AIRWAY WALL REMODELING IN ASTHMA: NOVEL MECHANISMS OF HUMAN BRONCHIAL SMOOTH MUSCLE CELLS IN THE INDUCTION OF ANGIOGENESIS

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Laura Franziska Keglowich
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Auf Antrag von

Prof. Dr. Michael N. Hall

Prof. Dr. Michael Roth

Prof. Dr. Ruth Chiquet-Ehrismann

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Prof. Dr. Jörg Schibler

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Abbreviations

(b)FGF	(Basic) fibroblast growth factor
BALF	Broncho-alveolar lavage fluid
BM	Basement membrane
BOS	Bonchiolitis obliterans syndrome
BSA	Bovine serum albumin
BSM(C)	Bronchial smooth muscle (cell)
COPD	Chronic obstructive pulmonary disease
CXCR2	Chemokine (C-X-C motif) receptor 2
EC	Endothelial cells
ECGM	Endothelial cell growth medium
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
ENA-78	epithelial neutrophil-activating protein-78, epithelial cell-derived neutrophil attractant-78
FEV_1	Forced expiratory volume in 1 s
FGF	Fibroblast growth factor
GC	Glucocorticoid
GM-CSF	Granulocyte macrophage colony-stimulating factor
HDM	House dust mite
HGF	Hepatocyte growth factor
HMEC-1	Human microvascular endothelial cell line
IL	Interleukin
IPF	Idiopathic pulmonary fibrosis
LABA	Long acting β_2-agonist
LTB	Leukotrien B

MBP	Major basic protein
MCP	Monocyte chemotactic protein
NGF	Nerve growth factor
PGD	Prostaglandine
TGF	Transforming growth factor
TIMP	Tissue inhibitor of matrix metalloproteases
TNF-α	Tumor necrosis factor α
TRITC	Tetramethyl rhodamine isothiocyanate
VEGF	Vascular endothelial growth factor

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Summary

Asthma is a global major health concern and it affects estimated 300 million people. The prevalence of asthma is rising and there is no cure for asthma, only the symptoms can be controlled. Acute asthma attacks are characterized by severe symptoms such as breathlessness, wheezing, tightness of the chest, and coughing, which may lead to hospitalization or death. Besides the acute symptoms, asthma is characterized by persistent airway inflammation and airway wall remodeling. The term airway wall remodeling summarizes the structural changes in the airway wall: epithelial cell shedding, goblet cell hyperplasia, hyperplasia and hypertrophy of the bronchial smooth muscle (BSM) bundles, basement membrane thickening and increased vascular density. Airway wall remodeling starts early in the pathogenesis of asthma and today it is suggested that remodeling is a prerequisite for other asthma pathologies. Furthermore, novel invasive therapies used to treat severe asthma provide evidence that the BSMC is a major effector cell in the pathology of asthma.

In the present thesis novel mechanisms of BSMC regulation and their role in the induction of asthma-associated angiogenesis have been elucidated. Therefore, the differences in the angiogenic capacities of BSMC from asthma and non-asthma patients and their modification by different conditions, such as an (i) inflammatory microenvironment, (ii) the influence of low oxygen concentration, and (iii) the stimulation with the most potent asthma relevant allergen (house dust mite (HDM) allergen) on the angiogenic properties of BSMC have been studied.

A major finding of this thesis is the increased angiogenic potential of BSMC from asthma patients and the altered release of CXCR2 ligands in an *in vitro* inflammatory environment. It has been demonstrated that BSMC from asthma patients release significantly more of the CXCR2 ligands ENA-78, GRO-α and IL-8, which may explain the increased vascular density in the sub-epithelial cell layers observed in the airways of asthma patients. These finding adds to previous studies showing that BSMC are a source of angiogenic factors (e.g. VEGF) and that CXCR2 ligands are elevated in the airway lining fluids of asthma patients. In this thesis BSMC have been shown to be a potential source of CXCR2 ligands, which induced spout outgrowth from endothelial cell spheroids in an *in vitro* model of angiogenesis.

Furthermore, this thesis investigated the effect of hypoxia on BSMC. Local restricted hypoxia in the airways of asthma patients had only recently been suggested. The animal model based hypothesis that hypoxia directly causes BSMC hyperplasia was tested. This hypothesis was not confirmed in human BSMC but nonetheless it was demonstrated that hypoxia leads to increased release of inflammatory and angiogenic mediators; as conditioned medium from BSMC grown under hypoxia induced angiogenesis in an *in vitro* model via VEGF.

These findings suggest that different conditions or stimuli induce angiogenesis in asthma through different pathways and therefore, different therapeutic strategies might be needed.

In the third part of this thesis the effect of HDM allergen on the release of inflammatory and angiogenic mediators from BSMC was assessed. Animal models demonstrated that exposure to HDM allergens increased airway wall vascularization. No direct contribution of BSMC to HDM extract induced angiogenesis was observed. However, HDM extract proteases degraded ENA-78, which is an import chemokine for neutrophil recruitment into the inflamed lung. Thus HDM allergens may alter the bio-availability of ENA-78 in the airways of asthma patients and modulate the immune response.

The findings of this thesis add a small piece to the knowledge of asthma pathology, the mechanisms underlying airway wall remodeling and in particular BSMC hyperplasia and neovascularization. This might represent novel targets for treatment, especially for the prevention or reversal of airway wall remodeling.

General Introduction

Asthma – a global health concern

Definition

Asthma is a complex, heterogeneous disease of unknown etiology. The disease is defined by its clinical, physiological and pathological features. Clinically asthma presents as recurrent episodes of wheezing, breathlessness, tightness of the chest, and coughing. These symptoms occur particularly but not solely during nighttime or in the early morning. The clinical spectrum of asthma is highly variable but all asthma patients suffer from airflow obstruction, which limits expiratory airflow. Pathological features in the lungs of asthma patients are the altered presence of immune cells in comparison to non-asthma patients as well as several functional alterations of the epithelium and the sub-epithelial cell layers including bronchial smooth muscle cells (BSMC). The pathology is usually referred to as airway inflammation and airway wall remodeling and will be addressed in detail in separate chapters.

Due to the heterogeneous clinical manifestation, the unknown etiology, the variable responsiveness to treatments, and the variable long-term outcome the definition of asthma provided by the GINA (Global Initiative for Asthma) is descriptive, dealing with the functional consequences of this disease:

> "Asthma is a chronic inflammatory disorder of the airways in which many cells and cellular elements play a role. The chronic inflammation causes an associated increase in 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 that is often reversible either spontaneously or with treatment."

Prevalence and socio-economic impact

In the mid-1980s asthma became a recognized public health concern in many countries. Population studies showed that asthma prevalence ranged from 8 – 20% in western countries and that the prevalence increased by 50% every decade [1]. This suggests that asthma is and will continue to be a major health concern. In 2004 Masoli *et al*. [2] and the GINA combined data from two studies [3-5] and reported a worldwide prevalence ranging from 0,7 (Macau) to 18,4% (Scotland) illustrated in Figure 1. A more recent study included about 180000 adults (aged 18 – 45y) in 70 countries [6]. This study revealed a worldwide prevalence of 4.3 – 8.6%, dependent on the stringency of definition criteria, with a highly variable prevalence across different countries. This was the first standardized, representative survey presented and currently is the best study that included population-based data regarding respiratory symptoms and treatment.

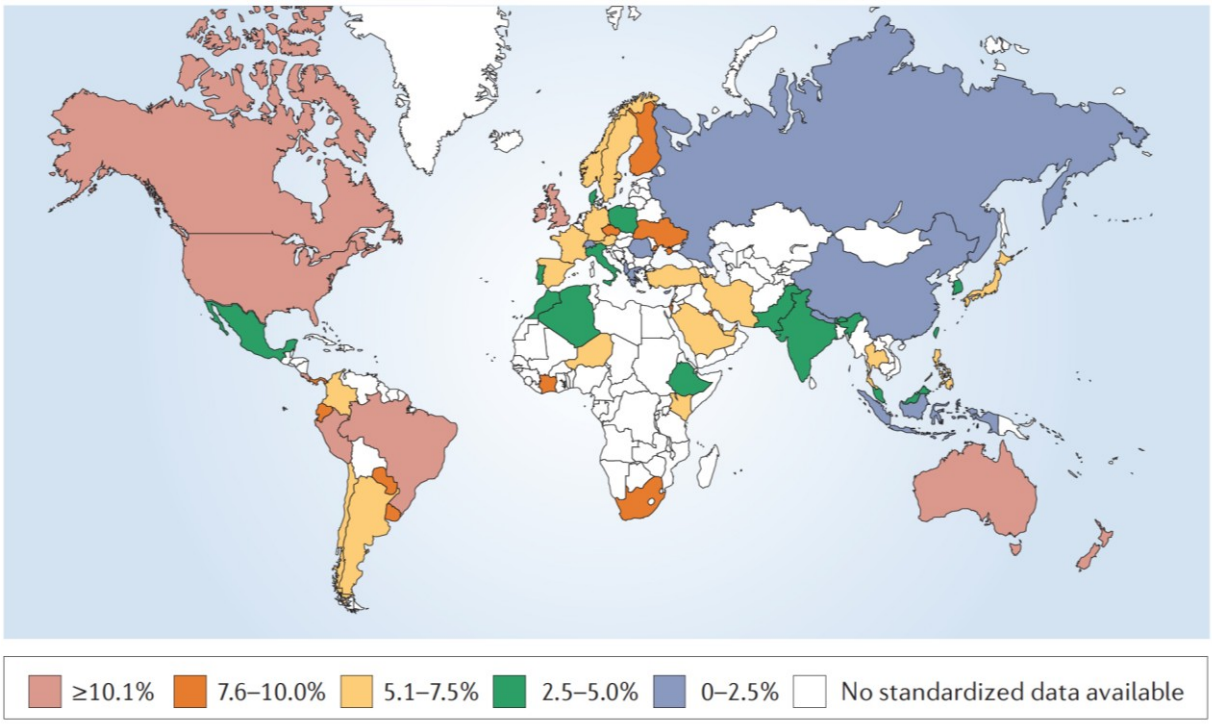

Figure 1: Worldwide prevalence of asthma. The highest prevalence of asthma (≥ 10.1% of total population) is found in North America, Peru and Brazil, the UK, Australia and New Zealand. [7].

The shortcoming of this study was that children, who represent more than 50% of all asthma patients, were not included. In children and young adults asthma is the most common chronic disorder. It is one of the major reasons for absence from school, loss of productivity, and responsible for vast health care costs [1, 8, 9]. In Europe asthma accounts for the majority of hospital admissions in children [10] and it appears that most children do

not “grow out of asthma” as it reappears in 30-50% of adults with a history of childhood asthma [11-13].

Undiagnosed, untreated or uncontrolled asthma has a severe impact on life quality and might be fatal [14]. Although the overall mortality rate of asthma is decreasing [2] a severe increase in the number of hospital admissions has been reported especially in children [15, 16]. This may reflect not only an increase in the severity of asthma but also poor disease management and low socio-economic status.

The economic costs caused by asthma exceed those of tuberculosis and HIV/AIDS combined (WHO factsheet 206: bronchial asthma) with an un-proportional contribution of severe asthma, which accounts for 10-20% of all cases but causes approximately 50% of all costs [16-19]. In addition to the economic burden, poorly controlled severe asthma causes work impairment, significant productivity losses and has a severe impact on social life [10, 20]. Diagnosis and treatment-availability is still challenging in resource-poor countries while the prevalence is rising sharply [1].

A better understanding of the pathology of asthma as well as new treatment options are needed to reveal the underlying cause(s) of the disease and reasons for its increasing prevalence.

Asthma phenotypes: Intrinsic *vs.* extrinsinc, atopic *vs*. non-atopic asthma, eosinophilic *vs.* neutrophilic

Asthma is a heterogeneous disease with different clinical manifestations, variable inter-individual severity of symptoms and degree of airflow obstruction, variable degrees of reversibility and loss of lung function, and variable responsiveness to therapy. Therefore, several approaches have been made to classify the different phenotypes of asthma. In general, those classifications provide the basis for clinicians to understand the characteristics of the disease in a patient in order to choose the right treatment.

In the early 20th century Rackemann was the first to sub-divide asthma into extrinsic and intrinsic asthma [21]. Intrinsic asthma is currently also known as atopic asthma and extrinsic asthma is synonymous with non-atopic asthma. Atopy, defined by increased levels of IgE is found in 60% of asthma patients [22, 23] and serum-IgE level correlated with asthma symptoms [24, 25]. To date the importance of atopy in childhood asthma is widely accepted, while its importance in adults has been questioned. A study analyzed the association of asthma and atopy and revealed only a weak and inconsistent association,

across populations as well as time periods [26]. The most important information from the classification as atopic or non-atopic asthma is in terms of disease onset and responsiveness to treatments. Atopic asthma has a peak of onset in children, whereas late-onset asthma is mainly non-atopic. Patients with atopy normally respond better to glucocorticoid (GC)-treatment whereas a substantial number of patients with non-atopic asthma do not respond to GC or only to very high doses [27]. For research(ers) however this classification leaves several unanswered questions, in particular why only 7-10 % of all atopic people (40% of the population in developed countries) develop asthma [26, 28]. This indicates that atopy as a cause of asthma might have been overestimated.

Additional classifications of asthma are based on asthma triggers such as: exercise-induced, aspirin-induced, and allergen-induced asthma.

The presence of distinct inflammatory cell types such as eosinophils or neutrophils in lung tissue or the airway lining fluids of asthmatic patients is another criterion to distinguish asthma phenotypes – eosinophilic and neutrophilic asthma. In mild asthma eosinophilic airway inflammation is present regardless of other characteristics such as atopy [29, 30]. Not all asthmatics display eosinophilia and some have an increased neutrophil number in the airways or airway lining fluids in absence of eosinophils [31-34]. Interestingly, neutrophilia has been linked to severe or fatal asthma [35, 36]. Furthermore it has been shown that most patients with non-eosinophilic asthma are non-atopic [27].

As asthma phenotypes overlap, a novel approach to classify asthma is the cluster analysis. Haldar *et al*. [37] used data from 371 patients, recruited from primary and secondary care. Plotting the degree of eosinophilic inflammation against the severity of symptoms they identified four clearly distinct asthma phenotypes as shown in Figure 2. Together with the definition of the phenotype the authors also provide treatment guidelines in accordance to the different phenotypes

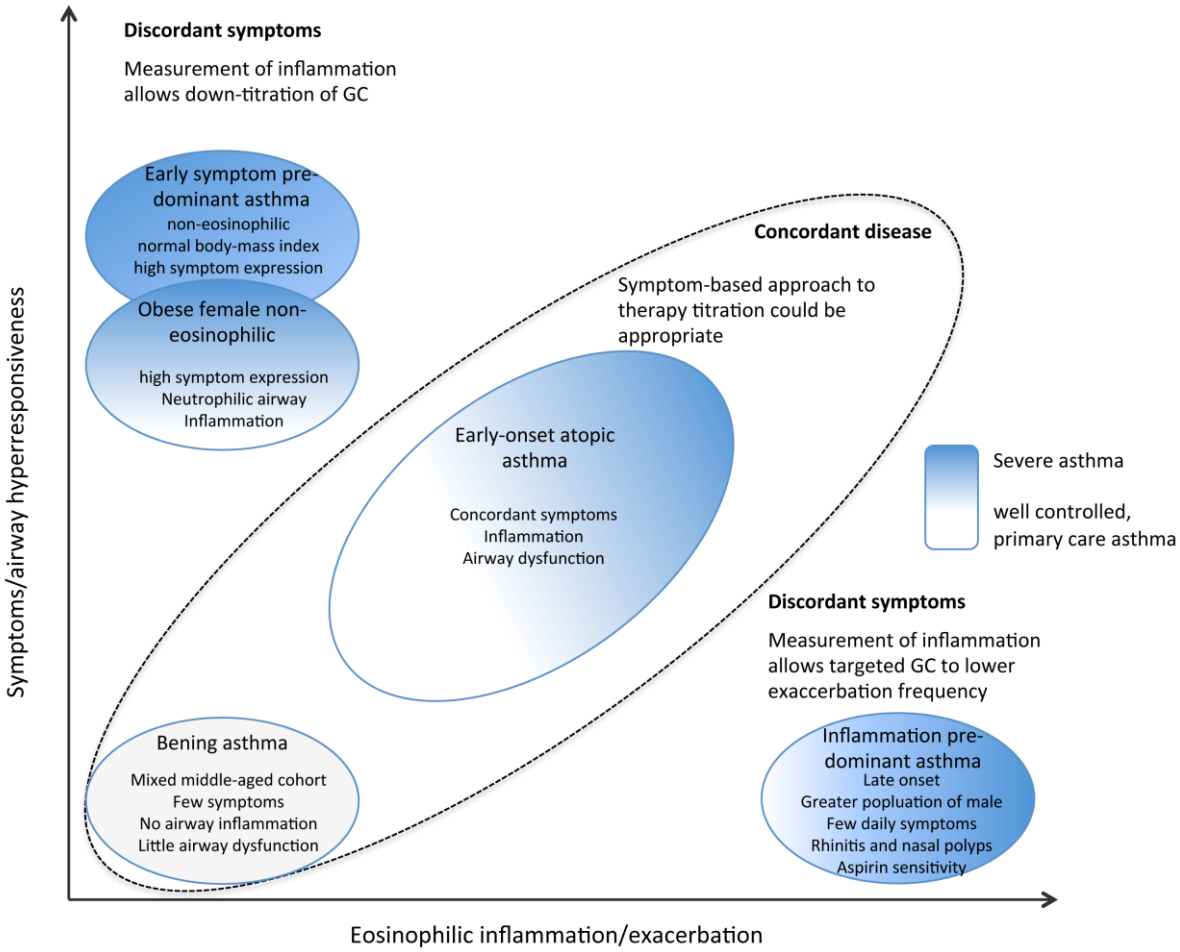

Figure 2 : Summary of asthma phenotypes identified by cluster analysis. Classification according symptom severity and observed inflammation reveals four asthma phenotypes (for details see text). Graph adapted from [38].

A second study using cluster analysis found similar phenotypes albeit different input parameters were used [39]. These two studies independently highlight the heterogeneity of the disease and the need for phenotype identification prior to start asthma therapy.

Treatment with GC might have an influence on the above-mentioned classification as GC-treated patients have reduced/no eosinophils and increased number of neutrophils [40]. Therefore, classification remains difficult and should preferably be done in naïve, untreated patients.

Asthma pathogenesis

Asthma and airway inflammation

Inflammation of the airway wall and the presence of immune cells in the lung tissue of asthma patients led to the classification of asthma as allergic hypersensitivity reactions of type I (acute asthma) and IV (chronic asthma). This view of asthma is usually referred to as the T_h2-paradigm and was postulated several decades ago. It defines asthma as an immunological disorder, with T_h2 cells as the pivotal immune cell to orchestrate, perpetuate and amplify chronic airway inflammation [41]. In this view chronic asthma develops as a result of an immune reaction driven by cytokines of the Interleukin (IL)-4 gene cluster located on chromosome 5. The cluster is comprised of the genes coding for IL-3, IL-4, IL-5, IL-9, IL-12, IL-13, granulocyte macrophage colony-stimulating factor (GM-CSF), all of which are known to contribute to asthma by inducing the IgE-isotype-switch of B cells, activation of mast cells, and proliferation and survival of eosinophils [42]. Mutations in this chromosomal region have been linked to atopy [43, 44]. The T_h2-paradigm may explain most of atopic/allergic inflammation but does not explain non-atopic asthma. Furthermore, it does not explain early occurring airway wall remodeling. Figure 3 shows the major effector cells of the immune response / system, their localization, interactions with each other and resident tissue-forming cells and the most important mediators.

