGFβ1 inhibited the expression of IL-17BR in fibroblasts. This finding may support the anti-inflammatory role often attributed to TGFβ1. Therefore, TNFα and TGFβ1 may affect a cell's sensibility to IL-17E.

On their own, IL-17E and IL-17A are not as potent as TNFα in inducing several types of cytokines (IL-6, IL-8, GM-CSF, etc), however, they are capable of enhancing an inflammatory reaction through a synergistic cooperation with TNFα. We show that IL-17E potentiates TNFα-mediated expression of GM-CSF and CXCL8 mRNA. This phenomenon might be explained by several mechanisms, either through upregulation of IL-17BR by TNFα, through activation of NF-kB or MAPK, since IL-17E and TNFα can these pathways [405,412] or because IL-17E, like IL-17A, could help stabilize TNFα-induced mRNA transcripts [164,364,367,374,381,450]. Indeed, IL-17A synergizes with TNFα to upregulate GM-CSF production in human colonic myofibroblasts through increased mRNA stabilization and possibly though increased mobilization of NF-kB [364]. However, there was no difference in CCL11 or CCL5 expression between TNFα-stimulated cells versus those treated with IL-17E+TNFα. Interestingly, while several reports show the synergistic effect of IL-17A coupled with TNFα, it can also antagonize TNFα-mediated secretion of chemokines such as CCL5 (RANTES) and CCL27 [385,386].

We show here that TGFβ1 downregulates IL-17BR mRNA in fibroblasts, and this could affect their response to IL-17E. We observe that TGFβ1 inhibits IL-17E-induced increase in CCL11 mRNA and does the same with GM-CSF, although it is only a transient effect observed at 4 h and not at 24 h. However, TGFβ1 synergized with IL-17E

to upregulate CXCL8 mRNA. Collectively these results suggest alternate pathways that either synergize or antagonize IL-17E-mediated cytokine production.

Chapter 4 - General discussion

4.1 Introductory remarks

There as been a longstanding interest in uncovering molecules that may be involved in the pathogenesis of asthma, and whether they contribute to the development or maintenance of this disease. Several newly discovered cytokines hold the potential to be targets of treatment. Concerning the IL-17 family of cytokines, the expression of its founding member, IL-17A, is increased in asthmatics and correlates with disease severity. Asthma is regarded as a condition where there is an imbalance between Th2 vs Th1 type cytokines. Among the Th2 cytokines now recognized, IL-17E appears to be one of the latest additions, mainly because of the asthma-like features it induces in mice. Importantly, cells that are known to infiltrate the airway mucosa such as Th2 cells, mastocytes and eosinophils express IL-17E. These leukocytes are in fact surrounded by resident structural cells and are likely targets of IL-17E in human airways. For this reason, we choose to investigate whether bronchial epithelial cells, lung fibroblasts and airway smooth cells can respond to IL-17E. This thesis contributes data that clarifies our understanding of the biology of IL-17E and its receptor (IL-17BR) *in vitro*.

4.2 Discussion of results

Our data shows that IL-17E is present in human airways and overlaps with positive eosinophil staining, suggesting this cell as a potential source of IL-17E in the airways. No data yet exists in the literature on the presence of the IL-17E receptor in the bronchial mucosa or the effect of its ligand on cultured structural cells of the airways. We show

here that IL-17BR is expressed by the bronchial epithelium and airway smooth muscle *in vivo*. Likewise, the IL-17R is present on the human lung epithelium. We demonstrated that IL-17BR was present at baseline in cultured airway smooth muscle cells, bronchial epithelial cells and lung fibroblasts. The expression of several cytokine and chemokine receptors can be modulated by cytokines such as TNFα, which increases CCR3 on ASMC [231], as well as the decoy IL-13 receptor, IL-13Rα2, but does not affect expression of the signaling IL-13Rα1 [340]. Similarly, TNFα upregulates the IL-17R transcript in human colonic myofibroblasts [364] but not in human chondrocytes or synovial fibroblasts [398]. IL-17BR mRNA is strongly increased in a rat model of intestinal inflammation [418], a condition in humans that is characterized by elevated levels of TNFα. In accordance with these data, we observed that TNFα consistently increased IL-17BR expression in all three strucutal cell types. Using ASMC, we have shown that the increase in IL-17BR by TNFα was very sensitive to the corticosteroid dexamethasone, as it efficiently downregulated IL-17BR mRNA in TNFα-treated cells. NF-kB is a chief downstream target of TNFα signaling and has been shown to be a target of corticosteroid action. Activation of NF-kB requires the phosphorylation of the inhibitory protein IkB by the IkB kinase (IKK) complex. The IKK2 subunit of the IkB kinase complex is though to play a crucial role in activating NF-kB. Using a specific inhibitor of IKK2 (AS602868) we show that the induction of IL-17BR mRNA by TNFα is totally dependent on NF-kB, and it is thus likely that IL-17BR gene transcription is dependent on the mobilization of NF-kB to its promoter.

