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List of abbreviations

ASM = airway smooth muscle

BSM = bronchial smooth muscle

C/EBP = CCAAT/enhancer binding protein

COPD = Chronic Obstructive Pulmonary Disease

CRT = calreticulin

ELISA = Enzyme Linked Immunosorbent Assay

FCS = fetal calf serum

HA = hemagglutinin

HDM = house dust mite

LDH = lactate dehydrogenase

LP = long peptide

SCM = smoke conditioned medium

SMA = smooth muscle actin

SP = short peptide

TCRS = translation control reporter system

TOP = tract of oligopyrimidines

uORF = upstream open reading frame

PAR = protease activated receptor

UTR = untranslated region

Summary

Both asthma and COPD are respiratory diseases and a major global health problem with increasing prevalence. Airway inflammation is a characteristic and important hallmark in both diseases and therefore, in the past, investigations focused strongly on the immunological aspect of these disorders.

In recent years, it has been shown that resident cells of the airways, in particular airway smooth muscle (ASM) cells, would be pivotal in understanding the mechanisms underlying asthma, since they are able to secrete pro-inflammatory cytokines and exert a major effector function in airway constriction. Especially the abnormal expression in ASM cells in asthmatic patients of the cell cycle regulator and pro-inflammatory gene transcription factor C/EBPα may account for many asthma-specific phenotypes (increased proliferation and increased bulk of ASM cells, increased release of inflammatory mediators).

In a first phase, we analyzed the translation of the *CEBPA* mRNA with a translation control reporter system (TCRS), which is able to monitor translation regulation of the C/EBPα. We found an impaired translation re-initiaion in ASM cells of asthmatic patients, which coincided with decreased levels of eIF4E, an important protein for translation initiation.

In a second part of this thesis, we investigated the interaction of ASM cells with house dust mite extract, a potent airborne allergen. We found that HDM extract (i) reduces C/EBPα expression in ASM cells of asthma patients, (ii) enhances the release of IL-6 and (iii) induces cell proliferation. The reduction of the C/EBPα protein is achieved trough up-regulation of calreticulin, a repressor of *CEBPA* mRNA translation. Therefore, the direct, not immune-mediated interaction of HDM extract with the ASM cells is able to trigger an inflammatory response in these cells and to induce an enhanced proliferation, which may finally lead to the characteristic increased muscle mass observed in the airway of asthmatic patients. These findings may be of particular importance to explain non-atopic, intrinsic asthma, which affects 30% - 50% of asthmatic subjects. In the light of these findings, new therapeutic strategies targeting regulatory mechanisms of *CEBPA* mRNA translation should be considered in order to restore a balanced expression of the C/EBPα protein.

In a third part of this thesis, we investigated the effect of cigarette smoke on the expression levels of C/EBPα and C/EBPβ in primary lung fibroblast. Cigarette smoke affects both C/EBPα and C/EBPβ

expression via translational control mechanisms in primary lung fibroblasts. In serum-free environment, cigarette smoke increased both C/EBPα and -β expression at the translational level via the uORF mechanism. In the presence of FCS, cigarette smoke increased the levels of hnRNP E2, an inhibitor of C/EBPα translation. As a consequence, both C/EBPα and -β expression decreased with increasing concentration of cigarette smoke. In both conditions, cigarette smoke had a potent antiproliferative effect on fibroblasts. Furthermore, cigarette smoke increased the release of IL-8. We postulate that the cigarette smoke-induced imbalance of pro- and anti-proliferative signals provides a novel mechanism to explain many pathologies of COPD and emphysema, especially the tissue destruction defined as an imbalance between tissue injury and tissue repair. Furthermore, we showed that that the direct interaction of lung fibroblast with cigarette smoke triggers the release of pro-inflammatory mediators, contributing to the inflammatory environment that characterizes COPD.

I hope that the novel findings of this thesis add a small piece of knowledge to the complex mosaics called asthma and COPD and that the better understanding of these diseases will in future lead to effective cures for millions of patients.

Chapter 1: General Introduction

Asthma

Definition

Asthma is a chronic airway disease and a major global health problem. The term asthma origins form the greek word „asthmaino“ (αστημαινω), indicating gasping, and the term was first used by Hippocrates (460-377 BC) in the Corpus Hippocraticum [1].

The Global Initiative for Asthma (GINA) defines asthma as a “chronic inflammatory disorder of the airways in which many cells and cellular elements play a role. The chronic inflammation is associated with *airway hyperresponsiveness* that leads to recurrent episodes of wheezing, breathlessness, chest tightness and coughing, particularly at night or in the early morning. These episodes are usually associated with widespread but variable, *airflow obstruction* within the lung that is often reversible either spontaneously or by treatment” [2].

This unifying definition of asthma highlights the clinical hallmarks of the disease: (i) the *inflammatory process*, (ii) the *airway hyperresponsiveness*, (iii) the *obstruction in the normal airflow* and (iv*) increased airway remodeling.* It is becoming increasingly clear that asthma is a very heterogeneous disease as it includes immunopathology, clinical different phenotypes, non-uniform response to therapies and natural history (3). There is a growing group of scientists who considers asthma a syndrome with different risk factors, different prognosis, and different response to treatment [4, 5]. This tendency points to the necessity to overcome the historical simplification that defined asthma as merely an inflammatory disease of the lung.

Asthma, a major health issue

Asthma is a major health problem worldwide, with estimated 300 millions individuals suffering from this disease, and its prevalence varies from 1% to 18% of the population in different countries. Worldwide

250’000 people die from asthma yearly with a higher mortality in underdeveloped countries. In western countries asthma is also a major cause of absence from work and school. Therefore, asthma represents a substantial burden in terms of medical costs (hospital and medication), economical costs [6], and social impact (reduced quality of life, premature deaths, absence from school) [6-9].

The factors influencing the development of asthma are both host-depended and environmental. Host factors may have a genetic background and are inheritable, however the data analysis for genes associated with (i) increased production of IgE (atopy), (ii) airway hyperrresponsiveness, (iii) release of inflammatory mediators are rather inconsistent and provided no specific gene to be related to asthma. A number of chromosomal regions had been associated with asthma susceptibility and a co-inheritance of the tendency to produce elevated IgE serum level with airway hyperresponsiveness has been observed [10-13]. Genomic studies identified 79 genes differentially expressed in asthma and controls [14]. However, the expression of these genes is likely to be influenced in a complex interaction with multiple environmental factors contribution. Host risk factors are also obesity and male sex, with male children that present a two-fold higher prevalence for asthma prior to the age of 14 [15].

Environmental factors influencing the development of asthma are indoor and outdoor allergens such as house dust mite, cockroach allergens, cat and dog dander or Aspergillus mold. Especially exposure to this allergens during childhood (up to 3 years) seems to be crucial for developing asthma-like symptoms [16-25]. Other risk factors are infections of the airways during childhood [26-29], occupational sensitizers [30-33], tobacco smoke [34-39], and the diet [40, 41]. There is a certain overlap between these risk factors leading to the development of asthma and factors that trigger asthma symptoms. The role of house dust mite will be discussed in more detail in a separate paragraph. However, factors that trigger asthma are not to be equated with factors that cause asthma. On one side many asthmatic subjects are atopic (60% of asthmatic adults, 80% of asthmatic children), but it is also true that not all atopic subjects develop asthma. Furthermore 30% - 50% of asthmatic subjects are not atopic, that means that no circulating IgE against one or more common allergens can be detected. Therefore, IgE-mediated mast cell degranulation is not necessary or sufficient for the development of asthma [42-44].

Pathogenesis of asthma

Airway inflammation; classical view

Airway inflammation is a multicellular process involving mainly Th2 lymphocytes, eosinophils, activated mast cells, neutrohphils, macrophages and basophils.

In atopic asthma, the airway responds to airborne inhaled allergens by a Th2 response with the release of the typical array of cytokines (Th2 paradigm) [3].

At the beginning of the inflammatory cascade, dendritic cells in the airway epithelium and the submucosa capture inhaled allergens internalize and process them, and then present them to T lymphocytes. The sensitized T cells then produce cytokines, in particular the interleukins IL-4, IL-5, IL-6, IL-9 and IL-13, can be found elevated in the bronchoalveolar lavage (BAL) fluid and in the serum of asthma patients indicating a predominantly Th2 mediated inflammatory response [45-47]. The cytokine production is leading to a recruitment of secondary effectors cells such as macrophages, basophils and eosinophils into the inflammatory area while IL-4 promotes the immonuglobulin isotype switching of B cells towards IgE sythesis (Fig. 1.1).

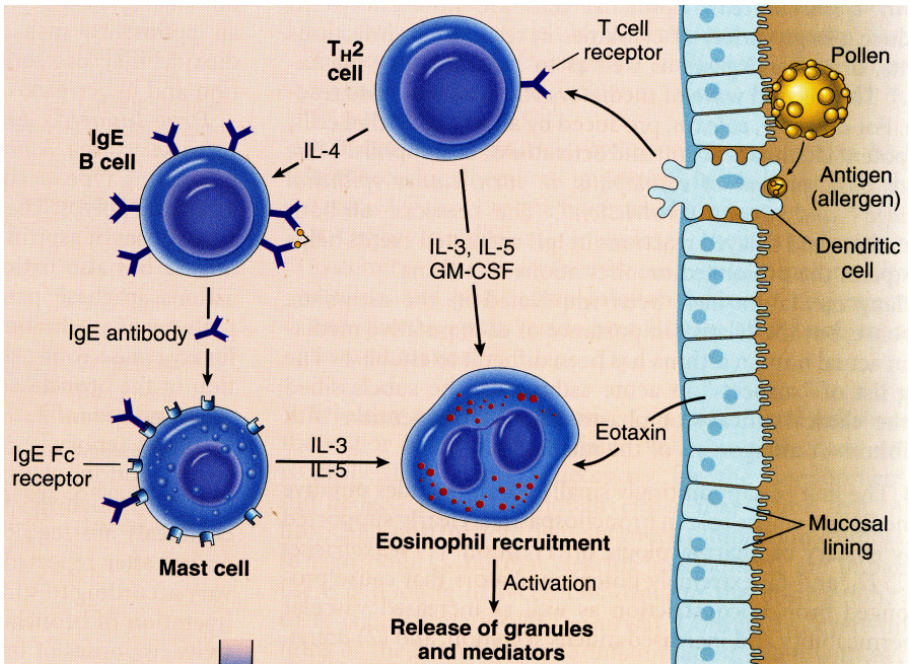

Figure 1.1: Initiation of the inflammatory cascade after allergen (pollen) inhalation and interaction with airway cells. (© Vinay Kumar; Abul Abbas, Nelson Fausto; Robbins and Contran pathologic basis of disease, Sevent Edition, Elsevier Saunders, 2005)

The *early reaction* to allergen inhalation is *mast-cell* dependent with mast-cells infiltrating the mucosa and the deeper airways [48]. Interestingly, in chronic asthma mast cells and smooth muscle (ASM) cells are both increased in small and large airway. Mast-cells are activate after binding of IgE to the highaffinity IgE receptor (FCεRI) leading to the release of TNF-α, IL-4 and IL-5. Mast cells act on airway ASM cells by the release of the bronchioconstrictive mediators leukotriene $(LT)D_4$, prostaglandin $(PG)D_2$ and histamine, which act as potent ASM cell contractile agents [49, 50]. Vice versa, ASM cells can produce stem cell factor, other chemokines, cytokines, and growth factors such as SCF, CXCL8, and CXCL10 that all may act in the recruitment, differentiation, and retention of mast cells [51, 52]. Also important in this context is mast cell tryptase, a protease that acts on the protease activated-receptor type 2 (PAR2) present among others ASMC and its activation induces bronchoconstriction by stimulating muscle contraction [53].

Eosinophils are a prominent cell population involved in allergic asthma [54]. It is mostly the Th2 cell released IL-5 contributing to the maturation of the eosinophils from $CD34^+$ precursors [55]. Eosinphils are a major source of basic proteins, peroxidase, eicosanoides, leukotrienes and superoxide that can damage the airway epithelium. They furthermore releases TGF-β(1) as well as other important cytokines which lead to a direct activation of epithelium and mesenchymal cells that are considered to drive asthma related airway remodeling [56].

The exact role of neutrophils, monocytes and basophils in the context of asthma is poorly understood. Neutrophils are found in increased numbers in airways and sputum of patients with severe asthma and smoking induced asthma [57] (Figure 1.2).

Recently it has been shown by profiling of the cytokine mRNA levels in asthma subjects and controls that in severe asthma there is a significant increased expression of the Th1 cytokine IFN-γ and this finding is questioning the primacy of the Th2 response in more severe forms of the disease [58].

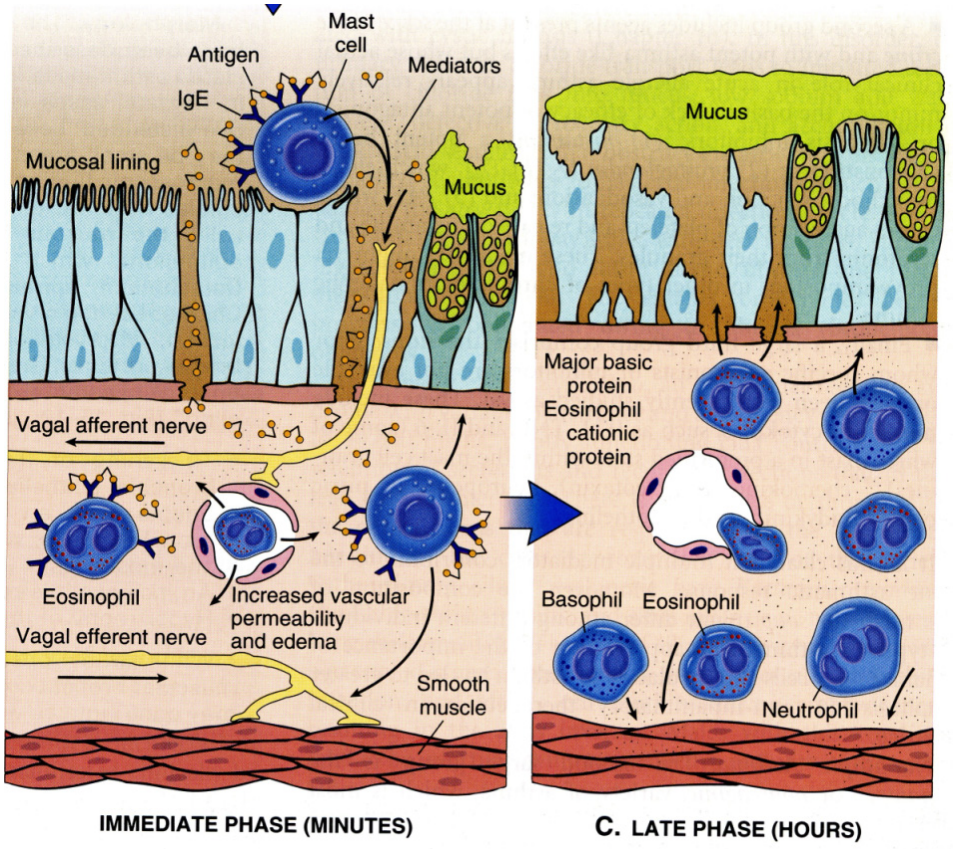

Figure 1.2: Early and late phase reactions after allergen (pollen) inhalation and interaction with airway cells. (© Vinay Kumar; Abul Abbas, Nelson Fausto; Robbins and Contran pathologic basis of disease, Sevent Edition, Elsevier Saunders, 2005)

Airway hyperresponsiveness

Airway hyperresponsivness is defined as a characteristic functional lung abnormality of asthma resulting in airway narrowing in response to a plethora of different stimuli that would be innocuous in non-asthmatic people [2]. In consequence, asthma patients suffer from *airflow limitation*. Airway hyperresponsivenss is linked to both airway inflammation and and airway remodeling and is partially reversible by bronchodilators that relax the airway smooth muscle. Airway hyperresponsivness can be explained by excessive contraction of the ASMC and by thickening of the airway wall, which will be discussed later. Additionally, broncho-constriction can be triggered by sensory nerves, which were sensitized by inflammation in response to sensory stimuli. As rewied by Veres, the importance of the neuron-immune interactions in asthma is underlined by the fact that current anti-asthmatic medications

are also directed against bronchoconstriction, such as β2 agonists and anti-cholinergics which are targeting several neural pathways [59].

Airway remodelling

Airway remodeling in asthma includes thickening of the reticular basement membrane (RBM), epithelium fragility, hypertrophy of mucus secreting glands, hypertrophy and hyperplasia of airway smooth muscle and increased deposition of extracellular matrix.

Thickening of the subepithelial lamina reticularis or RBM is a morphological hallmark of asthma. It is due to the increased deposition of Ig, collagen I and III, tenascin, and fibronectin, [60] but not of laminin. These proteins are likely produced by activated myofibroblasts [61] leading to a so-called subepithelial fibrosis. The thickening is greater in atopic than nonatopic forms of asthma. RBM thickening has been positively correlated with airway hyperresponsiveness, the frequency of asthma attacks, and the numbers of fibroblasts and "myofibroblasts" adjacent to the RBM [61-63].

Damage and shedding of the airway epithelium is another important histological characteristic of asthma. Asthmatics patients present clusters of epithelial cells (Creola bodies) in sputum, increased numbers of epithelial cells in bronchoalveolar lavage fluid, and loss of the surface epithelium in biopsy specimens [64, 65]. Upregulation of epidermal growth factor receptors (EGFRs), impaired proliferation reduced expression of proliferative markers and upregulation of the cyclin inhibitor, nuclear $p21^{wat}$ indicated that the epithelium in chronically injured and the repair mechanism is deregulated [66, 67]. Furthermore, the epithelium of asthmatic patients is more fragile due to the disruption of the tight junctions [3, 68] and in consequence, the airway epithelium enters into a chronic “wound scenario” that leads to the development of an abnormal epithelium-mesenchymal interaction ending in the release of pro-inflammatory cytokines and growth factors, such as epidermal growth factor (EGFR) [69].

Asthmatics subjects also present increased numbers of goblet cells that secrete viscous mucus with a reduction of the ciliated cells. The mucus composition is altered with an increase of the 5AC mucin that is responsible for the unusual high viscosity and problematic sputum expectorations in asthma [70]. This augmented mucus secretion has been relatively undervalued in asthma compared with airway

inflammation. However, mucus plugging contributes to airflow limitation, the airway hyperresponsiveness, to morbidity, and to mortality in asthma.

Under the effects of epithelial-derived growth factors, mesenchymal cells produce collagen, reticular and elastic fibers, as well as proteoglycans and glycoproteins of the extracellular matrix (ECM), all of which contribute to the thickened airway wall of asthmatic subjects, all leading to an enhanced proteoglycan deposition in the subepithelial tissue layer wall [71]. It is therefore likely that ECM production and deposition is under the control of the epithelial-mesenchymal unit, leading to the development of structural alterations localized in the inner tissue layer of the airway wall (the tissue between the luminal surface and the smooth muscle layer). The increase of the volume of the inner airway wall may have dramatic functional consequences in terms of luminal changes in response to a given stimulus by smooth muscle contraction. Fibroblasts and myofibroblasts can contribute to tissue remodeling by releasing ECM components such as elastin, fibronectin, and laminin [72]. An increased numbers of myofibroblasts are found in the airways of asthmatic patients. On the other hand, mast-cell derived serine protease is a potent stimulant of fibroblast and ASM cells proliferation, and it is capable of stimulating synthesis of type I collagen by human fibroblasts [73].

Airway wall remodeling in asthma includes also increased vascularity; evidence suggests that the number and size of bronchial vessels is increased in patients with asthma compared with normal controls [72, 74].

Finally, asthma patients show airway muscle hyperplasia and hypertrophy [75]. The role of ASM cells in the context of asthma will be discussed in the next chapter. A model of airway remodeling in asthma can be found in figure 1.3.

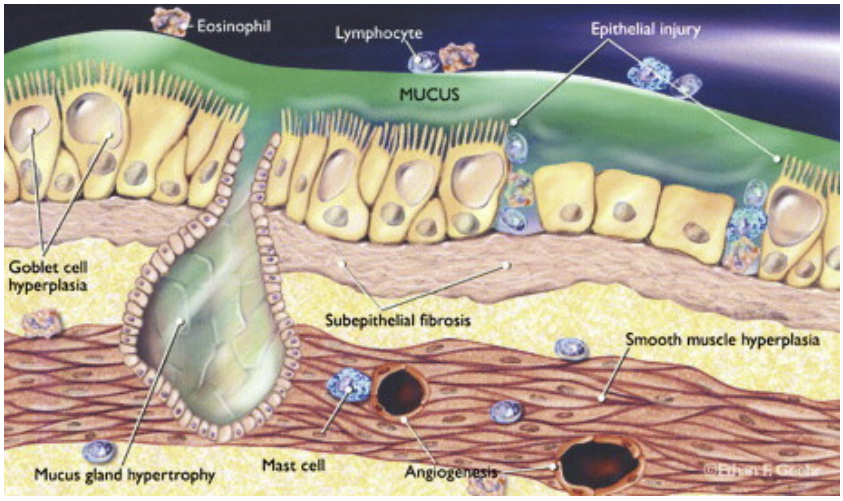

Figure 1.3: Model of the airway remodeling with inflammatory cells, epithelial damage, goblet cell hyperplasia, subepitelial fibrosis, mucus hypersecretion and smooth muscle cell hypertrophy and hyperplasia. [© Panettieri RA Jr, Covar R, Grant E, Hillyer EV, Bacharier L., Natural history of asthma: persistence versus progression-does the beginning predict the end? J Allergy Clin Immunol. 2008 Mar;121(3):607-13]

Asthma as a disease of the airway smooth muscle cells

One of the most striking aspects of the pathology of airway remodeling in asthma is the increased number and size of airway smooth muscle (ASM) cells, which had already been reported by Huber and Koesser in 1922 (Figure 1.4) [76]. Then, this smooth muscle bundle abnormality was considered to be the main cause of the airway hyperresponsiveness and the exaggerated constriction in asthma [76]. Interestingly, an increasing number of studies points back to the pathologic airway smooth muscle cell as a major cause of asthma. What properties of the airway smooth muscle cell would support this idea? As airway smooth muscle is the effector controlling airway caliber, it is reasonable to consider that dysfunction of ASM contributes to the pathophysiology of asthma. However, the predominant view in the past decades defined airway smooth muscle primarily as an effector whereas airway inflammation was thought to be the causal pathophysiological mechanism underlying airway hyperreponsivenss and remodeling. As described in the previous chapters, asthma is defined as chronic inflammatory disease of the lung with a increased Th2-like response and with high levels of IL-4, IL-5, IL-13 [2].

Studies of childhood asthma showed that the increased mass of airway smooth muscle exists already in very young children and does not necessarily correlate with the severity and duration of the disease as it

was assumed earlier [77-82]. Furthermore, airway inflammation is not present in all patients with childhood asthma, while remodelling is [79, 83].

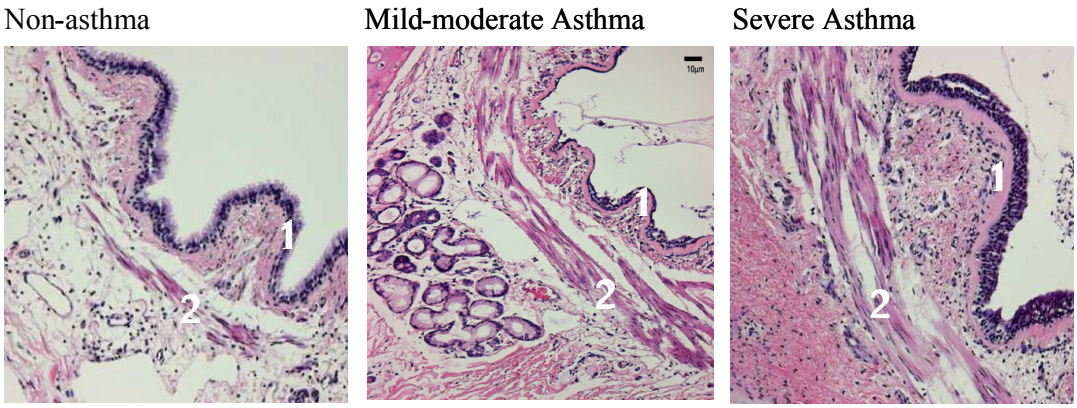

Figure 1.4: Histology of a representative airway of an non-asthma control (left panel), a patient with mild-to-moderate asthma (middle panel), and a patient with asthma who died of status asthmaticus (right panel). The asthmatic airway present a thickening of the basement membrane (1), and the increased mass of smooth muscle cells (2) [Borger P, Tamm M, Black JL, Roth M. Asthma: is it due to an abnormal airway smooth muscle cell? Am J Respir Crit Care Med. 2006 Aug 15;174(4):367-72]

As rewied by Borger (84), several findings are questioning the primacy of an immnune-mediated mechanism as the only cause of asthma. First, studies using cyclosporine to block T-cell activation and thereby the release of IL-2, IL-4 and IL-5 showed only an effect on the late asthmatic response, suggesting that early asthmatic response is not T-cell mediated [85, 86]. Also the use of anti-IL-5 monoclonal antibodies, even though reducing dramatically eosinophils (>80%) in the airways and in the blood of asthmatic subjects, did not result in any clinical outcome measures of asthma [87]. Interestingly the reduction of ASM cells mass by bronchial thermoplasty, a novel experimental procedure involving the application of controlled heat from a radiofrequency source to reduce airway smooth muscle in the airway wall, significantly improved asthma control such as diminished exacerbations over a period of 3 years by now [88].