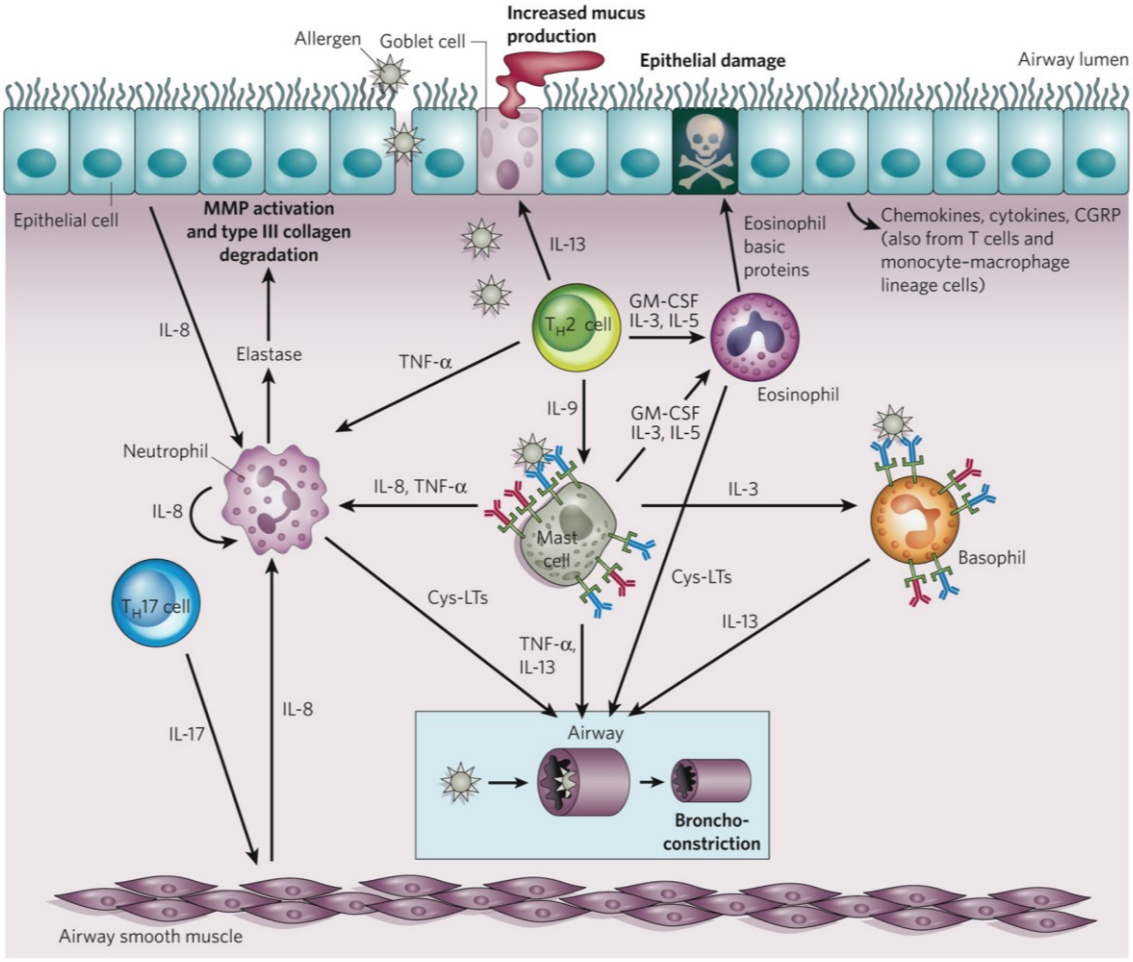

Figure 3: Airway wall inflammation (after allergen challenge). The most important effector cells of the innate and adaptive immune system and their mediators and the resident lung cells contributing to inflammation. [45].

Lymphocytes

Lymphocytes are divided into two major subsets: T cells and B cells. Cells belonging to the T cell linage can be either $CD8^+$ (cytotoxic T cells) or $CD4^+$ (T helper cells). The major function of cytotoxic T cells is the clearance of infected/dysfunctional somatic cells and therefore, they have only little contribution to asthma pathology. The contribution of B cells to airway inflammation is mainly by the production of IgE (in response to IL-4 stimulation), which is a major mediator in atopic asthma.

A better understanding of the underlying immunological mechanisms of airway inflammation came from the discovery of two distinct T helper cell subsets: T_h1 and T_h2 cells. In rodent models of airway inflammation, T_h2 cells were the dominating cell type in the lung tissue [46]. T_h2 cells dominated airway inflammation was also observed in the airways of humans suffering from asthma [24]. The predilection towards a T_h2-mediated immune response including the T_h2 cytokine profile in asthma can explain the observed

eosinophilic inflammation and/or the overproduction of IgE by B cells [47]. Cytokines of the T_h2 panel have various effects and act on several target cells. IL-4 is an important cytokine released from T_h2 cells. It acts in a positive feedback loop on T_h2 cells and is, together with IL-13, crucial for initiation of the isotype switch in B cells. IL-3, IL-5 and GM-CSF are essential for the proliferation and maturation of eosinophils.

Recently a third subset of T helper cells was defined: T_h17 cells. Like T_h1 and T_h2 cells, T_h17 cells belong to the $CD4^+$ T cell subset. They are named after their major cytokine: IL-17, which induces cytokine and chemokine release from bronchial epithelial cells, vein endothelial cells, fibroblasts and eosinophils and thereby influences recruitment of monocytes and neutrophils to inflamed tissue. IL-17 was upregulated in lungs of asthmatic patients and was linked to disease severity [48, 49]. In bronchial biopsies of asthmatics increased numbers of T_h17 cells were reported [50] but the contribution of T_h17 cells to asthma pathogenesis requires further investigation.

Another $CD4^+$ T cell subset, regulatory T cells $CD4^+/CD25^+$ (T_{reg}), is implicated in asthma. The function of T_{reg} is the mediation of the immune tolerance and the prevention of immune responses towards harmless antigens and host responses to (auto-) antigens. Still, the data on T_{reg} in asthma are controversial and incomplete, but recent *in vitro* studies suggest an important role of T_{reg} in asthma. It was shown that T_{reg} in asthmatic patients decreased and presence of T_{reg} could be positively correlated with the FEV_1 (forced expiratory volume in 1 s) [51, 52].

It was demonstrated that an appropriate T_h1/T_h2-balance prevented T_h2-driven inflammation [53] and T_{regs} can suppress exaggerated T_h2 responses via IL-10 [54]. This led to the hypothesis that T_h2-driven diseases like atopic asthma can be either caused by an increase in T_h2 cells (with the resulting decrease in T_h1) or a decrease of T_{reg} cells.

Lymphocytes do no only mediated acute and chronic inflammation but are also involved in angiogenesis [55, 56]. Angiogenesis, as well as inflammation involves a complex network of mediators and cellular interactions. In a murine model of airway remodeling it was demonstrated that mice lacking B cells had significantly reduced angiogenesis [57]. Furthermore, many of the cytokines released by T cells are implicated in angiogenesis as well. Angiogenesis-related cytokines of the T_h1 profile are mainly anti-angiogenic [56] whereas many T_h2 cytokines (IL-4, IL-6, IL-13) promote angiogenesis. IL-17 has been shown to promote angiogenesis in a murine model of breast cancer [58] and might be also involved in remodeling in asthma. The induction of angiogenesis can be mediated directly

by the release of mediators involved in endothelial cell (EC) survival, migration, proliferation, apoptosis or indirectly by inducing the release of cytokines and modulate receptor expression on target cells [56]

Myeloid Cells

Several cell types of the innate immune system are recognized to contribute to asthma pathogenesis. Mast cells are located in all vascularized tissues but most prominently in tissues forming interfaces with the environment, e.g. the lung. In normal human bronchi mast cells are located in the submucosal connective tissues. In asthmatics, additional mast cells are found within the bronchial smooth muscle bundles and in the epithelium. This pathology seems to be specific for asthma, since these mast cells are not found in other inflammatory pulmonary diseases [29, 59, 60]. In allergic asthmatics, mast cells are highly responsive to inhaled allergens [61, 62]. Allergen-bound IgE crosslinks IgE-receptors expressed on the mast cell surface and this is the trigger for the release of preformed granules containing histamine, several proteases (amongst others chymase and tryptase), heparin and some pro-inflammatory and angiogenic cytokines (TNF-α, IL-4, IL-5) [55, 63-69]. Mast cells cause the contraction of BSMC and increased microvascular permeability leading to edema through the production of eicosanoids and the release of histamine [70]. Mast cell-derived mediators account for the so-called early or immediate response to allergens and for the late response by recruitment of other immune cells and perpetuation of a T_h2-like airway inflammation [71]. Many of the mediators released from mast cells also promote angiogenesis and thus, mast cells are an important link between inflammation and angiogenesis [55, 67, 68].

Neutrophils and eosinophils are the best-studied polymorphonuclear granulocytes in asthma. Similar to mast cells they contain granules with preformed mediators [72, 73]. Eosinophils are recruited to sites of inflammation by cytokines, chemokines and lipid mediators (e.g. IL-3, GMC-SF, eotaxin 1-3, monocyte chemotactic protein (MCP)-3, MCP-4, prostaglandin (PGD) 2, leukotrien B (LTB) 4) and complete their maturation after stimulation with IL-5 [74-76]. In many asthmatics it was shown that the eosinophil number in the airways or airway lining fluids is elevated [34, 77, 78] and that treatment with GC significantly reduced their number [79, 80]. Eosinophils have long been regarded as key effector cells in asthma but their role in mediating the disease has been challenged [81]. Nonetheless, eosinophils are known be pro-angiogenic by the release of several cytokines such as vascular endothelial cell growth factor (VEGF)-A, fibroblast growth factor (FGF)-

2, tumor necrosis factor (TNF)-α, GM-CSF, neurite growth factor (NGF), and IL-8 and might therefore contribute to the remodeling process in asthma pathology [82, 83].

Neutrophils are the most abundant type of white blood cells and effector cells in the innate immune response. They are the first cells recruited to the site of inflammation during the acute phase of the immune reaction. It has been reported that the number of activated neutrophils in symptomatic severe asthmatics is elevated [31, 84]. Although some studies reported elevated numbers of neutrophils in bronchoalveolar lavage fluid (BALF) of mild asthmatics [85, 86], others were not able to confirm these findings [87]. Additional research demonstrated increased numbers of neutrophils only in non-atopic asthmatics, whereas the number of neutrophils in atopic asthmatics was not elevated [88].

Neutrophils do not release cys-leukotriens, major basic protein (MBP) or cytokines of the T_h2 profile, nor do they contribute to eosinophilic inflammation directly. Still they are a source of several mediators, which modify the eosinophilic response [89]. Furthermore, neutrophils are a source of important angiogenic factors such as VEGF, IL-8, TNF-α, hepatocyte growth factor (HGF) and matrix metalloproteases [90-92]. Evidence for the implication of polymorphonuclear granulocytes in angiogenesis and tissue remodeling was provided in an animal model with impaired angiogenesis and wound healing after blocking neutrophil recruitment [93, 94].

The role of neutrophils in asthma requires further investigations, as treatment with inhaled GC is less effective in patients with neutrophilic inflammation compared to patients with eosinophilic inflammation [27].

In conclusion, these data shows that several immune cells play a role in airway inflammation in asthma and that those cells can also contribute to angiogenesis. The recruitment of a multitude of immune cells (mast cells, basophils, eosinophils, macrophages and lymphocytes) is a hallmark of the asthmatic lung.

Airway wall remodeling in asthma

The first described pathology of asthma was the thickening of the airway wall [95]. The increased thickness of the airway walls is caused by alterations of several airway wall forming tissues and some studies found correlations with disease severity [96-98]. The most striking features of airway wall remodeling are: epithelial cell shedding and thickening, goblet cell hyperplasia, mucus gland hypertrophy, basement membrane thickening, hyperplasia and hypertrophy of BSMC, and increased vascularization [67, 99-102]. Figure 4 illustrates the alterations of the remodeled airway wall in asthma patients.

The aberrant behavior of mesenchymal cells is crucial to understand the pathology of asthma and this hypothesis is almost 100 years old [95]. However, this hypothesis became less important after eosinophilia was observed in asthmatic lungs and as the T_h2-paradigm arose. Thus, in the past 25 years the research and the treatment of asthma were carried out with a focus on the allergic component of this disease. Although the T_h2-paradigm is still important, it is acknowledged that non-T_h2 immune cells and resident cells of the lung are important to develop asthma [103]. It is recognized that many celltypes (like epithelial cells, lung fibroblasts or BSMC) are sources of "asthma-determining" T_h2 cytokines such as IL-4, IL5, IL-9, IL-13 [104-107]. Today, it is again a matter of debate whether the immune system is the key player of asthma pathogenesis, or if the disease originates within the lung itself [108]. The hypothesis that tissue-forming lung cells induce asthma is strengthened by the fact that airway wall remodeling is observed in preschool children without clinical signs of inflammation [109-116]. Remodeling of the airway wall contributes to the hallmark feature of asthma - the airflow obstruction- in a fixed manner.

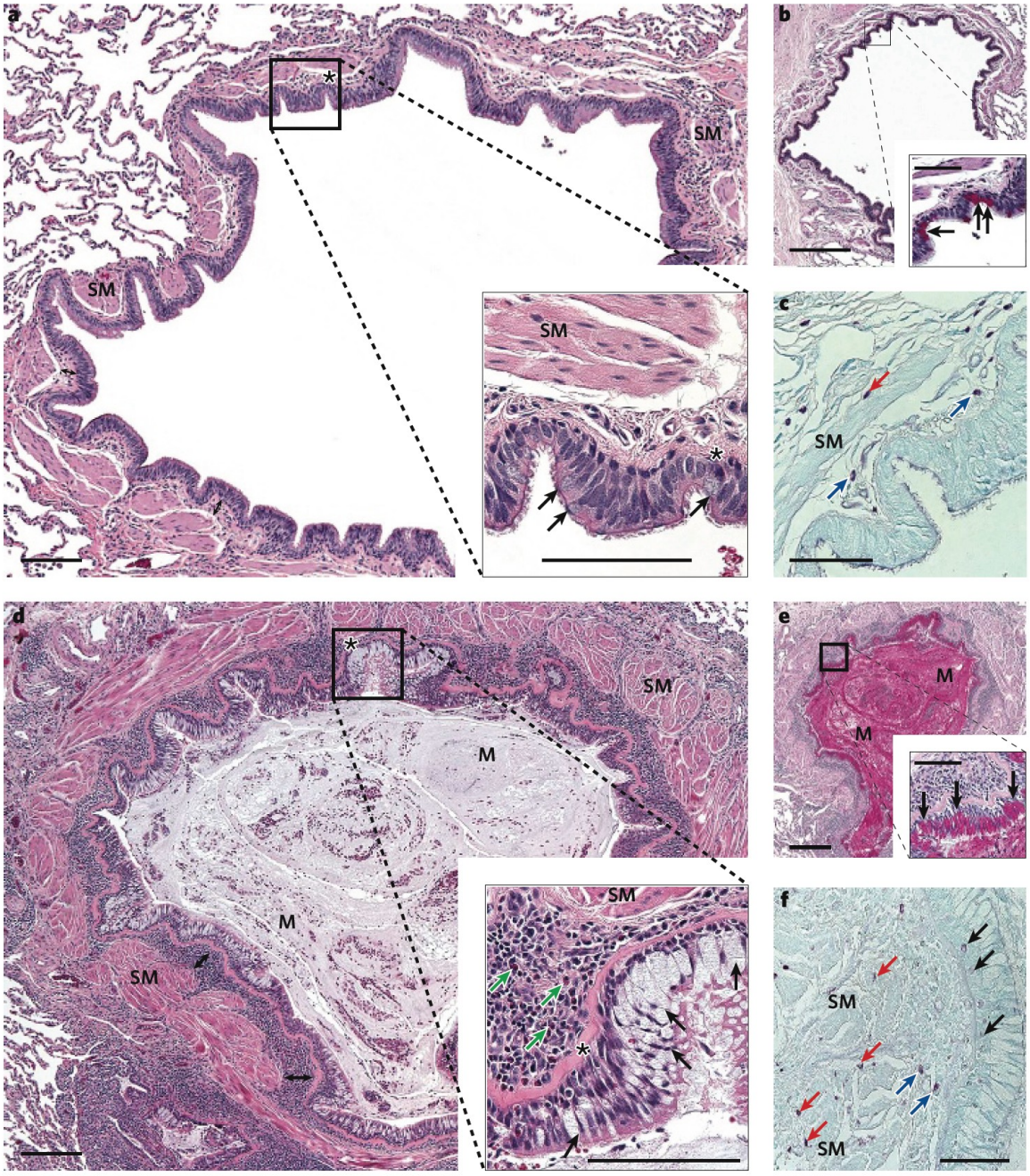

Figure 4: Alteration of the airway walls of healthy (a-c) and asthmatic (d-f) subjects. Tissue sectioned stained with haematoxylin and eosin (**a** and **d**), periodic acid–Schiff with diastase (**b** and **e**), or pinacyanol erythrosinate (**c** and **f**). Scale bars: 500 µm (**a** and **d**), 100 µm (insets **a, b, d**, **e** and **c** and **f**), 400 µm (**b** and **e**). In the normal bronchus are few goblet cells (black arrows) in the epithelium. The basement membrane and underlying *lamina reticularis* (* in **a**) is hardly visible. The submucosa (double-headed arrow) in **a)** contains few leukocytes and mast cells (blue arrows in **c**). Few adjacent mast cells (red arrow in **c**) are found in the smooth muscle (SM). **d–f**, In the bronchus of the asthma patient the airway lumen is filled with mucus (M) (**d** and **e**). There are many goblet cells (black arrows in insets) and intra-epithelial mast cell (black arrows in **f**). The *lamina reticularis* (* in **d**) is markedly thickened and thus visible. Many eosinophils and other leukocytes (green arrows in inset in **d**) as well as mast cells (blue arrows in **f**) are found in the submucosa (double-headed arrows in **d**). The layer of BSM is thickened with an increased number of mast cells (red arrows in **f**). (Figure courtesy of G. J. Berry, Stanford University, California.). [45]

Airway wall remodeling includes both, inflammatory cells and tissue-forming cells of the lung. Both celltypes considerably contribute to airway wall remodeling in asthma. Furthermore, it is clear that some cells of the immune system have various functions in the pathogenesis of asthma. In the following, the potential role of immune cells and tissue-forming lung cells in the process of airway wall remodeling is described:

Immune Cells

Connective tissue mast cells

In asthma the number of connective tissue mast cells in deeper regions of the lung is markedly increased and they might be involved in "programming" the adjacent BSMC of asthmatics [59, 64, 117-119]. In contrast to the mast cells of the submucosa and the epithelium, the mast cells within the BSM bundles are always tryptase and chymase positive (MC_{TC}) and their number has been linked to the severity of asthma [120] and airway hyperresponsiveness [59]. Mast cells are known to be one of the major sources of pro-angiogenic factors and the number of tryptase-positive mast cells has been correlated with angiogenesis in human endometrial cancer [121]. Recent studies suggested a role of mast cells in the vascular component of airway wall remodeling in asthma [122, 123].

Eosinophils

As mentioned in chapters before eosinophils have long been regarded the most important immunomodulatory cell in asthma. Eosinophils are also important in airway wall remodeling, demonstrated by the finding that eosinophil-depletion caused altered composition of the basement membrane and therefore eosinophils might have more influence on remodeling then assumed so far [124, 125]. Furthermore, eosinophils produce TGF-β1 and thereby promote proliferation of fibroblasts, myo-fibroblast maturation and collagen synthesis [126]. Eosinophils are the source of many angiogenic factors [127, 128] and can therefore induce angiogenesis in the asthmatic lung. All these findings suggest multiple functions of eosinophils in asthma pathogenesis.

These findings illustrate the interconnection of the immune system, inflammation and resident lung cells in the pathology of asthma.

Resident, tissue-forming lung cells

Before the discovery that asthma is not always associated with eosinophilia and/or T_h2 cell accumulation, the disease was mainly regarded as an immunological disorder. The observation that tissue-forming lung cells also produce mediators of inflammation and

cytokines prompted asthma researcher to rethink the role of tissue-forming lung cells in the pathogenesis of asthma.

Epithelial cells

Epithelial cells were considered merely a mechanical barrier protecting the body from the environment. It is the first line barrier against all influences from outside (allergens, pollutants, temperature, etc.) and has been reported to be damaged and metaplastic (pseudostratisfied) in asthma. Epithelial cells produce a vast spectrum of cytokines and chemokines [129, 130]. It has been shown that epithelial cell proliferation in asthmatics is impaired, which was attributed to altered level of several markers of proliferation such as proliferating cell nuclear antigen (PCNA), Ki67, and $p21_{wat}$ [131-133]. These findings suggest a chronic injury with reduced ability to repair [133]. The composition of the extracellular matrix (forming hemi-desmosomes with the epithelial cells) is altered and the expression of tight-junction proteins is reduced in asthmatics [134-136]. This might be causative for the epithelium in asthmatics to display an increased sensitivity to oxidant pollution like ozone, tobacco smoke, or ambient air pollution [137].

The impaired proliferation of cells forming a frequently renewing tissue and the impaired mechanical properties of the epithelium, as it occurs in the airways of asthmatics, might lead to the production of a variety of cytokines and chemokines reminiscent of chronic wound healing [133]. Several studies demonstrated the altered expression and secretion of chemokines and cytokines by airway epithelial cells from asthmatics compared to controls [138, 139].

The airway epithelium is a source of mediators of inflammation and remodeling (including angiogenesis), its shedding might enable allergens to access the lung tissue, and the metaplasia of the mucus producing cells directly causes airflow limitations.

Bronchial smooth muscle cells

The most obvious feature of airway wall remodeling in asthma is related to the BSM. A marked increase in BSM mass is observed in the asthmatic airway wall, which is due to hyperplasia and hypertrophy of BSMC.

Over the past 20-25 years the view on BSMC and their role in the pathology of asthma has significantly changed. Bronchoconstriction is the most severe symptom of an asthma attack and the cells of the BSM are the major effector cells and the proximal cause of the excessive airway narrowing [140]. The constriction it is completely or partially reversible,

but sometimes only with the help of bronchodilating drugs. The two characteristics of BSMC that contribute to bronchoconstriction in asthma patients are summarized as airway hyperresponsiveness (AHR) [141]. One characteristic is airway hypersensitivity, which describes the response of BSMC of asthma patients to low doses of stimuli. The other alteration of BSM is airway hyperreactivity, which describes an abnormal strong bronchoconstrictive response. The reason for AHR remains unclear, but it is suggested that it is due to fundamental changes within the muscle cell itself, due to external changes (e.g. reduced mechanical load) or a combination of both. *In vitro,* the maximal shortening of smooth muscles is approximately 70% of the optimal length. In a physiological context the contraction of a muscle is abrogated as soon as there is a balance between the contractile force of the muscle and the load against which the muscle contracts. The load-setting factors are the elasticity of the airway wall, the tethering forces of the lung-parenchyma and other contractile cells.