In ASMC, IFNγ decreases the basal level of IL-17BR and also inhibits TNFα from increasing the receptor mRNA. Furthermore, IFNγ also has an inhibitory effect on IL-

17BR expression by human monocytes [426]. Unlike IL-17BR, IL-17R appears unsensitive to IFNγ in bronchial epithelial cells and keratinocytes [351,384]. IFNγ has also been shown to decrease the expression of IL-4Rα and the chemokine receptor CCR2 [451,452]. Interestingly, both IL-4R and CCR2 are downregulated by IFNγ through an increase in mRNA decay rather than a decrease in transcription. Inhibition of IL-17BR mRNA in ASMC by IFNγ was completely reverted by preventing ERK1/2 phosphorylation through the use of the U0126 compound. U0126 inhibits MEK1/2, a kinase that phosphorylates ERK1/2. p38 or JNK phosphorylation inhibitors had no effect. Thus IFNγ-induced downregulation of the receptor is mediated at least in part through the MEK-ERK pathway. A similar observation has been made in PBMCs, treatment with PMA leads to a decrease in the production the amyloid precursor protein mRNA and this can be reversed by addition of U0126 [453].

IL-17E appears to be profibrotic in ASMC as it promotes collagen I gene expression. Interestingly, ASMC have been shown to be a potent source of ECM [308]. Ligation of IL-17BR on mouse embryonic fibroblasts stimulates an increase in TGFβ1 mRNA [405] and TGFβ1 is known to strongly promote collagen I expression in fibroblasts and ASMC. IL-17E might directly induce collagen I expression, or indirectly through TGFβ1 which might then act in an autocrine/paracrine fashion on ASMC and enhance its synthesis [240]. There seems to be a profibrotic role for members of the IL-17 family, IL-17A induces the expression of the profibrotic cytokines IL-6 and IL-11 in lung fibroblasts [297] and its expression is correlated with an increased collagen I and III deposition in asthmatic airways [150].

We have also shown by antibody array that IL-17E-treated ASMC respond by

releasing more cytokines such as IL-6, IL-8, IL-10, GROα, and GM-CSF. IL-17E is associated with increased IL-6 and IL-8 release from eosinophils, epithelial cells and human articular cartilage [393,407,412]. Moreover, IL-10, GROα and to a lesser extent GM-CSF are upregulated in the kidneys and lungs of IL-17E transgenic mice [414]. IL-8 and GROα are known neutrophil chemoattractants, and while IL-17E is known to induce eosinophilia in mice, some have noted an increase in neutrophils as well [413,414]. It is possible that IL-17E could have a dual effect on both types of granulocytes. A key action of IL-17A *in vivo* is to attract neutrophils through the release of CXC chemokines (IL-8, GROα) and this effect is efficiently diminished by a blocking antibody to IL-17A [367,454]. Unexpectedly, blocking IL-17A enhances IL-5 levels in the BAL and blood and worsens bronchial eosinophilia induced by allergen in mice [454]. IL-17A could thus have a regulatory role in controlling airway eosinophilia. However, sputum expression of IL-17A and IL-5 mRNA correlates in asthmatics [356]. Taken together, these results suggest that the regulation of neutrophil and eosinophil influx into the airways by IL-17A or IL-17E is likely to be more complex than first thought.