In the light of these findings, it has to be taken in consideration that ASM cells may not be only effectors leading to airway hyperresponsiveness as a consequence of the inflammatory process, but they may be initiator or co-initiator of the disease's onset and progress. In line with this hypothesis, it is known that ASM cells are able to secret a broad range of cytokines, chemokines and growth factors [89, 90]. Our group was the first to demonstrate that isolated ASM cells of asthmatic patients also proliferate faster than cells from control subjects under defined conditions [91]. This *in vitro* observed increased

proliferative capacity could explain the *in-vivo* observed augmentation of smooth muscle cell mass in the asthmatic airway. Importantly, the phenotype is maintained through multiple weeks in culture and many passages, that means long after any inflammatory mediators present in the tissue and therefore an inflammation dependent effect should have been washed out. Furthemore, our group found that ASM cells from asthma patients are primed for IL-6 release [92, 93]. Thus, the airway smooth muscle from asthmatic patients is *intrinsically* different from normal airway smooth muscle. We can therefore speak of an *activated phenotype* of ASM cells in asthmatic subjects. Interestingly, ASM cells from asthmatic patients show a significant decreased level of CCAAT/enhancer binding protein (C/EBP) α, am important transcription factor and regulator of cell proliferation and inflammation [94]. C/EBPα can be expressed in several isoforms, p42, p40 and p30, which are regulated at the level of translation by the presence of alternative translation initiation sites [95]. C/EBPα is regulating proliferation trough the induction of the cell cycle inhibitor $p21^{waf/cip21}$. In normal cells, β-mimetics and steroids activate $p21^{waf/cip21}$ via C/EBPα forming a complex with the glucocorticoid receptor (GR) [96-100]. In absence or low levels of C/EBPα the complex with the GR can not be formed in a sufficient amount to activate the $p21^{waf/cip21}$ gene. Therefore, the absence of the C/EBPα could explain the increased in vitro proliferation of ASM cells. Furthermore, C/EBPα has the potential to inhibit activation of proinflammatory gene by interaction with the transcritption factor (NF)-κB [101, 102]. Diminished expression of C/EBPα may abolish this inhibitory effect and initiate airway inflammation by increased release of proinflammatory mediators. ASM cells are able to produce IL-1, IL-2, IL-5, IL-6, IL-11 and IL-12 and, interestingly also TARC, a cytokine that induces Th2 migration and recruitment [103-105]. Taken togheter, deregulated, active ASM cells are able to initiate and orchestrate an inflammatory environment by the release of proinflammatory mediators and as the consequence induce the recruitement of inflammatory cells into the lung. This capacity may be enhanced by a crosstalk with the airway epithelium, which plays important role in the airway inflammation, as described before.

Finally, diminished C/EBPα expression could also explain the increased airway hyperresponsiveness observed in asthmatic patients. An excessive decrease in airway luminal area via bronchoconstriction is one of the final pathways to asthma. However, very little is understood about the molecular mechanics of smooth muscle in airway hyperresponsiveness and asthma. Ma *et al.* showed a significant increase in

both shortening capacity and velocity in endobronchial biopsies in correlation with an increase in mRNA for the myosin light chain kinase (smMLCK) [106]. Interestingly, the promoter that regulates the kinase contains several C/EBP consensus-binding sequences in the 5' untranslated region. A lack of C/EBPα could therefore lead to an increase in the myosin light chain kinase (smMLCK) expression, leading to an increased velocity of ASM cells shortening and finally, to an increased airway narrowing [107].

A model of the central role of ASM cells in asthma can be found in figure 1.5.

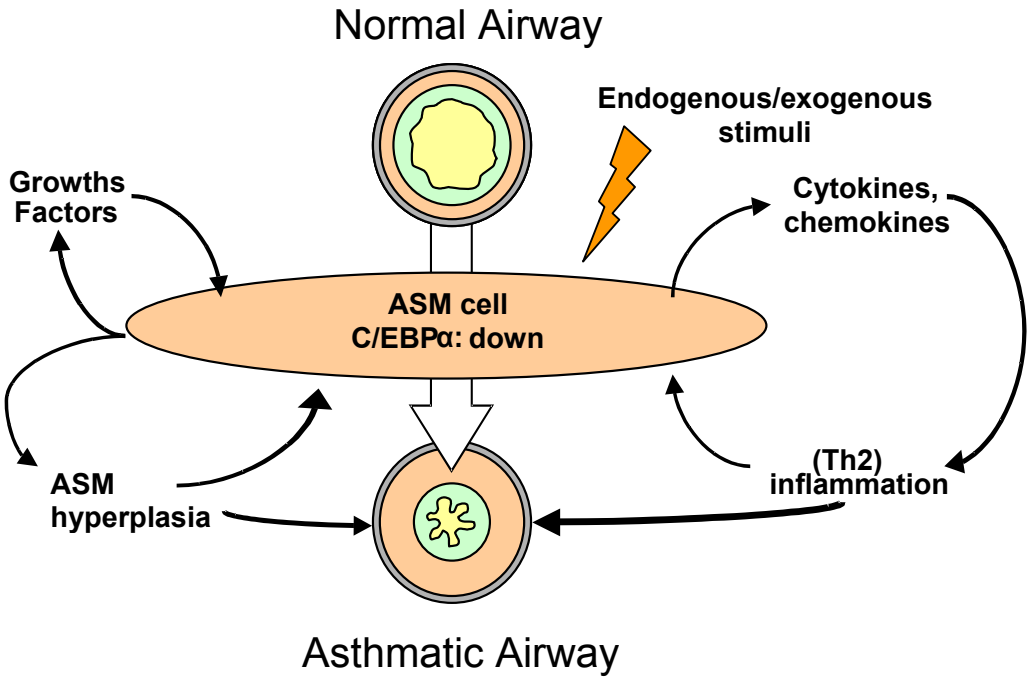

Figure 1.5: Model with the proposed central role of the airway smooth muscle (ASM) cell in airway inflammation and remodeling. Due to predisposition and/or environmental stimuli ASM cells of patients with asthma express decreased levels of the C/EBPα. [Adapted from Borger P, Tamm M, Black JL, Roth M., Asthma: is it due to an abnormal airway smooth muscle cell? Am J Respir Crit Care Med. 2006 Aug 15;174(4):367-72].

Asthma treatement

There is no curative therapy for asthma. Today's standard therapy consists mainly in inhaled glucocorticosteroids such as budenoside, ciclenoside and fluticasone that control airway inflammation [108] with beneficial effects in terms of asthma symptoms, improvement of lung function [109], decreased airway hyperresponsivness [110, 111]. The anti-inflammatory action of glucocorticoids is through a transactivation of anti-inflammatory mediators or transrepression of pro-inflammatory

mediators. Corticosteroids enter the cell and bind to the glucocorticoid receptor, which translocates them into the nucleus, where transcription of target genes is regulated. The binding of the activated glucocorticoid receptor homodimer to a glucocorticoid regulatory element (GRE) DNA sequence in the promoter region of steroid-sensitive genes leads to the transcription of genes encoding anti-inflammatory mediators (annexin-1, secretory leukoprotease inhibitor (SLPI), IL-10, and the inhibitor of nuclear factor-kB (IkBα).

In addition, the glucocorticoid receptor –corticosteroid complex interacts with large co-activater molecules such as the regulators of intrinsic histone acetyltransferase (HAT) activity, which are activated by pro-inflammatory transcription factors, such as NF-kB or AP-1, thus switching off the expression of inflammatory genes [112, 113].

Inhaled β2-adrenoceptor agonists are divided into short-acting and long-acting β2-adrenoceptor agonists (SABAs and LABAs). SABAs such as salbutamol and turbutaline bind to the β2-adrenoceptor, activate the adenylate cyclase and increase production of cyclic adenosine 3'5'-monophosphate (cAMP) by the signal-transducing G_s, finally activating protein kinase A. This leads to muscle relaxation and relieves the bronchocontriction trough the phosphorylation of myosin light-chain kinase and opening of Ca^{2+} channels. LABAs such as formoterol and salmeterol induce bronchiodilatation for at least 12 hours [114]. The combination of LABAs and corticosteroids represents the main therapy in asthma today.

Some recent therapeutic concepts focus on the immunological mediators of the disease. Omalizumab, a humanized anti-IgE-specific antibody binding to free IgE thereby decreasing cell-bound IgE, has been developed for the treatment of severe allergic asthma [115]. This concept improved symptom control and allowed the reduction of the corticosteroids doses, but up to 16 weeks of treatment are required before any clinical improvements can be recognized. Administration of humanized anti-IL 5 antibodies lead to a consistent reduction of circulating and sputum eosinophils (>80%), but was without significant beneficial effects in terms of clinical outcomes [87, 116, 117]. The use of a soluble, recombinant, human IL-4 receptor (altrakincept) gave first promising results in patients with mild to moderate asthma, but failed in larger trials (118, 119). As an alternative therapy for adult patients with mild persistent asthma leukotriene modifiers such as cysteinyl-leukotriene 1 (CysLT1) receptor antagonists (montelukast, pranlukast) and 5-lipoxygenase inhibitors (zileuton) are used with good results [120-123].

House dust mites and asthma

House dust mites (HDM) are animals of the phylum Arthropoda, class Arachnida and they are closely related to spiders and scorpions. Their size varies from 20 to 320 µm, depending on the species and developmental stage. HDM are present in all humid areas around the world [124]. Thirteen different species of mites are found in common house dust, but mainly three species produce the majority of mite allergens: D. pteronyssinus, D. farinae and Euroglyphus maynei [125].

In 1967, Voorhorst's article provided the first conclusive report that a little recognized species of house-mites, Dermaphagoides pteronyssinus, was the origin of the elusive, but ubiquitous HDM allergens [126-128]. It turned out that the mite faces are the major source of house dust allergens. Today, HDM are recognized as one of the most common causes of allergy worldwide, against which more than 50% of all allergic patients and 80% of asthmatic children are sensitized [129]. The importance of HDM allergens during the development of asthma in western countries is emphasized by the fact that people spent an average 87% of their time indoors [130].

In a mouse model, chronic exposure to HDM-extract has been shown to lead to persistent airway inflammation, AHR and airway remodeling [131]. The exact mechanisms by that HDM affects the human system are largely unknown. To study the biological action of HDM allergens in the pathogenesis of chronic allergic diseases such as asthma, HDM-extracts are made from an aqueous solution of a variable mixture of the HDM, fecal pellets, eggs, and spent culture media [129, 132]. The analysis of these HDM extracts indicated over 30 different proteins, which were categorized into 21 different groups, listed by the Allergen Nomenclature Sub-Committee of the International Union of Immunoligical Societies. The members of the various groups of proteins have been characterized due to their capacity to induce IgE antibody production in patients allergic to HDM and by extensive investigations about their biological function as well as by their 3-dimensional structure [133]. The Group 1 HDM-allergens, which also contains the first ever described HDM allergen, Der p 1 [134], display a mixed cysteine/serine proteolytic activity [136], while groups 3, 6, and 9 are serine proteases [135-138].

HDM extract also contains biological active non-protein compounds such as lipo-polysaccharide (LPS), which are potent stimulators of the innate immune response. This may be important in the light of the

recent findings, that skeletal muscle cells express multiple Toll-like receptors [139]. It has been shown that HDM-allergens induced Toll-like receptor 4 thereby triggering airway structural cells in mice to release innate pro-allergic cytokines, thymic stromal lymphopoietin, granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-25 and interleukin-33 [140]. Group 15 contains chitinase, which is normally located in the gut of the HDM. The different HDM-allergens have different IgE binding capacity, within which the group 1, 2 and 14 showed the highest IgE reactivity and account for 80-100% of HDM-allergies.

Most studies investigated a single biologically active protein of the whole HDM-extract. Especially the serine proteases Der p1, Der p 3, Der p 6 have been studied for their contribution to the triggering mechanism on HDM-induced asthma. It is still an open question if it is preferable to use whole HDM-extracts or single compounds in studies of their biological activity. It is a considerable challenge to identify and investigate the effect of a single allergic compound in the complex mixture of 3000 different HDM proteins of which 5% are thought to provoke allergic responses [141]. However, in daily life subjects are exposed to the whole HDM protein mixture and there may be many different proteins in addition to the proteases contributing to the HDM-induced asthmatic response. Therefore, the use of a single allergic HDM compound may contribute to gain a very specific knowledge, whereas whole HDM extracts may reflect a more realistic picture of the responses to HDM exposure.

The majority of the investigations on HDM-derived allergens focused on their proteolytic activity. A first important finding was that HDM-extract disrupted the integrity of the tight junctions between epithelium cells resulting in cell desquamation of the airways [142]. It is reasonable to assume that once the barrier function of the epithelium is disturbed, as it is observed in asthmatic airways, HDM allergens and other particles may easier find their way through the lamina propria and therefore penetrate deeper into the airways. By this mechanism allergens may even directly interact with fibroblasts and airway smooth muscle cells.

Der p 1, 3 and 9 have been shown to induce the release of GM-CSF, IL-6, IL-8 and eotaxin from bronchial epithelial cells [143, 144]. In these first studies the mechanism of action was thought to be mediated by the protease-dependent receptor 2 (PAR)-2 [144-146]. However, most recently also

protease-independent mechanisms have been reported. Studies done by Kauffman et al. [147], demonstrated that HDM allergens activated human airway-derived epithelial cells by both protease-dependent and protease-independent mechanisms. Der p 1 and Der p 5 induced a dose-dependent, but protease-independent release of IL-6 and IL-8. Similar results were reported by Adam et al [148], which lead them to conclude that the Der p 1-induced IL-8 production by epithelial cells was independent of PAR2 activation and therefore independent of the protease activity. Both Der p 2 and Der p 5 lack protease activity, but are major IgE binding proteins [147]. Heijink showed that the proteolytic activity of Der p allergens induced TARC expression in bronchial epithelial cells via the activation of MAP kinases and the subsequent activation of NF-κB [149]. Together, the direct contact of HDM-allergens with the bronchial epithelium and its proteolytic activity may contribute to the inflammatory response of the lung and lead to an increased recruitment of T-cells into the airways. The non-proteolytic Der p 2 induced a dose-dependent up-regulation of GM-CSF, IL-6, IL-8 by the activation of NF-kappaB and MAP kinase pathways in bronchial epithelial cells [150]. In addition, in a mouse model it had been shown that HDM-extract exposure resulted in vascular remodeling in the sub-mucosa, and in proliferation of smooth muscle cells and endothelial cells, as well as in enhanced pro-collagen I synthesis, and increased collagen deposition [151].

In isolated primary airway smooth muscle (ASM) cells, Der p 2 induced high level of pro-inflammatory cytokines by activation of NF-kappaB and phosphorylation of ERK1/2 MAP kinase [152]. Der p 1 has been shown to trigger an enhanced constriction and impaired relaxation of rabbit ASM cells. Therefore, Der p1 induced a direct change in airway responsiveness [153]. This pro-asthmatic effect of Der p 1 depended on its proteolytic activity and was associated with the activation of ERK1/2 and p38 MAPK kinase signaling pathways. In infant male rhesus monkeys postnatal, periodic exposure to HDM over a period of 5 months led to a significant increase of total smooth muscle mass and of average muscle bundle size in the conducting airways [154]. Therefore, repeated exposure to HDM alters the postnatal morphogenesis of the airway smooth muscle, which indicated a pivotal role of this cell type in the pathogenesis of asthma. In addition, histological analysis of the monkey airway showed a taller epithelium cell layer, larger mucous goblet cells and more infiltrated inflammatory cells. Furthermore, the study reported a thickening of the basement membrane. The induction of the asthma phenotype in

this rhesus monkey model is of particular interest since the branching pattern and the distribution of airways in the rhesus monkey are more similar to humans than in rodent asthma models.

These findings showed clearly that the classical view of asthma has largely underscored the role of direct allergen contact with tissue forming resident cells of the airways in the pathogenesis of asthma. Therefore it is reasonable to postulate that a misfunction of epithelial cells and/or ASM cells under the influence of HDM-allergens may well account for the chronic nature of airway wall inflammation and remodeling observed in the lungs of asthma patients.

It was one aim of this thesis, to investigate the effect of the direct interaction of house dust mite extract with primary human ASM cells.

C/EBPα, translation control and asthma

As we described in a previous chapter the decreased expression level of the CCAAT enhancer binding protein (C/EBP) α plays a very important role in the development of an asthmatic phenotype of airway smooth muscle (ASM) cells. C/EBPα is a DNA binding transcription factor, capable of interacting with the CCAAT box motif present in several gene promoters [155]. It belongs to a family of six proteins, C/EBPα, C/EBPβ, C/EBPδ, C/EBPγ, C/EBPε, C/EBPζ, which are characterized by two transactivation domains, a basic DNA binding domain and a leucine zipper motif that mediates dimerization between same (homo-dimers) or other C/EBPs (heterodimers) [156, 157].

C/EBP proteins are involved in the transcription of several genes that are modulated during inflammatory processes or which control cell proliferation. In addition, C/EBPs control cell differentiation and metabolism. Their ability to control differentiation in different progenitors cell types in a context-specific manner is achieved trough their interaction with other transcription factors, such as the peroxisome proliferator activated receptor-γ (PPAR-γ) during adipocyte differentiation, demonstrating the combinatorial nature of cell lineage direction by CEBPs [158, 159]. The biology of CEBPs is more complex as there exists a functional redundancy of the different C/EBP-isoforms that accounts for unchanged phenotypes in certain Cebp- null mice [158]. High levels of C/EBPα mRNA can be found in

the liver, fat, intestine, lung, adrenal gland, myeloid and placental cells and is limited to fully differentiated cells [160].

C/EBPα plays a crucial role in cell growth arrest and cell differentiation, showing an expression pattern that is inversely related to the proliferative state of cells [161]. As reviewed by Johnson et al [162], initial evidence for the anti-proliferative function of C/EBPα was observed when the activation of a chimeric C/EBPα-estrogen-receptor (ER) by estrogen arrested pre-adipocytes in the G0/G1 phase [163]. More recently C/EBPα has been implicated in the development of a form of acute myelogenous leukemia (AML1-ETO) where C/EBPα expression is suppressed or strongly decreased, leading to leukemogenesis and impaired neutrophil differentiation due to an inhibition of cell cycle exit [164]. In another form of leukemia (AME) due to the oncogenic fusion protein BCR-ABL and AML1-MDS1-EVI1, an inhibited C/EBPα translation by increased calreticulin (CRT) levels was observed and this led to the malignant phenotype [165]. In general, the down-regulation of C/EBPα protein appears crucial in myeloid leukemia. In the lung, C/EBPα is also a master regulator of airway epithelial differentiation and probably functions as a tumor suppressor in non-small lung cancer [166]. These findings show that CEBPα protein plays an important role in both (i) cell-cycle arrest and (ii) cell differentiation.

There are different models of C/EBPα induced growth arrest. In cell lines, C/EBPα can associate with the cyclin-dependent kinase (CDK) inhibitor p21 [167], which in turn binds and inhibits the activity of CDKs. On the other hand, it was reported that C/EBPα induced cell-cycle exit in p21-deficient mouse embryo fibroblast [168]. These contrasting results show once again how much experimental findings depend on the setting and on the cell type or on the animal model used.

C/EBPα can also bind directly, in vitro, to CDK2 and CDK4 thereby inhibiting them to phosphorylate their substrate [169]. In normal ASM cells stimulated with β-mimetics and steroids, C/EBPα forms a complex with the glucocorticoid receptor (GR), which then activates the $p21^{(Waf/Cip1)}$ promoter [96-98]. The $p21^{(Waf/Cip1)}$ protein is a strong anti-mitotic protein, hence providing an alternative GC-inducible pathway to inhibit proliferation. Furthermore, C/EBPα can directly repress S-phase driving genes either forming a complex with EF2 or binding directly to the CCAAT consensus site in these genes suppressing their transcription [162]. Taken together, the different anti-proliferative actions C/EBPα can be divided

into a direct or p21$^{(Waf/Cip1)}$-dependent inhibition of CDK activity and/or repression of S-phase driving genes.

Importantly in humans, C/EBPs are predominantly regulated at the translational level. To explain this particular type of regulation I will focus on that of C/EBPα. The same mechanisms operate also for C/EBPβ. As shown in figure 1.6 (below), after transcription various C/EBPα protein isoforms can be translated from a single *C/EBPA* mRNA by initiation of translation at 3 different Kozak sequences [95].

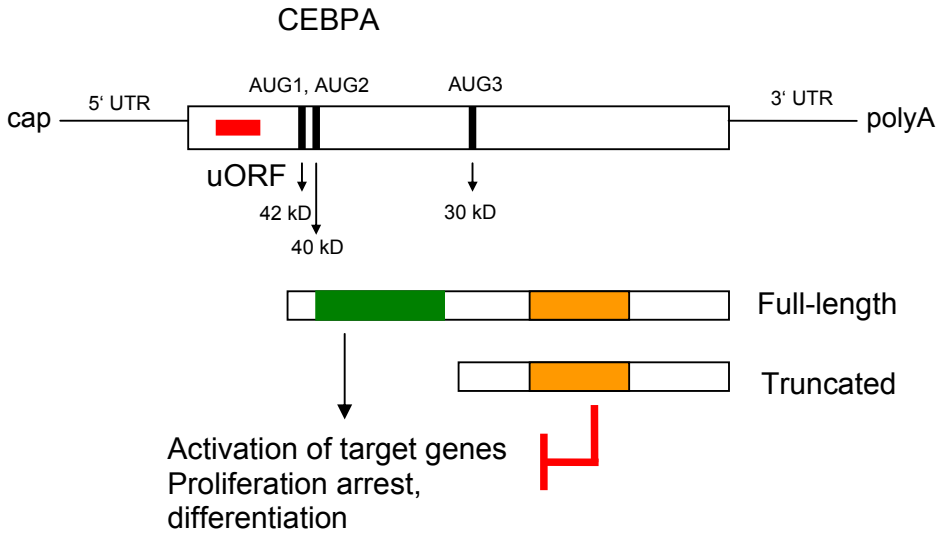

Figure 1.6: Schematic representation of the CEBPA mRNA, the position of the translation start codons (AUG), and the translation products (red: upstream open reading frame; green: transactivation domain; orange: DNA binding domain; uORF: upstream open reading frame)

The different C/EBPα isoforms retain different parts and functional domains of the amino terminus and display different, even contrasting functions, in regard to gene regulation and proliferation control (see figure). The full-length CEBPα is a transcriptional activator, and contains the transactivation domain, while the truncated C/EBPα has ittle transactivation activity and may counteract the function of the full-length isoform [170-173].

To understand the mechanism of translation control of C/EBPα it is necessary to start with the analysis of the C/EBPA mRNA sequence. Comparison in various vertebrates showed several potential translation initiation sites, defined as sequence with an AUG start codon and critical nucleosides at position -3 and +4 corresponding to the optimal Kozak consensus sequence [95, 174]. In human C/EBPα, 3 different

translation initiation sites were found, giving rise to a 42kD, 40kD and 30 kD protein isoform. Experiments with translation initiation sites null mutations abrogated the expression of the corresponding C/EBPα isoform. It was noticed that mutations that abolished the expression of the full-length isoforms enhanced the expression of the truncated C/EBPα (95). Intriguingly, all vertebrates contain an additional translation initiation site upstream of the 3th initiation site from which a small upstream open reading frame (uORF) can be translated into a small protein. The salient feature of this uORF is that it is always out of frame with respect to the C/EBP coding frame. Mutation of the uORF abolished the translation of the truncated isoform and enhanced the expression of the full-length, demonstrating that the uORF is essential for differential translation initiation. Moreover, increasing the strength of the Kozak sequence in this small uORF shifts the ratio towards more of the truncated isoform.