The active and passive forces limit the narrowing of the airways in healthy individuals even upon challenge and breathing remains easy. In asthmatics the muscle itself is not stronger, but the BSM is hyperplastic and hypertrophic [142, 143]. Due to this pathology the contraction might occur faster rather than stronger compared to healthy individuals and the relaxation might be impaired [144, 145].

The increase in BSM mass has been observed in young adults (17-23y) [146] and in children without any signs of eosinophilic inflammation [111, 147]. These observations suggest that the increased BSM mass is rather the cause of asthma then a consequence of progressing disease. Besides their effector role in bronchoconstriction BSMC are synthetically active cells, which are able to produce a variety of inflammatory and angiogenic mediators such as: GM-CSF, IL-1β, IL-2, IL-5, IL-6, IL-8, IL-11, IL10, Eotaxin, bFGF, PDGF-BB and VEGF [148]. It has been show that BSMC of asthmatics respond differentially (quantitatively and qualitatively) compared to healthy subjects. For instances, BSMC of asthmatics release more IL-6 and IL-8 after stimulation with house dust mite (HDM) extracts or after rhinovirus (RV) infection [149, 150].

The release of many of the aforementioned cytokines can be induced by several stimuli or in paracrine and autocrine regulated mechanisms. This shows that tissue-forming cells of the airways provide a mechanism how inflammation may be amplified and/or perpetuated without the involvement of immune cells.

In addition, BSMC are an important source of extracellular matrix (ECM) components. It has been demonstrated that serum derived from asthma patients leads to increased generation of fibronectin, lamin γ, and proteoglycans from BSMC [151-153]. BSMC from asthmatics also produce more connective tissue growth factor (CTGF), which may further contribute to the alterations of the basement membrane (described in the following chapter) [154, 155].

These findings identified BSMC as attractive and promising targets in airway biology research. Moreover, the interaction and interconnection of BSMC and immune cells and of BSMC and ECM components highlight the strong interdependence of the systems discussed in this chapter. Insights into disease specific alterations might open the door for new therapeutics.

ECM/Basement membrane thickening

When airway remodeling was reported for the first time a thickening of the basement membrane (BM) was described [95]. Decades after this first description of BM thickening electron-microscopy revealed that the thickened layer is not the BM itself but a zone adjacent to it – the *lamina reticularis* [156]. Since most publications call this phenomenon "basement/basal membrane thickening" this term will be used throughout this thesis.

Several studies demonstrated that the BM is thickened, even in young asthmatic children and this thickening is comparable to that observed in adults [114, 134, 157, 158]. In non-diseased subjects the BM is 4-5 μm thick, whereas in asthmatics it varies between 7 and 23 μm [159]. Among chronic inflammatory lung diseases, which are also accompanied by ECM alterations (like cystic fibrosis or chronic obstructive pulmonary disease (COPD)) BM thickening (including alterations in dimension and composition) is a unique pathology of asthma [160]. The thickening of the BM has also impact on the efficacy of treatment, as it was correlated with a limited short-term responsiveness to GC treatment [161].

There are multiple processes and cell types, which potentially contribute to the increased and/or altered deposition of ECM components in the BM. As mentioned earlier BSMC produce a variety of ECM components. In asthma, the ECM deposition is increased and BSMC mass correlates with BM thickening. This indicates that alterations in ECM composition and subsequent BM thickening may also be BSMC dependent [162]. Furthermore, the ECM is a reservoir for secreted factors, which can be released upon degradation/reconstruction of the ECM within the tissue. VEGF, for instance binds to the

ECM through heparan sulphate proteoglycans and is released upon matrix degradation processes [163, 164].

Taken together, these findings illustrate the importance of tissue-forming cells in perpetuating and amplifying airway inflammation and airway wall remodeling. Therefore, asthma should not be solely regarded as an inflammatory disease, but rather as a complex disease where alterations of the microenvironment and cellular structure lead to airway inflammation and airway wall remodeling. Resident tissue-forming lung cells are important target cells as potential drug targets, biomarkers, or for the assessment of therapy efficacy [165, 166].

In summary, airway wall remodeling is a key feature of asthma and its occurrence in very young patients [114, 134, 158] suggests it to be a necessary precursor to the onset of asthma [109, 111, 112, 116, 167-170].

Environmental factors in asthma

Asthma is a complex, multi-factorial disease and the diseases development depends on genetic pre-deposition as well as environmental factors. Environmental factors have a crucial role as cause and as triggers of asthma(-exacerbations). A considerable overlap between environmental factors that cause asthma and influence the development of asthma exists. Nonetheless causative factors cannot always be identified in a patient nor can causative and triggering factors be equated.

Allergens

Allergens are complex, biochemically active molecules including enzymes, enzyme inhibitors and proteins involved in molecular transport, regulation and cell and tissue structure. They are recognized by the immune system and provoke an immune response although they are harmless. Among the different classes of allergens, ingestionallergens and most importantly aeroallergens contribute to the development of asthma and asthma exacerbation. Food allergies have been implicated in the development of asthma [171] and peanuts or seafood might cause asthma symptoms in allergic subjects [23]. Exposure to indoor and outdoor allergens (HDM, cockroach, cat and dog dander, pollen or fungal spores) early in life was shown to play a considerable role in the development of asthma [172, 173]. Furthermore it was demonstrated that inhalation of allergens can cause bronchoconstriction and, as allergens are ubiquitously present they also contribute to airway wall remodeling. Torrego *et al.* showed that allergen challenge of atopic asthmatics results in altered composition of the basement membrane, increase of eosinophils and neutrophils, and an altered release of $TGF\beta_{1/2}$ [139]. In rodent models of allergic asthma it was demonstrated that repeated allergen-challenge results in increased release of pro-inflammatory mediators as well as (vascular) remodeling [174-179]. Doyle *et al.* show that the microvascular density in mice is increased after allergen-challenge and that this is in part due to the release of angiogenic mediators from vascular endothelial progenitor cells [180]. These findings are in accordance with another study revealing increased number and diameter of vessels in endobronchial biopsies of asthmatics after allergen challenge [181].

In Europe the most important aeroallergen are allergens derived from house dust mites (HDM). Common house dust comprises of 13 different species of HDM. The major sources of allergens are three different HDM species: the European house dust mite (*Dermatophagoides pteronyssinus),* the American house dust mite (*Dermatophagoides farinae)* and a third species *Euroglyphus maynei* [182-184]. The species *D. pteronyssinus*

and *D. farinae* are predominating and they coexist in most geographical regions [185]. About 50% of allergic adults and 80% of allergic children are sensitized against HDM-derived allergens and HDM allergens are a major trigger of asthma exacerbation [186]. HDM-derived allergens are the mites themselves (in all stages of development) and most importantly their fecal pellets, which contain the majority of immunogenic compounds [187]. The mode of action how HDM allergens contribute to the development of asthma and acute asthma attacks are incompletely understood yet. This in part due to the fact, that HDM extracts contain of a pleiotropy of different proteins and more than 20 of them were identified to induce IgE-mediated immune reactions [188]. Although all HDM-derived allergens have IgE-binding capacities, the absorption of IgE from sera of allergic subjects varies highly between the different proteins. Der p1 and p2 absorb 50% of IgE and Der p4, p5 and p6 absorb another 30% of the IgE present in the sera [189].

Besides the immunogenicity of the HDM-derived proteins they have also enzymatic functions, which can affect the immune/tissue response. Protease activity is a common feature of many allergens (fungi, animal dander, pollen). In HDM extracts the cysteine protease Der p1 cleaves epithelial tight junctions and thereby opens the door for the allergens to penetrate into the tissue [190]. This allows an uptake and processing of the HDM-derived proteins by tissue-resident dendritic cells. A recent study provided evidence that the impaired epithelial barrier is crucial for HDM-mediated pathological features of asthma (eosinophilic inflammation, goblet cell hyperplasia and AHR) but that this can be mediated independently from Ser- and Cys-protease activity [191]. It is reasonable that cleavage of tight junctions will also allow protein delivery to tissue-forming cells like BSMC. The proteases do not only have a direct effect by cleavage of tight junctions-forming proteins but they can also activate protease-activated receptors (PAR), which leads to lymphocyte infiltration to the airways [192] and results in BSMC contraction and proliferation [149, 193].

HDM extract challenge in a sheep model of chronic asthma increased the vascular density in the lungs in response to the stimulus. Furthermore, this study provided evidence of a direct correlation of airway wall vascularity and thickness revealing a direct link between angiogenesis and remodeling [194].

In order to study the effects of HDM in *in vitro* and *in vivo* systems aqueous solutions of 95% house dust mites (in all developmental stages) and 5% fecal pellets or purified/chemically synthesized Der p or Der f proteins are used. The use of purified

proteins elicits very specific functions and effects, but in daily life patients are exposed to the complete mixture of immunogenic, proteolytic and several other proteins. Therefore, the use of total HDM extract might reflect better the contribution of HDM allergens to the pathogenesis of asthma.

Pathogens

A considerable overlap between the aforementioned allergens, their implication in asthma pathogenesis, and the pathogens discussed in this chapter exists. LPS and endotoxins detected in whole HDM extracts are derived from bacteria of the Bartonella species [195]. A recent study provided evidence that recurrent wheezing in young children was associated with bacterial infections [196] and bacterial infections of the lower respiratory tract in asthmatics have been linked to symptom frequency and severity [197]. Despite the fact that several studies demonstrated the implication of bacterial infections in the pathogenesis of asthma it is not yet clear how bacterial infection influence the disease. Initiation of the innate immune response by activation of toll-like receptors (TLR) followed by the release of pro-inflammatory cytokines is one possible mechanism. Another possible mechanism is the release of TGF-β induced by bacterial LPS. Increased concentrations of TGF-β can activate fibroblasts and thereby induce an increased production of ECM proteins, which causes airway wall thickening.

Several different viruses have been associated with the development of asthma or asthma exacerbation [198]. Viral infections during early childhood have been identified as risk factors to develop asthma later in life [199, 200]. Furthermore, it has been shown that infections of human epithelial cells and fibroblasts with RV caused the release of pro-angiogenic and remodeling mediators from these cell-types and thereby caused angiogenesis *in vitro* [201, 202]. Although it is not fully understood yet how viral infections and development of asthma or asthma exacerbation are linked the results of several studies suggest that viral infections are not a cause of asthma *per se,* but rather suggest a susceptibility to both, viral infections and asthma. The tissue damage due to viral clearance is supposed to cause an environmental injury, which in turn alters the epithelial cells and thereby causes inflammation and at a later stage remodeling.

A third group of pathogens are fungi. A recent study identified 3 different fungi to play important role in the pathology of asthma (*Aspergillus, Cladosporium, Alternia*). This study provides evidence that exposure to indoor fungal spores is worsening asthma symptoms, whereas exposure to outdoor occurring spores led to symptom impairment

[203]. Due to their allergic potential fungal spores also belong to the extrinsic factors mentioned before.

Others

Besides the aforementioned factors, several other, naturally occurring stimuli can induce asthma and/or cause asthma exacerbations/attacks. Cold air, exercise, ozone, tobacco smoke or exhaust fumes are only some of those stimuli and the mechanisms how they cause bronchoconstriction are mainly unrevealed yet. The existance of many different provoking agents/circumstances further illustrates the complexity of the disease and the constant and/or unavoidable exposure to many of them highlights the need for new treatment options and curative drugs.

Genetics of asthma

Asthma is a heterogeneous disease with a variety of clinical manifestations and unknown etiology. Several approaches have been made to identify asthma susceptibility genes or the genetic background for the disease. Asthma could not be linked to a single gene or to a distinct chromosomal region. Rather, genetic studies confirm asthma to be a complex disorder. The use of genome wide associated studies (GWAS) contributed significantly to the identification of several candidate genes. In GWAS many single nucleotide polymorphisms (SNP) are compared hypothesis-free across the entire genome. The candidate genes identified by several GWAS and other types of genetic studies (candidate gene association studies and genome-wide linkage studies) can be divided into four groups:

The first group includes genes that encode proteins to sustain epithelial barrier function [204-206]. The second group is comprised of genes encoding proteins involved environmental sensing and immune detection [207-211]. Genes encoding proteins of the T_h2 mediated response and recruitment of eosinophils are represented in group three [42, 43, 208, 210, 212-216] and genes encoding proteins implicated in the tissue response are filed in group four [217-219].

A disintegrin and metalloprotease (ADAM)-33, a gene of group IV has been identified as an asthma susceptibility gene in several studies [217, 218, 220]. A recent study focused on function of ADAM-33 in airway wall remodeling in the asthmatic lung. It was demonstrated that soluble ADAM-33 can promote angiogenesis and that this effect is enhanced by environmental factors causing epithelial damage [221]. In the scope of angiogenesis-related genes SNP in the VEGF gene were identified related to atopy and asthma [222-225].

The effect of a single gene variant seems to be small and therefore suggest a complex network between interaction/combination of multiple genetic variants, environmental factors and epigenetic regulations [226]. Therefore, it is important to keep in mind that the aim of genetic studies is to identify groups/combinations of variants that reliably predict the risk of susceptibility and/or severity. Furthermore, it is important to consider the influence of environmental factors for the onset and progression of asthma.

In summary the development and exacerbation of asthma is a complex, multifactorial process, which involves genetic predisposition, epigenetic mechanism as well as environmental factors.

Angiogenesis

General

Angiogenesis is the process of blood vessel formation from existing vessels and angiogenesis depends on the balance of positive and negative modulators. During adolescence angiogenesis occurs in physiologically growing organs but in normal tissues in adults, vascular quiescence is mediated [227]. Nonetheless, quiescent endothelial cells (EC) remain their capacity to proliferate, migrate and synthesize pro-angiogenic factors and are thereby able to form new blood vessels if required or induced during pathogenesis of diseases. In healthy subjects the formation of blood vessels occurs only in the female reproductive cycle [228, 229]. Apart from that, angiogenesis is induced in wound healing after injury. It also occurs in diseases like rheumatoid arthritis (RA), solid tumors, and in chronic airway diseases such as asthma, COPD or bronchiolitis obliterans syndrome (BOS). In diseases associated with a pathological increase in vasculature, the net balance is shifted towards pro-angiogenic factors. This can be achieved through a reduction of angiostatic factors, the increase of angiogenic factors or a combination of both.

The formation of new blood vessels is a complex process involving multiple factors that either promote (angiogenic) or counteract/inhibit (angiostatic) angiogenesis. Mediators involved in the regulation of angiogenesis come from seven classes of molecules (Figure 5). VEGF, angiopoietin-1 (Ang-1), bFGF, TGF-β, PDGF; TNF-α are amongst the best studied angiogenic proteins [230, 231]. More recent studies showed that a so far not noticed class of molecules is also involved in the formation of new blood vessels - chemokines and their receptors. Chemokines are small (8 - 12 kDA) proteins that attract circulating immune cells to sites of inflammation/injury. They were first identified in 1977 with the purification of CXCL4 [232] and comprise to date of more than 50 members divided into 4 families (in regard to conserved cysteine-residues): C-, CC-, CXC-, and CX_2C-chemokines [233]. In contrast to other cytokines, all chemokines are signaling via G protein coupled receptors (GPCR). Based on the sequence of the amino acids the angiogenesis-related chemokines are either of the CC-[234] or of the CXC-[235-237] family of chemokines. The CXC-chemokines are further divided into chemokines with or without a conserved amino acid-sequence Glu-Leu-Arg (ELR) – the ELR^+ and ELR^- chemokines. Among those, ELR^+-chemokines are in favor to promote angiogenesis whereas ELR^--chemokines inhibit it. ELR^+-chemokines are signaling via one of two

receptors CXCR1 and CXCR2 but mediate their angiogenic effects through CXCR2 [238-240].

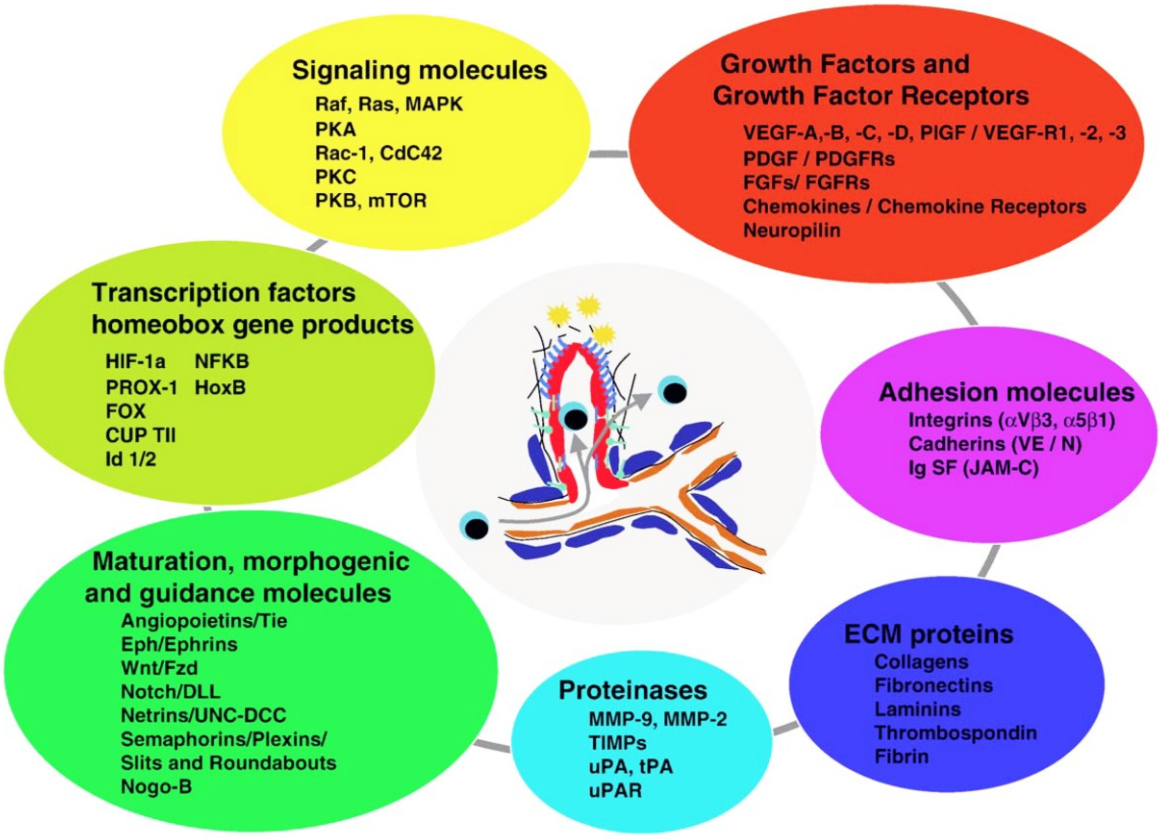

Figure 5: Classes of molecules involved in the regulation of angiogenesis. From [241]

The implication of CXC-chemokine and their corresponding receptor in neovascularization has been mainly described in the context of cancer. It has a critical role in the progression of many cancers including breast cancer, bronchogenic carcinomas, malignancies of the gastrointestinal tract, prostate cancer, ovarian cancer, glioblastoma as well as head and neck cancer [242-247]. Furthermore, it has been shown the CXC-chemokines are involved in several lung diseases, which involve altered angiogenesis such as idiopathic pulmonary fibrosis (IPF), BOS or acute respiratory distress syndrome (ARDS) [248-250].

Angiogenesis in asthma

Airway wall remodeling in asthma comprises of several features of which one is angiogenesis. The first incidence that vascular alterations occur in the airways of asthma patients was published in 1960. Besides other features of airway wall remodeling, Dunhill reported the dilatation of blood vessels in lung tissue from asthma patients [251]. It has been shown *ex vivo* as well as *in vivo* that the sub-epithelial layers of the lungs of asthmatic patients exhibit an increased vascular density [134, 252-254]. Besides the increased vascular density other prominent alterations of the bronchial vasculature have been described. Several studies in humans and in animal models of asthma show that the blood flow in the asthmatic lung is increased [255-258]. Furthermore, not only the vessel number is increased in asthmatics it has also been also demonstrated that the vasculature in asthmatics is more permeable, which in turn led to edema [259]. The increased permeability of vessels has another important effect. Vessel leakage is an important prerequisite for tissue inflammation, as the extravasation of inflammatory cells to the tissue requires transmigration through the endothelium.

To date the factors and mechanisms that induce angiogenesis in the airways of asthma patients are not fully understood. Recent studies were able to provide evidence that VEGF is an important factor in mediating angiogenesis in asthmatics [128, 260-262]. Furthermore other pro-angiogenic factors (bFGF, angiogenin) could be attributed to vascular alterations occurring in the airways of asthma patients [128]. Besides the observation, that asthmatics have increased vessel number and size, several mediators of angiogenesis have been demonstrated to be elevated in asthma. It has been demonstrated that the submucosa of asthma patients contains more VEGF, bFGF and angiogenin compared to control subjects [128].

The aforementioned alterations in the bronchial vasculature contribute significantly to the pathology of asthma. They are correlated to asthma severity by influencing airflow limitation [252, 263, 264] and bronchial hyperresponsiveness [264-266]

The correlations between the increased vascularization, airway wall remodeling, and disease pathology remain incompletely understood but recent studies provide evidence that vascular density and asthma severity are linked [128, 267-269].