In human lung fibroblasts, IL-17E also induces RANTES and eotaxin mRNA, in addition to IL-8 and GM-CSF. Our observation of increased RANTES and eotaxin in IL-17E-treated fibroblasts might account for the pro-eosinophilic effect of IL-17E in mouse models, as both are chemotactic for eosinophils. Moreover, GM-CSF prolongs eosinophil survival [455,456] and can prime them to increase their release of eosinophil cationic protein and eosinophil-derived neurotoxin [288,457]. The combination of IL-17A and TNFα offer strong synergistic effects on the mRNA levels and protein release of several cytokines including GM-CSF. Similarly, in lung fibroblasts, the combination of IL-17E and TNFα

appears to induce more GM-CSF than with TNFα alone. However, addition of IL-17E to TNFα-treated fibroblasts does not further increase IL-8 release. The potentiating effect of IL-17E might occur via TNFα-mediated increase in IL-17BR in fibroblasts, rendering the cells more responsive to IL-17E. This phenomenon can be seen in the synergistic antiproliferative effect of TNFα and IFNγ on HT-29 epithelial adenocarcinoma cells. Ruggiero *et al.* demonstrated that pretreating cells with IFNγ upregulated the TNF receptor and rendered them much more responsive to TNFα [265]. It is also quite likely that IL-17E stabilizes the GM-CSF mRNA transcript induced by TNFα. This mechanism of increased transcript stability has often been reported for IL-17A [164,364,371]. We have also reported that TGFβ1 downregulates the basal expression of IL-17BR mRNA in fibroblasts and downregulates IL-17E-induced eotaxin mRNA. Yet, TGFβ1 is synergistic in combination with IL-17E on IL-8 message levels. Conversely, TGFβ1 antagonizes eotaxin mRNA induced by IL-17E, though it is synergistic with the Th2 cytokine IL-13 on the induction of eotaxin in lung fibroblasts [331].

The human bronchial epithelium stains positively for IL-17BR and is thus a likely target for IL-17E *in vivo*. Cultured bronchial epithelial cells, isolated from biopsies taken from non-asthmatics (control) and mild asthmatics donors, also reveal basal expression of IL-17BR mRNA. We detected more IL-17BR transcript in unstimulated cells from the asthmatics, but this difference was not significant. Nevertheless, it does not preclude the possibility that IL-17BR might be increased in cells from moderate or severe asthmatics. In accordance with our findings in ASMC, fibroblast and A549 cells, IL-17BR mRNA is increased by treatment with TNFα in bronchial epithelial cells from both control and

asthmatics, but unexpectedly, cells fron controls responded much more to TNFα stimulation than asthmatic cells. We tested the levels of the CXC family chemokine GROα as well as IL-6 in epithelial cells. These mediators were elevated in the supernatants from IL-17E-treated in ASMC, as assessed by the antibody array. GROα and IL-8 share a very similar sequence and function in attracting neutrophils. Interestingly, GROα is also produced by human eosinophils and might be released in asthmatic airways [458]. In a murine model of allergic airway disease, GROα is increased in the early phase of the inflammatory response after allergen challenge and remains elevated throughout the experimental period [459]. Similarly, IL-17E induces the release of IL-8 in a renal epithelial cell line as well as by eosinophils [407,412]. GROα is upregulated by IL-17A and IL-17F in bronchial epithelial cells [293,368,460], we show however that IL-17E on its own has almost no effect on the level of GROα and IL-6 message in A549 cells. Nonetheless, IL-17E can synergize with TNFα to increase GROα message, but not with IL-6. IL-17E has been shown to physically bind IL-17BR using techniques that isolated both molecules and tested them outside of an *in vitro* or *in vivo* biological context [342,412]. So far, no data in the literature has shown that blocking the receptor or using a competitor abrogates IL-17E's biological activity. For that reason, we incubated IL-17E with recombinant IL-17BR before adding it to the cells in combination with TNFα. We observed that this abrogated the effect caused by IL-17E. Moreover, this effect is specific, as incubating IL-17A with IL-17BR had no effect on its biological activity. As discussed earlier, it is probable that IL-17E enhances TNFα-induced GROα by stabilization of its mRNA. In line with our findings, GROα was also elevated in IL-

17E transgenic mice [414] and could in part account for the increase in circulating neutrophil numbers [413,414].