The initiation of translation is controlled by different pathways: (i) glycogen-synthase kinase 3 (GSK3), (ii) phosphoinostitol 3-kinase (PI3K), and (iii) mammalian target of rapamaycin (mTOR) pathway. The RNA-dependent protein kinase (PKR) is part of the GSK3-pathway and affects translation initiation by phosphorylation-induced inactivation of the rate-limiting eIF-2. eIF-2 is part of the ternary eIF2/GTP/Met-tRNA-$_i^{Met}$ complex needed for translation re-initiation [175]. This process facilitates the recognition of the AUG-codon and initiates protein synthesis. Together with PKR three additional eIF2 kinases, namely (i) haem-regulated inhibitor kinase (HRI), (ii) PKR-like endoplasmic-recticulum kinase (PERK) and (iii) GCN2, have been identified [176]. Constitutive activation of the eIF-2 pathway resulted in a high eIF-2 activity in PKR mutant cells and shifted the C/EBPα expression towards the truncated isoform. Similarly, activation of the mTOR pathway increased eIF-4E expression, a protein of the cap-binding complex [177]. mTOR phosphorylates and inhibits the phosphatase PP2A, which in turn keeps the inhibitory 4E-BP1 protein in an active non-phosphorylated state [178-181]. 4E-BP1 in turn was shown to inhibit eIF4E [182]. Binding of the eIF4E to the mRNA-cap is a rate-limiting step of the assembly of the eIF4E complex and of the initiation of translation [183]. Over-expression of eIF4E shifted translation CEBPα towards the truncated isoform, while the inhibition of mTOR by rapamycin reduced the expression of the truncated C/EBPα isoform [95]. Taken together, these important findings showed that the under optimal condition, which is high eIF-2 and eIF-4E activitiy, C/EBPs are predominantly expressed as the truncated isoforms.

Since the deletion of the uORF under high eIF-4E activity abolished the expression of the truncated CEBPα, it can be assumed that the regulatory activity of the uORF is crucial to modulate the ratio of C/EBPα isoforms and thus the re-initiation of its translation. In this model, illustrated in fig. 1.7 (below), at moderate translation activity the uORF initiation codon allows part of the ribosomes to read trough the uORF and to initiate translation at the proximal AUG-codon, generating a full-length C/EBPα isoform (Fig 1.7 B). Poor efficiency of translation re-initiation at the distal site further decreased the expression of the small isoform. In contrast, increased translation activity led the ribosomes to recognize and translate the uORF, terminated scanning and re-initiated translation at the distant down-stream AUG-codon, giving rise to the small isoform (Fig. 1.7. A) [184]. The efficiency of translation re-initiation depends on the reloading of the eIF2/GTP/Met-tRNA$_i^{Met}$ complex whereas the eIF4E complex is needed for efficient scanning and re-initiation after uORF translation [95, 185]. As a consequence of this type of translational regulation, C/EBPα isoforms shift their ratio from truncated to full-length protein. This shift in the isoform ratio finally allows the fine-tuning of the cellular response to external stimuli, and determines if the cell will further proliferate or exit the cell cycle and differentiate.

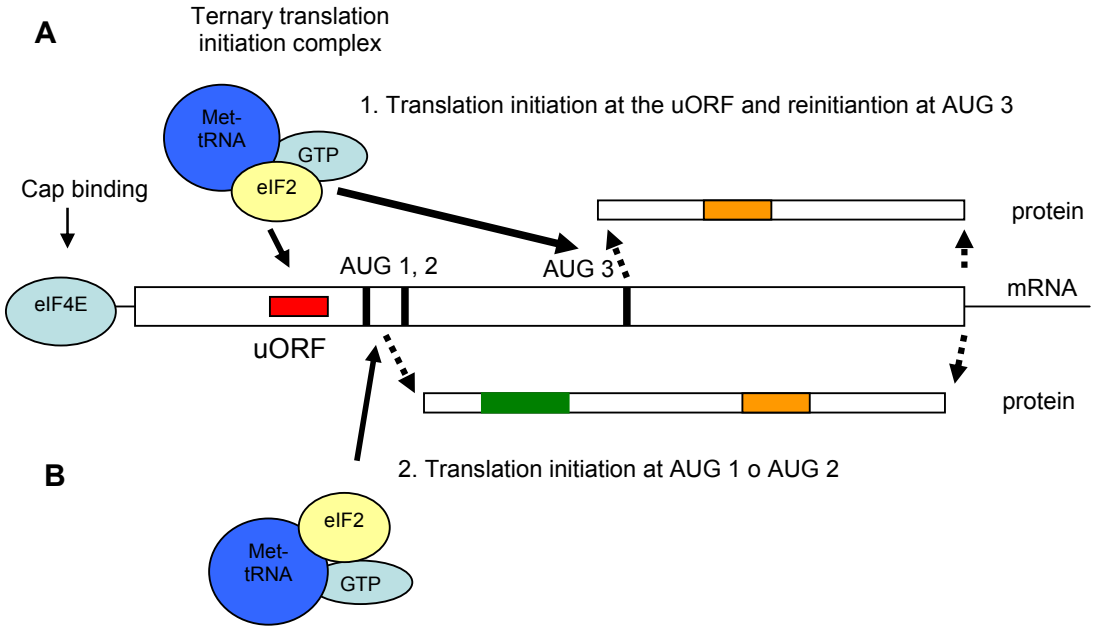

Figure 1.7: Schematic representation of the CEBPA mRNA translation mechanism, leading to the formation of the truncated or full-length C/EBPα protein. For truncated C/EBPα the translation starts at the uORF, and stops after translation of the uORF. Then it reinitiates at AUG 3, generating the truncated isoform (A). For the generation of the full length C/EBPα the translation starts at the start codon AUG 1 or AUG 2 and reads trough (B). (red: upstream open reading frame; green: transactivation domain; orange: DNA binding domain)

Small uORFs that act as cis-regulatory mRNA elements of translation initiation have been found in various regulatory genes, including the transcription factors *SCL/Tal1* [186] and *ATF-4* [187], thrombopoietin (TPO) [188], the cyclin *CLN3* mRNA [189] and the beta-Secretase (BACE-1) [190].
The C/EBPα uORF has been used to develop a translation control reporter system (TCRS) which allows to monitor the ratio of two different length peptides with start codons down-stream of the uORF. The TCRS makes use of the regulatory function in translation control of the uORF described before and therefore allows to determine and to quantify variations or disturbances of the translation re-initiation efficiency [184, 191].

In recent years, mutations in the genes encoding the proteins of the translation control machinery have been linked to the etiologies of several human diseases and deregulation of translation was associated with a wide range of proliferative disorders, including cancers [192]. Mutations of the PERK kinase which regulates the eIF2 cycle led to the Wolcott-Rallison Syndrome (WRS), characterized by the loss of pancreatic β-cells that causes permament diabetes [193-195]. Mutations in the eIF2B are linked with an inheritable brain disease, leukoencephalopathy with vanishing white matter (VWM) [196]. VWM patients who experience fever can fall in coma or may die due to their inability to alter translation activity during mild stress, such as an increased body temperature. In the chronic myeloid leukaemia (CML: BCR-ABL) cells, the expression of the RNA-binding protein hnRNP is abnormally high. hnRNP binds to the 5' UTR of the C/EBPA mRNA thereby inhibiting its translation [197]. Increased levels of eIF4E have been found in several cancers [198], including colon adenoma and carcinoma [199], breast carcinoma [200, 201], non-Hodgkin's lymphoma [202] and primary bladder cancer [203]. The regulation of the eIF4E activity is under control of both the PI3K and the mTOR pathway. Therefore, deregulated translation as a result of the disturbed signaling contributes to oncogenesis.

In addition, the deregulation of eIF2 is frequently observed in cancer cells [202]. Enhanced eIF2 or eIF4E with concomitant up-regulation of the truncated C/EBPα isoforms were reported in mammary-epithelial and intestinal-epithelial cancer cells [173, 199, 204]. Hereditary thromboythaemia, a disease characterized by sustained proliferation of mega-karyocytes in the bone marrow leading to an increased number of blood platelets, is caused by a mutation in the uORF of the cytokine thrombopoietin (TPO) [188].

As an additional regulatory mechanism, C/EBPα expression can be repressed by calreticulin. It has been shown that *CEBPA* mRNA folds an internal stem loop formed by a GC rich motif, which is a strong binding site for calreticulin, which repressed the translation into the corresponding protein [205]. An inverse relationship of C/EBPα and calreticulin has been demonstrated in adipogenesis where calreticulin promoted adipogenesis by repressing the expression of C/EBPα and PPARα. In a form of leukemia (AME) which was linked to the occurrence of the onocgenic fusion proteins BCR-ABL and AML1-MDS1-EVI1 an inhibited C/EBPα translation was due to increased calreticulin levels leading potentially to the cancerogenic phenotype [206].

Finally, in a recent study it has been show that there is an additional regulatory mechanism of the translation control of the C/EBPA mRNA. In adipocyte differentiation, the protein hematopoietic zinc-finger (Hzf) interacts with the 3' un-translated region of the C/EBPα enhancing its translation [207]. Therefore, it is likely that the translational regulation of the CEBPA mRNA is a complex mechanism involving several proteins in the 5' and 3' un-translated regions.

The finding that the translation control of C/EBPα in ASMC of asthma patients was faulty originated from the observation that ASM cells of asthma patients showed normal levels of *CEBPA* mRNA but the expression of the corresponding protein was missing or heavily reduced [94, 208]. The reason for this difference between ASMC of patients with asthma and healthy people remained an open question and it was one of the aims of the thesis to find the mechanism(s) to explain it.

Chronic obstructive pulmonary disease (COPD)

Chronic obstructive pulmonary disease (COPD) is a common inflammatory lung disease and a major cause of illness and death throughout the world.

The Global Initiative for Chronic Obstructive Lung Disease defines COPD as "a pulmonary disease characterized by *airflow limitation* that is not fully reversible. The airflow limitation is usually progressive and associated with an *abnormal inflammatory response* of the lung to noxious particles or gases " [209].

The abnormal inflammatory response is a pathology of COPD which it shares with asthma. In COPD this inflammatory process leads to a co-presence of (i) *small airway diseases (obstructive bronchiolitis)* with fibrosis and obstruction, (ii) *parenchymal lung tissue destruction (emphysema)*, loss of lung elasticity and closure of small airways and (iii) *chronic bronchitis*, characterized by cough and mucous hypersecretion. As a consequence, the airways undergo (iv) *structural changes* with further loss of lung elasticity and (v*) airflow limitation*. These pathological mechanisms manifest at different degrees in COPD patients [210].

Despite its high global prevalence, there is still a fundamental lack of knowledge about the cellular, molecular and genetic causes of COPD and no available therapy which may reduce the disease progression or mortality [211].

COPD, the fourth-leading cause of death in the world

COPD affects > 10% of the world population over the age of 40 years [212] and every year almost 3 million people die of this disease [213, 214]. According the WHO, in 2007 COPD was the 4th cause of death worldwide and it is predicted to become the 3rd leading cause of death by 2030 [215]. COPD is the 13th cause of morbidity today and will become the 5th cause of morbidity by 2020 [213, 216]. Therefore, the economical and social burden of the disease is immense today and will dramatically increase in the future, also considering that at present the disease is under-estimated, it is insufficiently recognized and is poorly diagnosed. In the European Union, COPD accounts for 56% of the cost for respiratory diseases [217]. For an individual, of course, the disease may dramatically lower the quality of life. Paradoxically,

despite its global impact and compared to asthma, research in COPD is less progressing and highly under-funded.

COPD is a complex, multi-factorial pathology and both environmental and host-depended factors are needed for the clinical manifestation of the disease. However, cigarette smoking is undoubtedly the major causative environmental risk factor for COPD. It accounts for approximately 90% of all cases and a dose-dependent relationship between tobacco consumption and the development and severity of COPD has been observed. Important is the age at which a person started smoking, the numbers of packages of cigarettes smoked per year, and the current smoking status. Passive exposure to cigarette smoke is another risk factor that is increasing the total amount of inhaled particles into the lung [218-221]. However, only 10-20% of the smokers develop clinical symptoms of COPD and susceptibility and other environmental factors are therefore crucial for the pathology of COPD [222]. It is not known which genes make an individual smoker susceptible to develop COPD, but family and twin studies have demonstrated an important genetic component [223, 224]. Additional environmental risk factors are occupational dust and chemical exposure [225-228], indoor and outdoor air pollution, bacterial and viral infections [229-231], the socioeconomic status [232], and asthma [233]. Although there is no conclusive evidence, adults with asthma are found to have a twelvefold higher risk of acquiring COPD than subjects without asthma [233]. Like many other diseases, COPD is a polygenic disease and gene-environment interactions are critical for the development of this disorder. So far, the best investigated genetic cause for the development of COPD is the hereditary deficiency of the of alpha-1 antitrypsin, an inhibitor of serine proteases. The lack of this protein is leading to the development of emphysema and decline in lung function due to digestion of the lung forming extracellular matrix and cell-cell interactions [234].

Pathogenesis of COPD

Pathological changes in COPD include chronic inflammatory processes and airway remodeling, both localized in the proximal and peripheral airways, in the lung parenchyma and in pulmonary vasculature [210]. Cigarette smoking is by far the most prominent cause for COPD. The inflammatory processes and the airway remodeling increase with disease severity and persist after cessation of smoking. Therefore, it

is assumed that after a certain threshold of disease severity is passed, simply quitting smoking may not be sufficient to prevent disease progression [235].

In recent years, investigations on COPD led to a major expansion of paradigms explaining the pathobiology of the disease and many different models are proposed today. For example, COPD can be seen as a disease of accelerated lung aging, since many cellular pathological processes are due to the accumulation of reactive oxygen species [211]. There is also a group of scientist that define COPD as an auto-immune disease which response to antigens (e.g. elastin) released after smoke induced tissue or cell injuries [236]. Although other mechanisms that may be involved in COPD have been investigated and potential targets for a therapeutic approach proposed, an efficient cure for this disease is still not available today.

In the next paragraphs we will summarizes mechanisms leading to the pathogenesis of COPD.

Airway inflammation

COPD and asthma are both characterized by chronic airway inflammation, but the composition of the immune cells involved in the inflammatory process are different and show a disease-specific pattern. While in asthma the predominant inflammatory cells are eosinophils, Th2 cells and mast cells, in COPD they are neutrophils, macrophages, and CD8 positive T-cells. Especially, macrophages seem to play a pivotal role in COPD. COPD patients with emphysema show a 25-fold increase in the numbers of macrophages in the tissue and in the alveolar space when compared to normal smokers [229] and there is a correlation between macrophage numbers in the airways and the severity of COPD [237]. The key inflammatory mediators in asthma are IL-4, IL-5, IL-13 and LTD4, while the most important inflammatory factor in COPD are IL-8, LTB4 and TNFα. Furthermore, in asthma the inflammation is localized in the proximal airways, while in COPD it occurs mainly in the peripheral airways (bronchioles), the lung parenchyma and in addition the pulmonary vessels are affected. The bronchioles are obstructed and present with fibrosis [238-243]. In COPD, the typical inflammatory cascade is triggered by noxious air-borne particles, mainly by oxidants derived from cigarette smoke, that activate macrophages to release IL-8, TNF-α and matrix metalloproteinases (MMP). The release of these factors

is promoted by the inactivation of histone deacetylase (HDAC) leading to the transcription of NF-κB-inducible cytokines [244, 245].

Protease-antiprotease imbalance

MMPs display enzymatic capacity which causes morphological changes in the lung structure and contribute significantly to the state of COPD. Increased concentrations of MMP-1, -2, -9, -12 have been found in bronchoalveolar lavage samples of COPD patients when compared with that of non-COPD individuals [246, 247]. Since MMP-12 degrades elastin and is predominantly produced by alveolar macrophages, it is considered the leading proteinase responsible for the occurrence of pulmonary emphysema [248]. Neutrophiles, recruited into the lung along gradients of TNF-α, IL-8 and MMPs are activated and release neutrophil elastase and proteinase-3, which enhance the matrix-degrading effect of MMPs, by digesting also their inhibitors, the tissue inhibitor metalloproteinanses (TIMPS) and α1-antitrypsin [249, 250]. Therefore, the destruction of tissue in the pulmonary alveoli leading finally to emphysema in COPD patients is not the result of a single proteinase or a single inflammatory cell, but a combination of cells and effector molecules finally leading to a protease-antiprotease imbalance. An important target of tissue degradation is elastin, a major connective tissue component of the lung parenchyma [250, 251]. Furthermore, neutrophile elastase can induce epithelial cell damage and the loss of the ciliated epithelium. It also reduces the cilia beating frequency, and induces mucous gland hyperplasia [252].

Alpha-1 antitrypsin is the most important protease inhibitor in the lungs protecting the tissue against the proteolytic digestion of proteases. It is the only known genetic cause for COPD. The lack of this protein is leading to development of emphysema and decline in lung function, but it accounts for only about 2% of the cases of severe COPD, with a substantial higher risk in smokers [234]. Proteases that are inhibited by alpha-1 antitrypsin are capable of inducing emphysema in animal models, and thus it has been proposed that unopposed activity of proteases will induce lung damage that is similar to the tissue structure changes typical for the emphysematous lung.

Oxidative stress

Oxidative stress is defined as an excess of reactive oxygen species (ROS) that can not be neutralized by the antioxidant defense mechanisms and thus leading to cell damage. There is evidence that oxidative stress plays a major role in the pathogenesis of COPD [253, 254]. On one side, inflammatory cells are able to generate ROS, but also cigarette smoke itself contains ROS at high concentrations [235]. ROS activate NF-κB, which induces the transcription of multiple inflammatory genes leading to an inflammatory response in the lung (255). Interestingly, oxidative stress in COPD may be also linked to the poor response to corticosteroids in COPD patients. Oxidative stress impairs the translocation of the glucocorticoid receptors to the nucleus and the binding to its corresponding target DNA sequence [256, 257].

Airway remodeling

In COPD patients, increased airway remodeling leading to airflow limitation is observed preferentially in the small airways and in the lung parenchyma. Furthermore, mucus hypersecretion may lead to luminal airway obstruction. One of the major characteristics of airflow limitation in COPD is the fact that it is only partly reversible and progressive.

The small airways in COPD are narrowed due to an increased thickness of the airway walls as a result of high collagen deposition and mucosa thickening after containing an inflammatory exudate [258-262]. The observed fibrosis (defined as an increased ECM accumulation in a pathologic status) is thought to be a consequence of the chronic inflammatory process. Increased levels of TGF-β are typically observed in COPD patients lungs and are assumed to mediate the release of connective tissue growth factor (CTGF) which, in turn, stimulates collagen deposition in the airway wall [263-266]. Furthermore, airway wall inflammation contributes to the destruction of the alveolar walls which are attached to the airways, leading to airway wall deformation and narrowing of the airway lumen [267].

Emphysema is defined as an abnormal permanent enlargement of the air space distal to the terminal bronchioles, accompanied by the destruction of the bronchiole walls and the formation of emphysematous bullae which are surrounded by normal lung tissue [268]. Emphysema contributes to

airflow limitation by the loss of lung elasticity [269]. The molecular mechanisms of parenchymal destruction in emphysema remains enigmatic, the proteases-antiproteases imbalance hypothesis is today still the most supported. An alternative mechanism has been proposed by Kasahara and coworkers. They hypothesized that emphysema may be due to an accelerated apoptosis of endothelial and epithelial cells. Experimental findings showing that the blockade of vascular endothelial growth factor (VEGF) receptors is able to induce cell apoptosis and emphysema in alveolar septae and suggests a role of apoptosis in the pathogenesis of the disease [270]. Interestingly, the tissue destruction characteristic for emphysema may be accompanied by an increase in the collagen deposition, suggesting that there is active alveolar wall fibrosis in emphysematous lungs [271].

Mucus hypersecretion is expressed in chronic bronchitis and may be a risk factor leading to the decline in lung function in COPD [272, 273]. Chronic mucus hypersecretion may be a consequence of the inflammatory process occurring at the submucosal glands [274]. It might be due to the increased numbers of neutrophils and mast cells, and to their secreted serine proteases and mast cell chymase, both are potent mucus secretagogues factors [275, 276]. Patients with COPD and chronic mucus hypersecretion have a higher probability to die from pulmonary infections than subjects without chronic mucus hypersecretion [277].

Cigarette smoke and COPD

According to WHO projections, there will be 2 billion smokers and 10 million smoke-related deaths per year by 2050. Cigarette smoke is the leading cause for COPD as it accounts for accounts for ~80–90% of COPD cases [278]. Despite many prevention programs to encourage people to stop cigarette smoking or to prevent people from starting to smoke, tobacco-associated diseases are increasing. In Europe, Greece has the highest numbers of smokers, Sweden has the lowest [279]. It is estimated that in the future tobacco addiction will increase especially in developing countries, while for Western countries it is expected to decrease, due to high taxes on tobacco products [280] and a restrictive legislation with respect to smoking in public rooms in an increasing number of nations.

Cigarette smoke is a complex mixture that consists of over 4000 chemical components [281, 282], including ~10^{15} reactive species in the gas phase alone [283]. Mainstream cigarette smoke is inhaled by puffing, while the side stream smoke is released into the environment and defined as environmental tobacco smoke (ETS). Today, two kinds of approaches are used to study the biological effect of tobacco smoke. One approach is single compound driven (e.g. nicotine), while the second is based in the generation of cigarette smoke extracts (CSE) or smoke conditioned medium (SCM), which are then used to incubate cells. The use of a single compound may not detect compounds interactions while CSE/SCM is limited in quality due to a poor standardization method [284]. In addition, animal models with experimental cigarette smoke exposure are frequently used.

Cigarette smoke is the main trigger of the inflammatory process characterizing COPD. Smoke extract has been shown to stimulate lung fibroblasts to release neutrophil and monocyte chemoactractants such as IL-8, granulocyte colony-stimulating factor (GM-CSF), and monocyte chemotactic protein-1 (MCP-1) [285]. Chronic cigarette smoke increases the number of inflammatory cells, especially neutrophils, lymphocytes, and macrophages in the BAL and lung tissue of both humans and animals [286, 287]. It triggers therefore an inflammatory response, which potentially causes alveoli destruction. In addition, significant retention of activated neutrophils in the lung has been documented after inhalation of cigarette smoke [288]. Furthermore, in a mouse model, exposure to cigarette smoke is associated with increased desmosine detection in BAL, which is an indicator of elastin fiber breakdown [289]. Compared to non-smokers, exhaled breath condensate of healthy smokers show increased levels of IL-1beta, IL-6, IL-8, IL-10, and TNF-α [290]. In rodent models acute and repeated (chronic) cigarette smoke exposure leads to increased levels of TNF-α, IL-1beta, IL-8, and MCP-1 which is associated with an increase of mononuclear cells and neutrophils in the BAL [291]. Especially TNF-α plays a pivotal role in cigarette smoke-induced emphysema since transgenic overexpression of TNF-α led to the development of emphysema and of alveolar inflammation [292], while TNF-α receptor knockout mice showed significant protection against cigarette smoke-induced emphysema [293] Finally, cigarette smoke is able to transcriptionally upregulate MUC5AC expression, amplifying the expression of respiratory mucins [294] and therefore enhances airflow limitation.

It was one aim of the thesis to investigate the effect of cigarette smoke on the expression of the important cell-cycle regulators and pro-inflammatory gene transcription factors C/EBPα and C/EBPβ.

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Objectives of the thesis

Both asthma and COPD are chronic inflammatory respiratory diseases and present a major global health problem with increasing prevalence. Airway inflammation is a characteristic and important hallmark in both diseases. As a consequence, in the past investigations focused on the immunological aspect of these disorders; and therefore asthma has been defined as a Th-2 driven disease (Th-2 paradigm). However, in recent years, it has been shown that resident cells of the airways, in particular airway smooth muscle cells, are pivotal in understanding the pathogenesis underlying asthma and it is tempting to see asthma as a disease of an abnormal airway smooth muscle (ASM) cells biology. Especially the abnormal expression of the cell cycle regulator and pro-inflammatory gene transcription factor C/EBPα in asthmatic ASM cells may account for many asthma-specific pathologies such as increased proliferation, increased release of inflammatory mediators. In this thesis, I focused on of resident airway cells, namely bronchial smooth muscle cells and lung fibroblasts. Of special interest was the effect of external stimuli such as house dust mite extract and cigarette smoke on the expression and regulation of C/EBPα. The focus of the included studies was predominantly at the control mechanisms of translation of *CEBPA* mRNA.

The specific objectives of this thesis were:

I. To investigate the translational mechanisms of C/EBPα in ASM cells of asthma patients and non-asthma controls.
II. To assess the effect of house dust mite extracts on the (IIa) proliferation and pro-inflammatory characteristics, (IIb) the C/EBPα protein expression, and (IIc) the C/EBPα translation mechanisms in ASM cells of both asthma patients and controls.
III. To investigate the effect of cigarette smoke on the (IIIa) proliferation and pro-inflammatory characteristics, (IIIb) the C/EPBα and C/EBPβ protein expression, and (IIIc) the C/EBPα and C/EBPβ translation mechanisms in primary human lung fibroblasts.

Chapter 2: Impaired translation of *CEBPA* mRNA in bronchial smooth muscle cells of asthma patients.

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Abstract

Background: Bronchial smooth muscle (BSM) cells of asthma patients have an impaired expression of CCAAT/Enhancer Binding Protein (CEBP)α, which is associated with an increased proliferation.

Objective: We sought to assess the translational regulation of *CEBPA* mRNA in cultured BSM cells of healthy controls (n=11) and asthma patients (n=12).