The increased number of vessels adjacent to the BM and within the *lamina propria* might contribute to remodeling and asthma pathology in different ways. In response to vasodilating stimuli and in an inflammatory condition the increased number of vessels will

leak, causing tissue edema and thereby narrowing of the airways. The increased vascular permeability leads to plasma exudation into the surrounding tissue and might thereby submerge factors of inflammation and remodeling into the lung tissue. This effect might be amplified by proteoglycans, which have been deposited during BM thickening. These components of the ECM are able to sequestrate the plasma and thereby "store" mediators of inflammation as well as amplifying edematous effects. Last but not least an increased number of vessels results in increased blood supply to the lungs. This might supply the hyperplastic BSM layer with the required nutritions and oxygen.

Hypoxia

General

Hypoxia describes the diminished availability of oxygen in body tissues. It is observed in many physiological conditions such as embryonic development and differentiation or wound healing. Hypoxia may also occur as the consequences of several pathologies including tumor progression, interstitial lung diseases, ARDS, COPD, neoplasms, arteriosclerosis and asthma [270-272]. Hypoxia is always associated with the activation of proteins of the hypoxia inducible factor (HIF)-family. The activation of the HIF-pathway allows the cells to adapt to external factors (in this case the low oxygen concentration). The major component of hypoxia signaling is the transcription factor HIF, which is comprised of two subunits (HIF-α and HIF-β). The HIF-1β subunit is constitutively expressed and hetero-dimerizes with one out of its three potential binding partners HIF-1α, HIF-2α or HIF-3α. All HIF-α proteins have a very short half-life under normal oxygen conditions (normoxia) and are, by hydroxylation of two proline-residues in the oxygen-dependent degradation domain (ODDD), constantly targeted for proteasomal degradation [273]. Ubiquitinylation of hydroxylated HIFα is mediated by the von Hippel-Lindau protein (VHL) [274]. Under low oxygen concentrations hydroxylation is impaired and results in increased levels of HIF-α. HIF-α is then translocated to the nucleus, dimerizes with the HIF-β subunit and the active transcription factor binds to hypoxia response elements (HRE) in the promotor regions of downstream target genes [273]. Roughly 100 targets genes of the oxygen-sensing pathway are known and their corresponding proteins are implicated in tissue angiogenesis, erythropoiesis ventilation, glycolysis, and autophagy [275]. The activation of the HIF-dependent pathway is illustrated in Figure 6.

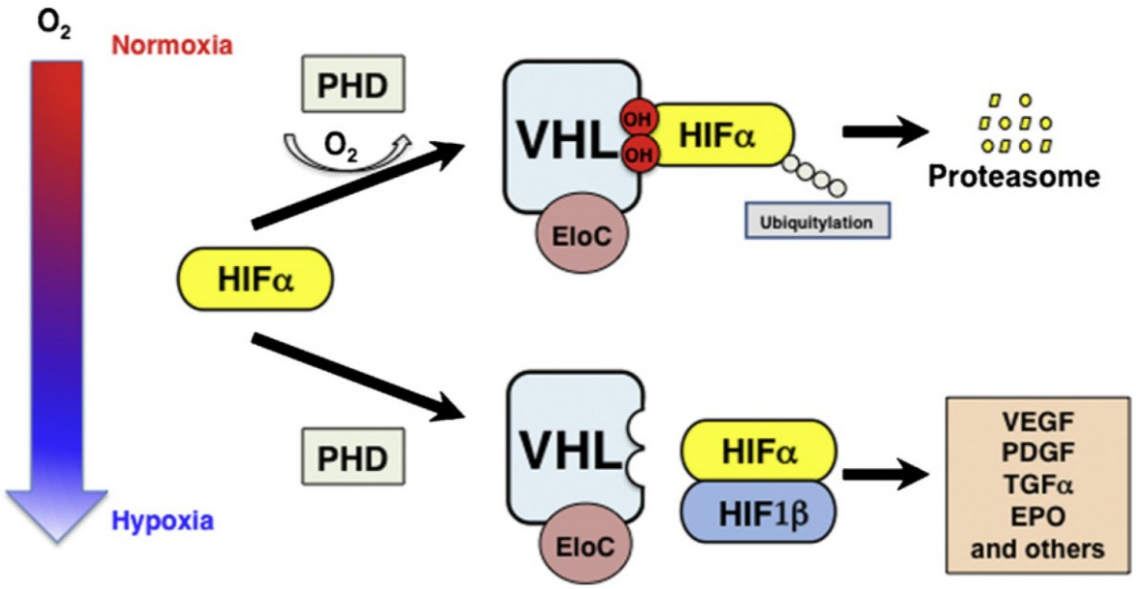

Figure 6: Schematic illustration of the HIF oxygen-sensing pathway. Under normal oxygenation HIF-α is hydroxylated, ubiqitinylated and finally proteasomal degraded. Low oxygen concentrations result in HIF stabilization and transcription of HIF target genes. Image from [276].

Potential role of hypoxia in asthma

A role for hypoxia in the pathogenesis of asthma has been acknowledged only recently. In a rat model of asthma HIF is pivotal in promoting airway inflammation and elevated levels of this factor have been detected in endobronchial biopsies and BALF of asthmatics [271]. Furthermore, increased levels of the "oxygen-dependent" HIF subunits (HIF-1α and HIF-1β) have been observed in lung tissues, epithelial cells and BALF of asthmatic patients. Here, the HIF expression and VEGF level were correlated [277].

Although little is known about the impact or the role of hypoxia in the pathology of asthma or remodeling processes in asthma, hypoxia has been recognized in other (inflammatory) pulmonary diseases. Pulmonary disease associated with reduced ventilation, airway obstruction, intra-alveolar exudates and/or edematous septal thickening can lead to impaired gas exchange and thereby to reduced tissue and/or blood oxygenation. Cystic fibrosis (CF) is an autosomal recessive disorder cause by mutation in the Cystic fibrosis transmembrane conductance regulator (CFTR) gene coding for a chloride-channel. The disease is characterized by altered mucus composition, which results in impaired mucociliar clearance and the subsequent formation of mucus plaques. For CF it is suggested that, besides the increased energy consumption by bacteria (comorbidity), the mucus restricts the O_2 diffusion and thereby causing hypoxia [278]. An example how reduced ventilation drive and airway obstruction might cause hypoxia is COPD. The

decreased oxygen transport leads to hypoxia in the alveoli of COPD patients and is accompanied by several alteration of the lung tissue including vascular alterations [272].

Although several studies in animal models and from humans demonstrated an increased activation of hypoxia signaling cascade and its target genes the mechanisms of hypoxia-induced signaling and its contribution to asthma pathology are far from understood. To understand hypoxia and angiogenesis in the remodeling process observed in the lung of asthma patients further investigation is needed.

Current asthma therapies

First and foremost it should be noted that there is no cure for asthma. Fortunately most of the symptoms can be controlled quite effectively using several regimes of drugs. To date the standard therapy for asthmatic patients is inhaled or orally administered GC or a combination of GC and long acting β_2 adrenergic receptor agonists (LABA).

GC reduced the ongoing inflammation [79, 279, 280], improved the lung function [279], and lessened AHR [281, 282]. The mode of action of GC is via trans-activation of anti-inflammatory as well as via trans-repression of pro-inflammatory mediators [14, 283]. LABA caused muscle relaxation and reduced bronchoconstriction by increasing cAMP level. LABA are used as part of the combination therapy in severe or difficult to treat asthmatics. In combination with LABA, the efficacy of GC can be enhanced, and the patient needs less GC to reach the same relief. Reducing the dose of GC by the use of combination therapy also reduces the systemic side effects such as osteoporosis, arterial hypertension, glaucoma, skin thinning, etc., which high doses of GC might have [284]. Although GC are quite effective in reducing the ongoing airway inflammation in asthmatics they do not affect BSMC proliferation [285]. The effect of GC on the airway epithelium is a controversial issue. Some studies showed a beneficial effect of GC on the epithelium [286] but others could not confirm these findings [287, 288]. Another controversial issue is the influence of GC on BM alterations. Some studies showed a reversion of BM thickening [289] and other did not find an effect [290]. Treatment of bronchial epithelial cells with GC and/or LABA *in vitro* caused a decrease in the release of pro-inflammatory and pro-angiogenic mediators [291, 292]. The beneficial effects of GC on vascular remodeling have been addressed in a murine model showing a significant inhibitory effect on angiogenesis [293, 294]. GC treatment has similar effects in asthmatic patients. Reduced vessels numbers, sprouting, and VEGF level have been observed in GC-treated asthma patients [295, 296]. In summary the use of GC is only partially stopping/reversing the remodeling and some features of remodeling persist.

The previously described heterogeneity of asthma is also reflected by another important fact: Asthma patients react differently to the existing asthma medications. In about 10% of the cases the available therapies do not have a satisfying effect [17].

Several alternative strategies to treat severe or steroid resistant asthma exist or are in clinical trials. One example is the use of a humanized, neutralizing IgE-specific antibody in severe allergic asthma. This antibody binds free IgE and thereby reduces cell-bound IgE. It

has been shown that under this treatment the dose of GC could be reduced [297] and on long term the emergency visit rate was decreased as well as asthma exacerbation [298]. Another strategy, the administration of IL-4 receptor or IL-5 reducing antibodies failed in large clinical trials [299, 300] or did not have beneficial effects on the clinical outcome [81, 301, 302]. Currently there is a clinical trial going on testing a CCR4-specifc antibody in asthmatics. This antibody had beneficial effects in T cell leukemia by depletion of the T cells. As T cell recruitment play an important role in asthma pathogenesis it efficacy in the treatment of asthma will be tested in the future [303].

Although Leukotriene-receptor-antagonists and 5-lipooxygenase inhibitors are no first-line treatment in asthma they are used with good results in difficult-to-treat patients [304-306]. Furthermore, anti-inflammatory drugs like muscarinic receptor antagonists, which have been used in the treatment of COPD for instance, have been shown in key clinical trials to have beneficial effects in asthma [307].

A new, invasive therapy option has been use in cases of uncontrolled asthma: thermoplasty. With this treatment locally restricted heating of the muscle tissue significantly reduces the mass of BSM with a laser. This technique has been used in several cases of severe asthma and the symptoms in those patients could be markedly reduced [308]. The efficacy of this therapy emphasizes the role of BSMC in the pathogenesis of asthma and a non-invasive reduction of BSM or slow down/reversal of remodeling processes might provide new therapeutic strategies. A long-term follow up of patients receiving thermoplasty revealed a stable lung function [308]. The beneficial effect of BSM mass reduction seems to be an attractive strategy to treat asthma patients.

In conclusion, several treatment options exist, targeting almost all features that contribute to the pathology of asthma. Furthermore, several new treatments are in clinical trials either to use existing drugs or to study the effect of new compounds. Unfortunately there is no cure for asthma yet and treatment regimens do not meet the needs of every patient. Therefore, studies are need to prove efficacy of existing therapies, to provide guidelines/biomarkers to estimate treatment efficacy in a single patient and the search for a cure for the disease still goes on.

In summary, this introduction points out the complexity of asthma with emphasis on different cellular aspects and mechanisms involved in its pathology. In summary, asthma

can be described as an environmental mediated disorder with a strong impact of genetic predeposition on the development and progression of the disease. The immune system and its mediating cells play an important role in the perpetuation of asthma symptoms and in acute exacerbations, but the impact of the resident cells of the lung tissue, as well as the components of the extracellular matrix is increasingly recognized. It is important to keep in mind that asthma is a complex, heterogeneous syndrome with several different clinical manifestations and varying severity. To date there is no cure for the disease although symptoms can be controlled in about 90% of the cases. This increasing complexity of the disease makes asthma a disease that is very hard to understand. The Lancet summarized this as follows:

> "Progress in understanding asthma is slow; treatment can be difficult and response unpredictable; and prevention and cure are still a pipedream." (The Lancet, 2008)

In conclusion, the cells and mechanisms described throughout the introduction may all contribute to the onset, persistence and outcome of asthma. Treatment of the disease, or the possibility of developing cures, should take the heterogeneity into account, or focus on holistic approaches that integrate these mechanisms.

References

1. Braman SS. The global burden of asthma. Chest. 2006;130(1 Suppl):4S-12S.

2. Masoli M, Fabian D, Holt S, Beasley R. The global burden of asthma: executive summary of the GINA Dissemination Committee report. Allergy. 2004;59(5):469-78.

3. Worldwide variations in the prevalence of asthma symptoms: the International Study of Asthma and Allergies in Childhood (ISAAC). The European respiratory journal : official journal of the European Society for Clinical Respiratory Physiology. 1998;12(2):315-35.

4. Variations in the prevalence of respiratory symptoms, self-reported asthma attacks, and use of asthma medication in the European Community Respiratory Health Survey (ECRHS). The European respiratory journal : official journal of the European Society for Clinical Respiratory Physiology. 1996;9(4):687-95.

5. Worldwide variation in prevalence of symptoms of asthma, allergic rhinoconjunctivitis, and atopic eczema: ISAAC. The International Study of Asthma and Allergies in Childhood (ISAAC) Steering Committee. Lancet. 1998;351(9111):1225-32.

6. To T, Stanojevic S, Moores G, Gershon AS, Bateman ED, Cruz AA, et al. Global asthma prevalence in adults: findings from the cross-sectional world health survey. BMC public health. 2012;12:204.

7. Devereux G. The increase in the prevalence of asthma and allergy: food for thought. Nature reviews Immunology. 2006;6(11):869-74.

8. Doull IJ, Williams AA, Freezer NJ, Holgate ST. Descriptive study of cough, wheeze and school absence in childhood. Thorax. 1996;51(6):630-1.

9. Newacheck PW, Halfon N. Prevalence, impact, and trends in childhood disability due to asthma. Archives of pediatrics & adolescent medicine. 2000;154(3):287-93.

10. Lodddenkemper R. European Lung White Book. The first comprehensive survey on respiratory health in Europe.2003.

11. Martin AJ, Landau LI, Phelan PD. Asthma from childhood at age 21: the patient and his disease. Br Med J (Clin Res Ed). 1982;284(6313):380-2.

12. Gerritsen J, Koeter GH, Postma DS, Schouten JP, Knol K. Prognosis of asthma from childhood to adulthood. The American review of respiratory disease. 1989;140(5):1325-30.

13. Gerritsen J, Koeter GH, Postma DS, Schouten JP, van Aalderen WM, Knol K. Airway responsiveness in childhood as a predictor of the outcome of asthma in adulthood. The American review of respiratory disease. 1991;143(6):1468-9.

14. Holgate ST, Polosa R. Treatment strategies for allergy and asthma. Nature reviews Immunology. 2008;8(3):218-30.

15. Mitchell EA. International trends in hospital admission rates for asthma. Archives of disease in childhood. 1985;60(4):376-8.

16. Beasley R. The burden of asthma with specific reference to the United States. The Journal of allergy and clinical immunology. 2002;109(5 Suppl):S482-9.

17. Reddy RC. Severe asthma: approach and management. Postgraduate medical journal. 2008;84(989):115-20; quiz 9.

18. Wenzel S. Severe asthma in adults. American journal of respiratory and critical care medicine. 2005;172(2):149-60.

19. Wenzel S. Severe asthma: from characteristics to phenotypes to endotypes. Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology. 2012;42(5):650-8.

20. Papiris S, Kotanidou A, Malagari K, Roussos C. Clinical review: severe asthma. Crit Care. 2002;6(1):30-44.

21. Rackemann FM. A clinical study of one hundred and fifty cases of bronchial asthma. Archives of Internal Medicine. 1918;XXII(4):517-52.

22. McFadden ER, Gilbert IA. Asthma. New England Journal of Medicine. 1992;327(27):1928-37.

23. Arbes SJ, Jr., Gergen PJ, Vaughn B, Zeldin DC. Asthma cases attributable to atopy: results from the Third National Health and Nutrition Examination Survey. The Journal of allergy and clinical immunology. 2007;120(5):1139-45.

24. Robinson DS, Hamid Q, Ying S, Tsicopoulos A, Barkans J, Bentley AM, et al. Predominant TH2-like bronchoalveolar T-lymphocyte population in atopic asthma. The New England journal of medicine. 1992;326(5):298-304.

25. Kovac K, Dodig S, Tjesic-Drinkovic D, Raos M. Correlation between asthma severity and serum IgE in asthmatic children sensitized to Dermatophagoides pteronyssinus. Archives of medical research. 2007;38(1):99-105.

26. Pearce N, Pekkanen J, Beasley R. How much asthma is really attributable to atopy? Thorax. 1999;54(3):268-72.

27. Green RH, Brightling CE, Woltmann G, Parker D, Wardlaw AJ, Pavord ID. Analysis of induced sputum in adults with asthma: identification of subgroup with isolated sputum neutrophilia and poor response to inhaled corticosteroids. Thorax. 2002;57(10):875-9.

28. Beasley R, Pekkanen J, Pearce N. Has the role of atopy in the development of asthma been over-emphasized? Pediatric pulmonology. 2001;Suppl 23:149-50.

29. Brightling CE, Symon FA, Birring SS, Bradding P, Wardlaw AJ, Pavord ID. Comparison of airway immunopathology of eosinophilic bronchitis and asthma. Thorax. 2003;58(6):528-32.

30. Bochner BS, Busse WW. Allergy and asthma. The Journal of allergy and clinical immunology. 2005;115(5):953-9.

31. Wenzel SE, Schwartz LB, Langmack EL, Halliday JL, Trudeau JB, Gibbs RL, et al. Evidence that severe asthma can be divided pathologically into two inflammatory subtypes with distinct physiologic and clinical characteristics. American journal of respiratory and critical care medicine. 1999;160(3):1001-8.

32. Tsoumakidou M, Papadopouli E, Tzanakis N, Siafakas NM. Airway inflammation and cellular stress in noneosinophilic atopic asthma. Chest. 2006;129(5):1194-202.

33. Wark PA, Johnston SL, Moric I, Simpson JL, Hensley MJ, Gibson PG. Neutrophil degranulation and cell lysis is associated with clinical severity in virus-induced asthma. The European respiratory journal : official journal of the European Society for Clinical Respiratory Physiology. 2002;19(1):68-75.

34. Lemiere C, Ernst P, Olivenstein R, Yamauchi Y, Govindaraju K, Ludwig MS, et al. Airway inflammation assessed by invasive and noninvasive means in severe asthma: eosinophilic and noneosinophilic phenotypes. The Journal of allergy and clinical immunology. 2006;118(5):1033-9.

35. Tonnel AB, Gosset P, Tillie-Leblond I. Characteristics of the Inflammatory response in bronchial lavage fluids from patients with status asthmaticus. International archives of allergy and immunology. 2001;124(1-3):267-71.

36. Carroll N, Carello S, Cooke C, James A. Airway structure and inflammatory cells in fatal attacks of asthma. The European respiratory journal : official journal of the European Society for Clinical Respiratory Physiology. 1996;9(4):709-15.

37. Haldar P, Pavord ID, Shaw DE, Berry MA, Thomas M, Brightling CE, et al. Cluster analysis and clinical asthma phenotypes. American journal of respiratory and critical care medicine. 2008;178(3):218-24.

38. Pavord ID. Complex airway disease: an approach to assessment and management. The Lancet. 2013;1(1):84-90.

39. Moore WC, Meyers DA, Wenzel SE, Teague WG, Li H, Li X, et al. Identification of asthma phenotypes using cluster analysis in the Severe Asthma Research Program. American journal of respiratory and critical care medicine. 2010;181(4):315-23.

40. Nguyen LT, Lim S, Oates T, Chung KF. Increase in airway neutrophils after oral but not inhaled corticosteroid therapy in mild asthma. Respiratory medicine. 2005;99(2):200-7.

41. Homer RJ, Elias JA. Airway remodeling in asthma: therapeutic implications of mechanisms. Physiology (Bethesda). 2005;20:28-35.

42. Barnes PJ. The cytokine network in asthma and chronic obstructive pulmonary disease. The Journal of clinical investigation. 2008;118(11):3546-56.

43. Kabesch M, Tzotcheva I, Carr D, Hofler C, Weiland SK, Fritzsch C, et al. A complete screening of the IL4 gene: novel polymorphisms and their association with asthma and IgE in childhood. The Journal of allergy and clinical immunology. 2003;112(5):893-8.

44. Basehore MJ, Howard TD, Lange LA, Moore WC, Hawkins GA, Marshik PL, et al. A comprehensive evaluation of IL4 variants in ethnically diverse populations: association of total serum IgE levels and asthma in white subjects. The Journal of allergy and clinical immunology. 2004;114(1):80-7.

45. Galli SJ, Tsai M, Piliponsky AM. The development of allergic inflammation. Nature. 2008;454(7203):445-54.

46. Kucharewicz I, Bodzenta-Lukaszyk A, Buczko W. Experimental asthma in rats. Pharmacological reports : PR. 2008;60(6):783-8.

47. Cohn L, Elias JA, Chupp GL. Asthma: mechanisms of disease persistence and progression. Annual review of immunology. 2004;22:789-815.

48. Molet S, Hamid Q, Davoine F, Nutku E, Taha R, Page N, et al. IL-17 is increased in asthmatic airways and induces human bronchial fibroblasts to produce cytokines. The Journal of allergy and clinical immunology. 2001;108(3):430-8.

49. Al-Ramli W, Prefontaine D, Chouiali F, Martin JG, Olivenstein R, Lemiere C, et al. T(H)17-associated cytokines (IL-17A and IL-17F) in severe asthma. The Journal of allergy and clinical immunology. 2009;123(5):1185-7.