Hypertrophy and hyperplasia of the airway epithelium was among the biological changes noticed in mice receiving IL-17E [408,414], although no direct association was made between IL-17E and enhanced epithelial proliferation. We demonstrate that bronchial epithelial cells isolated from normal and mild asthmatic donors proliferate when stimulated with IL-17E and moreso in asthmatic-derived epithelial cells. Though, these cells also proliferate more in the presence of TNFα, suggesting that BAE cells might be more proliferative in response to mitogens, rather than this being a specific response to IL-17E. In support for our observations, IL-17E promotes the proliferation of the B cell line BaF3 and enhances the survival of eosinophils [405,461]. By comparison, IL-17A has recently been shown to induce the proliferation of human tracheal epithelial cells [448] in addition to enhancing vascular endothelial growth factor (VEGF)-induced endothelial cell proliferation [370,462]. It is likely that *in vivo*, in addition to inducing cytokine expression, IL-17E is a pro-proliferative and/or pro-survival factor for various cell types.

So far, only *in vivo* data show distinct differences in the biological behavior of IL-17E versus IL-17A or IL-17F, namely its Th2-promoting effects. In contrast, epithelial hyperplasia/hypertrophy [353,415], as well as mucus production [353] appear to be common observations. *In vitro*, our data on the functional role of IL-17E on structural cells do not depart radically from observations made with either recombinant IL-17A or IL-17F, that is, its synergistic effect with TNFα, and a possible pro-fibrotic role [150,344]. Moreover, we and others, have shown that like IL-17A, IL-17E also induces IL-6 and IL-8 (in ASMC).

So far, none of the IL-17's are able to promote pro-eosinophilic chemokines like eotaxin or RANTES, as we have demonstrated for IL-17E in lung fibroblasts [384].

4.3 Summary

We have observed for the first time the IL-17E receptor on human structural cells of the airways both *in vivo* and *in vitro*. Airway smooth muscle cells, fibroblasts and bronchial epithelial cells constitutively express IL-17BR. TNFα consistently increases its expression in these cells. We thus suggest that in asthma, Th2 lymphocytes mast cells and eosinophils that are infiltrated in the airway mucosa might act as sources of IL-17E and that the local inflammatory environment characterized by increased TNFα could upregulate IL-17BR on airway structural cells and render these more responsive to IL-17E. Furthermore, we have observed that IL-17E promotes pro-collagen I synthesis by ASMC, as well as the increased synthesis of proinflammatory mediators by ASMC (IL-8, GROα, GM-CSF, IL-6), lung fibroblasts (eotaxin, RANTES, GM-CSF and IL-8) and A549 epithelial cells (GROα). In asthmatic airways, it is possible that IL-17E induces the release of pro-inflammatory, pro-eosinophilic and pro-neutrophilic mediators by these structural cells. In this respect, IL-17E might contribute yo the influx and survival of inflammatory cells and maintain a state of inflammation in asthmatic airways. IL-17E could also promote the increased deposition of collagen I by ASMC and thus contribute to increased subepithelial fibrosis that occurs in airway remodelling. In addition, we have observed that IL-17E supports the proliferation of bronchial epithelial cells isolated from control and mild asthmatic donors, in accordance with *in vivo* data in mice that shows hyperplasia of the airway and gut epithelium. In asthma, the epithelium shows signs of

increased proliferation and as such, IL-17E might participate in the repair of damaged or wounded bronchial mucosa.