Methods: Translation efficiency was studied using a translation control reporter system (TCRS) driven by the control elements present in the *CEBPA* mRNA. Translation efficiency was determined by the ratio of two artificial HA.11 proteins: p23 and p12. We also analyzed levels of proteins that control translation of CEBPA mRNA, namely: hnRNPE2, calreticulin, eIF4E and 4EBP.

Results: Compared to healthy controls, BSM cells of asthma patients proliferate faster (2.1-fold) and are primed for IL-6 secretion. Real-time RT-PCR showed BSM cells of asthma patients express normal levels of *CEBPA* mRNA, whereas they express lower levels of C/EBPα (p42) proteins. Transient transfections with the TCRS construct showed a disturbed p12/p23 ratio in BSM cells of asthma patients relative to healthy controls, which coincided with lower levels of eIF-4E.

Conclusion: BSM cells of asthma patients have normal levels of *CEBPA* mRNA but inadequately reinitiate the translation into C/EBPα proteins. Impaired translation control upstream of eIF-4E may underlie the observed increased proliferation and priming of BSM cells of asthma patients.

Clinical implications: Hyperplasia of BSM cells in the bronchi of asthma patients may result from a dysfunctional translation of the *CEBPA* mRNA. Novel strategies to counteract BSM hyperplasia at the translation level should be envisaged.

Capsule Summary

The report finds abnormal translation of CEBPA mRNA in BSM cells of asthma patients and may potentially explain pathological features of this disease, in particular BSM cell hyperplasia.

Abbreviations

BSM = bronchial smooth muscle

C/EBP = CCAAT/enhancer binding protein

FCS = fetal calf serum

UTR = untranslated region

TCRS = translation control reporter system

HA = hemagglutinin

uORF = upstream open reading frame

LP = long protein

SP = short protein

Keywords:

Asthma, bronchial smooth muscle cell hyperplasia, C/EBP-alpha messenger RNA translation,

INTRODUCTION

Pulmonary inflammation and airway wall remodeling are characteristic pathological features of asthma and are assumed to result from chronic airway inflammation.[1] Therefore, asthma research has mainly focused on the inflammatory component of the disease. In particular the studies on mast cells, eosinophils and T-lymphocytes have contributed to our knowledge on airway inflammation and how it aggravates asthma symptoms. The remodeled airways of asthma patients display an increase in BSM mass of which the etiology is unknown, but with a major effector function in airway constriction.[2] Recent studies showed that bronchial thermoplasty (a novel bronchoscopic strategy to reduce the mass of BSM) markedly improved asthma control in subjects with mild or severe asthma.[3] These findings are in line with our proposition that infiltrating cells of the immunological compartment may not be the cause of asthma, but rather that asthma may be regarded as the result of aberrant behavior of the BSM cell due to an imbalanced expression of alternative forms of C/EBPα, a transcription factor that controls cell proliferation, differentiation and inflammation. The biological effects of C/EBPα are exerted by a full length (p42) isoform, and counteracted by a truncated isoform (p30) that is generated at the translational level.[4] We and others reported that BSM cells obtained from asthma patients proliferate faster than their non-asthmatic counterparts [5,6], have an activated phenotype characterized by more severe contraction [2,7], and demonstrate increased mediator production.[8] The faster proliferation rate observed for BSM cells of asthma patients was associated with diminished expression of C/EBPα.[8,9] Understanding the origin of the hyper-reactive ("primed") phenotype of the asthmatic BSM cell may prove valuable for developing novel therapeutic strategies. Therefore, we investigated the translational regulation of *CEBPA* mRNA and the proliferative response of primary human non-transformed BSM cell lines obtained from asthma patients and non-asthmatic controls.

METHODS

Tissue specimens & cell cultures

Lung tissue specimens were obtained from the Department of Pathology, Royal Prince Alfred Hospital; the Cardiopulmonary Transplant Unit and the Department of Pathology, St Vincent's Hospital (Sydney, Australia), and the Department of Pathology, University Hospital Basel (Switzerland) after approval of the local Ethical Committees and written consent of all patients. BSM cells from controls and asthma patients were established as previously described.[5,10] Cells were grown in RPMI 1640 with and without 5% fetal calf serum (FCS).

Western analysis

Equal amounts of proteins were loaded on gradient polyacrylamide gels (4-20%) and immuno-blotting and DNA binding was performed as described previously.[5,10] C/EBPα antibodies were obtained from AVIVA systems biology (San Diego, CA), HA.11 antibodies were from Covance (Berkeley, CA).

RNA extraction, Reverse transcription

Cells were starved 24 hours and then incubated for 0, 6 and 24 hours in RPMI in the absence and presence of 5% FCS or PDGF-BB (10 ng/ml). RNA extraction was performed using the RNeasy Mini kit (Qiagen) including the DNA digestion step. The RNA was quantified with a NanoDrop spectrophotometer and reverse transcribed using the Omniscript RT kit (Qiagen).

Real-time polymerase chain reaction

C/EBPα and 18S expression were analyzed using a human C/EBPα Gene Expression Kit (Applied Biosystem) and an 18S Genomic control kit FAM-TAMRA (Eurogentec), respectively, according the manufacturers' protocols. Samples were run in duplicates using a

7500 Fast Real-Time PCR System (Applied Biosystems). Relative C/EBPα levels were expressed as means ± SEM after normalization to18S RNA.

Translation Control Reporter System

We used the translation control reporter system (TCRS) described in detail elsewhere.[9,10] In brief, to detect translational abnormalities we used a recently developed translation control reporter system (TCRS) that utilizes the *cis*-regulatory uORF from the *CEBPA* locus to monitor the translation of a dual reporter gene into two unique reporter peptides.[9,10] The reporter peptides contain a pre-pro-trypsin signal for secretion and three distinct immunogenic epitopes for detection and quantification purposes, including a *c-myc* tag, a *FLAG* tag, and an influenza *hemagglutinin (HA)* epitope. Secreted p23 and p12 reporter peptides can be detected using *FLAG* and *c-myc* antibodies, respectively. The *HA* epitope is present on both reporter peptides and an improved second-generation HA monoclonal antibody (HA.11) is sufficient to detect both p23 and p12 reporter peptides in a single immuno-blot.[9,10] To test the TCRS, we transiently transfected BSM cells with (i) the TCRS containing the wild-type *cis*-regulatory uORF from the *CEBPA* locus ($TCRS^{WT}$), (ii) a control TCRS construct with a non functional uORF ($TCRS^{\Delta uORF}$) which therefore can only express reporter peptide p23; (iii) a TCRS encoding solely the long peptide p23 ($TCRS^{LP}$), and (iv) a TCRS encoding solely the short peptide p12 ($TCRS^{SP}$). After 24 hours total protein extracts were analyzed for p23 and p12 expression using a HA.11 antibody. To test the hypothesis that a translational defect underlies aberrant expression of the *CEBPA* gene, we transfected BSM from normal and asthmatic patients with the $TCRS^{WT}$, which utilizes the upstream open reading frame (uORF) from C/EBPalpha in a cassette with two translation start (ribosome binding) sites. Translation from the first site acts as a control and produces a long peptide (LP) with a FLAG epitope; translation from the second site results in a short peptide (SP) with a Myc epitope. In addition, both LP (p23) and SP (p12) contain an HA

epitope and can be detected by HA.11 antibodies. Equally successful transfections result in 1) the FLAG or LP epitope of roughly equal intensity, and 2) different amounts of the SP or Myc epitopes depending on translational regulation. To identify translation efficiency by Western analysis, we used the SP/LP ratio, not the FLAG/myc ratio (which can be determined in supernatants of BSM cells). A small SP/LP ratio (<<1.0) indicates attenuation of translation. [10,11]

Transfections

Transfections with siRNA or TCRC constructs were performed as described elsewhere.[10,11, 12]

Data analysis & presentation

For the purpose of statistical analysis, the following groups were compared: controls in the absence and presence of 5% FCS, asthmatics in absence and presence of 5% FCS. For each group data were compared by analysis of variance (ANOVA, single factor analysis). Protein analyses are representative of at least 3 independent experiments. Signals were normalized to α-tubulin signals (as indicated).

RESULTS

BSM cells of asthma patients are primed for IL-6 release, proliferate faster, and express lower levels of C/EBPα

In accordance with previous reports [3,6,13], BSM cells obtained from asthma patients exhibited a significantly increased proliferation rate (2.1-fold) relative to control cells ($p<0.05$, Fig. 1a). Time course experiments showed that, in response to PDGF-BB, the BSM cells of asthma patients immediately and significantly enhanced the secretion of IL-6 protein, whereas non-asthmatic cell lines reached the same level only after ≥24 hours of stimulation ($p<0.05$, Fig. 1b). The accelerated rate of IL6 secretion observed in BSM cells of asthma patients was associated with decreased levels of the C/EBPα protein p42 compared to non-asthmatic controls (Fig. 1c).

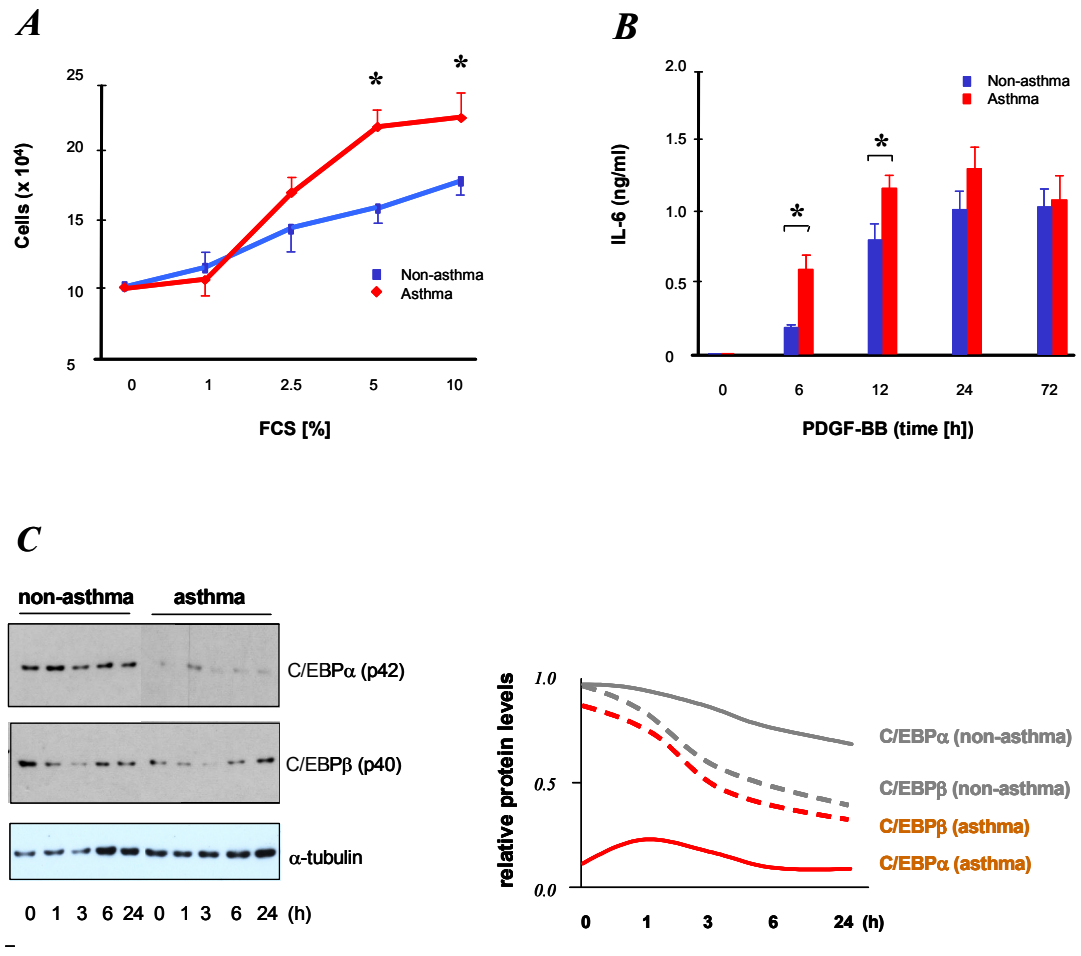


Figure 1. *Figure 1. Proliferation and proinflammatory characteristics of BSM Cells.* Asthma (n=11); non-asthma (n=12). a. Proliferation in response to Fetal Calf Serum (FCS). *, $p < 0.05$. b. PDGF-induced IL-6 secretion at: 0, 6, 12, 24 and 72 hours. *, $p < 0.05$. c. Time course for C/EBPα and -β protein expression in response to PDGF-BB (10 ng/ml).

BSM cells of asthma patients express normal levels of *CEBPA* mRNA

Real time RT-PCR revealed that BSM cells of asthma patients and controls expressed equal levels of *CEBPA* mRNA (Fig. 2.a). However, the same BSM cells of asthma patients expressed significantly less C/EBPα proteins (both p42 and p30), and the p42/p30 ratio was slightly higher in asthma patients compared to non-asthmatics (2.0 ± 0.21 *versus* 1.65 ± 0.13, Fig. 2.b). Together, the data presented in figure 2.a and 2.b suggest an impaired or deregulated translation

of the *CEBPA* mRNA underlying the observed low levels of the C/EBPα proteins in BSM cells of asthma patients.

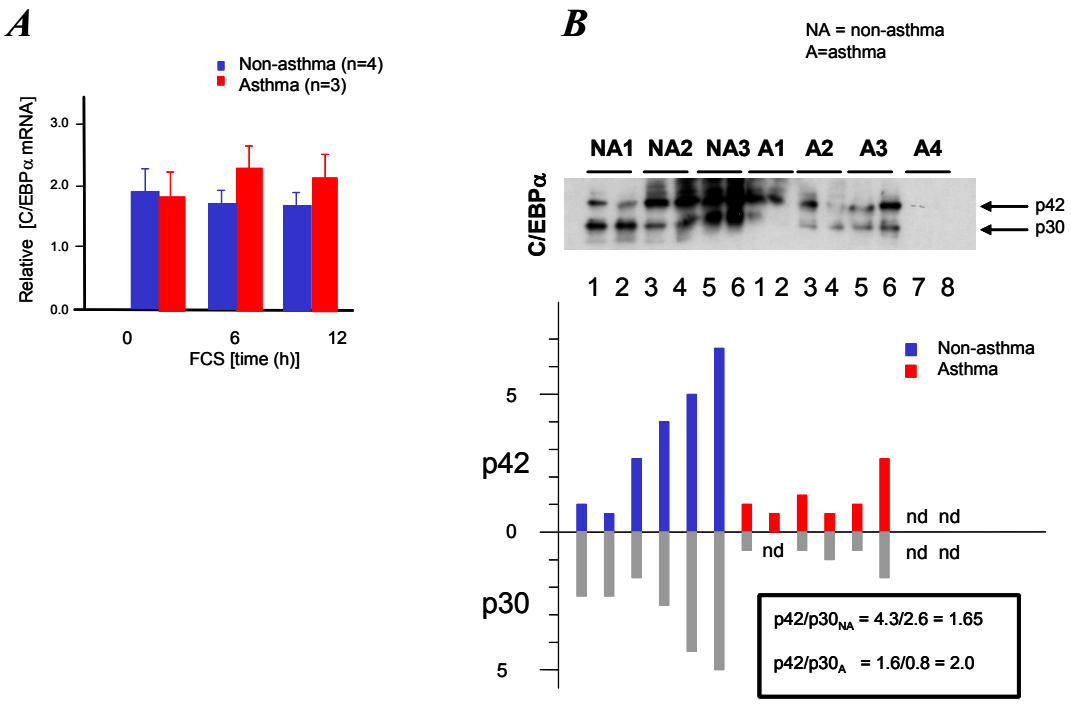
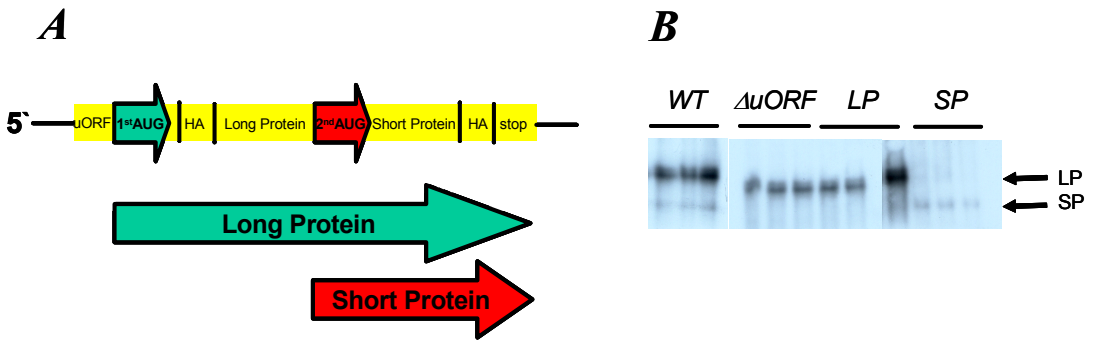


Figure 2. *mRNA and Protein expression of C/EBPα in BSM cells.* a. C/EBPα mRNA expression by quantitative real-time PCR after treatment with FCS. b. The quantified levels of C/EBPα p42/p30 ratios are expressed in the bar diagram and for BSM cells obtained from asthma patients and non-asthma controls are shown in the box-insert. nd=not detectable.

BSM cells of asthma patients exhibit impaired translation of *CEBPA* mRNA

To detect translational abnormalities BSM cells were transfected with the TCRS reporter constructs. The reporter peptides contain a pre-pro-trypsin signal for secretion and three distinct immunogenic epitopes for detection and quantification purposes, including a *c-myc* tag, a *FLAG* tag, and an influenza *hemagglutinin (HA)* epitope (Fig 3.a). In accordance with expectation [10,11], p23 (LP) and p12 (SP) bands were observed in BSM cells transfected with $TCRS^{WT}$, a single LP band was found in cells transfected with $TCRS^{\Delta uORF}$ or $TCRS^{LP}$, and a single SP band was found

in cells transfected with TCRS^{SP} (Fig. 3.b.). Next, BSM cell lines obtained from non-asthmatic controls and asthma patients that express low levels of C/EBPα after FBS treatment were transiently transfected with the TCRS^{WT}, and analyzed for C/EBPα (p42/p40), C/EBPα (p30), p23 and p12 after 24 hours. Proliferating BSM cells of asthma patients (24h, 5% FCS) consistently displayed low levels of the anti-proliferative C/EBPα (p42/40) proteins and lacked the p30 truncated variant, which coincided with small SP/LP ratios (Fig. 3.c A1, A2). Additional transfections with the TCRS^{WT} showed that 5/6 BSM cells of asthma patients were characterized by small SP/LP ratios, whereas this was only true for 1/7 of cells from control patients (figure 3.c., 3.d and 3.e).



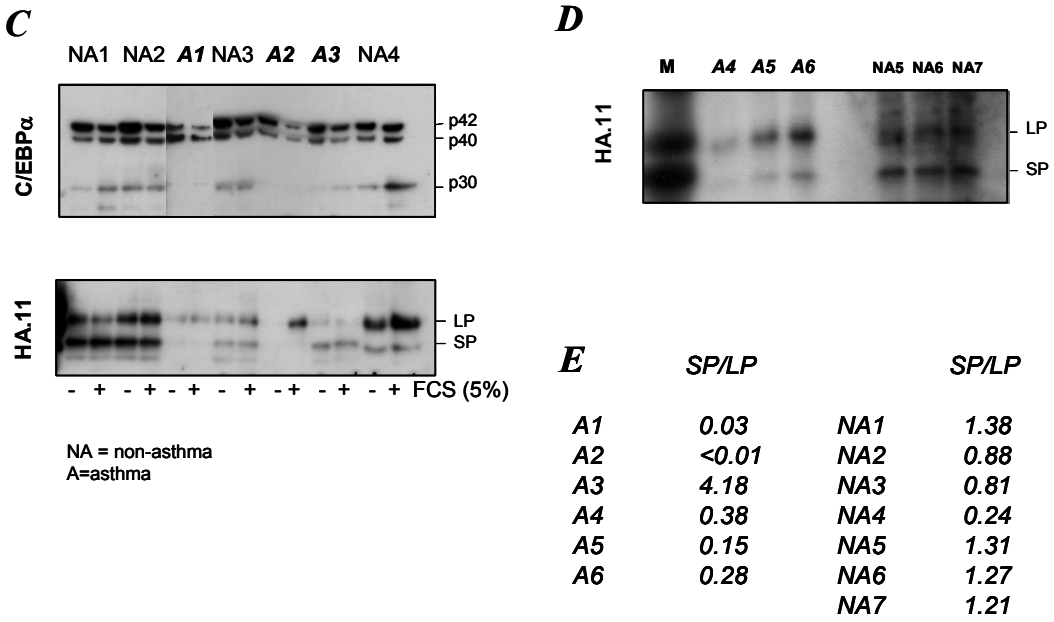


Figure 3. *Western analysis of impaired translation of C/EBPα in BSM cells of asthma patients*. **a.** Schematic representation of the Translation Control Reporter System (TCRS). For construct details see: Methods. **b.** Normal BSM cells transiently transfected with $TCRS^{WT}$, $TCRS^{\Delta uORF}$, $TCRS^{LP}$, and $TCRS^{SP}$. **c.** C/EBPα isoforms and SP/LP ratios expressed in $TCRS^{WT}$-transfected BSM cells. For comparison A1 required longer exposure (insert). **d.** SP/LP expression in $TCRS^{WT}$-transfected BSM cells (M=marker), and **e.** the corresponding ratios.

BSM cells of asthma patients exhibit lower levels of eIF-4E

Our data implied a deficiency with respect to the translation of normal levels of *CEBPA* mRNA and prompted us to investigate the signaling pathways involved in translation initiation of *CEBPA* mRNA. As demonstrated in figure 4A, BSM cells of asthma patients exhibited normal levels of 4EBP, calreticulin, and hnRNPE2, whereas they demonstrated diminished levels of eIF-4E ($n=3$; $p<0.05$). An siRNA specific for eIF4E was able to almost completely abrogate the *CEBPA* gene expression in proliferating BSM cells of both asthmatics and non asthmatics (5% FCS, 24 h) (figure 4B).

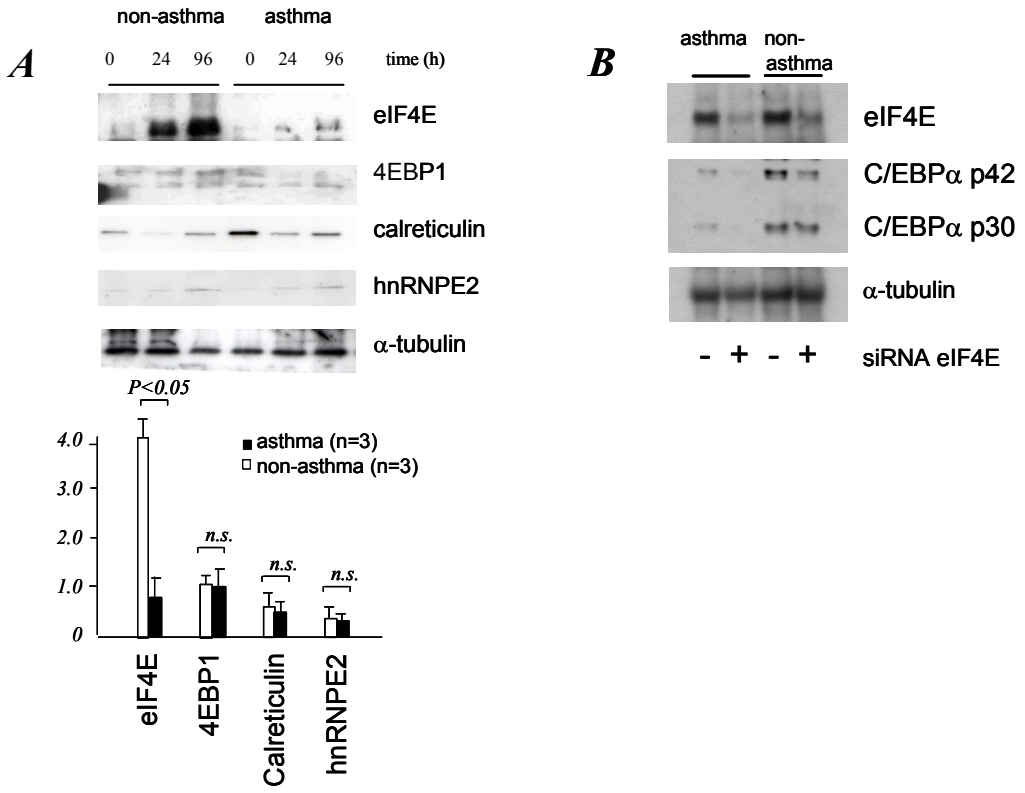


Figure 4. *Expression of translation control proteins in BSM cells*. a**.** Western analysis demonstrates significantly distinct expression patterns of eIF4EC/EBPα, but not of hnRNPE2, 4EBP1, and calreticulin. The data shown is typical of three independent experiments. Quantified protein levels are shown in the bar diagram. (n.s. = not significant). b**.** Down-regulation of eIF4E and C/EBPα in FCS stimulated BSM cells by eIF4E siRNA.