50. Pene J, Chevalier S, Preisser L, Venereau E, Guilleux MH, Ghannam S, et al. Chronically inflamed human tissues are infiltrated by highly differentiated Th17 lymphocytes. J Immunol. 2008;180(11):7423-30.

51. Hartl D, Koller B, Mehlhorn AT, Reinhardt D, Nicolai T, Schendel DJ, et al. Quantitative and functional impairment of pulmonary CD4+CD25hi regulatory T cells in pediatric asthma. The Journal of allergy and clinical immunology. 2007;119(5):1258-66.

52. Lin YL, Shieh CC, Wang JY. The functional insufficiency of human CD4+CD25 high T-regulatory cells in allergic asthma is subjected to TNF-alpha modulation. Allergy. 2008;63(1):67-74.

53. Chai SK, Altman GM, Yazdanbakhsh M, Tsuji J, Godat L, Takaro TK. Production of interleukin 10 and transforming growth factor beta in concomitant allergy and autoimmunity. Annals of allergy, asthma &

immunology : official publication of the American College of Allergy, Asthma, & Immunology. 2005;94(2):279-85.

54. Francis JN, Till SJ, Durham SR. Induction of IL-10+CD4+CD25+ T cells by grass pollen immunotherapy. The Journal of allergy and clinical immunology. 2003;111(6):1255-61.

55. Ono M. Molecular links between tumor angiogenesis and inflammation: inflammatory stimuli of macrophages and cancer cells as targets for therapeutic strategy. Cancer science. 2008;99(8):1501-6.

56. Naldini A, Pucci A, Bernini C, Carraro F. Regulation of angiogenesis by Th1- and Th2-type cytokines. Current pharmaceutical design. 2003;9(7):511-9.

57. Aurora AB, Baluk P, Zhang D, Sidhu SS, Dolganov GM, Basbaum C, et al. Immune complex-dependent remodeling of the airway vasculature in response to a chronic bacterial infection. J Immunol. 2005;175(10):6319-26.

58. Du JW, Xu KY, Fang LY, Qi XL. Interleukin-17, produced by lymphocytes, promotes tumor growth and angiogenesis in a mouse model of breast cancer. Molecular medicine reports. 2012;6(5):1099-102.

59. Brightling CE, Bradding P, Symon FA, Holgate ST, Wardlaw AJ, Pavord ID. Mast-cell infiltration of airway smooth muscle in asthma. The New England journal of medicine. 2002;346(22):1699-705.

60. Laitinen LA, Laitinen A, Haahtela T. Airway mucosal inflammation even in patients with newly diagnosed asthma. The American review of respiratory disease. 1993;147(3):697-704.

61. Heard BE, Dewar A, Nunn AJ, Kay AB. Heterogeneous ultrastructure of human bronchial mast cells: morphometric subdivision of cell types and evidence for a degranulation gradient. American journal of respiratory cell and molecular biology. 1990;3(1):71-8.

62. Pesci A, Foresi A, Bertorelli G, Chetta A, Olivieri D. Histochemical characteristics and degranulation of mast cells in epithelium and lamina propria of bronchial biopsies from asthmatic and normal subjects. The American review of respiratory disease. 1993;147(3):684-9.

63. Bradding P, Okayama Y, Howarth PH, Church MK, Holgate ST. Heterogeneity of human mast cells based on cytokine content. J Immunol. 1995;155(1):297-307.

64. Bradding P, Walls AF, Holgate ST. The role of the mast cell in the pathophysiology of asthma. The Journal of allergy and clinical immunology. 2006;117(6):1277-84.

65. Okayama Y, Petit-Frere C, Kassel O, Semper A, Quint D, Tunon-de-Lara MJ, et al. IgE-dependent expression of mRNA for IL-4 and IL-5 in human lung mast cells. J Immunol. 1995;155(4):1796-808.

66. Okayama Y, Tkaczyk C, Metcalfe DD, Gilfillan AM. Comparison of Fc epsilon RI- and Fc gamma RI-mediated degranulation and TNF-alpha synthesis in human mast cells: selective utilization of phosphatidylinositol-3-kinase for Fc gamma RI-induced degranulation. European journal of immunology. 2003;33(5):1450-9.

67. Ribatti D, Crivellato E. Immune cells and angiogenesis. Journal of cellular and molecular medicine. 2009;13(9A):2822-33.

68. Ribatti D, Crivellato E. Mast cells, angiogenesis, and tumour growth. Biochimica et biophysica acta. 2012;1822(1):2-8.

69. Wilson SJ, Shute JK, Holgate ST, Howarth PH, Bradding P. Localization of interleukin (IL) -4 but not IL-5 to human mast cell secretory granules by immunoelectron microscopy. Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology. 2000;30(4):493-500.

70. Inman MD, Watson R, Cockcroft DW, Wong BJ, Hargreave FE, O'Byrne PM. Reproducibility of allergen-induced early and late asthmatic responses. The Journal of allergy and clinical immunology. 1995;95(6):1191-5.

71. Gauvreau GM, Lee JM, Watson RM, Irani AM, Schwartz LB, O'Byrne PM. Increased numbers of both airway basophils and mast cells in sputum after allergen inhalation challenge of atopic asthmatics. American journal of respiratory and critical care medicine. 2000;161(5):1473-8.

72. Kariyawasam HH, Robinson DS. The eosinophil: the cell and its weapons, the cytokines, its locations. Seminars in respiratory and critical care medicine. 2006;27(2):117-27.

73. Nakagome K, Matsushita S, Nagata M. Neutrophilic inflammation in severe asthma. International archives of allergy and immunology. 2012;158 Suppl 1:96-102.

74. Luna-Gomes T, Bozza PT, Bandeira-Melo C. Eosinophil recruitment and activation: the role of lipid mediators. Frontiers in pharmacology. 2013;4:27.

75. Robinson DS, North J, Zeibecoglou K, Ying S, Meng Q, Rankin S, et al. Eosinophil development and bone marrow and tissue eosinophils in atopic asthma. International archives of allergy and immunology. 1999;118(2-4):98-100.

76. Williams TJ, Jose PJ. Role of eotaxin and related CC chemokines in allergy and asthma. Chemical immunology. 2000;78:166-77.

77. Wenzel SE. Eosinophils in asthma--closing the loop or opening the door? The New England journal of medicine. 2009;360(10):1026-8.

78. Kay AB. The role of eosinophils in the pathogenesis of asthma. Trends in molecular medicine. 2005;11(4):148-52.

79. Djukanovic R, Wilson JW, Britten KM, Wilson SJ, Walls AF, Roche WR, et al. Effect of an inhaled corticosteroid on airway inflammation and symptoms in asthma. The American review of respiratory disease. 1992;145(3):669-74.

80. Djukanovic R, Homeyard S, Gratziou C, Madden J, Walls A, Montefort S, et al. The effect of treatment with oral corticosteroids on asthma symptoms and airway inflammation. American journal of respiratory and critical care medicine. 1997;155(3):826-32.

81. Flood-Page PT, Menzies-Gow AN, Kay AB, Robinson DS. Eosinophil's role remains uncertain as anti-interleukin-5 only partially depletes numbers in asthmatic airway. American journal of respiratory and critical care medicine. 2003;167(2):199-204.

82. Puxeddu I, Alian A, Piliponsky AM, Ribatti D, Panet A, Levi-Schaffer F. Human peripheral blood eosinophils induce angiogenesis. The international journal of biochemistry & cell biology. 2005;37(3):628-36.

83. Puxeddu I, Ribatti D, Crivellato E, Levi-Schaffer F. Mast cells and eosinophils: a novel link between inflammation and angiogenesis in allergic diseases. The Journal of allergy and clinical immunology. 2005;116(3):531-6.

84. Radeau T, Chavis C, Damon M, Michel FB, Crastes de Paulet A, Godard PH. Enhanced arachidonic acid metabolism and human neutrophil migration in asthma. Prostaglandins, leukotrienes, and essential fatty acids. 1990;41(2):131-8.

85. Boulet LP, Turcotte H, Boutet M, Montminy L, Laviolette M. Influence of natural antigenic exposure on expiratory flows, methacholine responsiveness, and airway inflammation in mild allergic asthma. The Journal of allergy and clinical immunology. 1993;91(4):883-93.

86. Barbato A, Panizzolo C, Gheno M, Sainati L, Favero E, Faggian D, et al. Bronchoalveolar lavage in asthmatic children: evidence of neutrophil activation in mild-to-moderate persistent asthma. Pediatric allergy and immunology : official publication of the European Society of Pediatric Allergy and Immunology. 2001;12(2):73-7.

87. Frangova V, Sacco O, Silvestri M, Oddera S, Balbo A, Crimi E, et al. BAL neutrophilia in asthmatic patients. A by-product of eosinophil recruitment? Chest. 1996;110(5):1236-42.

88. Amin K, Ludviksdottir D, Janson C, Nettelbladt O, Bjornsson E, Roomans GM, et al. Inflammation and structural changes in the airways of patients with atopic and nonatopic asthma. BHR Group. American journal of respiratory and critical care medicine. 2000;162(6):2295-301.

89. Monteseirin J. Neutrophils and asthma. J Investig Allergol Clin Immunol. 2009;19(5):340-54.

90. Dubravec DB, Spriggs DR, Mannick JA, Rodrick ML. Circulating human peripheral blood granulocytes synthesize and secrete tumor necrosis factor alpha. Proceedings of the National Academy of Sciences of the United States of America. 1990;87(17):6758-61.

91. Bazzoni F, Cassatella MA, Rossi F, Ceska M, Dewald B, Baggiolini M. Phagocytosing neutrophils produce and release high amounts of the neutrophil-activating peptide 1/interleukin 8. The Journal of experimental medicine. 1991;173(3):771-4.

92. Grenier A, Chollet-Martin S, Crestani B, Delarche C, El Benna J, Boutten A, et al. Presence of a mobilizable intracellular pool of hepatocyte growth factor in human polymorphonuclear neutrophils. Blood. 2002;99(8):2997-3004.

93. Devalaraja RM, Nanney LB, Du J, Qian Q, Yu Y, Devalaraja MN, et al. Delayed wound healing in CXCR2 knockout mice. The Journal of investigative dermatology. 2000;115(2):234-44.

94. Cacalano G, Lee J, Kikly K, Ryan AM, Pitts-Meek S, Hultgren B, et al. Neutrophil and B cell expansion in mice that lack the murine IL-8 receptor homolog. Science. 1994;265(5172):682-4.

95. Huber Hl KK. The pathology of bronchial asthma. Archives of Internal Medicine. 1922;30(6):689-760.

96. Kuwano K, Bosken CH, Pare PD, Bai TR, Wiggs BR, Hogg JC. Small airways dimensions in asthma and in chronic obstructive pulmonary disease. The American review of respiratory disease. 1993;148(5):1220-5.

97. Little SA, Sproule MW, Cowan MD, Macleod KJ, Robertson M, Love JG, et al. High resolution computed tomographic assessment of airway wall thickness in chronic asthma: reproducibility and relationship with lung function and severity. Thorax. 2002;57(3):247-53.

98. Niimi A, Matsumoto H, Amitani R, Nakano Y, Mishima M, Minakuchi M, et al. Airway wall thickness in asthma assessed by computed tomography. Relation to clinical indices. American journal of respiratory and critical care medicine. 2000;162(4 Pt 1):1518-23.

99. Lambrecht BN, Hammad H. The airway epithelium in asthma. Nature medicine. 2012;18(5):684-92.

100. Westergren-Thorsson G, Larsen K, Nihlberg K, Andersson-Sjoland A, Hallgren O, Marko-Varga G, et al. Pathological airway remodelling in inflammation. The clinical respiratory journal. 2010;4 Suppl 1:1-8.

101. Lai HY, Rogers DF. Mucus hypersecretion in asthma: intracellular signalling pathways as targets for pharmacotherapy. Current opinion in allergy and clinical immunology. 2010;10(1):67-76.

102. Bai TR, Knight DA. Structural changes in the airways in asthma: observations and consequences. Clin Sci (Lond). 2005;108(6):463-77.

103. Bentley AM, Menz G, Storz C, Robinson DS, Bradley B, Jeffery PK, et al. Identification of T lymphocytes, macrophages, and activated eosinophils in the bronchial mucosa in intrinsic asthma. Relationship to symptoms and bronchial responsiveness. The American review of respiratory disease. 1992;146(2):500-6.

104. Grunstein MM, Hakonarson H, Leiter J, Chen M, Whelan R, Grunstein JS, et al. IL-13-dependent autocrine signaling mediates altered responsiveness of IgE-sensitized airway smooth muscle. American journal of physiology Lung cellular and molecular physiology. 2002;282(3):L520-8.

105. Hakonarson H, Grunstein MM. Autocrine regulation of airway smooth muscle responsiveness. Respiratory physiology & neurobiology. 2003;137(2-3):263-76.

106. Hakonarson H, Maskeri N, Carter C, Grunstein MM. Regulation of TH1- and TH2-type cytokine expression and action in atopic asthmatic sensitized airway smooth muscle. The Journal of clinical investigation. 1999;103(7):1077-87.

107. Seki E, Yoshizumi M, Tanaka R, Ryo A, Ishioka T, Tsukagoshi H, et al. Cytokine profiles, signalling pathways and effects of fluticasone propionate in respiratory syncytial virus-infected human foetal lung fibroblasts. Cell biology international. 2013.

108. Barrett NA, Austen KF. Innate cells and T helper 2 cell immunity in airway inflammation. Immunity. 2009;31(3):425-37.

109. Baena-Cagnani C, Rossi GA, Canonica GW. Airway remodelling in children: when does it start? Current opinion in allergy and clinical immunology. 2007;7(2):196-200.

110. Bossley CJ, Fleming L, Gupta A, Regamey N, Frith J, Oates T, et al. Pediatric severe asthma is characterized by eosinophilia and remodeling without T(H)2 cytokines. The Journal of allergy and clinical immunology. 2012.

111. Cokugras H, Akcakaya N, Seckin, Camcioglu Y, Sarimurat N, Aksoy F. Ultrastructural examination of bronchial biopsy specimens from children with moderate asthma. Thorax. 2001;56(1):25-9.

112. Jenkins HA, Cherniack R, Szefler SJ, Covar R, Gelfand EW, Spahn JD. A comparison of the clinical characteristics of children and adults with severe asthma. Chest. 2003;124(4):1318-24.

113. Martinez FD, Wright AL, Taussig LM, Holberg CJ, Halonen M, Morgan WJ. Asthma and wheezing in the first six years of life. The Group Health Medical Associates. The New England journal of medicine. 1995;332(3):133-8.

114. Saglani S, Malmstrom K, Pelkonen AS, Malmberg LP, Lindahl H, Kajosaari M, et al. Airway remodeling and inflammation in symptomatic infants with reversible airflow obstruction. American journal of respiratory and critical care medicine. 2005;171(7):722-7.

115. Saglani S, Payne DN, Zhu J, Wang Z, Nicholson AG, Bush A, et al. Early detection of airway wall remodeling and eosinophilic inflammation in preschool wheezers. American journal of respiratory and critical care medicine. 2007;176(9):858-64.

116. Tsartsali L, Hislop AA, McKay K, James AL, Elliot J, Zhu J, et al. Development of the bronchial epithelial reticular basement membrane: relationship to epithelial height and age. Thorax. 2011;66(4):280-5.

117. Kaur D, Saunders R, Berger P, Siddiqui S, Woodman L, Wardlaw A, et al. Airway smooth muscle and mast cell-derived CC chemokine ligand 19 mediate airway smooth muscle migration in asthma. American journal of respiratory and critical care medicine. 2006;174(11):1179-88.

118. Plante S, Semlali A, Joubert P, Bissonnette E, Laviolette M, Hamid Q, et al. Mast cells regulate procollagen I (alpha 1) production by bronchial fibroblasts derived from subjects with asthma through IL-4/IL-4 delta 2 ratio. The Journal of allergy and clinical immunology. 2006;117(6):1321-7.

119. Bradding P. Human lung mast cell heterogeneity. Thorax. 2009;64(4):278-80.

120. Balzar S, Fajt ML, Comhair SA, Erzurum SC, Bleecker E, Busse WW, et al. Mast cell phenotype, location, and activation in severe asthma. Data from the Severe Asthma Research Program. American journal of respiratory and critical care medicine. 2011;183(3):299-309.

121. Norrby K. Mast cells and angiogenesis. APMIS : acta pathologica, microbiologica, et immunologica Scandinavica. 2002;110(5):355-71.

122. Chetta A, Zanini A, Foresi A, D'Ippolito R, Tipa A, Castagnaro A, et al. Vascular endothelial growth factor up-regulation and bronchial wall remodelling in asthma. Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology. 2005;35(11):1437-42.

123. Zanini A, Chetta A, Saetta M, Baraldo S, D'Ippolito R, Castagnaro A, et al. Chymase-positive mast cells play a role in the vascular component of airway remodeling in asthma. The Journal of allergy and clinical immunology. 2007;120(2):329-33.

124. Flood-Page P, Menzies-Gow A, Phipps S, Ying S, Wangoo A, Ludwig MS, et al. Anti-IL-5 treatment reduces deposition of ECM proteins in the bronchial subepithelial basement membrane of mild atopic asthmatics. The Journal of clinical investigation. 2003;112(7):1029-36.

125. Kay AB, Phipps S, Robinson DS. A role for eosinophils in airway remodelling in asthma. Trends in immunology. 2004;25(9):477-82.

126. Halwani R, Al-Muhsen S, Al-Jahdali H, Hamid Q. Role of transforming growth factor-beta in airway remodeling in asthma. American journal of respiratory cell and molecular biology. 2011;44(2):127-33.

127. Hoshino M, Nakamura Y, Hamid QA. Gene expression of vascular endothelial growth factor and its receptors and angiogenesis in bronchial asthma. The Journal of allergy and clinical immunology. 2001;107(6):1034-8.

128. Hoshino M, Takahashi M, Aoike N. Expression of vascular endothelial growth factor, basic fibroblast growth factor, and angiogenin immunoreactivity in asthmatic airways and its relationship to angiogenesis. The Journal of allergy and clinical immunology. 2001;107(2):295-301.

129. Saenz SA, Taylor BC, Artis D. Welcome to the neighborhood: epithelial cell-derived cytokines license innate and adaptive immune responses at mucosal sites. Immunological reviews. 2008;226:172-90.

130. Newcomb DC, Sajjan US, Nagarkar DR, Goldsmith AM, Bentley JK, Hershenson MB. Cooperative effects of rhinovirus and TNF-{alpha} on airway epithelial cell chemokine expression. American journal of physiology Lung cellular and molecular physiology. 2007;293(4):L1021-8.

131. Puddicombe SM, Torres-Lozano C, Richter A, Bucchieri F, Lordan JL, Howarth PH, et al. Increased expression of p21(waf) cyclin-dependent kinase inhibitor in asthmatic bronchial epithelium. American journal of respiratory cell and molecular biology. 2003;28(1):61-8.

132. Bucchieri F, Puddicombe SM, Lordan JL, Richter A, Buchanan D, Wilson SJ, et al. Asthmatic bronchial epithelium is more susceptible to oxidant-induced apoptosis. American journal of respiratory cell and molecular biology. 2002;27(2):179-85.

133. Kicic A, Sutanto EN, Stevens PT, Knight DA, Stick SM. Intrinsic biochemical and functional differences in bronchial epithelial cells of children with asthma. American journal of respiratory and critical care medicine. 2006;174(10):1110-8.

134. Barbato A, Turato G, Baraldo S, Bazzan E, Calabrese F, Panizzolo C, et al. Epithelial damage and angiogenesis in the airways of children with asthma. American journal of respiratory and critical care medicine. 2006;174(9):975-81.

135. Montefort S, Roberts JA, Beasley R, Holgate ST, Roche WR. The site of disruption of the bronchial epithelium in asthmatic and non-asthmatic subjects. Thorax. 1992;47(7):499-503.

136. Shahana S, Jaunmuktane Z, Asplund MS, Roomans GM. Ultrastructural investigation of epithelial damage in asthmatic and non-asthmatic nasal polyps. Respiratory medicine. 2006;100(11):2018-28.

137. Truong-Tran AQ, Grosser D, Ruffin RE, Murgia C, Zalewski PD. Apoptosis in the normal and inflamed airway epithelium: role of zinc in epithelial protection and procaspase-3 regulation. Biochemical pharmacology. 2003;66(8):1459-68.

138. Lopez-Guisa JM, Powers C, File D, Cochrane E, Jimenez N, Debley JS. Airway epithelial cells from asthmatic children differentially express proremodeling factors. The Journal of allergy and clinical immunology. 2012;129(4):990-7 e6.

139. Torrego A, Hew M, Oates T, Sukkar M, Fan Chung K. Expression and activation of TGF-beta isoforms in acute allergen-induced remodelling in asthma. Thorax. 2007;62(4):307-13.

140. Dulin NO, Fernandes DJ, Dowell M, Bellam S, McConville J, Lakser O, et al. What evidence implicates airway smooth muscle in the cause of BHR? Clinical reviews in allergy & immunology. 2003;24(1):73-84.

141. Cain H. Bronchoprovocation testing. Clinics in chest medicine. 2001;22(4):651-9.

142. Ebina M, Takahashi T, Chiba T, Motomiya M. Cellular hypertrophy and hyperplasia of airway smooth muscles underlying bronchial asthma. A 3-D morphometric study. The American review of respiratory disease. 1993;148(3):720-6.