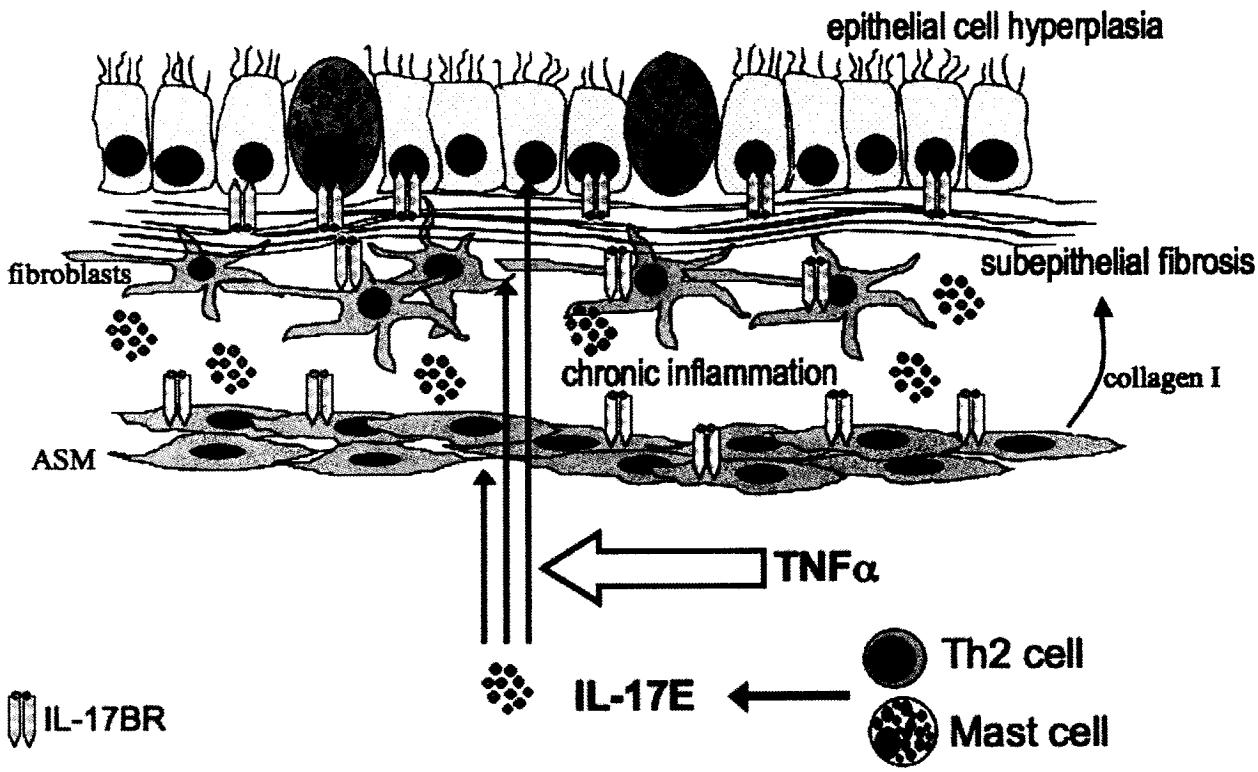
4.4 Future directions

It would be of considerable interest to assess IL-17E levels in non-asthmatic and asthmatics of different severities either in the BAL fluid or sputum and in bronchial tissue, as well as to acertain to cellular sources of this cytokine in the airways. Moreover, now that IL-17E knockout mice are available [416,417], it would be particularly interesting to use them as models of allergic airway disease to see if the absence of IL-17E results in a milder asthma phenotype.

4.5 Perspectives

IL-17E is part of a complex cytokine network and because of the paucity of information on it, it is difficult to evaluate its real life contribution in normal physiology and disease. Compounding the situation, cytokines are known to have redundant functions, as well as synergistic and antagonistic effects, depending on the conditions. In the case of the IL-17 family members, they share many *in vitro* as well as *in vivo* biological effects. Indeed, the problematic part when studying a cytokine *in vitro*, is to be able to appreciate its output in the context of a whole organism as well as to understand how it distinguishes itself from a sea of mediators by its unique biological effects. Interestingly, the same research institute that has contributed to the discovery of IL-17E

has recently found a novel Th2 cytokine, termed IL-33, which, very much like IL-17E, upregulates IL-4, IL-5 and IL-13, increases IgE, induces eosinophilia, promotes epithelium hypertrophy and mucus secretion in mice [463]. This brings about questions that are often asked in cytokine biology: what are the unique biological effects of these two seemingly similar interleukins and are they just molecules with redundant properties?



4.6 Schematic representation of the potential role of IL-17E in asthma. Th2 and mast cells are infiltrated in the bronchial mucosa in asthmatic airways and could locally produce IL-17E. Surrounding structural cells, such as airway smooth muscle cells (ASM), fibroblasts and epithelial cells express the IL-17E receptor (IL-17BR) and are thus likely targets for the action of IL-17E *in vivo*. IL-17E could act on these cell types to

induce the release of several cytokines involved in airway inflammation. IL-17E could participate in remodeling by enhancing the production of collagen I from ASM and increase the thickening of the basement membrane (subepithelial fibrosis). In addition, IL-17E could also promote a greater proliferative response in bronchial epithelial cells.

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