DISCUSSION

In our present study we provide further evidence that cultured BSM cells of asthma patients have abnormal proliferative characteristics. As reported earlier, BSM cells have low levels of the inhibitory protein C/EBPα (p42) and as a consequence they may be primed for an accelerated release of proinflammatory cytokines and growth factors that are regulated by C/EBP transcription factors. IL-6 secretion, which is controlled by CEBP transcription factors[14,15], provides an easy read-out for functional effects of inefficient translation of CEBPA mRNA. Since all C/EBP-isoforms compete with each other in promoter binding, it is the balance between the isoforms that regulates the ultimate effect.[4] We hypothesized that an altered expression of C/EBPα should be reflected in variable levels of secreted IL-6. IL-6 secretion time course experiments showed that in response to PDGF-BB the BSM cells of asthma patients immediately and significantly enhanced the secretion of IL-6 protein, whereas non-asthmatic cell lines reached the same level only after ≥24 hours of stimulation. Previously, we showed that impaired C/EBPα levels in asthmatic BSM cells did not affect end-stage IL-6 production levels after 48 hours of stimulation with FCS.[13] Here, we show that BSM cells of asthma patients were primed to rapidly secrete IL-6. This observation may be related to a diminished expression of C/EBPα in BSM cells of asthma patients. We surmise that in normal resting cells the promoter of the *IL6* gene is occupied by C/EBPα, keeping the gene in an inactive state. After the addition of FCS, C/EBPα is replaced by C/EBPβ which activates the gene in a similar way as has been described for the activation of the *COX2* gene.[16] If lower levels of C/EBPα are present, C/EBPβ may already occupy the promoter of the *IL6* gene and accelerate transcription.

Earlier, we reported that C/EBPα could not be detected in BSM cells of asthma patients[13] and that about one third of the BSM cell lines of asthma patients studied expressed low levels of distinct isoforms of C/EBPα.[9] Using a novel C/EBPα antibody (AVIVA), we now observed that

the detection of C/EBPα in BSM cells of asthma patients was dependent on the epitope recognized by the antibody. Antibodies that recognize an epitope of an internal region of C/EBPα (p42) of rat origin (Santacruz) were used in our previous studies [9,13], and did not detect human C/EBPα proteins (p42 and p30) in BSM cells of asthma patients. In contrast, antibodies raised against human C/EBPα (AVIVA) readily detected both C/EBPα (p42/40) and C/EBPα (p30) isoforms in protein extracts of both asthma and non-asthma BSM cells. In accordance with our previous reports, however, we found significantly lower levels of all C/EBPα isoforms (p42/40 and p30) in BSM cells obtained from asthma patients, which was further down-regulated after incubation with 5% FCS (Fig. 3.c). These findings suggest that the structure or shape of C/EBPα isoforms found in BSM cells of asthma patients may be different from that of control cells.

C/EBPα proteins are modular proteins, containing a C-terminal basic leucine-zipper domain required for dimerization and DNA binding in CCAAT-type *cis*-regulatory motifs in responsive genes, and N-terminal sequences involved in the trans-activation of genes and the regulation of the cell cycle.[17] C/EBPα can be expressed as full length proteins (p42/p40) with an inhibitory effect on cell proliferation, but also as a truncated (p30) polypeptide lacking the inhibitory function.[18-21] The C/EBPα (p42) and C/EBPα (p30) proteins arise from one unique *CEBPA* mRNA through different translation initiation sites. Enhanced expression of truncated C/EBPα isoforms caused abnormal proliferation characteristics in several cell types and the development of inflammatory disorders.[22] Full length C/EBPα (p42/p40) isoforms prevail in differentiating cells and reflect proliferation arrest. Truncated C/EBPα (p30) interfered with terminal differentiation and induced cell transformation [21-23], but did not have the anti-mitotic effect of full length C/EBPα.[21] The ratio of full length and truncated C/EBPα isoforms is strictly regulated by a specific class of *cis*-regulatory sequences in the *CEBPA* mRNA, known as

upstream open reading frames (uORFs). The uORF present in the 5' untranslated region (UTR) of the *CEBPA* mRNA senses subtle changes in the activity of the translation machinery as a response to intracellular signaling cascades activated by external stimuli, such as fluctuations in nutrients, growth factors, mitogens and factors that induce differentiation.[22] We hypothesize a deregulation of C/EBPα at this level occurs in BSM cells of asthma patients.

Moreover, in BSM cells of non-asthma patients the expression of full-length C/EBPα (p42/40) and reporter proteins was increased upon serum treatment, whereas in BSM cells of asthma patients serum treatment rapidly down-regulated the expression of both C/EBPα (p42/40) and reporter proteins. In addition, these asthma patient-derived cells were unable to properly translate C/EBPα (p30). The almost complete absence of p12 (SP) proteins from BSM cell lines of asthma patients confirmed an impaired translation in asthma patients only (Fig. 3.c (lower panel) and Figure 3.d). In many cell types, low levels of C/EBPα (p42/40) relative to C/EBPα (p30) (i.e. decreased p42/p30 ratio) were associated with hyperplasia and malignant transformation.[22] The *CEBPA* mRNA belongs to the group of 5'TOP mRNAs, which is translationally regulated by a 5'terminal oligopyrimidine motif [24], and the initiation of translation is the rate limiting step for the synthesis of protein.[25] Our observations in BSM cells of asthma patients could explain their increased proliferation rate in terms of dysfunctional translation control of *CEBPA* mRNA. Upstream signals for translation control of *CEBPA* mRNA can be provided by the hnRNPE2 [26], the mTOR-associated pathway [10] and calreticulin.[27] Wiesenthal and coworkers recently showed that the mTOR blocker rapamycin rapidly attenuated the expression of p12 (SP) from the $TCRS^{WT}$ and the data were interpreted as a failure to reinitiate translation.[10] Likewise, our data obtained in BSM cells of asthma patients suggest a failure to reinitiate the translation of *C/EBPA* mRNA, which would explain the attenuation of full length C/EBPα (p42/40) proteins and the complete lack of C/EBPα (p30).

Our data therefore suggest an important contribution of a disturbed balance of C/EBPα isoforms to airway remodeling and subsequently to the development and/or establishment of an asthmatic phenotype. Interestingly, rapamycin completely blocked the proliferation of lung fibroblasts and in a small set of BSM cells [28]. In addition, it has been shown that PI(3) kinase and mTOR activate p70S6K in airway myocytes leading to the accumulation of contractile apparatus proteins, differentiation, and growth of large, elongated contractile phenotype airway smooth muscle cells [29].

Finally, we found that the attenuated translation of C/EBPα in BSM cells of asthma patients was associated with diminished levels of eIF-4E, a rate-limiting initiation factor for *CEBPA* mRNA translation that is activated by mTOR via phosphorylation of 4EBP1. In contrast, the expression levels of calreticulin, 4EBP1 and hnRNPE2, proteins that have been reported to be involved in *CEBPA* mRNA translation, were not significantly affected. Currently it is unknown what initiates the disturbed translation control in BSM cells of asthmatic patients, but it may be related to exposure to and increased reactivity to allergens. Der p 1, the major allergen present in house dust mite extracts, elicits pro-asthmatic effects in isolated BSM cells of rat origin[30], and we recently observed that house dust mite allergens reduced C/EBPα protein levels exclusively in cultured human BSM cells obtained from asthma patients, but not in BSM cells of non-asthmatic controls.[31] In the light of the promising results obtained with bronchoscope-mediated thermoplastic reduction of the mass of BSM cells in the airway walls of asthma patients [1], our findings may open new ways to prevent BSM cells hyperplasia, and thus reduce or even cure asthma symptoms through modulation of the appropriate signals leading to balanced expression of full-length and truncated C/EBPα proteins. Considering our findings, less invasive strategies to counteract BSM remodeling should be envisaged.

Acknowledgement

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Chapter 3: House dust mite extract down-regulates C/EBPα protein in bronchial smooth muscle cells of asthma patients.

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Submitted

Abstract

Background: House dust mite allergens cause bronchoconstriction in asthma patients, induce an inflammatory response and airway remodeling. A reduced translation of CEBPA mRNA was associated with increased proliferation of bronchial smooth muscle cells of asthma patients

Objective: We sought to assess the effect of house dust mite extracts on the translational regulation of C/EBPα in isolated human bronchial smooth muscle cells of healthy controls (n=7) and asthmatic patients (n=6).

Methods: Bronchial smooth muscle cells were incubated (2, 24 or 48 hours) in the presence or absence of house dust mite extract (1 and 10 μg/ml) and the expression of C/EBPα, calreticulin and eIF4E were determined by immuno-blotting. Proliferation was determined by [3H]-thymidine incorporation and IL-6 release by ELISA.

Results: House dust mite extract significantly increased IL-6 protein, cell proliferation and reduced the C/EBPa expression in bronchial smooth muscle cells of asthma patients only ($p<0.05$). This effect coincided with significantly increased levels of calreticulin - an inhibitor of CEBPA mRNA translation. Furthermore, the effect of house dust mite extract was protease-dependent, could be mimicked by Protease Activated Receptor (PAR) 2 agonists, and was counteracted by blocking anti-PAR2 antibodies.

Conclusion: House dust mite extract reduced CEBPA mRNA translation in asthmatic bronchial smooth muscle cells and coincided with (i) up-regulated calreticulin, (ii) activated PAR2, (iii) increased IL-6 expression and (iv) proliferation of asthmatic bronchial smooth muscle cells. Hence, house dust mite exposure contributes to inflammation and remodeling by non-immune cell mediated mechanism via a direct interaction with bronchial smooth muscle cells.

Clinical implications

Hyperplasia of bronchial smooth muscle of asthmatic patients might result from a repressed translation of the *CEBPA* mRNA due to exposure to allergens. Novel intervention strategies to reduce hyperplasia should be considered.

Capsule Summary

C/EBPα is down-regulated specifically in BSM cells of asthma patients after exposure to HDM-extract. This may potentially explain several pathological features of this disease, in particular BSM cell hyperplasia.

Keywords:

Asthma, bronchial smooth muscle cells, C/EBP-alpha, house dust mite extract, mRNA translation

Abbreviations

BSM = bronchial smooth muscle

C/EBP = CCAAT/enhancer binding protein

CRT = calreticulin

ELISA = Enzyme Linked Immunosorbent Assay

HA = hemagglutinin

HDM = house dust mite

LP = long peptide

PAR = protease activated receptor

SMA = smooth muscle actin

SP = short peptide

TCRS = translation control reporter system

TOP = tract of oligopyrimidines

uORF = upstream open reading frame

INTRODUCTION

Although bronchial smooth muscle (BSM) hyperplasia is a prominent feature of airway remodeling in asthma and may be linked with the severity of asthma, its mechanism is still of unknown origin. Exposure to allergens during the early years of life leads to a persistent increase of BSM cells by an as yet unknown mechanism that was independent of the immune system.[1,2] Therefore, airway remodeling might not be a secondary event, but rather one of the fundamental pathological causes of asthma.[3-5]

In humans, house dust mite (HDM, *Dermatophagoides pteronissinus*) allergens are a major trigger of asthma exacerbation.[6, 7] HDM-extract modified the biology of airway structural cells by its proteolytic activity, thereby disrupting the integrity of the tight junctions between epithelium cells.[8] Once this barrier function of the epithelium is disturbed, as in asthmatic airways, allergens and other particles may easily find their way to deeper areas of the *lamina propria*.[9] Furthermore, the HDM allergen, Der p1 directly triggered a change in BSM cell responsiveness and activated the ERK1/2 mitogen-activated protein kinase (MAPK) signaling pathway.[10] In this context it should be noted that HDM-extract can elicit protease dependent and independent responses.[11] Together, these studies indicated a direct non immune-mediated effect of HDM allergens which induces or contributes to airway wall remodeling and inflammation in asthma.

In an asthma rhesus monkey model HDM-allergen exposure acts as a trigger for BSM cell sensitization.[12] Here HDM also induced airway remodeling in an early stage of life.[1] These observations fit well with the hypothesized role of BSM cells in embryonic lung development and its modification by inhaled allergens.[13] The studies were supported by clinical observations in difficult-to-control childhood asthma where BSM remodeling was always present, while inflammation was not.[4]

A persistent pathology of BSM cells in asthma is their low threshold towards mitogenic stimuli which is maintained *in vitro* and which was associated with decreased levels of C/EBPα and may also be linked to overactive mitochondria.[14-16] The lower C/EBPα protein levels in BSM cells of asthma patients resulted from impaired translation.[17] C/EBPα is a crucial controller of cell cycle progression and its protein expression is predominantly regulated by translation through 5'TOP by eIF4E and hnRNPE2 in humans.[17–19] Translation control might involve a GC-rich sequence in the *CEBPA* mRNA sequence which forms an internal stem loop and is the docking site for calreticulin which prevents translation.[20]

A deficiency in C/EBPα may also underlie reduced expression of α-smooth muscle actin (α-SMA) which is controlled by at least two C/EBP-isoforms, -β and -δ.[21,22] Only the expression of the full length C/EBPα maintains normal cell differentiation and function in other cell types.[23,24] Whether the deficient translation of *CEBPA* mRNA is an intrinsic characteristic of BSM cells in asthma or whether it can be acquired through exposure to external stimuli such as HDM-allergens was the major question addressed in this study.

METHODS

Tissue specimens & cell cultures

Lung tissue specimens were obtained from the Department of Internal Medicine, Pulmonology, University Hospital Basel, Switzerland and the Royal Prince Alfred Hospital (Sydney, Australia), with the approval of the local Ethical Committees and written consent of all patients. BSM cells were established as previously described[14] and grown in RPMI 1640 (Lonza, Basel; Switzerland) supplemented with 5% fetal calf serum (FCS, 8 mM L-glutamine, 20 mM HEPES, 1% MEM vitamin mix (Gibco, Paisley, UK). Neither antibiotics nor anti-mycotics were added at any time.

House dust mite (HDM) extracts

HDM-extract (gift from ALK-Abello, Hørsholm, Denmark) was prepared by dissolving the powder in RPMI-1640 medium at 20µg/ml and was sterile-filtered (0.22µm) (MN Sterilizer PES, Macherey-Nagel AG, Oensigen, Switzerland). Confluent cells were serum deprived for 24 hours and were then stimulated with 10µg/ml HDM-extract in the presence or absence of 5% FCS over 24 hours for protein expression.

Protein isolation and analysis by immuno-blot

Cellular protein was isolated from confluent cells by dissociation in lysis buffer [62,5 mM Tris-HCl (pH 6.8), 2% SDS, 2% β-mercaptoethanol, 10% gylcerol] and denaturation in sample buffer (3x Laemmli buffer with β-mercaptoethanol) and boiling for 5 min. Equal protein amounts were loaded onto a 4 – 12% PAGE-gel (Pierce Biotech, Thermo Fisher Scientific, Rockford, IL, USA) and were size fractionated by electrophoresis (1 hr, 100V, open Amp). The gel was sandwiched between two nitrocellulose membranes (Biorad, Reinach, Switzerland) and proteins were transferred [transfer buffer: 0.05 M NaCl, 2 mM Na-EDTA, 0.1 mM DTT, 10

mM, Tris HCl (pH 7.5)] overnight (50°C). Protein transfer and equal loading was confirmed by Ponceau's staining. The membranes were blocked (10min) in 3% bovine serum albumin (Roche, Rotkreuz, Switzerland) in 1x phosphate buffered saline with 0.05% Tween-20 (PBST). The membranes were incubated (1 hour) at room temperature (RT) with one of the antibodies to C/EBPα (AVIVA systems biology, San Diego, USA); or HA.11 (Covance, Berkley, USA); or calreticulin (Santa Cruz Biotech, Santa Cruz, USA); or α-smooth muscle cell actin (Signet Laboratories, Dedham, MA, USA);); or α-tubulin (Santa Cruz Biotech); or actin (Santa Cruz Biotech). Membranes were then washed (3x 5min) and incubated (1 hour, RT) with horseradish labeled species-specific antibodies (Santa Cruz Biotech). The membranes were washed (3x 5min) with and incubated (5min) with ECL-substrate (Pierce) and protein bands were visualized on x-ray films (Fuji Film, Medical x-ray film, Luzern, Switzerland). Protein bands were semi-quantified by an image analysis system (ImageJ) and protein expression was normalized to α-tubulin as internal control.[14]

Translation Control Reporter System (TCRS)

Cells were transiently transfected with TCRS in Tfx^{TM}-50 reagent Kit (Promega, Madison, USA). Cells (70% confluence) were incubated with 2.5µg/well TCRS construct for 1 hour (37°C). Then 5% FCS medium was added and the cells were incubated for 48 hours. Before experiments cells were kept 24 hours in serum-free medium. The ratio of the short protein (SP) to the long protein (LP) was determined by immuno-blot and analyzed by image analysis.[17]

Small inhibitory RNA (siRNA) treatment

Transfection with siRNA for calreticulin or negative control (Ambion, Austin, USA) was performed according manufacturer protocol. Cells (60% confluence) were plated into 6 well

plates and transiently transfected with siRNA (50nM) for 24 hours. Thereafter, fresh RPMI was added for 24 hours and the cells were collected for protein analysis.

Light microscopy

Cells were incubated 24 hours in HDM-extract (10µg/ml) and images were acquired with an Olympus IX50 microscope equipped with Cell^{P} image software.

Proliferation assay by [^{3}H]-thymidine incorporation

BSM cells were seeded in a 96 well plates (4,000 cells/well, 60% confluence) and allowed to adhere in growth medium over night before being serum deprived (24 hours) and stimulated with HDM-extract (1, 10 µg/ml) in the presence of 2 µCi/ml [^{3}H]-thymidine (Perkin Elmer, Boston, USA) at 37°C for 48 hours. After being washed with PBS and lysed in 0.1M NaOH, the DNA was collected onto glass fiber filters and cpm was counted in a Packard TOP COUNT NXT^{TM}.[14]

Cell viability and membrane integrity assay

The cytotoxic effect of HDM-extract (10µg/ml) was determined by membrane integrity assay after 24 hours in serum free medium. The release of lactate dehydrogenase (LDH) was determined according to the manufacturer's protocol (CytoTox-One^{TM}, Promega) and fluorescence was assessed at 560/590nm (Spectramax Gemini XS Microplate Spectrofluorometer, Molecular Devices Corporation, Sunnyvale, USA). Cytotoxicity was calculated as relative LDH increase compared to the untreated control cells.

IL-6 Enzyme-linked immunosorbent assay (ELISA)

Samples of cell culture medium were collected from sub-confluent BSM cells after stimulation (24 hrs) with HDM-extract (10 μg/ml) and IL-6 ELISA was performed according to the manufacturer's manual (R&D Systems, Abingdon, U.K.).

PAR agonists

PAR1 (SFLLRN) and PAR2 (SLIGKV) lyophilized agonist peptides (JPT, Berlin, Germany) were diluted in RPMI-1640 and added to cells (500μM), overnight (37°C). Cells were incubated (15min, RT) with 25 μg/ml anti-PAR2 antibody (Santa Cruz Biotech.) to block PAR2 agonists.

Statistics

Cytokine and proliferation data are presented as mean +/- SEM, immuno- blot analysis are shown as mean +/- SEM after densitometric image analysis (ImageJ software, National Institute of Mental Health, Bethesda, Maryland, USA.) of independent experiments. Paired/unpaired Student's t test was performed and P-values < 0.05 were considered significant.

RESULTS

HDM-extract dose-dependently induced the release of interleukin- 6 (IL-6) and increased the proliferation of BSM cells of asthma patients

BSM cells of asthma patients (n=5) and controls (n=5) were incubated with HDM-extract for 24 h in absence of FCS. HDM dose-dependently increased IL-6 release in both groups. Only in BSM cells of asthma patients a statistical significant increase of IL-6 ($p < 0.05$) was observed (Fig. 1A). A significantly increased dose-dependent proliferation in response to HDM-extract was observed only in BSM cells of asthma patients (Fig. 1B). As shown in Fig. 1C, HDM-extract did not induce LDH release, and therefore had no cytotoxic effect.

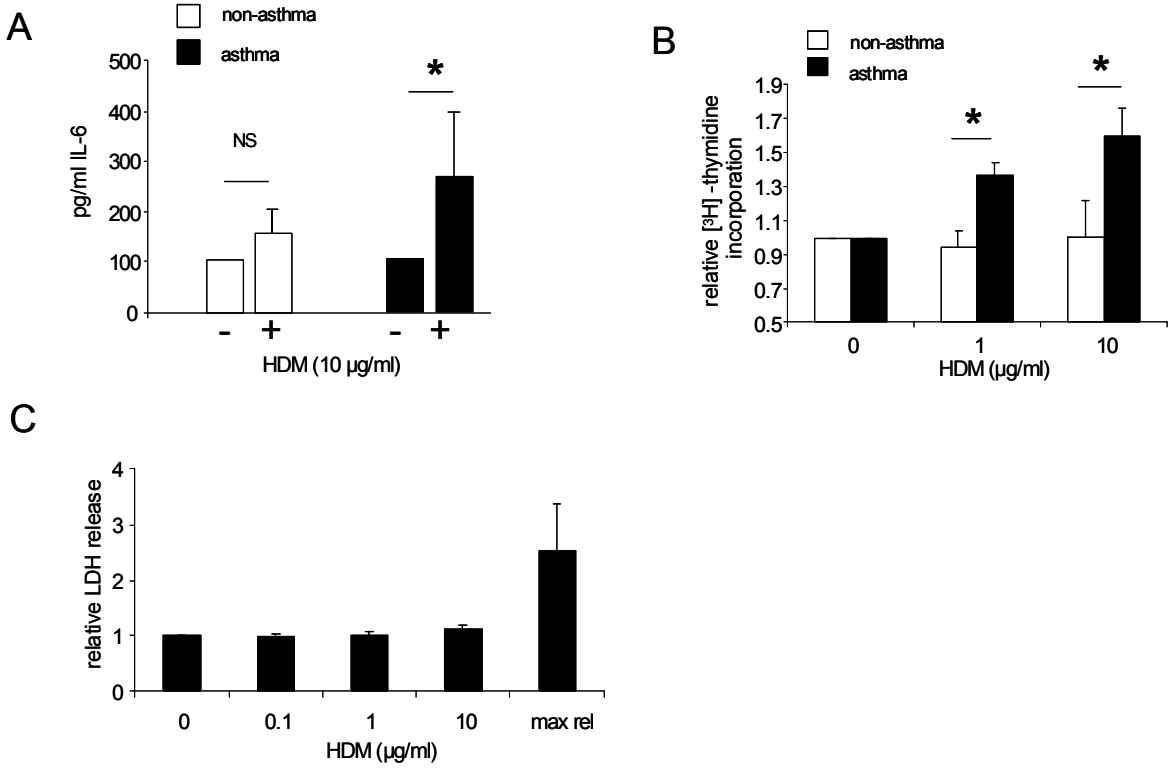

Figure 1. *Pro-inflammatory and proliferation characteristics of BSM cells after HDM-extract treatment.* **A.** HDM-induced IL-6 secretion after 24 hours (non-asthmatic n=5, asthmatic n=5), * $P < 0.05$. **B.** HDM-induced proliferation after 48 hours (non-asthmatic n=4, asthmatic n=4). *$P < 0.05$**. C.** HDM-induced LDH release after 24 hours (n=3).

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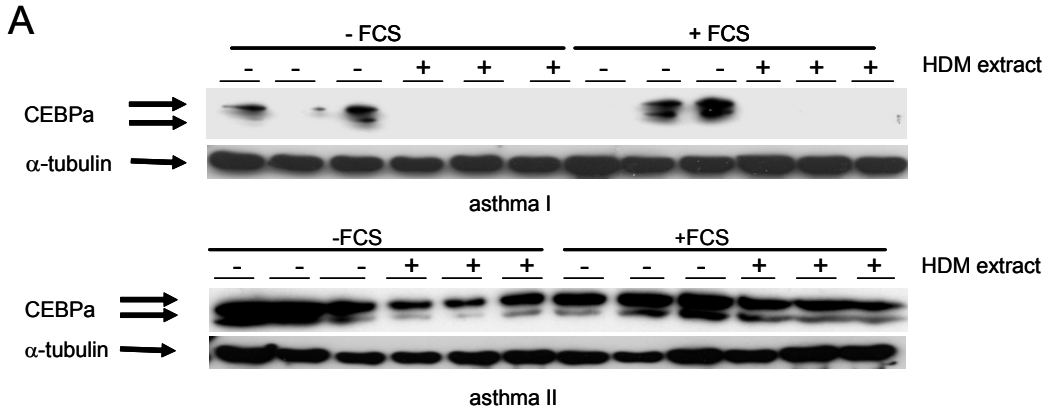
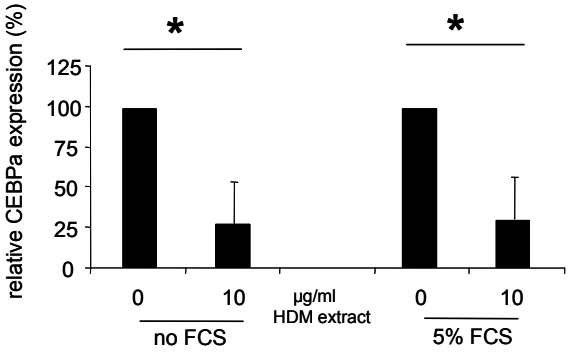
HDM-extract down-regulated C/EBPα expression in BSM cells of asthma patients

Independent of the presence of FCS, HDM-extract (10 µg/ml) significantly down-regulated the expression of C/EBPα in BSM cells of asthma patients, but not in cells of controls ($p < 0.05$, Fig 2A). Although the degree of the C/EBPα suppression was individual (range 50-100%), it was consistent in all experiments (n=6). Two representative immuno-blots are shown in figure 2A. In contrast, HDM-extract incubation of BSM cells from non-asthmatic controls (n=7) did not significantly affect the expression of C/EBPα (Fig. 2B). We used primary lung fibroblasts (n=2) to examine the cell specificity of the responses and these were incubated with HDM-extract (10 µg/ml) which did not have any effect on the expression of C/EBPα proteins (Fig. 2C).