143. Woodruff PG, Dolganov GM, Ferrando RE, Donnelly S, Hays SR, Solberg OD, et al. Hyperplasia of smooth muscle in mild to moderate asthma without changes in cell size or gene expression. American journal of respiratory and critical care medicine. 2004;169(9):1001-6.

144. Leguillette R, Lauzon AM. Molecular mechanics of smooth muscle contractile proteins in airway hyperresponsiveness and asthma. Proceedings of the American Thoracic Society. 2008;5(1):40-6.

145. Leguillette R, Laviolette M, Bergeron C, Zitouni N, Kogut P, Solway J, et al. Myosin, transgelin, and myosin light chain kinase: expression and function in asthma. American journal of respiratory and critical care medicine. 2009;179(3):194-204.

146. Bai TR, Cooper J, Koelmeyer T, Pare PD, Weir TD. The effect of age and duration of disease on airway structure in fatal asthma. American journal of respiratory and critical care medicine. 2000;162(2 Pt 1):663-9.

147. Pohunek P, Warner JO, Turzikova J, Kudrmann J, Roche WR. Markers of eosinophilic inflammation and tissue re-modelling in children before clinically diagnosed bronchial asthma. Pediatric allergy and immunology : official publication of the European Society of Pediatric Allergy and Immunology. 2005;16(1):43-51.

148. Hirst SJ. Regulation of airway smooth muscle cell immunomodulatory function: role in asthma. Respiratory physiology & neurobiology. 2003;137(2-3):309-26.

149. Miglino N, Roth M, Tamm M, Borger P. House dust mite extract downregulates C/EBPalpha in asthmatic bronchial smooth muscle cells. The European respiratory journal : official journal of the European Society for Clinical Respiratory Physiology. 2011;38(1):50-8.

150. Oliver BG, Johnston SL, Baraket M, Burgess JK, King NJ, Roth M, et al. Increased proinflammatory responses from asthmatic human airway smooth muscle cells in response to rhinovirus infection. Respiratory research. 2006;7:71.

151. Johnson PR. Role of human airway smooth muscle in altered extracellular matrix production in asthma. Clinical and experimental pharmacology & physiology. 2001;28(3):233-6.

152. Johnson PR, Black JL, Carlin S, Ge Q, Underwood PA. The production of extracellular matrix proteins by human passively sensitized airway smooth-muscle cells in culture: the effect of beclomethasone. American journal of respiratory and critical care medicine. 2000;162(6):2145-51.

153. Johnson PR, Roth M, Tamm M, Hughes M, Ge Q, King G, et al. Airway smooth muscle cell proliferation is increased in asthma. American journal of respiratory and critical care medicine. 2001;164(3):474-7.

154. Burgess JK, Johnson PR, Ge Q, Au WW, Poniris MH, McParland BE, et al. Expression of connective tissue growth factor in asthmatic airway smooth muscle cells. American journal of respiratory and critical care medicine. 2003;167(1):71-7.

155. Xie S, Sukkar MB, Issa R, Oltmanns U, Nicholson AG, Chung KF. Regulation of TGF-beta 1-induced connective tissue growth factor expression in airway smooth muscle cells. American journal of physiology Lung cellular and molecular physiology. 2005;288(1):L68-76.

156. Roche WR, Beasley R, Williams JH, Holgate ST. Subepithelial fibrosis in the bronchi of asthmatics. Lancet. 1989;1(8637):520-4.

157. Bossley CJ, Fleming L, Gupta A, Regamey N, Frith J, Oates T, et al. Pediatric severe asthma is characterized by eosinophilia and remodeling without T(H)2 cytokines. The Journal of allergy and clinical immunology. 2012;129(4):974-82 e13.

158. Fedorov IA, Wilson SJ, Davies DE, Holgate ST. Epithelial stress and structural remodelling in childhood asthma. Thorax. 2005;60(5):389-94.

159. Homer RJ, Elias JA. Consequences of long-term inflammation. Airway remodeling. Clinics in chest medicine. 2000;21(2):331-43, ix.

160. Jeffery PK. Remodeling in asthma and chronic obstructive lung disease. American journal of respiratory and critical care medicine. 2001;164(10 Pt 2):S28-38.

161. Bourdin A, Kleis S, Chakra M, Vachier I, Paganin F, Godard P, et al. Limited short-term steroid responsiveness is associated with thickening of bronchial basement membrane in severe asthma. Chest. 2012;141(6):1504-11.

162. James AL, Maxwell PS, Pearce-Pinto G, Elliot JG, Carroll NG. The relationship of reticular basement membrane thickness to airway wall remodeling in asthma. American journal of respiratory and critical care medicine. 2002;166(12 Pt 1):1590-5.

163. Bergers G, Brekken R, McMahon G, Vu TH, Itoh T, Tamaki K, et al. Matrix metalloproteinase-9 triggers the angiogenic switch during carcinogenesis. Nature cell biology. 2000;2(10):737-44.

164. Lee S, Jilani SM, Nikolova GV, Carpizo D, Iruela-Arispe ML. Processing of VEGF-A by matrix metalloproteinases regulates bioavailability and vascular patterning in tumors. The Journal of cell biology. 2005;169(4):681-91.

165. Bhakta NR, Woodruff PG. Human asthma phenotypes: from the clinic, to cytokines, and back again. Immunological reviews. 2011;242(1):220-32.

166. Holgate ST. Pathogenesis of asthma. Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology. 2008;38(6):872-97.

167. Malmstrom K, Pelkonen AS, Makela MJ. Remodeling, inflammation and airway responsiveness in early childhood asthma. Current opinion in allergy and clinical immunology. 2013;13(2):203-10.

168. O'Reilly R, Ullmann N, Irving S, Bossley CJ, Sonnappa S, Zhu J, et al. Increased airway smooth muscle in preschool wheezers who have asthma at school age. The Journal of allergy and clinical immunology. 2013;131(4):1024-32, 32 e1-16.

169. Plopper CG, Smiley-Jewell SM, Miller LA, Fanucchi MV, Evans MJ, Buckpitt AR, et al. Asthma/allergic airways disease: does postnatal exposure to environmental toxicants promote airway pathobiology? Toxicologic pathology. 2007;35(1):97-110.

170. Lex C, Zacharasiewicz A, Payne DN, Wilson NM, Nicholson AG, Kharitonov SA, et al. Exhaled breath condensate cysteinyl leukotrienes and airway remodeling in childhood asthma: a pilot study. Respiratory research. 2006;7:63.

171. Penard-Morand C, Raherison C, Kopferschmitt C, Caillaud D, Lavaud F, Charpin D, et al. Prevalence of food allergy and its relationship to asthma and allergic rhinitis in schoolchildren. Allergy. 2005;60(9):1165-71.

172. Lodge CJ, Allen KJ, Lowe AJ, Hill DJ, Hosking CS, Abramson MJ, et al. Perinatal cat and dog exposure and the risk of asthma and allergy in the urban environment: a systematic review of longitudinal studies. Clinical & developmental immunology. 2012;2012:176484.

173. Huss K, Adkinson NF, Jr., Eggleston PA, Dawson C, Van Natta ML, Hamilton RG. House dust mite and cockroach exposure are strong risk factors for positive allergy skin test responses in the Childhood Asthma Management Program. The Journal of allergy and clinical immunology. 2001;107(1):48-54.

174. Asosingh K, Hanson JD, Cheng G, Aronica MA, Erzurum SC. Allergen-induced, eotaxin-rich, proangiogenic bone marrow progenitors: a blood-borne cellular envoy for lung eosinophilia. The Journal of allergy and clinical immunology. 2010;125(4):918-25.

175. Jones CP, Pitchford SC, Lloyd CM, Rankin SM. CXCR2 mediates the recruitment of endothelial progenitor cells during allergic airways remodeling. Stem Cells. 2009;27(12):3074-81.

176. Su X, Taniuchi N, Jin E, Fujiwara M, Zhang L, Ghazizadeh M, et al. Spatial and phenotypic characterization of vascular remodeling in a mouse model of asthma. Pathobiology : journal of immunopathology, molecular and cellular biology. 2008;75(1):42-56.

177. Suzaki Y, Hamada K, Sho M, Ito T, Miyamoto K, Akashi S, et al. A potent antiangiogenic factor, endostatin prevents the development of asthma in a murine model. The Journal of allergy and clinical immunology. 2005;116(6):1220-7.

178. Tigani B, Cannet C, Karmouty-Quintana H, Ble FX, Zurbruegg S, Schaeublin E, et al. Lung inflammation and vascular remodeling after repeated allergen challenge detected noninvasively by MRI. American journal of physiology Lung cellular and molecular physiology. 2007;292(3):L644-53.

179. Lee SY, Cho JY, Miller M, McElwain K, McElwain S, Sriramarao P, et al. Immunostimulatory DNA inhibits allergen-induced peribronchial angiogenesis in mice. The Journal of allergy and clinical immunology. 2006;117(3):597-603.

180. Doyle TM, Ellis R, Park HJ, Inman MD, Sehmi R. Modulating progenitor accumulation attenuates lung angiogenesis in a mouse model of asthma. The European respiratory journal : official journal of the European Society for Clinical Respiratory Physiology. 2011;38(3):679-87.

181. Imaoka H, Punia N, Irshad A, Ying S, Corrigan CJ, Howie K, et al. Lung homing of endothelial progenitor cells in humans with asthma after allergen challenge. American journal of respiratory and critical care medicine. 2011;184(7):771-8.

182. Zock JP, Heinrich J, Jarvis D, Verlato G, Norback D, Plana E, et al. Distribution and determinants of house dust mite allergens in Europe: the European Community Respiratory Health Survey II. The Journal of allergy and clinical immunology. 2006;118(3):682-90.

183. Milian E, Diaz AM. Allergy to house dust mites and asthma. Puerto Rico health sciences journal. 2004;23(1):47-57.

184. Walshaw MJ, Evans CC. The effect of seasonal and domestic factors on the distribution of Euroglyphus maynei in the homes of Dermatophagoides pteronyssinus allergic patients. Clinical allergy. 1987;17(1):7-14.

185. Arlian LG, Morgan MS, Neal JS. Dust mite allergens: ecology and distribution. Current allergy and asthma reports. 2002;2(5):401-11.

186. Nelson RP, Jr., DiNicolo R, Fernandez-Caldas E, Seleznick MJ, Lockey RF, Good RA. Allergen-specific IgE levels and mite allergen exposure in children with acute asthma first seen in an emergency department and in nonasthmatic control subjects. The Journal of allergy and clinical immunology. 1996;98(2):258-63.

187. Tovey ER, Chapman MD, Platts-Mills TA. Mite faeces are a major source of house dust allergens. Nature. 1981;289(5798):592-3.

188. Thomas WR, Smith WA, Hales BJ, Mills KL, O'Brien RM. Characterization and immunobiology of house dust mite allergens. International archives of allergy and immunology. 2002;129(1):1-18.

189. Thomas WR, Hales BJ, Smith WA. House dust mite allergens in asthma and allergy. Trends in molecular medicine. 2010;16(7):321-8.

190. Wan H, Winton HL, Soeller C, Tovey ER, Gruenert DC, Thompson PJ, et al. Der p 1 facilitates transepithelial allergen delivery by disruption of tight junctions. The Journal of clinical investigation. 1999;104(1):123-33.

191. Post S, Nawijn MC, Hackett TL, Baranowska M, Gras R, van Oosterhout AJ, et al. The composition of house dust mite is critical for mucosal barrier dysfunction and allergic sensitisation. Thorax. 2012;67(6):488-95.

192. Reed CE, Kita H. The role of protease activation of inflammation in allergic respiratory diseases. The Journal of allergy and clinical immunology. 2004;114(5):997-1008; quiz 9.

193. Hauck RW, Schulz C, Schomig A, Hoffman RK, Panettieri RA, Jr. alpha-Thrombin stimulates contraction of human bronchial rings by activation of protease-activated receptors. The American journal of physiology. 1999;277(1 Pt 1):L22-9.

194. Van der Velden J, Barker D, Barcham G, Koumoundouros E, Snibson K. Increased vascular density is a persistent feature of airway remodeling in a sheep model of chronic asthma. Experimental lung research. 2012;38(6):307-15.

195. Valerio CR, Murray P, Arlian LG, Slater JE. Bacterial 16S ribosomal DNA in house dust mite cultures. The Journal of allergy and clinical immunology. 2005;116(6):1296-300.

196. Bisgaard H, Hermansen MN, Bonnelykke K, Stokholm J, Baty F, Skytt NL, et al. Association of bacteria and viruses with wheezy episodes in young children: prospective birth cohort study. BMJ. 2010;341:c4978.

197. Zhang Q, Illing R, Hui CK, Downey K, Carr D, Stearn M, et al. Bacteria in sputum of stable severe asthma and increased airway wall thickness. Respiratory research. 2012;13:35.

198. Kusel MM, de Klerk NH, Kebadze T, Vohma V, Holt PG, Johnston SL, et al. Early-life respiratory viral infections, atopic sensitization, and risk of subsequent development of persistent asthma. The Journal of allergy and clinical immunology. 2007;119(5):1105-10.

199. Jackson DJ, Evans MD, Gangnon RE, Tisler CJ, Pappas TE, Lee WM, et al. Evidence for a causal relationship between allergic sensitization and rhinovirus wheezing in early life. American journal of respiratory and critical care medicine. 2012;185(3):281-5.

200. Sigurs N, Aljassim F, Kjellman B, Robinson PD, Sigurbergsson F, Bjarnason R, et al. Asthma and allergy patterns over 18 years after severe RSV bronchiolitis in the first year of life. Thorax. 2010;65(12):1045-52.

201. Kuo C, Lim S, King NJ, Bartlett NW, Walton RP, Zhu J, et al. Rhinovirus infection induces expression of airway remodelling factors in vitro and in vivo. Respirology. 2011;16(2):367-77.

202. Psarras S, Volonaki E, Skevaki CL, Xatzipsalti M, Bossios A, Pratsinis H, et al. Vascular endothelial growth factor-mediated induction of angiogenesis by human rhinoviruses. The Journal of allergy and clinical immunology. 2006;117(2):291-7.

203. Pongracic JA, O'Connor GT, Muilenberg ML, Vaughn B, Gold DR, Kattan M, et al. Differential effects of outdoor versus indoor fungal spores on asthma morbidity in inner-city children. The Journal of allergy and clinical immunology. 2010;125(3):593-9.

204. Palmer CN, Ismail T, Lee SP, Terron-Kwiatkowski A, Zhao Y, Liao H, et al. Filaggrin null mutations are associated with increased asthma severity in children and young adults. The Journal of allergy and clinical immunology. 2007;120(1):64-8.

205. Paternoster L, Standl M, Chen CM, Ramasamy A, Bonnelykke K, Duijts L, et al. Meta-analysis of genome-wide association studies identifies three new risk loci for atopic dermatitis. Nature genetics. 2012;44(2):187-92.

206. Palmer CN, Irvine AD, Terron-Kwiatkowski A, Zhao Y, Liao H, Lee SP, et al. Common loss-of-function variants of the epidermal barrier protein filaggrin are a major predisposing factor for atopic dermatitis. Nature genetics. 2006;38(4):441-6.

207. Eder W, Klimecki W, Yu L, von Mutius E, Riedler J, Braun-Fahrlander C, et al. Toll-like receptor 2 as a major gene for asthma in children of European farmers. The Journal of allergy and clinical immunology. 2004;113(3):482-8.

208. Genuneit J, Cantelmo JL, Weinmayr G, Wong GW, Cooper PJ, Riikjarv MA, et al. A multi-centre study of candidate genes for wheeze and allergy: the International Study of Asthma and Allergies in Childhood Phase 2. Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology. 2009;39(12):1875-88.

209. Kormann MS, Depner M, Hartl D, Klopp N, Illig T, Adamski J, et al. Toll-like receptor heterodimer variants protect from childhood asthma. The Journal of allergy and clinical immunology. 2008;122(1):86-92, e1-8.

210. Li X, Howard TD, Zheng SL, Haselkorn T, Peters SP, Meyers DA, et al. Genome-wide association study of asthma identifies RAD50-IL13 and HLA-DR/DQ regions. The Journal of allergy and clinical immunology. 2010;125(2):328-35 e11.

211. Smit LA, Siroux V, Bouzigon E, Oryszczyn MP, Lathrop M, Demenais F, et al. CD14 and toll-like receptor gene polymorphisms, country living, and asthma in adults. American journal of respiratory and critical care medicine. 2009;179(5):363-8.

212. Munthe-Kaas MC, Carlsen KH, Haland G, Devulapalli CS, Gervin K, Egeland T, et al. T cell-specific T-box transcription factor haplotype is associated with allergic asthma in children. The Journal of allergy and clinical immunology. 2008;121(1):51-6.

213. Potaczek DP, Nishiyama C, Sanak M, Szczeklik A, Okumura K. Genetic variability of the high-affinity IgE receptor alpha-subunit (FcepsilonRIalpha). Immunologic research. 2009;45(1):75-84.

214. Pykalainen M, Kinos R, Valkonen S, Rydman P, Kilpelainen M, Laitinen LA, et al. Association analysis of common variants of STAT6, GATA3, and STAT4 to asthma and high serum IgE phenotypes. The Journal of allergy and clinical immunology. 2005;115(1):80-7.

215. Weidinger S, Gieger C, Rodriguez E, Baurecht H, Mempel M, Klopp N, et al. Genome-wide scan on total serum IgE levels identifies FCER1A as novel susceptibility locus. PLoS genetics. 2008;4(8):e1000166.

216. Wu X, Li Y, Chen Q, Chen F, Cai P, Wang L, et al. Association and gene-gene interactions of eight common single-nucleotide polymorphisms with pediatric asthma in middle china. The Journal of asthma : official journal of the Association for the Care of Asthma. 2010;47(3):238-44.

217. Blakey J, Halapi E, Bjornsdottir US, Wheatley A, Kristinsson S, Upmanyu R, et al. Contribution of ADAM33 polymorphisms to the population risk of asthma. Thorax. 2005;60(4):274-6.

218. Foley SC, Mogas AK, Olivenstein R, Fiset PO, Chakir J, Bourbeau J, et al. Increased expression of ADAM33 and ADAM8 with disease progression in asthma. The Journal of allergy and clinical immunology. 2007;119(4):863-71.

219. Van Eerdewegh P, Little RD, Dupuis J, Del Mastro RG, Falls K, Simon J, et al. Association of the ADAM33 gene with asthma and bronchial hyperresponsiveness. Nature. 2002;418(6896):426-30.

220. Koppelman GH, Sayers I. Evidence of a genetic contribution to lung function decline in asthma. The Journal of allergy and clinical immunology. 2011;128(3):479-84.

221. Puxeddu I, Pang YY, Harvey A, Haitchi HM, Nicholas B, Yoshisue H, et al. The soluble form of a disintegrin and metalloprotease 33 promotes angiogenesis: implications for airway remodeling in asthma. The Journal of allergy and clinical immunology. 2008;121(6):1400-6, 6 e1-4.

222. Park HW, Lee JE, Shin ES, Lee JY, Bahn JW, Oh HB, et al. Association between genetic variations of vascular endothelial growth factor receptor 2 and atopy in the Korean population. The Journal of allergy and clinical immunology. 2006;117(4):774-9.

223. Sharma S, Murphy AJ, Soto-Quiros ME, Avila L, Klanderman BJ, Sylvia JS, et al. Association of VEGF polymorphisms with childhood asthma, lung function and airway responsiveness. The European respiratory journal : official journal of the European Society for Clinical Respiratory Physiology. 2009;33(6):1287-94.

224. Simpson A, Custovic A, Tepper R, Graves P, Stern DA, Jones M, et al. Genetic variation in vascular endothelial growth factor-a and lung function. American journal of respiratory and critical care medicine. 2012;185(11):1197-204.

225. Balantic M, Rijavec M, Skerbinjek Kavalar M, Suskovic S, Silar M, Kosnik M, et al. Asthma treatment outcome in children is associated with vascular endothelial growth factor A (VEGFA) polymorphisms. Molecular diagnosis & therapy. 2012;16(3):173-80.

226. Eichler EE, Flint J, Gibson G, Kong A, Leal SM, Moore JH, et al. Missing heritability and strategies for finding the underlying causes of complex disease. Nature reviews Genetics. 2010;11(6):446-50.

227. Ribatti D, Nico B, Crivellato E, Roccaro AM, Vacca A. The history of the angiogenic switch concept. Leukemia. 2007;21(1):44-52.

228. Oliver G, Alitalo K. The lymphatic vasculature: recent progress and paradigms. Annual review of cell and developmental biology. 2005;21:457-83.

229. Carmeliet P. Angiogenesis in life, disease and medicine. Nature. 2005;438(7070):932-6.

230. Pinedo HM, Verheul HM, D'Amato RJ, Folkman J. Involvement of platelets in tumour angiogenesis? Lancet. 1998;352(9142):1775-7.

231. Seljelid R, Jozefowski S, Sveinbjornsson B. Tumor stroma. Anticancer research. 1999;19(6A):4809-22.

232. Wu VY, Walz DA, McCoy LE. Purification and characterization of human and bovine platelet factor 4. Preparative biochemistry. 1977;7(6):479-93.