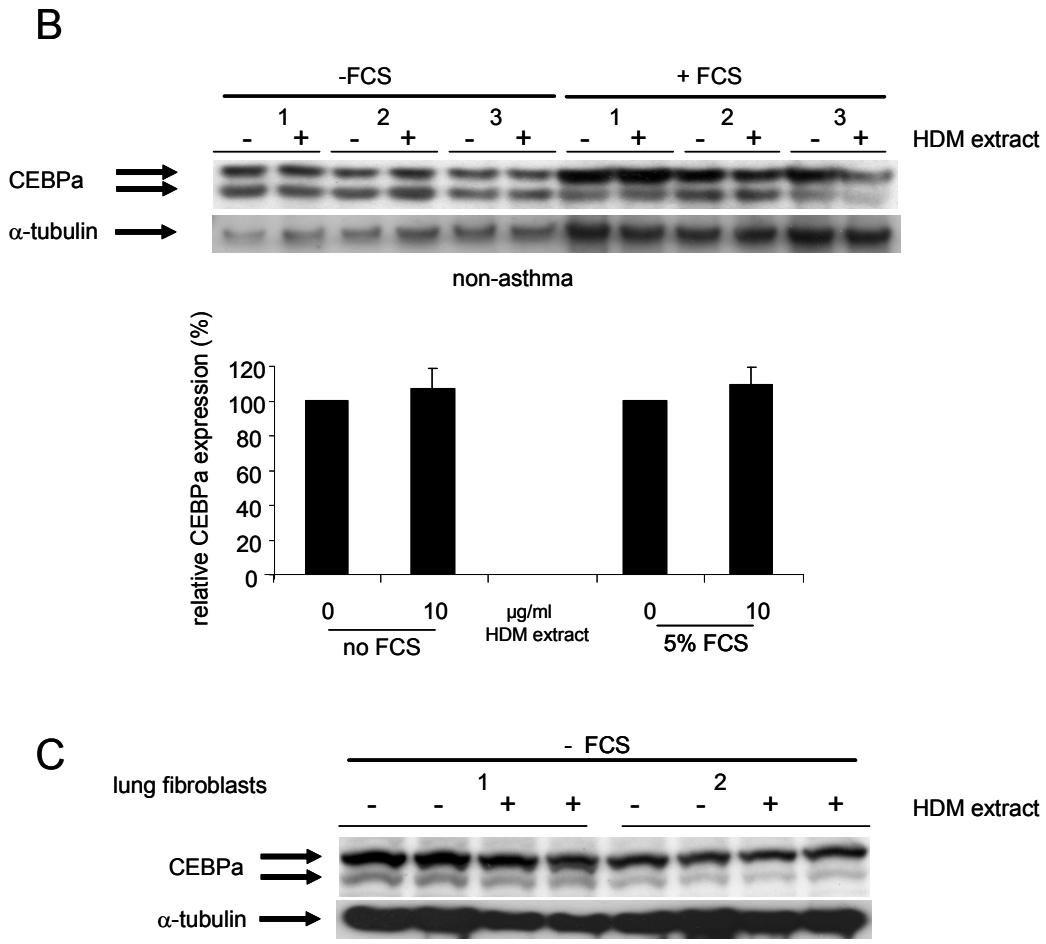


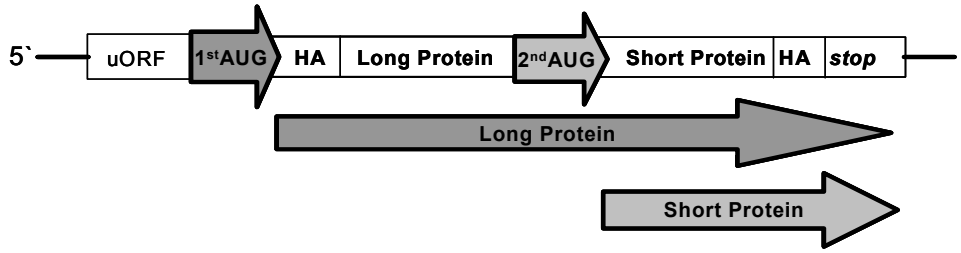
Figure 2. *HDM-extract effects on C/EBPα expression in asthma and control BSM cells.* **A.** The two most extreme protein expression patterns of C/EBPα (in triplicates). Bar-diagram: Densitometry of all asthma patients (n=6); * $p < 0.05$. **B.** C/EBPα expression in non-asthmatic controls (n=3). Bar-diagram: Densitometry of all non-asthma controls (n=7) **C.** C/EBPα expression in fibroblasts (n=2; in duplicates).

HDM-extract did not affect the re-initiation of 5'TOP mRNA translation and had no effect on eIFE4 expression

The re-initiation of 5'TOP mRNA *CEBPA* translation was monitored by TCRS, the principle of which is depicted in Fig. 3A. The construct generates a long (p23) and a short (p12) peptide of which the ratio (p12/p23) is a measure for translation reinitiation.[17] As shown in figure 3B, HDM-extract did not significantly change the SP/LP ratio indicating that the re-initiation of translation of 5'TOP mRNAs was not affected by HDM-extract. Translation of *CEBPA* mRNA is regulated by several proteins, including eIF4E, hnRNPE2 and calreticulin (Fig. 3C).

Consistent with the unchanged p12/p23 ratio the expression of eIF4E of asthmatic cells (n=3) was unaffected after incubation with HDM-extract (10µg/ml), indicating that a different mechanism controls translation (Fig. 3D).

A



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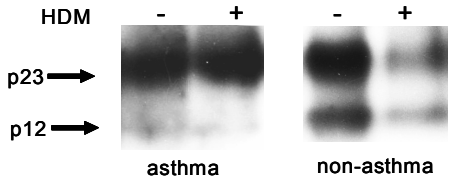

SP/LP ratio

	asthma control	asthma HDME 10		non-asthma control	non-asthma HDME 10	
A1	0.69	0.80	NA1	0.98	0.94	
A2	0.21	0.19	NA2	0.64	0.88	
A3	0.76	0.75	NA3	0.69	0.63	
Mean	**0.55**	**0.58**		**0.77**	**0.82**	
+/-SEM	**0.30**	**0.34**		**0.18**	**0.16**	**not significant**

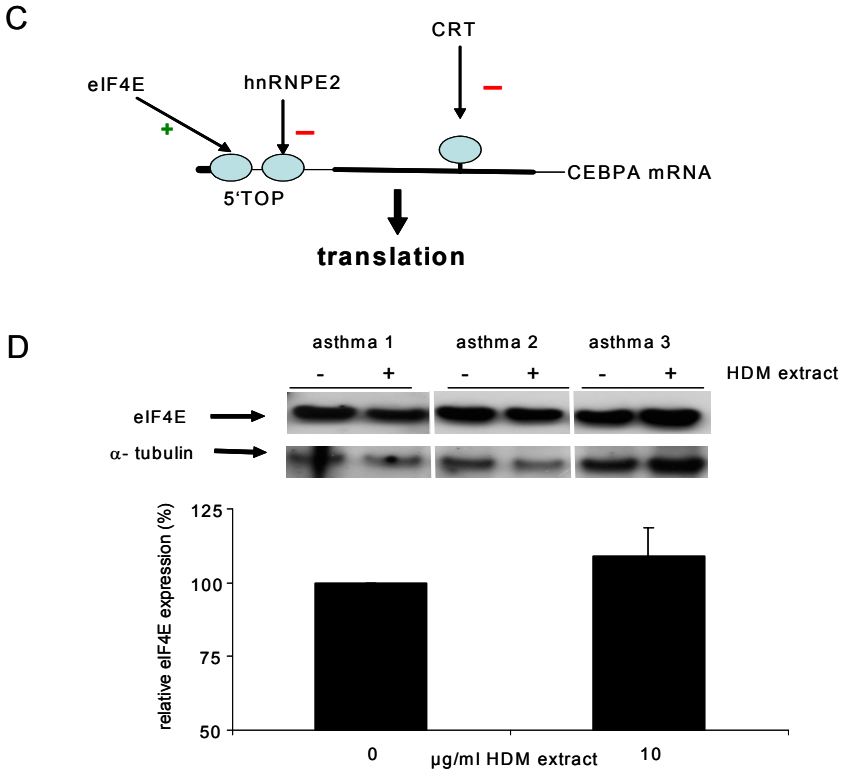

Figure 3. *Translation control of C/EBPα.* A. Schematic representation of the TCRS. For details see reference 17. B. Representative LP/SP expression pattern in asthma and non-asthma. Lower panel: SP/LP ratios (asthma n=3, non-asthma n=3). C. Scheme of regulatory proteins for translation control CEBPA mRNA. D. eIF4E expression in BSM cells of asthma patients (n=3) incubated 24 hours with HDM-extract (10µg/ml).

HDM-extract up-regulated the expression of calreticulin in BSM cells of asthma patients

HDM-extract (10µg/ml) significantly increased the relative expression of calreticulin in BSM cells of asthma patients (n=3) in a time dependent manner, but not in control cells (n=3). A 1.8-fold ($p < 0.05$) increase of calreticulin was detected after 2 hrs of incubation with HDM-extract in asthma BSM cells compared to time point 0 (Fig. 4A), whereas the calreticulin level in non-asthma control cells was not significantly affected (Fig. 4A). The specificity of this finding was assessed by incubating BSM cells of both groups with calreticulin specific siRNA, which revealed an inverse relationship between the expression of calreticulin and that of C/EBPα protein (Fig. 4B).

Figure 4 A

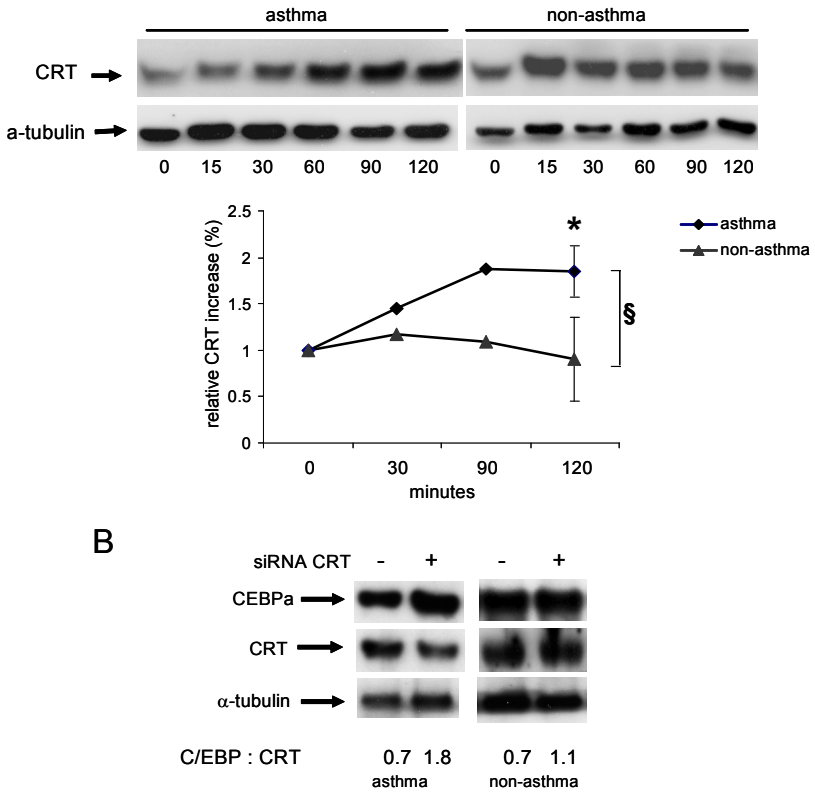

Figure 4. *Calreticulin expression in asthmatic and non-asthmatic BSM* cell. **A.** HDM-extract time-dependently and significantly induced CRT only in asthma BSM cells (n=3). * $P < 0.05$, asthma BSM cells 0 minutes vs 120 minutes. § $P < 0.05$, asthma vs non-asthma BSM cells at 120 minutes. **B.** C/EBPα in asthmatic patients and non-asthma after transfection with CRT siRNA and CEBPα/CRT ratios.

HDM-extract induced protease-dependent morphological changes in BSM cells

HDM-extract contains high levels of proteolytic activity and the effect on cell-desquamation and the involvement of protease activated receptor (PAR)-1 and PAR-2 was assessed. HDM-extract dose-dependently induced morphological changes in BSM cells within 24 hours (Fig. 5A). A partial detachment of BSM cells was observed in the presence of HDM-extract (10µg/ml), but this was insufficient to induce a complete cell desquamation. In the presence of 5% FCS, the BSM cell detachment was reversed, indicating the involvement of a protease-dependent mechanism. Therefore, the effect of HDM-extract, PAR1-, and PAR2-agonists on the expression of C/EBPα protein was assessed in BSM cells of asthmatics and controls (n=3). In BSM cells of

asthma patients the expression of C/EBPα protein in response to 24 hours treatment with HDM-extract or to a PAR2 agonist was dramatically reduced, whereas the PAR1 agonist had no effect (Fig. 5B). The involvement of PAR2 in the HDM-extract-induced down regulation of C/EBPα was completely reversed by a specific PAR2 blocking antibody (Fig. 5B).

Figure 5C demonstrates that HDM-extract specifically down-regulated α-smooth muscle actin (α-SMA) in two cell lines obtained from asthma patients and this result coincided with the above described diminished levels of C/EBPα.

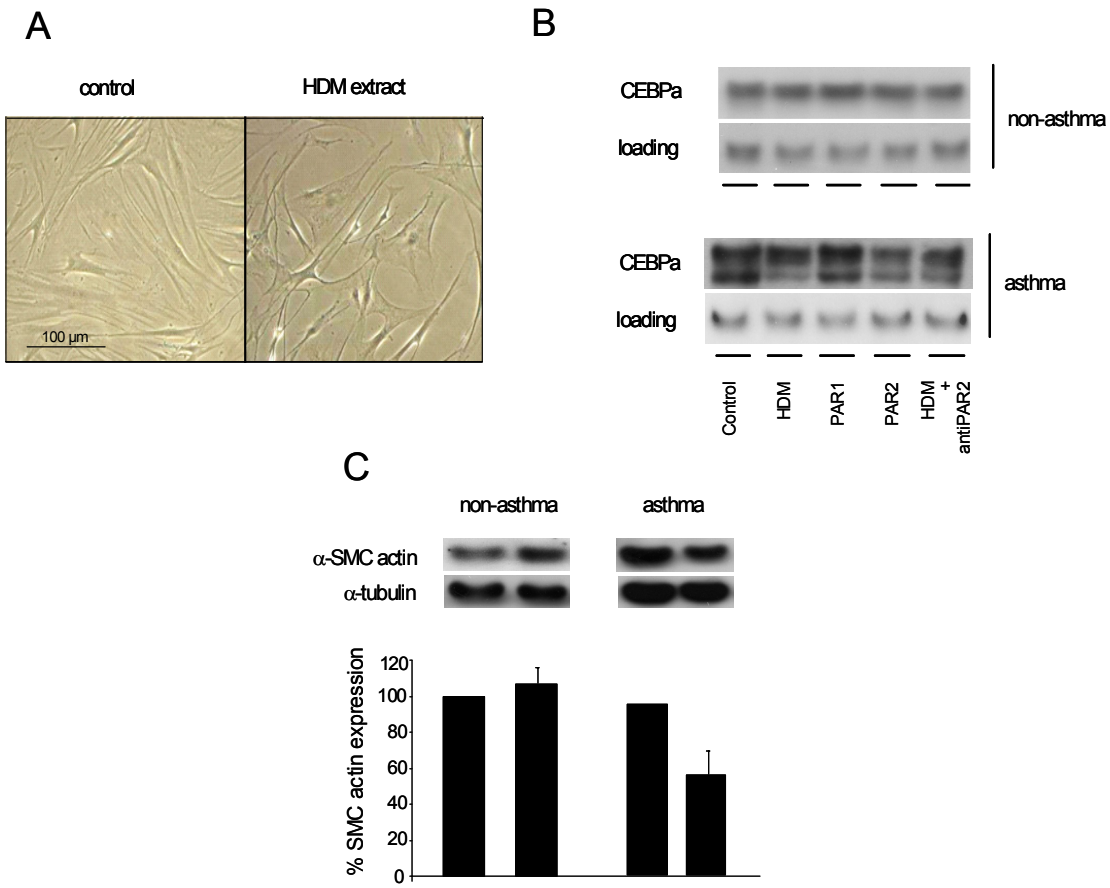

Figure 5. *Protease-dependent and –independent effects of HDM* **A.** HDM-extract-induced detachment of BSM cells (scale bar: 100 µm)**. B.** Effects of HDM, PAR1 and PAR2 agonists, and PAR2 blocking antibody on C/EBPα in asthma and non-asthma. **C.** α-Smooth muscle cell actin expression in BSM cells of a non-asthmatic control and an asthma patient (HDM-treated, 24 hours). Bar-diagram: Densitometry, n=2.

DISCUSSION

In this study, we showed that HDM-extract down-regulated C/EBPα protein expression in BSM cells of asthma patients only. The down-regulation was mediated via two mechanisms, which were independent of the immune system. The first mechanism was mediated via calreticulin, the second involved PAR2. In addition to its disease specificity the BSM cell response to HDM-extract was also cell-type specific, because lung fibroblasts did not respond.

These findings further substantiate the importance of C/EBPα translation in asthma pathology and how BSM cells hyperplasia may be triggered by external stimuli, in particular by HDM. Together with our previous findings, which showed diminished C/EBPα protein levels associated with increased proliferative capacity of BSM cell of asthma patients, this may explain an increased bulk of smooth muscle cells as found in the airways of asthma patients. [15,17, 25] In addition, in response to HDM-extract BSM cells of asthma patients showed an increased proliferation and IL-6 secretion. Thus HDM has the capacity to elicit an inflammatory response and induce airway remodeling as the result of a direct action on resident cells of the lung – independent of the immunological compartment. Of course, we realize that an HDM-IgE driven immune response is of great importance to understand atopic asthma, but it should also be realized that not all asthma is associated with IgE – in particular intrinsic asthma.[26]

Previously, we found that an impaired initiation of the translation of *CEBPA* mRNA in BSM cells of asthma patients was associated with the decreased expression of the translation regulator eIF4E.[17] Here, we could not detect an impaired translation using the translation reporter construct, [27,28] indicating that a different mechanism may be involved. We proposed that calreticulin, a protein initially identified as an endoplasmatic reticulum luminal chaperone that controls the regulation of intracellular Ca^{2+} homeostasis[29], could be pivotal in the HDM-induced down-regulation of C/EBPα. It was recently shown that binding of calreticulin inhibited the

translation of the *CEBPA* mRNA, as a result of a direct interaction of calreticulin and the *CEBPA* transcript. Calreticulin was shown to bind the stem loop within the *CEBPA* mRNA, which is formed by internal base-pairing of the GCN-repeat motif.[20] This loop then functions as the docking site for calreticulin and prevents the translation of CEBPA mRNA into protein. An inverse relationship of C/EBPα and calreticulin had been demonstrated in adipocytes where calreticulin promoted adipogenesis by repressing the expression of C/EBPα[30], an observation that was also reported in acute myeloid leukemia.[31] Here we demonstrated that the same mechanism operates in BSM cells of asthma patients as transient suppression of calreticulin by siRNA restored C/EBPα levels. Therefore, we propose that the decrease of the C/EBPα protein level in BSM cells of asthma patients is due to HDM-extract-induced sequestration of the corresponding mRNA by calreticulin.

HDM allergens are omnipresent and by far the most important indoor IgE-triggering compound[7] and have a potent pro-inflammatory and desquamating effect on airway epithelial cells.[11] Here, we also provide evidence that HDM-extract affected the behavior of BSM cells of asthma patients by down-regulating *CEBP*A gene expression, which involved a mechanism using PAR2. Our data show that a PAR2 agonist decreased C/EBPα expression in BSM cells of asthma patients, but not in controls. Hindering the access of HDM allergens to PAR2 by blocking antibodies counteracted this down-regulation of C/EBP α . In line with this finding the HDM allergen Der p1 and Der p5 activated human airway-derived epithelial cells by a protease-dependent and -independent mechanism.[11] In our present study, similar protease-dependent and -independent mechanisms may be involved, because in the presence of FCS, which is a potent protease activity blocker, HDM allergens still partially down-regulated C/EBPα proteins. This suggested that the pathway involving calreticulin is independent of the activation of PARs.

Taken together these results indicate that the HDM-induced down-regulation of CEBPα is specific for BSM cells of asthma patients and involves PAR-dependent and independent mechanisms – the latter mechanism involving an induction of calreticulin.

The down-regulation of C/EBPα in BSM cells by HDM-extract may be a first indication that there is a link between the pathologies in atopic and non-atopic asthma. An integrated schematic overview of our ideas on protease dependent and independent mechanisms and how they are involved in airway wall remodeling is shown in figure 6. In the light of our present findings new C/EBPα tailored asthma therapies that directly target the resident cells of the airway wall could be envisaged.

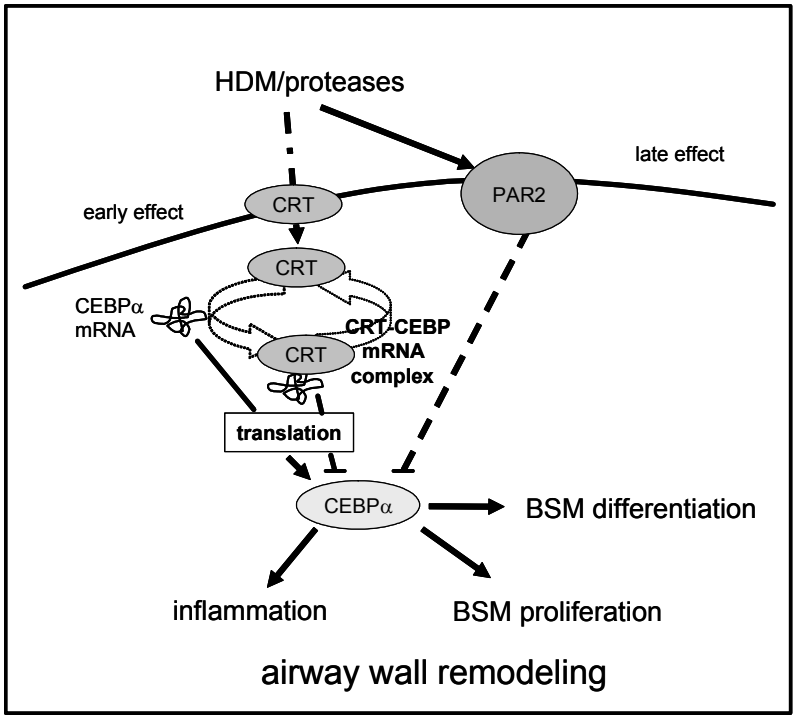

Figure 6. *Schematic overview over putative mechanisms involved in C/EBPα expression after stimulation of BSM cells with HDM allergens.*

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Chapter 4: Cigarette smoke modulates the translation of *CEBPA* and *CEBPB* mRNA in primary lung fibroblasts: is COPD a translation control disease?

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Submitted

Abstract

Rationale: Cigarette smoke is the major cause of COPD and emphysema. Although, cigarette smoke represses proliferation and induces cellular senescence in several cell types, the molecular mechanisms underlying the diseases are unknown. As decisive regulators of cell cycle progression, proliferation and pivotal transcription factors for pro-inflammatory genes, an imbalance of the translation controlled C/EBP proteins may be involved.

Objectives: We assessed the effect of cigarette smoke on proliferation, inflammatory parameters, and on the expression and translational regulation of C/EBPα and C/EBPβ in primary human lung fibroblasts (n=6).

Methods: Primary human lung fibroblasts were exposed to cigarette smoke (10% and 20%, 24 hours) in absence and presence of 5% FCS. Proliferation was determined by [^{3}H]-thymidine incorporation and IL-8 release by ELISA. C/EBPα, C/EBPβ, calreticulin and hnRNP-2E were determined by immuno-blotting. Translation was monitored by a Translation Control Reporter System (TCRS).

Results: Cigarette smoke significantly reduced the proliferation of fibroblasts and induced IL-8 secretion in the absence and presence of FCS. In serum-free cells, cigarette smoke significantly up-regulated C/EBPα and C/EBPβ expression, whereas in the presence of FCS C/EBPα and C/EBPβ expression were down-regulated. Both effects were translationally controlled but involved distinct mechanisms. In absence of FCS, the mechanism is a re-initiation of the mRNA translation, whereas in presence of FCS hnRNP-E2 was induced.

Conclusions: Cigarette smoke inhibited proliferation of fibroblasts and induced IL-8 release, which may be attributed to a differentially translated C/EBPα and C/EBPβ mRNA expression. As a consequence, tissue turn-over may be disturbed and pro-inflammatory genes may become activated.

At a glance commentary:

Scientific knowledge on the subject: Altered tissue structure is a key factor in COPD and emphysema.

What this study adds to the field:

Cigarette smoke reduces the proliferation of human primary lung fibroblasts and initiates an inflammatory responses which are both associated with impaired translation of *CEBPA* and *CEBPB* mRNA. These findings may provide novel strategies for therapeutic interventions.

Abbreviations

C/EBP = CCAAT/enhancer binding protein

CRT = calreticulin

ELISA = Enzyme Linked Immunosorbent Assay

HA = hemagglutinin

LDH = lactate dehydrogenase

LP = long peptide

SCM = smoke conditioned medium

SP = short peptide

TCRS = translation control reporter system

TOP = tract of oligopyrimidines

uORF = upstream open reading frame

Keywords:

COPD, emphysema, human lung fibroblast, C/EBP-alpha, C/EBP-beta, smoke, mRNA translation control

Introduction

Chronic obstructive pulmonary disease (COPD) is characterized by chronic inflammation of the lung and airway remodeling leading to a fixed narrowing of the small airways and destruction of the alveolar wall (emphysema) (1). COPD is a global health problem that affects more than 10% of the world population over the age of 40 years (2) and it will become the 5th cause of morbidity by 2020 (3, 4). Cigarette smoke is the most important risk factor for the development of COPD (5). The most widely accepted hypothesis to explain the pathology of COPD and emphysema is an imbalance of proteases and their inhibitors which leads to the degradation of elastin and other structural components of the lung tissue (6, 7). Alternatively, the inflammation and the loss of tissue in end-stage COPD (emphysema) may derive from an imbalance of tissue turnover when proliferation is unable to compensate for tissue loss.

Lung fibroblasts maintain the integrity of the lung parenchyma as they contribute to the repair of lung injuries through synthesis and secretion of the main components of the extracellular matrix such as proteoglycans and collagens. Importantly, lung fibroblasts also produce elastin, an essential component of the alveolar extracellular matrix that provides the lung tissue with elasticity (8). An impaired capacity of lung fibroblasts to execute tissue repair has been found in COPD patients (9). Cigarette smoke inhibits proliferation of normal fibroblasts (10, 11), induces cellular senescence (12) and inhibits alveolar repair (13). In the context of tissue maintenance and cell proliferation, CCAAT/enhancer binding proteins (C/EBP) could be of interest. C/EBPs have both stimulatory and inhibitory effects on the proliferation of many cell types and appear to be crucial for the regulation of inflammatory responses. C/EBPs are an important family of transcription factors that regulates cell differentiation, cell cycle progression and the expression of many cytokines and chemokines relevant to COPD and emphysema (14, 15, 16). In addition, CCAAT/enhancer binding protein (C/EBP)β was shown to inhibit the transcription of elastin (17) and it was elevated in emphysema lungs (18). In airway smooth muscle (ASM) cells of

asthma patients, the expression of C/EBPα was reduced, due to an impaired translation of the mRNA which associated with a faster proliferation rate (19, 20) and may be related to the hyperplasia of ASM cells in the lungs of asthma patients (21). Here we show that the exposure of lung fibroblasts to cigarette smoke alters the expression of C/EBPα and C/EBPβ, which may disturb tissue homeostasis and increase the inflammatory response to external stimuli.

Methods

Tissue specimens & cell cultures

Lung tissue specimens were obtained from the Departments of Thoracic Surgery and of Internal Medicine, Pneumology, University Hospital Basel, Switzerland with the approval of the local Ethical Committees and written consent of all patients. Fibroblast cells were established from small sections of lung parenchymal tissue and grown in RPMI 1640 (Lonza, Basel; Switzerland) supplemented with 5% fetal calf serum (FCS, 8 mM L-glutamine, 20 mM HEPES, 1% MEM vitamin mix (Gibco, Paisley, UK).

Cigarette Smoke conditioned medium (SCM)

SCM was prepared by leading cigarette smoke equivalent of 1 cigarette from a commercially available brand (Gauloises Blondes, Altadis, Switzerland) through 25 ml of RPMI medium. This cigarette smoke concentration was defined as 100%. The generated SCM was sterile-filtered to remove larger particles (0.22µm syringe filter) (MN Sterilizer PES, Macherey-Nagel AG, Oensigen, Switzerland). The chemical analysis of the SCM was performed by Gas Chromatography/Mass Spectroscopy full scan and the nicotine content was determined for standardization purposes (EMPA, Dübendorf, Switzerland).

Protein isolation and analysis by immuno-blot

Total cellular protein extracts were isolated using a crude cell lysis buffer [62,5 mM Tris-HCl (pH 6.8), 2% SDS, 2% β-mercaptoethanol, 10% gylcerol] and proteins were denatured in sample buffer (3x Laemmli buffer; 98°C, 5 min). Equal protein amounts were loaded onto a 4–12% PAGE-gel (Pierce Biotech, Thermo Fisher Scientific, Rockford, IL, USA) and were size fractionated by electrophoresis (1 hr, 100V, open Amp). The gel was sandwiched between two nitrocellulose membranes (Biorad, Reinach, Switzerland) and the proteins were transferred

[transfer buffer: 0.05 M NaCl, 2 mM Na-EDTA, 0.1 mM DTT, 10 mM, Tris HCl (pH 7.5)] overnight (50°C). Protein transfer and equal loading was confirmed by Ponceau's staining. The membranes were blocked (10min) in 3% bovine serum albumin (Roche, Rotkreuz, Switzerland) in 1x phosphate buffered saline with 0.05% Tween-20 (PBST). Membranes were incubated (1 hour) at room temperature (RT) with one of the primary antibodies to C/EBPα (AVIVA, San Diego, USA); or C/EBPβ (AVIVA); or HA.11 (Covance, Berkley, USA); or calreticulin (Santa Cruz Biotech, Santa Cruz, USA); or hnRNP E2 (Santa Cruz Biotech); or β-tubulin (Santa Cruz Biotech). Membranes were then washed (3x 5min) and incubated (1 hour, RT) with horseradish labeled species specific antibodies (Santa Cruz Biotech). The membranes were washed with PBST (3x 5 min), incubated (5 min) with ECL-substrate (Pierce), and protein bands were visualized on X-ray films (Fuji Film, Medical x-ray film, Luzern, Switzerland). Protein bands were quantified by an image analysis system (ImageJ) and protein expression was normalized to α-tubulin (22).

Translation Control Reporter System (TCRS)

Cells were transiently transfected with TCRS (gift from Dr Calkhoven, Jena, Germany) in Tfx^{TM}-50 reagent Kit (Promega, Madison, USA). Cells (70% confluence) were incubated with 2.5 µg/well TCRS construct for 1 hour (37°C). Then medium containing 5% FCS was added and the cells were incubated for 48 hours. Before further experiments, the cells were kept for 24 hours in starving conditions (0.1% FCS). The ratio of the short protein (SP) to the long protein (LP) was determined by immuno-blot and analyzed by image analysis as previously described (20).

Proliferation assay by [^{3}H]-thymidine incorporation

Fibroblasts were seeded in a 96 well plates (4,000 cells/well, 60% confluence) and allowed to adhere in growth medium over night before being serum deprived (24 hours) and stimulated with SCM (0, 10, 20, 30, 40%) in the presence of 2 μCi/ml [^{3}H]-thymidine (Perkin Elmer, Boston, USA) at 37°C for 24 hours. After being washed with PBS and lysed in 0.1 M NaOH, the DNA was collected onto glass fiber filters and cpm was counted in a Packard TOP COUNT NXT™ (22).

Cell viability and membrane integrity assay

The cytotoxic effect of SCM was determined by membrane integrity assay after 24 hours in serum free medium. The release of lactate dehydrogenase (LDH) was determined according to the manufacturer's protocol (CytoTox-One™, Promega, Madison; WI, USA) and fluorescence was assessed at 560/590nm (Spectramax Gemini XS Microplate Spectrofluorometer, Molecular Devices Corporation, Sunnyvale, USA). Cytotoxicity was calculated as LDH increase relative to the untreated control cells.

IL-8 Enzyme-linked immunosorbent assay (ELISA)

Samples of cell culture medium were collected from sub-confluent fibroblasts after incubation (24 hrs) with SCM (10%) and IL-8 ELISA was performed according to the manufacturer's manual (Anibiotech Orgenium Laboratories, Vantaa, Finland).

Statistics

Cytokine and proliferation data are presented as mean +/- SD, immuno-blot analyses are shown as mean +/- SD after densitometric image analysis (ImageJ software, National Institute of Mental Health, Bethesda, Maryland, USA.) of at least three independent experiments in cell

lines of different subjects. Paired/unpaired Student's t test was performed and P-values < 0.05 were considered significant.

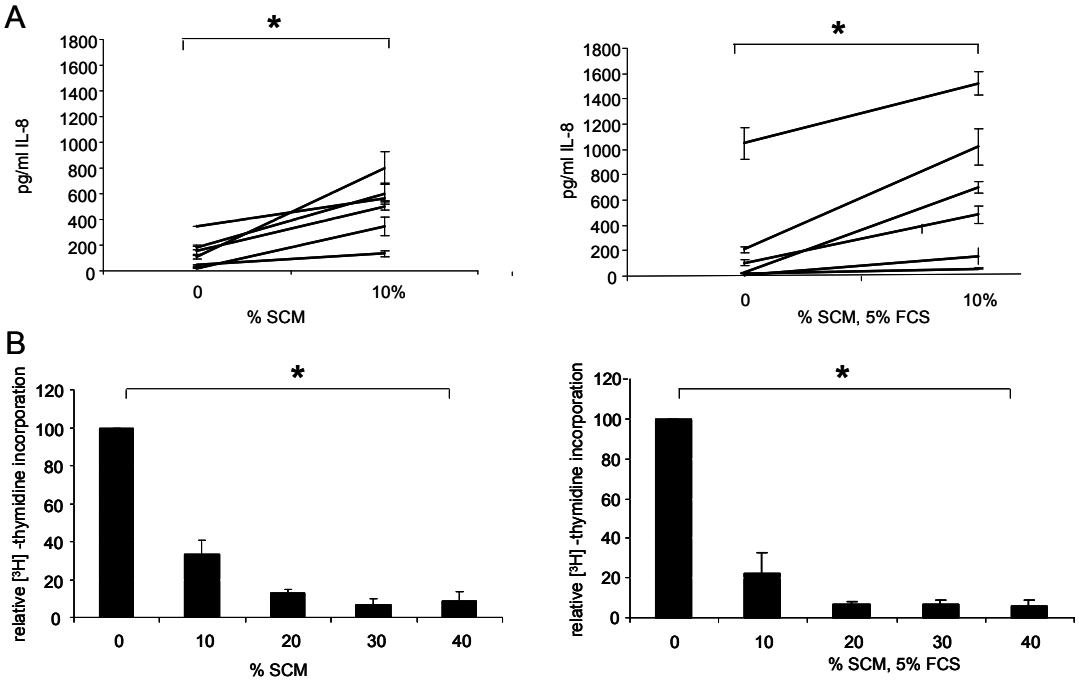
Results

SCM induced the release of interleukin-8 (IL-8) and decreased the proliferation of primary lung fibroblasts.

In order to asses the effect of SCM on the inflammatory response of primary lung fibroblasts, cells were incubated for 24h in absence or presence of FCS. SCM (10%) significantly increased IL-8 release, both in the presence or absence of FCS (Fig. 1A). Used at concentrations >10 % IL-8 release started to decline (data not shown).

To asses the effect of SCM on the proliferation capacity of primary lung fibroblast, cells were incubated for 24 h in absence or presence of FCS. As shown in figure 1B, SCM dose-dependently reduced the proliferation and the inhibitory effect was significant at all measured concentrations ($p < 0.05$, n=3).

As shown in Fig. 1C, SCM did not induce LDH release in fibroblasts within 24 hours, demonstrating that SCM does not have significant cytotoxic effects in the concentration range tested (10-20%)



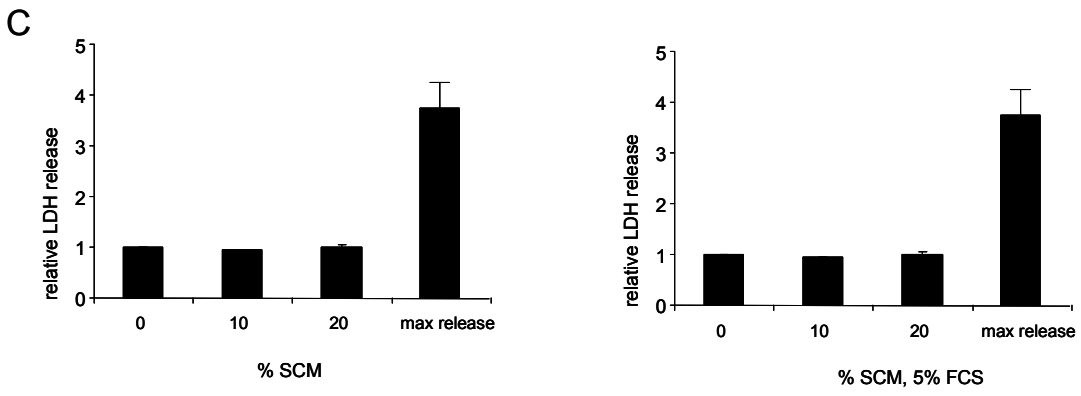


Figure 1: *Pro-inflammatory and proliferation characteristics of primary lung fibroblasts exposed to smoke conditioned medium (SCM).* **A.** SCM (10%) significantly increased IL-8 secretion after 24 hours in primary lung fibroblasts in both absence and presence of FCS (n=6), * $P < 0.05$. **B.** SCM in (dilution range from 10% to 40%) significantly inhibited cell proliferation after 24 hours in primary lung fibroblasts in both absence and presence of FCS (n=3), *$P < 0.05$**. C.** LHD release after 24 hours incubation with SCM (n=4)

Differential effects of SCM on C/EBPα and CEBPβ expression in primary lung fibroblasts depended on the presence of FCS

In the absence of FCS, SCM (20%) significantly induced the expression of C/EBPα ($p < 0.05$, n=6, Fig. 2A). In the presence of FCS, SCM decreased the expression of C/EBPα, reaching statistically significance at concentrations of 20% ($p < 0.05$, n=6, Fig 2A).

As observed for C/EBPα, SCM (20%) significantly induced the expression of C/EBPβ in absence of FCS ($p < 0.05$, n=6, Fig. 2B). In the presence of FCS, SCM (10% and 20%) significantly down-regulated the expression C/EBPβ ($p < 0.05$, n=6, Fig 2B).

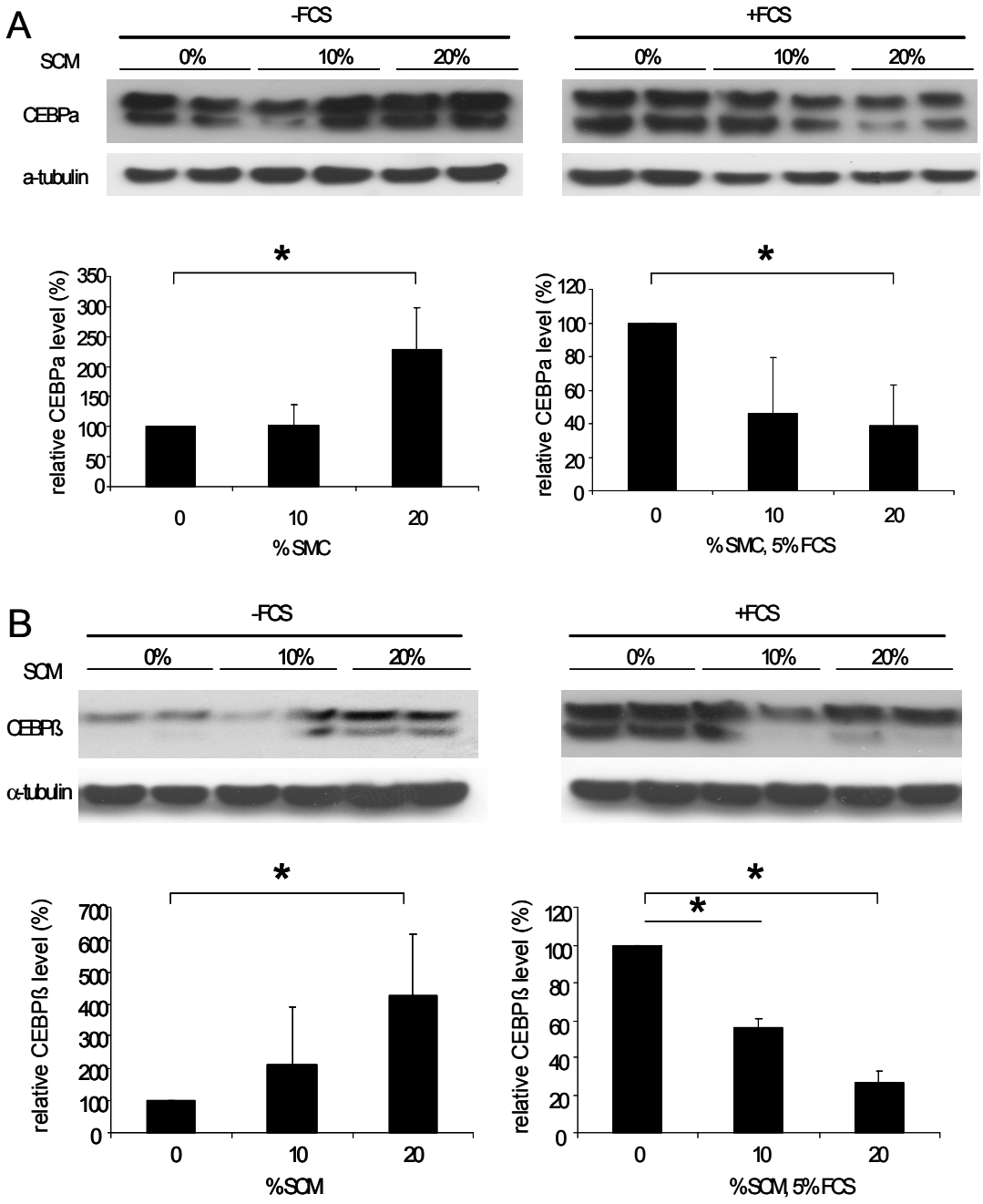
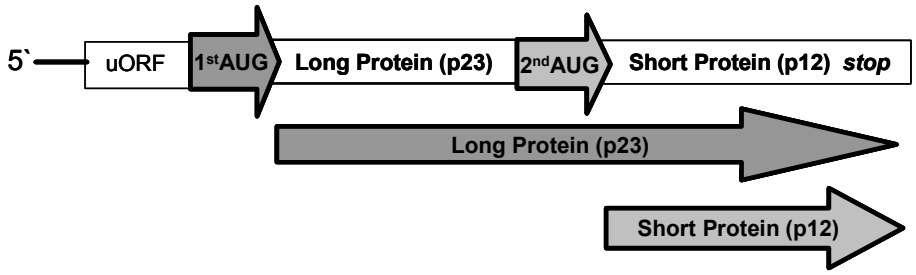


Figure 2:*Western blot analysis of C/EBPα and C/EBPβ expression after exposure to SCM in absence or presence of FCS.* **A.** Representative western blots of the C/EBPα expression in a lung fibroblast cell line incubated for 24 hours in SCM (10% and 20%). Bar diagram = densitometric analysis (n=6); * $P < 0.05$. **B.** Representative western blots of the C/EBPβ expression in a lung fibroblast cell line incubated for 24 hours in SCM (10% and 20%). Bar diagram = densitometric analysis (n=6); * $P < 0.05$.

In the absence of FCS, SCM modulated the translation re-initiation of C/EBPA and C/EBPB mRNAs via the upstream open reading frame (uORF)

Previously we demonstrated that *CEBPA* gene expression is predominantly regulated at the translational level (20). Here we used the same translation control reporter system (TCRS) to detect changes with respect to translational regulation. The principle of the TCRs is depicted in Fig. 3A. The construct generates a long (p23) and a short (p12) peptide with a hemagglutinin epitope (HA) of which the ratio (p12/p23) is a measure of the translation re-initiation efficiency (28, 29). In absence of FCS, SCM significantly decreased the SP/LP ratio ($p < 0.05$; figure 3B abd 3C), indicating that the re-initiation of translation of 5'TOP mRNAs was shifted to the full-length form of the protein. This coincided with a shift of the CEBPβ expression from the truncated to the full-length isoform in the corresponding fibroblast cell line shown in figure 3D, and confirms that SCM controlled the translation of *CEBP* mRNAs via the uORF mechanism. In contrast, in the presence of FCS the p12/p23 ratio was unaffected and demonstrated that the uORF did not play a role. This indicated that a distinct mechanism may be responsible for the SCM-mediated effects on the expression of CEBP proteins (Fig. 3B and 3C).

A



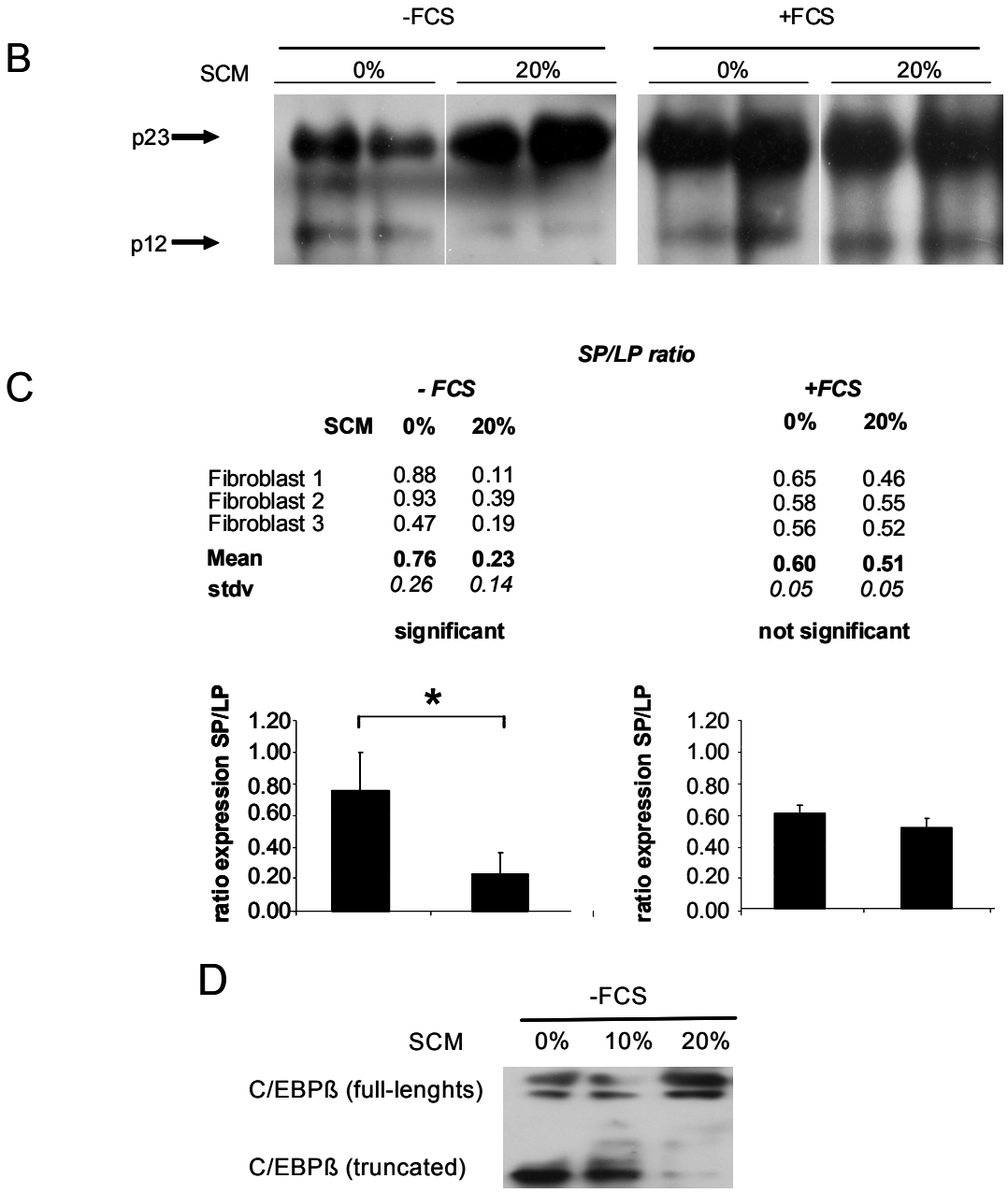
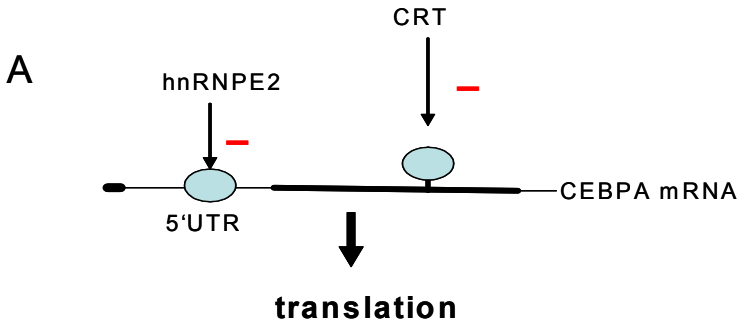


Figure 3: *Western blot analysis of translation control of C/EBPα and C/EBPβ***. A.** Schematic representation of the translation control reporter system (TCRS). Impaired translation leads to lower reinitiation at the start codon 2 and as a consequence to decreased expression of the short peptide (uORF: regulatory upstream open reading frame, HA: hemagglutinin epitope)**. B.** Representative western blot of the expression of the small (p12) and long peptide (p23) in a primary lung fibroblast exposed to SCM (20%). The figure shows duplicates of two independent experiments **C. C**alculated p12/p23 ratios of three different fibroblast cell lines. All experiments were performed in duplicates**. D**. Shift from the truncated to the full-lengths C/EBPβ observed in a fibroblast cell line after exposure to SCM (20%).