233. Ruffini PA, Morandi P, Cabioglu N, Altundag K, Cristofanilli M. Manipulating the chemokine-chemokine receptor network to treat cancer. Cancer. 2007;109(12):2392-404.

234. Salcedo R, Ponce ML, Young HA, Wasserman K, Ward JM, Kleinman HK, et al. Human endothelial cells express CCR2 and respond to MCP-1: direct role of MCP-1 in angiogenesis and tumor progression. Blood. 2000;96(1):34-40.

235. Luan J, Shattuck-Brandt R, Haghnegahdar H, Owen JD, Strieter R, Burdick M, et al. Mechanism and biological significance of constitutive expression of MGSA/GRO chemokines in malignant melanoma tumor progression. Journal of leukocyte biology. 1997;62(5):588-97.

236. White ES, Flaherty KR, Carskadon S, Brant A, Iannettoni MD, Yee J, et al. Macrophage migration inhibitory factor and CXC chemokine expression in non-small cell lung cancer: role in angiogenesis and prognosis. Clinical cancer research : an official journal of the American Association for Cancer Research. 2003;9(2):853-60.

237. Arenberg DA, Kunkel SL, Polverini PJ, Glass M, Burdick MD, Strieter RM. Inhibition of interleukin-8 reduces tumorigenesis of human non-small cell lung cancer in SCID mice. The Journal of clinical investigation. 1996;97(12):2792-802.

238. Addison CL, Daniel TO, Burdick MD, Liu H, Ehlert JE, Xue YY, et al. The CXC chemokine receptor 2, CXCR2, is the putative receptor for ELR+ CXC chemokine-induced angiogenic activity. J Immunol. 2000;165(9):5269-77.

239. Brat DJ, Bellail AC, Van Meir EG. The role of interleukin-8 and its receptors in gliomagenesis and tumoral angiogenesis. Neuro-oncology. 2005;7(2):122-33.

240. Vandercappellen J, Van Damme J, Struyf S. The role of CXC chemokines and their receptors in cancer. Cancer Lett. 2008;267(2):226-44.

241. Ruegg C, Hasmim M, Lejeune FJ, Alghisi GC. Antiangiogenic peptides and proteins: from experimental tools to clinical drugs. Biochimica et biophysica acta. 2006;1765(2):155-77.

242. Sharma B, Nawandar DM, Nannuru KC, Varney ML, Singh RK. Targeting CXCR2 enhances chemotherapeutic response, inhibits mammary tumor growth, angiogenesis, and lung metastasis. Molecular cancer therapeutics. 2013;12(5):799-808.

243. Hertzer KM, Donald GW, Hines OJ. CXCR2: a target for pancreatic cancer treatment? Expert opinion on therapeutic targets. 2013;17(6):667-80.

244. Dwyer J, Hebda JK, Le Guelte A, Galan-Moya EM, Smith SS, Azzi S, et al. Glioblastoma cell-secreted interleukin-8 induces brain endothelial cell permeability via CXCR2. PloS one. 2012;7(9):e45562.

245. Bolitho C, Hahn MA, Baxter RC, Marsh DJ. The chemokine CXCL1 induces proliferation in epithelial ovarian cancer cells by transactivation of the epidermal growth factor receptor. Endocrine-related cancer. 2010;17(4):929-40.

246. Wolff HA, Rolke D, Rave-Frank M, Schirmer M, Eicheler W, Doerfler A, et al. Analysis of chemokine and chemokine receptor expression in squamous cell carcinoma of the head and neck (SCCHN) cell lines. Radiation and environmental biophysics. 2011;50(1):145-54.

247. Saintigny P, Massarelli E, Lin S, Ahn YH, Chen Y, Goswami S, et al. CXCR2 expression in tumor cells is a poor prognostic factor and promotes invasion and metastasis in lung adenocarcinoma. Cancer research. 2013;73(2):571-82.

248. Keane MP, Arenberg DA, Lynch JP, 3rd, Whyte RI, Iannettoni MD, Burdick MD, et al. The CXC chemokines, IL-8 and IP-10, regulate angiogenic activity in idiopathic pulmonary fibrosis. J Immunol. 1997;159(3):1437-43.

249. Belperio JA, Keane MP, Burdick MD, Gomperts B, Xue YY, Hong K, et al. Role of CXCR2/CXCR2 ligands in vascular remodeling during bronchiolitis obliterans syndrome. The Journal of clinical investigation. 2005;115(5):1150-62.

250. Keane MP, Donnelly SC, Belperio JA, Goodman RB, Dy M, Burdick MD, et al. Imbalance in the expression of CXC chemokines correlates with bronchoalveolar lavage fluid angiogenic activity and procollagen levels in acute respiratory distress syndrome. J Immunol. 2002;169(11):6515-21.

251. Dunnill MS. The pathology of asthma, with special reference to changes in the bronchial mucosa. J Clin Pathol. 1960;13:27-33.

252. Vrugt B, Wilson S, Bron A, Holgate ST, Djukanovic R, Aalbers R. Bronchial angiogenesis in severe glucocorticoid-dependent asthma. The European respiratory journal : official journal of the European Society for Clinical Respiratory Physiology. 2000;15(6):1014-21.

253. Wilson JW, Robertson CF. Angiogenesis in paediatric airway disease. Paediatric respiratory reviews. 2002;3(3):219-29.

254. Tanaka H, Yamada G, Saikai T, Hashimoto M, Tanaka S, Suzuki K, et al. Increased airway vascularity in newly diagnosed asthma using a high-magnification bronchovideoscope. American journal of respiratory and critical care medicine. 2003;168(12):1495-9.

255. Csete ME, Chediak AD, Abraham WM, Wanner A. Airway blood flow modifies allergic airway smooth muscle contraction. The American review of respiratory disease. 1991;144(1):59-63.

256. Kumar SD, Emery MJ, Atkins ND, Danta I, Wanner A. Airway mucosal blood flow in bronchial asthma. American journal of respiratory and critical care medicine. 1998;158(1):153-6.

257. Mendes ES, Campos MA, Hurtado A, Wanner A. Effect of montelukast and fluticasone propionate on airway mucosal blood flow in asthma. American journal of respiratory and critical care medicine. 2004;169(10):1131-4.

258. Wanner A, Chediak AD, Csete ME. Airway mucosal blood flow: response to autonomic and inflammatory stimuli. The European respiratory journal Supplement. 1990;12:618s-23s.

259. Nagy JA, Benjamin L, Zeng H, Dvorak AM, Dvorak HF. Vascular permeability, vascular hyperpermeability and angiogenesis. Angiogenesis. 2008;11(2):109-19.

260. Simcock DE, Kanabar V, Clarke GW, Mahn K, Karner C, O'Connor BJ, et al. Induction of angiogenesis by airway smooth muscle from patients with asthma. American journal of respiratory and critical care medicine. 2008;178(5):460-8.

261. Simcock DE, Kanabar V, Clarke GW, O'Connor BJ, Lee TH, Hirst SJ. Proangiogenic activity in bronchoalveolar lavage fluid from patients with asthma. American journal of respiratory and critical care medicine. 2007;176(2):146-53.

262. Tuder RM, Yun JH. Vascular endothelial growth factor of the lung: friend or foe. Current opinion in pharmacology. 2008;8(3):255-60.

263. Li X, Wilson JW. Increased vascularity of the bronchial mucosa in mild asthma. American journal of respiratory and critical care medicine. 1997;156(1):229-33.

264. Orsida BE, Li X, Hickey B, Thien F, Wilson JW, Walters EH. Vascularity in asthmatic airways: relation to inhaled steroid dose. Thorax. 1999;54(4):289-95.

265. Kanazawa H, Hirata K, Yoshikawa J. Involvement of vascular endothelial growth factor in exercise induced bronchoconstriction in asthmatic patients. Thorax. 2002;57(10):885-8.

266. Kanazawa H, Asai K, Hirata K, Yoshikawa J. Vascular involvement in exercise-induced airway narrowing in patients with bronchial asthma. Chest. 2002;122(1):166-70.

267. Hashimoto M, Tanaka H, Abe S. Quantitative analysis of bronchial wall vascularity in the medium and small airways of patients with asthma and COPD. Chest. 2005;127(3):965-72.

268. Chetta A, Zanini A, Torre O, Olivieri D. Vascular remodelling and angiogenesis in asthma: morphological aspects and pharmacological modulation. Inflammation & allergy drug targets. 2007;6(1):41-5.

269. Grigoras A, Caruntu ID, Grigoras CC, Mihaescu T, Amalinei C. Relationship between immunohistochemical assessment of bronchial mucosa microvascularization and clinical stage in asthma. Romanian journal of morphology and embryology = Revue roumaine de morphologie et embryologie. 2012;53(3):485-90.

270. Bull TM, Clark B, McFann K, Moss M. Pulmonary vascular dysfunction is associated with poor outcomes in patients with acute lung injury. American journal of respiratory and critical care medicine. 2010;182(9):1123-8.

271. Huerta-Yepez S, Baay-Guzman GJ, Bebenek IG, Hernandez-Pando R, Vega MI, Chi L, et al. Hypoxia inducible factor promotes murine allergic airway inflammation and is increased in asthma and rhinitis. Allergy. 2011;66(7):909-18.

272. Wrobel JP, Thompson BR, Williams TJ. Mechanisms of pulmonary hypertension in chronic obstructive pulmonary disease: a pathophysiologic review. The Journal of heart and lung transplantation : the official publication of the International Society for Heart Transplantation. 2012;31(6):557-64.

273. Safran M, Kaelin WG, Jr. HIF hydroxylation and the mammalian oxygen-sensing pathway. The Journal of clinical investigation. 2003;111(6):779-83.

274. Kallio PJ, Pongratz I, Gradin K, McGuire J, Poellinger L. Activation of hypoxia-inducible factor 1alpha: posttranscriptional regulation and conformational change by recruitment of the Arnt transcription factor. Proceedings of the National Academy of Sciences of the United States of America. 1997;94(11):5667-72.

275. Weidemann A, Johnson RS. Biology of HIF-1alpha. Cell death and differentiation. 2008;15(4):621-7.

276. Richard S, Gardie B, Couve S, Gad S. Von Hippel-Lindau: how a rare disease illuminates cancer biology. Seminars in cancer biology. 2013;23(1):26-37.

277. Lee SY, Kwon S, Kim KH, Moon HS, Song JS, Park SH, et al. Expression of vascular endothelial growth factor and hypoxia-inducible factor in the airway of asthmatic patients. Annals of allergy, asthma & immunology : official publication of the American College of Allergy, Asthma, & Immunology. 2006;97(6):794-9.

278. Worlitzsch D, Tarran R, Ulrich M, Schwab U, Cekici A, Meyer KC, et al. Effects of reduced mucus oxygen concentration in airway Pseudomonas infections of cystic fibrosis patients. The Journal of clinical investigation. 2002;109(3):317-25.

279. Meijer RJ, Kerstjens HA, Arends LR, Kauffman HF, Koeter GH, Postma DS. Effects of inhaled fluticasone and oral prednisolone on clinical and inflammatory parameters in patients with asthma. Thorax. 1999;54(10):894-9.

280. Jatakanon A, Kharitonov S, Lim S, Barnes PJ. Effect of differing doses of inhaled budesonide on markers of airway inflammation in patients with mild asthma. Thorax. 1999;54(2):108-14.

281. Aldridge RE, Hancox RJ, Robin Taylor D, Cowan JO, Winn MC, Frampton CM, et al. Effects of terbutaline and budesonide on sputum cells and bronchial hyperresponsiveness in asthma. American journal of respiratory and critical care medicine. 2000;161(5):1459-64.

282. van Rensen EL, Straathof KC, Veselic-Charvat MA, Zwinderman AH, Bel EH, Sterk PJ. Effect of inhaled steroids on airway hyperresponsiveness, sputum eosinophils, and exhaled nitric oxide levels in patients with asthma. Thorax. 1999;54(5):403-8.

283. Adcock IM, Ito K, Barnes PJ. Glucocorticoids: effects on gene transcription. Proceedings of the American Thoracic Society. 2004;1(3):247-54.

284. Gupta R, Jindal DP, Kumar G. Corticosteroids: the mainstay in asthma therapy. Bioorganic & medicinal chemistry. 2004;12(24):6331-42.

285. Girodet PO, Ozier A, Bara I, Tunon de Lara JM, Marthan R, Berger P. Airway remodeling in asthma: new mechanisms and potential for pharmacological intervention. Pharmacology & therapeutics. 2011;130(3):325-37.

286. Laitinen LA, Laitinen A, Haahtela T. A comparative study of the effects of an inhaled corticosteroid, budesonide, and a beta 2-agonist, terbutaline, on airway inflammation in newly diagnosed asthma: a randomized, double-blind, parallel-group controlled trial. The Journal of allergy and clinical immunology. 1992;90(1):32-42.

287. Erjefalt JS, Erjefalt I, Sundler F, Persson CG. In vivo restitution of airway epithelium. Cell and tissue research. 1995;281(2):305-16.

288. Dorscheid DR, Wojcik KR, Sun S, Marroquin B, White SR. Apoptosis of airway epithelial cells induced by corticosteroids. American journal of respiratory and critical care medicine. 2001;164(10 Pt 1):1939-47.

289. Hoshino M, Takahashi M, Takai Y, Sim J. Inhaled corticosteroids decrease subepithelial collagen deposition by modulation of the balance between matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-1 expression in asthma. The Journal of allergy and clinical immunology. 1999;104(2 Pt 1):356-63.

290. Mauad T, Bel EH, Sterk PJ. Asthma therapy and airway remodeling. The Journal of allergy and clinical immunology. 2007;120(5):997-1009; quiz 10-1.

291. Skevaki CL, Christodoulou I, Spyridaki IS, Tiniakou I, Georgiou V, Xepapadaki P, et al. Budesonide and formoterol inhibit inflammatory mediator production by bronchial epithelial cells infected with rhinovirus. Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology. 2009;39(11):1700-10.

292. Volonaki E, Psarras S, Xepapadaki P, Psomali D, Gourgiotis D, Papadopoulos NG. Synergistic effects of fluticasone propionate and salmeterol on inhibiting rhinovirus-induced epithelial production of remodelling-associated growth factors. Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology. 2006;36(10):1268-73.

293. Sun Y, Wang J, Li H, Sun L, Wang Y, Han X. The effects of budesonide on angiogenesis in a murine asthma model. Archives of medical science : AMS. 2013;9(2):361-7.

294. Sun Y, Wang JR, Han XZ, Li HB, Sun LF, Chen X, et al. [Effects of budesonide on HIF-1alpha and VEGF expression and airway remodeling in an asthmatic mouse model]. Zhongguo dang dai er ke za zhi = Chinese journal of contemporary pediatrics. 2012;14(8):622-7.

295. Feltis BN, Wignarajah D, Reid DW, Ward C, Harding R, Walters EH. Effects of inhaled fluticasone on angiogenesis and vascular endothelial growth factor in asthma. Thorax. 2007;62(4):314-9.

296. Wang K, Liu CT, Wu YH, Feng YL, Bai HL. Budesonide/formoterol decreases expression of vascular endothelial growth factor (VEGF) and VEGF receptor 1 within airway remodelling in asthma. Advances in therapy. 2008;25(4):342-54.

297. Holgate ST, Djukanovic R, Casale T, Bousquet J. Anti-immunoglobulin E treatment with omalizumab in allergic diseases: an update on anti-inflammatory activity and clinical efficacy. Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology. 2005;35(4):408-16.

298. Holgate S, Buhl R, Bousquet J, Smith N, Panahloo Z, Jimenez P. The use of omalizumab in the treatment of severe allergic asthma: A clinical experience update. Respiratory medicine. 2009;103(8):1098-113.

299. Borish LC, Nelson HS, Lanz MJ, Claussen L, Whitmore JB, Agosti JM, et al. Interleukin-4 receptor in moderate atopic asthma. A phase I/II randomized, placebo-controlled trial. American journal of respiratory and critical care medicine. 1999;160(6):1816-23.

300. Borish LC, Nelson HS, Corren J, Bensch G, Busse WW, Whitmore JB, et al. Efficacy of soluble IL-4 receptor for the treatment of adults with asthma. The Journal of allergy and clinical immunology. 2001;107(6):963-70.

301. Leckie MJ, ten Brinke A, Khan J, Diamant Z, O'Connor BJ, Walls CM, et al. Effects of an interleukin-5 blocking monoclonal antibody on eosinophils, airway hyper-responsiveness, and the late asthmatic response. Lancet. 2000;356(9248):2144-8.

302. Phipps S, Flood-Page P, Menzies-Gow A, Ong YE, Kay AB. Intravenous anti-IL-5 monoclonal antibody reduces eosinophils and tenascin deposition in allergen-challenged human atopic skin. The Journal of investigative dermatology. 2004;122(6):1406-12.

303. Subramaniam JM, Whiteside G, McKeage K, Croxtall JC. Mogamulizumab: first global approval. Drugs. 2012;72(9):1293-8.

304. Dicpinigaitis PV, Dobkin JB, Reichel J. Antitussive effect of the leukotriene receptor antagonist zafirlukast in subjects with cough-variant asthma. The Journal of asthma : official journal of the Association for the Care of Asthma. 2002;39(4):291-7.

305. Mastalerz L, Nizankowska E, Sanak M, Mejza F, Pierzchalska M, Bazan-Socha S, et al. Clinical and genetic features underlying the response of patients with bronchial asthma to treatment with a leukotriene receptor antagonist. European journal of clinical investigation. 2002;32(12):949-55.

306. Barnes NC, Miller CJ. Effect of leukotriene receptor antagonist therapy on the risk of asthma exacerbations in patients with mild to moderate asthma: an integrated analysis of zafirlukast trials. Thorax. 2000;55(6):478-83.

307. Lipworth BJ. Emerging role of long acting muscarinic antagonists for asthma. British journal of clinical pharmacology. 2013.

308. Thomson NC, Rubin AS, Niven RM, Corris PA, Siersted HC, Olivenstein R, et al. Long-term (5 year) safety of bronchial thermoplasty: Asthma Intervention Research (AIR) trial. BMC pulmonary medicine. 2011;11:8.

309. GINA Report (Internet), From the Global Strategy for Asthma Management and Prevention, Global Initiative for Asthma (GINA) (updated December 2011; cited May 2012). Available from http://www.ginasthma.org. [Internet]; Available from: http://www.ginasthma.org.

310. Grainge CL, Lau LC, Ward JA, Dulay V, Lahiff G, Wilson S, et al. Effect of bronchoconstriction on airway remodeling in asthma. The New England journal of medicine. 2011;364(21):2006-15.

311. Postma DS, Timens W. Remodeling in asthma and chronic obstructive pulmonary disease. Proceedings of the American Thoracic Society. 2006;3(5):434-9.

312. Lloyd CM, Robinson DS. Allergen-induced airway remodelling. The European respiratory journal : official journal of the European Society for Clinical Respiratory Physiology. 2007;29(5):1020-32.

313. Borger P, Miglino N, Baraket M, Black JL, Tamm M, Roth M. Impaired translation of CCAAT/enhancer binding protein alpha mRNA in bronchial smooth muscle cells of asthmatic patients. The Journal of allergy and clinical immunology. 2009;123(3):639-45.

314. Borger P, Matsumoto H, Boustany S, Gencay MM, Burgess JK, King GG, et al. Disease-specific expression and regulation of CCAAT/enhancer-binding proteins in asthma and chronic obstructive pulmonary disease. The Journal of allergy and clinical immunology. 2007;119(1):98-105.

315. Roth M, Johnson PR, Borger P, Bihl MP, Rudiger JJ, King GG, et al. Dysfunctional interaction of C/EBPalpha and the glucocorticoid receptor in asthmatic bronchial smooth-muscle cells. The New England journal of medicine. 2004;351(6):560-74.

316. Bentley JK, Hershenson MB. Airway smooth muscle growth in asthma: proliferation, hypertrophy, and migration. Proceedings of the American Thoracic Society. 2008;5(1):89-96.

317. Wilson JW, Hii S. The importance of the airway microvasculature in asthma. Current opinion in allergy and clinical immunology. 2006;6(1):51-5.

318. Abdel-Rahman AM, el-Sahrigy SA, Bakr SI. A comparative study of two angiogenic factors: vascular endothelial growth factor and angiogenin in induced sputum from asthmatic children in acute attack. Chest. 2006;129(2):266-71.

319. Asai K, Kanazawa H, Kamoi H, Shiraishi S, Hirata K, Yoshikawa J. Increased levels of vascular endothelial growth factor in induced sputum in asthmatic patients. Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology. 2003;33(5):595-9.

320. Ammit AJ, Moir LM, Oliver BG, Hughes JM, Alkhouri H, Ge Q, et al. Effect of IL-6 trans-signaling on the pro-remodeling phenotype of airway smooth muscle. American journal of physiology Lung cellular and molecular physiology. 2007;292(1):L199-206.

321. Raidl M, Sibbing B, Strauch J, Muller K, Nemat A, Schneider PM, et al. Impaired TNFalpha-induced VEGF expression in human airway smooth muscle cells from smokers with COPD: role of MAPkinases and histone acetylation--effect of dexamethasone. Cell Biochem Biophys. 2007;49(2):98-110.

322. Strieter RM, Burdick MD, Mestas J, Gomperts B, Keane MP, Belperio JA. Cancer CXC chemokine networks and tumour angiogenesis. Eur J Cancer. 2006;42(6):768-78.