In the presence of FCS, SCM induced an up-regulation of hnRNP E2, a repressor of CEBPA mRNA translation

To further elucidate the translation control mechanisms presented in figure 4A, we determined calreticulin (CRT) levels in response to SCM. CRT is a repressor protein that reduces CEBP mRNA expression by binding to an internal GC-rich region in the mRNA stem loop (38). As shown in figure 4B, SCM had no effect on the expression level of the CRT at all. Finally, we analyzed the expression of hnRNP E2, a repressor *CEBPA* mRNA translation by binding to the 5' untranslated region (39). As shown in figure 4C, SCM significantly increased the expression of hnRNP E2 ($n=3$, $P < 0.05$) in FCS stimulated cells only.



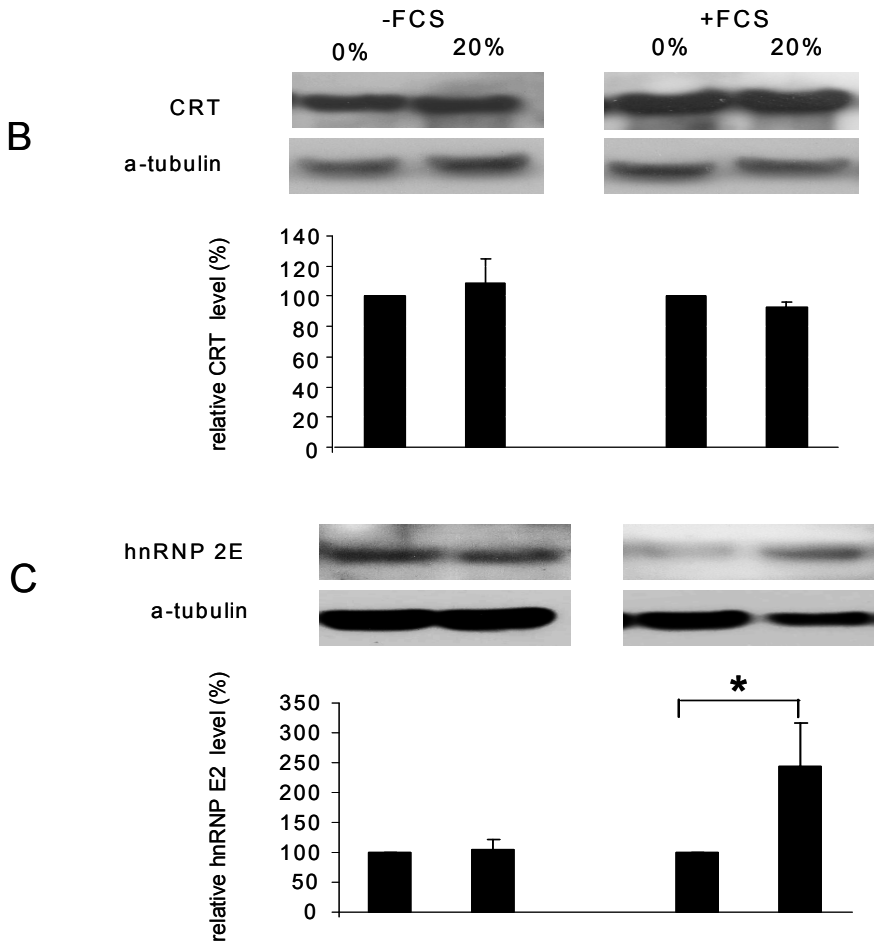


Figure 4:
Western blot analysis of calreticulin (CRT) and hnRNP E2 expression after exposure of primary lung fibroblast to SCM. **A** Schematic representation of regulatory proteins and their docking sites of the translation mechanisms for C/EBPα**. B.** Expression of CRT in primary lung fibroblast after exposure to SCM (20%). Bar diagram = densitometric analysis (n=3). **C.** Expression of hnRNP E2 in primary lung fibroblast after exposure to SCM (20%). Bar diagram = densitometric analysis (n=3). * $P < 0.05$.

Discussion

In this study, we showed that SCM had differential effects on the expression of the cell cycle regulators and transcription factors C/EBPα and C/EBPβ, which depended on the presence of FCS. These effects were controlled at the translational level, but involved distinct mechanisms. In the absence of FCS, the regulation of translation operates via the uORF of *CEBPA* and *CEBPB* mRNA, leading to an increased full-length protein expression and therefore suppresses proliferation. In presence of FCS, the translation is regulated via an increased expression of the hnRNP E2 protein, a suppressor of C/EBPα translation. Because the gene structure of C/EBPβ is similar to that of C/EBPα, a similar mechanism may be involved in C/EBPβ expression control. The ultimate expression of C/EBPα and C/EBPβ depended on the proliferation status of the cells, but coincided with an increased IL-8 release and a reduced proliferation rate. We hypothesize that in cigarette smoke susceptible subjects this smoke-induced translational modulation of *CEBP* mRNAs may contribute to an impaired lung tissue renewal process, a diminished protein turn-over, and may underlie the inflammation and destruction of the lung parenchyma.

A cigarette smoke-induced imbalance of pro- and anti-proliferative signals may hence provide a novel mechanism to explain many features of COPD and emphysema. C/EBPs are important regulator of cell cycle progression and proliferation and a disturbed balance in their expression has been linked to many proliferative disorders (14, 15, 16, 23). CEBPα and -β are modular proteins containing C-terminal basic leucine-zipper domain required for homo- and heterodimerization and binding of the DNA in CCAAT-type-cis-regulatory motifs in responsive genes and N-terminal sequences containing transactivation domains (24). Different full-length or truncated isoforms stem from one single mRNA transcript due to alternative translation initiation at different translation start sites within the open reading frame of the *CEBPA* and

CEBPB mRNAs. The translation initiation of both C/EBPs is predominantly regulated by a small upstream open reading frame (uORF) (27). Importantly, the truncated C/EBP isoforms miss the trans-activation domain and are therefore unable to activate gene transcription and inhibit cell proliferation. In airway smooth muscle cells of asthma patients an impaired translation of the *CEBPA* mRNA was observed, which coincided with lower levels of CEBPα protein and increased levels of IL-6 (20; 25). In a similar way, smoke exposure may prime lung fibroblasts to release more pro-inflammatory cytokines and decrease the proliferation due to an balance-shift of C/EBPα and -β.

In order to elucidate the mechanism underlying the imbalanced expression of C/EBPα and C/EBPβ by cigarette smoke exposed fibroblasts, we used a novel translation control reporter system (TCRS) which senses translation control and is regulated via an uORF (27), which is present in both *CEBPA* and *CEBPB* mRNA. The TCRS generates two peptides (p12 and p23), and their expression ratio (p12/p23) is a quantitative measure of translation initiation efficacy regulated by the uORF (28, 29). High initiation efficiency results in an increased expression of the small peptide, whereas poor translation re-initiation shifts the expression toward the long peptide. Similarly, low poor translation re-initiation shifts the expression of both C/EBPα and C/EBPβ toward the full-length isoforms. We found that cigarette smoke significantly decreased the p12/p23 ratio in lung fibroblasts, indicating impaired translation. In accordance with this finding, the expression of full-length C/EBPα and β isoforms was increased by cigarette smoke. We also observed a shift from the truncated to the full-length C/EBPβ isoform in fibroblasts which may account for the increase IL-8 level, as IL-8 is positively regulated by C/EBPβ (26)

C/EBPα plays a decisive role in cell growth arrest, showing an expression pattern that is inversely related to the proliferative state of many cell types (30). Here, in absence of FCS the

cigarette smoke-induced an up-regulation of C/EBPα, which may account for the strong anti-proliferative effect of SCM on lung fibroblasts. This effect may also underlie the impaired tissue repair and renewal and, eventually, lead to a tissue loss typical as observed in the emphysema lungs. The increased C/EBPβ expression after exposure to SCM is in accordance with a mouse model where increased C/EBPβ levels occurred in emphysema lungs following daily exposure to cigarette smoke over a period of 6 months (18). Interestingly, C/EBPβ was shown to be a negative regulator of elastin expression, a structural component of the lung alveoli (17). Proteolytic degradation of elastin resulted in airspace enlargement and development of emphysema (31, 32). Therefore, our observation that SCM induced the up-regulation of C/EBPβ may be responsible for an impaired resynthesis of elastin.

The SCM-induced up-regulation of C/EBPβ expression may also explain the increased IL-8 release, because the promoter of the IL-8 gene contains a CCAAT consensus sequence (33). IL-8 is a strong chemotactic agent for neutrophils, which are the main cells infiltrating the lung during the inflammatory process observed in COPD (1). In this way, cigarette smoke-induced C/EBPβ expression may activate pro-inflammatory genes to create an inflammatory environment required to recruit more pro-inflammatory cells in a positive feedback loop.

Interestingly, the opposite effect of cigarette smoke on the translation control mechanisms of C/EBPα and C/EBPβ expression depended on the presence of FCS. Similar FCS-dependent effects have been reported with respect to extracellular matrix deposition by primary human lung fibroblasts treated with corticosteroids and long-acting β_2-agonists (34) and steroid-receptor complexes (35). A serum free environment may represent non-inflammatory conditions, whereas 5% FCS may reflect inflammatory conditions characterized by vessel leakage, serum infiltration and activation of tissue repair. This model was confirmed by reports

that in response to 10 % FCS primary human neonatal foreskin fibroblast increased the expression of genes related to the wound healing process, cell migration and proliferation, inflammation and angiogenesis (36). In the presence of FCS, which is a condition that mimics a process of ongoing inflammation, SCM significantly decreased the expression of both C/EBPα and -β. In spite of these findings, SCM inhibited cell proliferation and triggered IL-8 release, indicating that different control mechanisms are involved in serum-free conditions. Interestingly, the presence of FCS did not alter the p12/p23 ratio, indicating that the regulation of the translation did not occur via the uORF. Therefore we tested two additional regulatory mechanisms of C/EBPα translation control. The first one is involving CRT, which inhibits the translation of the *CEBP* mRNAs into protein due to a direct interaction of CRT to an internal stem loop of both *CEBPA* and *CEBPB* mRNA (37). Alternatively, the protein hnRNP E2 can bind to the 5' untranslated region of the *CEBPA* mRNA and inhibit its translation, which has been shown in the context of leukemia (38). In the presence of FCS we found an up-regulation of hnRNP-E2 after exposure to SCM. Therefore, depending on the degree of inflammation, two distinct translational control mechanisms are involved in the control of C/EBPα and –β protein expression. In the presence of FCS, IL-8 expression may result from a cooperative interplay of a multitude of independently activated positive signaling pathways, which may become to dominate the IL-8 protein expression and cannot be counteracted by the observed downregulation of C/EBPβ – but at the same time C/EBPβ levels are still sufficient to generate an IL-8 driven inflammatory response. Similar findings were reported for IL-8 production by airway epithelial cell lines where the transcription of the IL-8 gene dependent highly on the presence of different transcription actived complexes (39).

In summary, our data indicate that exposure of primary lung fibroblasts to cigarette smoke in non-inflammatory condition repressed the translational of both *CEBPA* and *CEBPB* mRNA via

the regulatory uORF, which shifted the protein expression towards the full-length C/EBP isoforms. As a consequence, pro-inflammatory genes (including IL-8) may become up-regulated and this may reflect an early stage of COPD. At the same time, a reduction of cell proliferation may lead to an impaired regeneration of lung tissue. At a later stage of the disease with chronic inflammation present, which our model mimicked by FCS, this mechanism seems to be less important, while the translation of C/EBPs was predominantly controlled by an up-regulation of hnRNP 2E (figure 5). However, this was insufficient to counteract the cigarette smoke-induced effect on proliferation and inflammatory response. Insight in aberrant translational control mechanisms of C/EBP expression may potentially lead to new therapeutic strategies for COPD and emphysema. In the light of these findings, an early intervention to restore the imbalanced expression of C/EBPα and C/EBPβ in COPD (emphysema) should be envisaged.

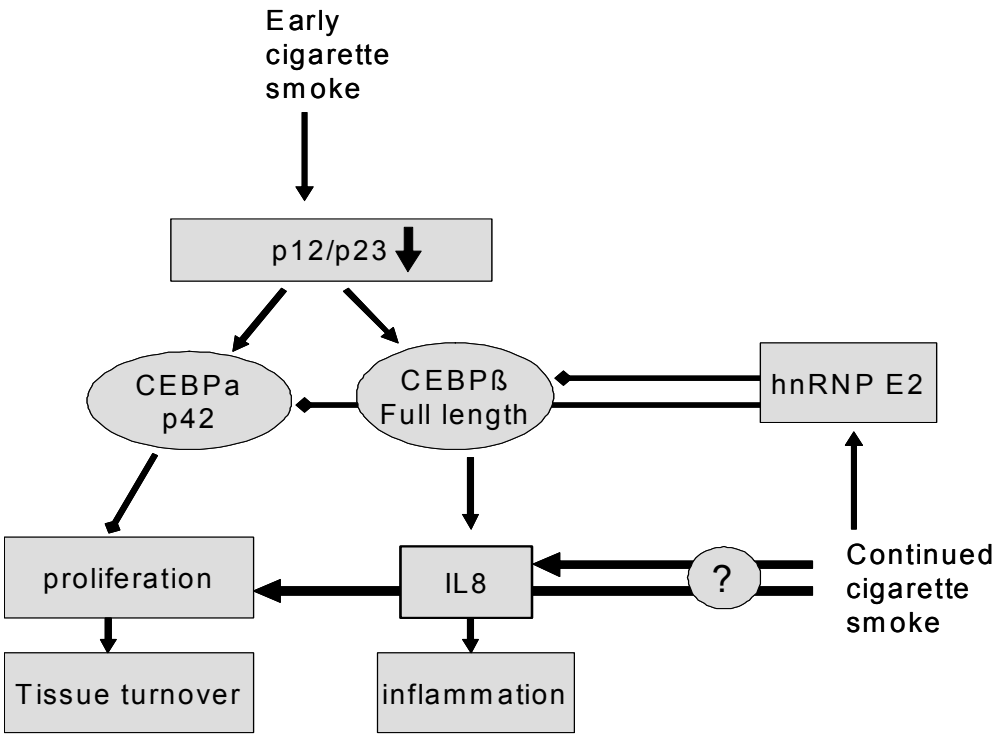


Figure 5:
Schematic overview of putative mechanisms involved in the early and continued exposure to cigarette smoke and how this might be relate to the pathogenesis of COPD/emphysema.

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Key findings, conclusion and future perspectives

In this thesis I showed that house dust mite extracts and cigarette smoke affect the expression level of C/EBPα via distinct translational control mechanisms, in primary human airways smooth muscle (ASM) cells and in lung fibroblasts respectively. Therefore, I was able to answer the three original objectives.

Key findings:

- ASM cells of patients with asthma show an impaired *CEBPA* mRNA translation leading to a decreased or abolished expression of the C/EBPα protein. C/EBPA mRNA was expressed at comparable levels in ASM cells obtained from asthma patients and healthy controls, while its translation re-initiation was impaired due to decreased levels of eIF4E in asthmatic cells. This mechanism operates via the uORF of the *CEBPA* mRNA.

- House dust mite extracts significantly and specifically reduces C/EBPα expression in ASM cells of asthma patients. Interestingly, this reduction is not achieved via the uORF mechanisms which predominantly regulates the translation re-initation of the *CEBPA* mRNA, but via increased levels of calreticulin, an inhibitor of C/EBPα translation. The decreased C/EBPα expression coincides with increased cellular proliferation and enhanced release of IL-6. Furthermore, a second independent mechanism leads to decreased C/EBPα levels trough activation of the PAR-2 receptor. Therefore HDM extract triggers both protease-dependent and protease-independent mechanisms that regulate C/EBPα.

- Cigarette smoke affects both C/EBPα and C/EBPβ expression via translational control in primary lung fibroblasts. In serum-free environment, cigarette smoke increases both C/EBPα and -β expression at the translational level via the uORF mechanism.

  In the presence of FCS, a different mechanism is leading to a decrease of both C/EBPα and -β expression with increasing concentration of cigarette smoke conditioned medium. The cigarette smoke concentration increases the levels of hnRNP E2, an inhibitor of C/EBPα translation.

  In both conditions, cigarette smoke has a potent antiproliferative effect on fibroblasts. Furthermore, cigarette smoke increases the release of IL-8.

Conclusions

This thesis provides for the first time experimental evidence that impaired translation of the cell cycle regulator C/EBPα may play a crucial role in the pathogenesis of asthma. Our group has shown in previous investigations that in ASM cells of asthmatics the expression of the C/EBPα is significantly reduced or missing (1). Here a mechanism which may explain this reduced C/EBPα expression was also linked to the translational regulation of *CEBPA* mRNA. As discussed in the previous chapter, an increasing number of diseases are recognized as being based on translation disorders and here we show that in ASM cells the impaired translation of C/EBPα may play a pivotal role in the pathogenesis of asthma.

Furthermore, we show that a direct interaction of ASM cells of asthma patients with house dust mite allergens specifically reduces C/EBPα expression, induces cell proliferation and stimulates the release of pro-inflammatory cytokines. These findings further substantiate the importance of C/EBPα translation and ASM cells in asthma pathology. First, the HDM-induced increased proliferation may explain the increased bulk of smooth muscle cells typically observed in the airways of asthma patients (2). Second, the HDM-dependent

release of IL-6 shows that ASM cells are able to directly trigger an inflammatory cascade in response to external stimuli. It has been shown by Grunstein *et al.* (3) that a component of the house dust mite, Der p 1 is able to elicit a pro-asthmatic effect (enhanced constrictor responses and impaired relaxation responses) in the airway responsiveness in isolated rabbit airway tissues. Taken together, these results show that HDM has the capacity to induce pro-asthmatic effects and induce airway remodeling as the result of a direct action on resident cells of the lung, namely on ASM cells, independent of the immune system. The importance of these findings is in fact that they offer a mechanism for the pathogenesis of asthma which is not mediated by the immune system, and this may be of particular interest to explain non-atopic, intrisinc asthma which affects 30% - 50% of asthmatic subjects.

It has been shown in a rhesus monkey model that exposure to allergens (including HDM) during the early years of life leads to a persistent increase of ASM cells by a mechanism that was independent of the immune system (4). Furthermore, recent studies showed that bronchial thermoplasty (a novel bronchoscopic strategy to ablate muscles from the airway of asthma patient) markedly improved asthma control in subjects with mild or severe asthma (5). Therefore, we think that airway remodeling, especially the ASM cells hyperplasia, might not be a secondary event, but rather a fundamental pathology that causes asthma. Therefore, ASM cells may be an initiator and not a bystander in inflammatory or allergic events in asthma. In this regard, we present a molecular mechanism that links external stimuli (HDM extracts) to an impaired translation of the C/EBPα protein and which may explain the hyperplasia and increased inflammatory response of ASM cells in asthma patients.

Interestingly, HDM repressed the C/EBPα translation by the induction of CRT, a repressor of *CEBPA* mRNA translation (6), rather than trough the uORF mechanism, which predominantly regulates C/EBPA translation re-intiation (7). A similar mechanism underlies

a form of leukemia, where increased CRT levels inhibit C/EBPα translation leading to a malignant phenotype (8). These observations indicate a link between deregulated C/EBPα translation and proliferative disorders and in this context asthma can be seen as a disease of over-proliferative, activated ASM cells.

We also found an effect of cigarette smoke on the expression of both C/EBPα and C/EBPβ in primary lung fibroblasts. Also in this case the expression of C/EBPs was regulated at the level of the translation, but two different mechanisms were involved, which were further affected by the presence or absence of serum. Therefore, also in COPD variations in the expression level of C/EBPα and C/EBPβ may play an important role in the pathogenesis of the disease. In the absence of serum, cigarette smoke induced an up-regulation of the full-length C/EBP isoforms, which coincided with decreased proliferation and increased IL-8 release. Our data indicates that the increased expression of both C/EBPs was achieved at the translational level trough the uORF regulatory mechanism. Full-length C/EBPα has a potent antiproliferative effect (9) and the cigarette smoke-induced increase in its expression may explain the reduced proliferation of fibroblasts. This effect may be crucial in the tissue homeostasis and lung repair mechanisms, where fibroblasts play an important role and this mechanism could finally contribute to the destruction of the tissue and the increased airway remodeling which is characteristic in COPD lungs (10-12). This effect may be further enhanced by the increased expression of C/EBPβ, which is a negative regulator of elastin transcription (13). Elastin is a structural component of the lung alveoli which is important for the elasticity of the tissue. Proteolytic degradation of elastin results in airspace enlargement and development of emphysema. Therefore, our observation that cigarette smoke induced the up-regulation of C/EBPβ may be responsible for the impaired re-synthesis of elastin. Taken together, I postulate that the cigarette smoke-induced imbalance

of pro- and anti-proliferative signals provides a novel mechanism to explain many pathologies of COPD and emphysema, especially the tissue destruction defined as an imbalance between tissue injury and tissue repair. Interestingly, cigarette smoke also induced IL-8 release showing that fibroblasts are capable to create a pro-inflammatory environment. IL-8 is a chemo-attractant for neutrophils, which are the characteristic lung infiltrating immune cells in COPD. Finally, the studies provide the first experimental evidence that cigarette smoke affects the translation of crucial proteins controlling cell cycle progression and transcription of pro-inflammatory genes. As in the case of asthma, we propose a link between external stimuli and the modified C/EBPα and C/EBPβ expression in resident tissue forming cells of the airway, which may explain characteristics pathology of COPD.

Future perspectives

For many years asthma research mainly focused on the inflammatory component of the disease and contributed to our knowledge on airway inflammation and how it aggravates asthma symptoms. The work presented in this thesis highlights the importance of the direct interaction of allergens with resident tissue forming cells of the airways, namely the ASM cells. This direct effect is not immune-system mediated, and affects the expression level of C/EBPα via translation regulation and may account for the main characteristic pathologies in asthma finally leading to an over expression of inflammatory mediators which in turn attract and activate immune cells into the airways.

In the light of these findings, novel therapeutic strategies may be considered. Today, no therapies for COPD are available while in the case of asthma, the use of inhaled glucocorticosteroids and β2-adrenoceptor agonists is controlling asthma symptoms, but is

not curative. Strategies to prevent the ASM cells hyperplasia in asthma and restore expression C/EBPα by targeting signaling pathways controlling the *CEBPA* mRNA translation may reduce or even cure asthma symptoms.

After three decades of studies based on the inflammation/protease-antiprotease hypothesis, COPD research experienced a strong expansion of paradigms, involving oxidative stress, apoptosis, aging and senesce in the lung. We propose a model where cigarette smoke directly affects the expression level of both C/EBPα and C/EBPβ via translation regulation. As a consequence, an imbalance between tissue injury and tissue repair may finally lead to tissue destruction.

Therefore, restoring the balanced expression of both C/EBPα and C/EBPβ by intervention at the level of translation control may be beneficial in COPD patients.

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