323. Johnson PR, Armour CL, Carey D, Black JL. Heparin and PGE2 inhibit DNA synthesis in human airway smooth muscle cells in culture. The American journal of physiology. 1995;269(4 Pt 1):L514-9.

324. Ades EW, Candal FJ, Swerlick RA, George VG, Summers S, Bosse DC, et al. HMEC-1: establishment of an immortalized human microvascular endothelial cell line. The Journal of investigative dermatology. 1992;99(6):683-90.

325. Timmins NE, Nielsen LK. Generation of multicellular tumor spheroids by the hanging-drop method. Methods Mol Med. 2007;140:141-51.

326. Philippova M, Banfi A, Ivanov D, Gianni-Barrera R, Allenspach R, Erne P, et al. Atypical GPI-anchored T-cadherin stimulates angiogenesis in vitro and in vivo. Arteriosclerosis, thrombosis, and vascular biology. 2006;26(10):2222-30.

327. Bradley ME, Bond ME, Manini J, Brown Z, Charlton SJ. SB265610 is an allosteric, inverse agonist at the human CXCR2 receptor. Br J Pharmacol. 2009;158(1):328-38.

328. Salchow K, Bond ME, Evans SC, Press NJ, Charlton SJ, Hunt PA, et al. A common intracellular allosteric binding site for antagonists of the CXCR2 receptor. Br J Pharmacol. 2010;159(7):1429-39.

329. Ahuja SK, Murphy PM. The CXC chemokines growth-regulated oncogene (GRO) alpha, GRObeta, GROgamma, neutrophil-activating peptide-2, and epithelial cell-derived neutrophil-activating peptide-78 are potent agonists for the type B, but not the type A, human interleukin-8 receptor. The Journal of biological chemistry. 1996;271(34):20545-50.

330. Asai K, Kanazawa H, Otani K, Shiraishi S, Hirata K, Yoshikawa J. Imbalance between vascular endothelial growth factor and endostatin levels in induced sputum from asthmatic subjects. The Journal of allergy and clinical immunology. 2002;110(4):571-5.

331. Kristan SS, Malovrh MM, Silar M, Kern I, Flezar M, Kosnik M, et al. Airway angiogenesis in patients with rhinitis and controlled asthma. Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology. 2009;39(3):354-60.

332. Liu Z, Kobayashi K, van Dinther M, van Heiningen SH, Valdimarsdottir G, van Laar T, et al. VEGF and inhibitors of TGFbeta type-I receptor kinase synergistically promote blood-vessel formation by inducing alpha5-integrin expression. Journal of cell science. 2009;122(Pt 18):3294-302.

333. Borish L, Culp JA. Asthma: a syndrome composed of heterogeneous diseases. Annals of allergy, asthma & immunology : official publication of the American College of Allergy, Asthma, & Immunology. 2008;101(1):1-8; quiz -11, 50.

334. Keane MP, Belperio JA, Burdick MD, Lynch JP, Fishbein MC, Strieter RM. ENA-78 is an important angiogenic factor in idiopathic pulmonary fibrosis. American journal of respiratory and critical care medicine. 2001;164(12):2239-42.

335. Heidemann J, Ogawa H, Dwinell MB, Rafiee P, Maaser C, Gockel HR, et al. Angiogenic effects of interleukin 8 (CXCL8) in human intestinal microvascular endothelial cells are mediated by CXCR2. The Journal of biological chemistry. 2003;278(10):8508-15.

336. Chapman RW, Phillips JE, Hipkin RW, Curran AK, Lundell D, Fine JS. CXCR2 antagonists for the treatment of pulmonary disease. Pharmacology & therapeutics. 2009;121(1):55-68.

337. Trian T, Benard G, Begueret H, Rossignol R, Girodet PO, Ghosh D, et al. Bronchial smooth muscle remodeling involves calcium-dependent enhanced mitochondrial biogenesis in asthma. The Journal of experimental medicine. 2007;204(13):3173-81.

338. Shifren A, Chen A, Castro M. Point: efficacy of bronchial thermoplasty for patients with severe asthma. Is there sufficient evidence? Yes. Chest. 2011;140(3):573-5; discussion 8.

339. Cox PG, Miller J, Mitzner W, Leff AR. Radiofrequency ablation of airway smooth muscle for sustained treatment of asthma: preliminary investigations. The European respiratory journal : official journal of the European Society for Clinical Respiratory Physiology. 2004;24(4):659-63.

340. Miller JD, Cox G, Vincic L, Lombard CM, Loomas BE, Danek CJ. A prospective feasibility study of bronchial thermoplasty in the human airway. Chest. 2005;127(6):1999-2006.

341. O'Byrne PM, Naji N, Gauvreau GM. Severe asthma: future treatments. Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology. 2012;42(5):706-11.

Objective of the thesis

Asthma is a chronic inflammatory respiratory disorder, which affects estimated 300 million people worldwide. Thus, asthma is a major health concern and currently there is no cure for asthma. Remodeling of the airway wall is a hallmark pathology of asthma manifesting as tissue structural changes. Amongst others, increased vascularity of the airway wall and an increased bulk of BSMC have been observed. The thickening of the basement membrane in asthmatic airway walls is one of the most prominent tissue structural changes linked to airway wall remodeling in asthma. Together with the increased BSMC mass and the inflammatory environment this may lead to a locally restricted hypoxic environment for the cells of the sub-epithelial cell layers in the airway wall of asthma patients. In my thesis the contribution of BSMC to asthma associated airway wall angiogenesis was assessed, comparing cells obtained from asthma patients to those of non-asthma subjects under different conditions.

The specific objectives of my thesis were:

I) Comparison of the angiogenic potential of BSMC obtained from asthmatics compared to non-asthmatics and to identify factors that are produced by BSMC and support (induce) angiogenesis of the airway wall.

II) To investigate the effect of hypoxia on the release of angiogenic factors by BSMC and on the proliferation of BSMC.

III) Effects of HDM allergens on the angiogenic properties of BSMC and their potential role in allergen-mediated angiogenesis..

In summary this thesis aims to contribute to the understanding of the pivotal role of BSMC in the pathogenesis of airway wall remodeling with a focus on the triangle of inflammation - resident tissue - forming lung cells (BSMC) - angiogenesis and the influence of low oxygen concentration and HDM allergens on the studied parameters. The findings may help to develop novel therapeutic strategies to inhibit the pathological effects of airway wall remodeling.

Bronchial smooth muscle cells of asthmatics promote angiogenesis through elevated secretion of CXC-chemokines

Laura Keglowich, MSc[1]; Michael Roth, PhD[2]; Maria Philippova, PhD[1], Thérèse Resink, PhD[1], Gavin Tjin, BSc[3]; Brian Oliver, PhD[3]; Didier Lardinois, MD[4], Sophie Dessus-Babus, PhD[5]; Reinoud Gosens, PhD[6]; Katrin Hostettler Haack, PhD[1,2]; Michael Tamm, MD[1,2]; Peter Borger, PhD[1]

Affiliations:

1) Pulmonary Cell Research & Signal Transduction, Department of Biomedicine, University of Basel, Basel, Switzerland
2) Department of Pneumology, University Hospital Basel, Basel, Switzerland
3) Department of Pharmacology, Sydney Medical School and Cell Biology, Woolcock Institute of Medical Research, The University of Sydney, Sydney, Australia.
4) Department of Thoracic Surgery, University Hospital Basel, Basel, Switzerland
5) Functional Genomics, Friedrich Miescher Institute for Biomedical Research, Basel, Switzerland
6) Department of Molecular Pharmacology, University of Groningen, Groningen, The Netherlands.

Running head: In asthma CXCR2 ligands promote angiogenesis

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Abstract

Background: Airway wall remodelling is a key pathology of asthma. It includes thickening of the airway wall, hypertrophy and hyperplasia of bronchial smooth muscle cells (BSMC), as well as an increased vascularity of the sub-epithelial cell layer. BSMC are known to be the effector cells of bronchoconstriction, but they are increasingly recognized as an important source of inflammatory mediators and angiogenic factors.

Objective: To compare the angiogenic potential of BSMC of asthma and non-asthma patients and to identify asthma-specific angiogenic factors.

Methods: Primary BSMC were isolated from human airway tissue of asthmatic and non-asthmatic patients. Conditioned medium (CM) collected from BSMC isolates was tested for angiogenic capacity using the endothelial cell (EC)-spheroid *in vitro* angiogenesis assay. Angiogenic factors in CM were quantified using a human angiogenesis antibody array and enzyme linked immunosorbent assay.

Results: Induction of sprout outgrowth from EC-spheroids by CM of BSMC obtained from asthma patients was increased compared with CM of control BSMC (twofold, $p < 0.001$). Levels of ENA-78, GRO-α and IL-8 were significantly elevated in CM of BSMC from asthma patients ($p < 0.05$ *vs*. non-asthmatic patients). SB 265610, a competitive antagonist of chemokine (CXC-motif) receptor 2 (CXCR2), attenuated the increased sprout outgrowth induced by CM of asthma patient-derived BSMC.

Conclusions: BSMC isolated from asthma patients exhibit increased angiogenic potential. This effect is mediated through the CXCR2 ligands (ENA78, GRO-α and IL-8) produced by BSMC.

Implications: CXCR2 ligands may play a decisive role in directing the neovascularization in the sub-epithelial cell layers of the lungs of asthma patients. Counteracting the CXCR2-mediated neovascularization by pharmaceutical compounds may represent a novel strategy to reduce airway remodeling in asthma.

Abbreviations

bFGF	*Basic fibroblast growth factor*
BSA	Bovine serum albumin
BSMC	Bronchial smooth muscle cell
CEBPα	CCAAT/enhancer binding protein
CM	Conditioned medium
CXCR2	Chemokine (C-X-C motif) receptor 2
ECGM	Endothelial cell growth medium
ELISA	Enzyme-linked immunosorbent assay
ENA-78	Epithelial neutrophil-activating protein 78; epithelial cell-derived neutrophil attractant-78
FCS	Fetal calf serum
FEV_1	Forced expiratory volume in 1 second
GRO-α	Growth regulated oncogene-*alpha*
HMEC-1	Human microvascular endothelial cell line
IgG	Immunoglobulin G
IL	Interleukin
MCP-1	Monocyte chemotactic protein-1
PBS	Phosphate buffered saline
TBST	Tris buffered saline (with 0.1% Tween)
TIMP	Tissue inhibitor of metalloproteinases
TRITC	Tetramethyl Rhodamine Isothiocyanate
VEGF	Vascular endothelial growth factor

Introduction

Asthma is a chronic inflammatory airway disease affecting over 300 million people worldwide with an expected increase of a further 100 million by 2025 [1, 2]. Although airway inflammation in asthma can be controlled, there is currently no cure for the disease and airway wall remodeling is unaffected by any asthma therapy. The etiology of asthma remains obscure and the pathology of asthma involves genetic predisposition and environmental factors. Increasing evidence suggests that inflammation is not the only cause of asthma and airway remodeling may be equally important [3]. Airway wall remodeling refers to persistent cellular and structural changes in the airway wall. In progressive disease, airway remodeling includes epithelial goblet cell hypertrophy, enhanced collagen deposition and airway wall hyperplasia [4-6].

It has been shown that bronchial smooth muscle cells (BSMC) isolated from asthma patients release more pro-inflammatory mediators than BSMC from control subjects [7-9]. These findings suggest that BSMC of asthma patients exhibit a hyper-reactive "primed" phenotype, which may be explained, at least in part, by an aberrant expression of the transcription regulator CCAAT/enhancer binding protein α (C/EBPα) [7, 10-12].

Histological studies of airways in adults and children with asthma have variously provided evidence for increased microvessel density/vascularity and increased numbers of BSMC [13-17]. Increased airway vascularity has also been demonstrated *in vivo* in asthmatic patients by bronchovideoscopy [18]. Vascular endothelial growth factor (VEGF), a mediator of microvascular leakage, EC proliferation and vascular remodeling, was found in the airways of asthma patients [19, 20]. Furthermore, increased levels of angiogenin and monocyte chemotactic protein-1 (MCP-1) were also found in the airways and airway lining fluids (broncho-alveolar lavage fluid, sputum) of asthma patients [21]. More recently it was reported that BSMC were a source of angiogenic factors [22-24] and that BSMC derived from asthma patients could initiate and sustain angiogenesis *in vitro* through release of VEGF [24]. These data suggest that BSMC may direct neovascularization in sub-epithelial cell layers in the airways of asthma patients. In addition, CXCR2 ligands have been implicated in angiogenesis but mainly in the context of tumor neovascularization [25]. Here we hypothesize that CXCR2 ligands may also be involved in asthma associated airway wall angiogenesis. Better knowledge of the spectrum of potential angiogenic factors expressed by BSMC is crucial to therapy of angiogenesis-driven airway remodeling in asthma. Using *in vitro* angiogenesis assay, angiogenesis antibody array,

enzyme linked immunosorbent assay (ELISA) and a competitive CXCR2-selective antagonist, this study demonstrates that BSMC derived from asthma patients exhibit increased angiogenic potential compared to controls that is mediated by CXCR2-ligands.

Methods

Ethics statement

Human airway tissue was obtained from explanted and resected lungs and post mortem organ donors with ethical approval from The University of Sydney and participating hospitals (Concord Repatriation General Hospital, Sydney South West Area Health Service and Royal Price Alfred Hospital) for sample collection. All volunteer, or their next of kin, provided written informed consent. The use of human primary BSMC was approved by local ethical committees (University Hospital, Basel, Switzerland, and University Hospital, Groningen, The Netherlands). Written informed consent was provided by each patient.

Histochemistry of human airway tissue

Human airway tissue was obtained from asthmatic patients and from healthy organ donors whose lungs were deemed unfit for use in a transplant procedure (for the samples used as non-diseased controls). Airway tissues were fixed in 4% phosphate-buffered formalin (pH 7.2) and embedded in paraffin. Sections of 3 µm thickness were stained with Milligan's trichrome and imaged using an Olympus BX60 microscope equipped with a DP71 camera (Olympus, Hamburg, Germany).

Isolation of primary BSMC from human airway tissue and preparation of conditioned medium

Human airway tissues from 8 non-asthmatic (NA) and 9 asthmatic (A) patients were obtained either by endobronchial biopsy or therapeutic lung resection. BSMC were isolated from each individual tissue as described before [26, 27]. BSMC isolates were normally grown in BSMC growth medium (RPMI 1640 supplemented with 5% fetal calf serum (FCS), 1x antibiotics-antimycotics and 1x modified Eagle`s medium vitamin mix (Invitrogen, Lubio, Luzern, Switzerland)) under normoxic conditions (20% O_2, 5% CO_2, 37 °C). BSMC were used at passages 3-10.

For the preparation of conditioned medium (CM), BSMC were seeded at 10^5 cells/well in 6-well plates and grown in normal growth medium for 24 h. Cells were subjected to a 24 h period of serum-deprivation and then further cultured for 24 h and 72 h under resting (serum-deprived) or normal growth (5% FCS-containing) conditions. Proliferation experiments showed that cell numbers between asthmatics and non-asthmatics did not significantly differ in our experimental setting (fold increase in cell number after 72 h: asthmatics: 2.05 ± 0.15, non-asthmatics: 1.98 ± 0.21; p = 0.78). Culture supernatants/CM

were harvested, centrifuged to remove cells and stored at -80 °C until use. Every BSMC isolate was used for preparation of CM. All experiments (except endothelial cell tube formation assay) were performed with cell culture supernatant (24 h and 72 h, serum-deprived) and CM (24 h and 72 h, 5% FCS). For any given BSMC isolate the experimental protocols for preparation of CM were performed on at least two independent occasions and in duplicate for each condition.

Endothelial tube-formation assay

Human microvascular endothelial cell (EC) line HMEC-1 [28] was normally maintained in EC growth medium (ECGM, Provitro, Bioconcept, Allschwil, Switzerland) supplemented with 10% FCS under normoxic conditions. Spheroids composed of 500 HMEC-1 cells were prepared using the hanging drop method [29]. The tube-forming (sprout outgrowth) assay was performed as previously described [30]. At least 10 spheroids per gel were embedded within fibrin gels in 48-well plates. Gels were overlaid with a 1:1 mixture of ECGM supplemented with 2% FCS and either normal BSMC growth medium (to determine spontaneous sprout outgrowth) or CM obtained from FCS-stimulated BSMC (to measure BSMC-dependent sprout outgrowth). CM (t = 72 h) of BSMC isolates from 3 different asthmatic patients and 3 different controls were tested. To block the chemokine (CXC-motif) receptor 2 (CXCR2) the competitive antagonist SB 265610 [31, 32] was included within the fibrin gel and medium overlay. After incubation for 24 h, spheroids were fixed in-gel, stained with TRITC-conjugated phalloidin and sprout outgrowth from each spheroid was quantitated by morphometric analysis of the length of outgrowing tubules [326]. For each well the mean of the 10 longest tubules per spheroid was quantitated by morphometric analysis of the length of outgrowing tubules using AnalySIS software (Soft Imaging System GmbH, Munich, Germany). This value was used to calculate the mean ± S.E.M.

Viability and proliferation assays

HMEC-1 were seeded at $7.5x10^4$ cells/well in 48-well plates, grown for 24 h, serum-deprived for 24 h and then further cultured in ECGM/10% FCS without or with inclusion of SB 265610. Viability was examined after 24 h by Trypan blue staining and manual cell counting using a Neubauer chamber. Proliferation was measured after 48 h by enzymatic disaggregation and cell enumeration using a Beckman Coulter particle counter Z1 (Nyon, Switzerland).

Immunocytochemistry

HMEC-1 were grown in 24-well-plates to 70% confluency and fixed in 4% PFA (20 min). Cells were permeabilized by incubation (5 min) in PBS containing 0.5% Triton X-100 and 1% bovine serum albumin (BSA) and unspecific binding was blocked by incubation (1 h) in PBS containing 5% BSA. Cells were incubated for 2 h with either mouse anti-CXCR2 IgG (Abcam, Lucerna-Chem, Luzern, Switzerland) or non-immune mouse IgG (DAKO, Baar, Switzerland), washed (PBS containing 0.05% Triton X-100) and then incubated with FITC-conjugated secondary anti-mouse IgG (Southern Biotech, Bioconcept, Allschwil, Switzerland). Nuclei were counterstained using Hoechst 33342 (200 ng/ml in PBS, 5 min). Images were taken with an Olympus IX50 inverted microscope (Olympus, Hamburg, Germany) equipped with a Color View II camera; exposure time was constant for both conditions.

Angiogenesis Antibody Array

To identify angiogenic factors in the CM of FCS-stimulated BSMC the Human Angiogenesis Antibody Array G Series 1 (Raybiotech, Lucerna-Chem, Luzern, Switzerland) specific for 20 angiogenesis-relevant proteins (antibody array map provided in Figure 3) was used. The array test was performed on four separate occasions; on any given occasion CM and cell culture supernatant of BSMC-derived from asthmatic and non-asthmatic patients were tested in parallel. BSMC isolates from 4 different asthmatic and 4 different non-asthmatic patients were used. 100 µl aliquots of undiluted CM/cell culture supernatant were applied to each sub-array and the expression levels of angiogenesis-relevant factors were determined according to the manufacturer's instructions. Cy3-fluoresence was measured using a NimbleGen MS 200 microarray Scanner (Roche, Basel, Switzerland) and signal intensities were analyzed with AIDA software (Raytest, Straubenhardt, Germany). Control experiments with BSMC growth or starving medium respectively were performed and revealed no unspecific signals due to the culture medium (no FCS or 5% FCS). Intensity ratios between asthmatic and control samples were calculated (normalized to the internal reference positive control); a ratio of ≥ 1.3 was considered significantly different as indicated by the manufacturer.

Cytokine-ELISA

ELISA kits for epithelial neutrophil-activating protein 78 (ENA-78), growth regulated oncogene α (GRO-α), and VEGF-A were from R&D (Abingdon. UK). ELISA kit for IL-8 was from Orgenium (Anibiotech, Vantaa, Finland). ELISAs were performed according to

the respective manufacturer´s instructions. For these experiments, we used CM of BSMC isolates from 6 different asthmatic patients and 6 different controls.

RT-PCR

HMEC-1 cells were plated in a 25 cm^2 cell culture flask and grown to confluency. Cells were washed 2x with DPBS and total RNA was isolated using RNeasy Mini kit (Qiagen, Hombrechtikon, Switzerland) according to the manufacturer`s instructions. RNA concentration was determined by spectroscopy (NanoDrop, Witec, Luzern, Switzerland). First strand DNA was synthesized with m-MLV Reverse Transcriptase (Promega, Dübendorf, Switzerland) from 2.5 µg of total RNA. The obtained cDNA was subjected to amplification with HotStarTaq Plus DNA polymerase (Qiagen, Hombrechtikon, Switzerland) using the following CXCR2-specific primers: forward 5`-CAG TTA CAG CTC TAC CCT GCC–3, reverse 5`-CCA GGA GCA AGG ACA GAC CCC–3 generating a 451 bp spanning fragment. PCR conditions were: 5 min 95°C; 32x: 30 sec 98°C, 30 sec 58°C, 1 min 72°C; 10 min 71°C. PCR products were size-fractioned on a 1% agarose gel, stained with ethidium bromide and visualized under UV light.

Statistics

Data was analyzed using Microsoft Excel and Graph Pad Prism software. Student`s t-test (Spheroid analysis) and Mann-Whitney test (ELISA) have been performed. A p-value of < 0.05 was considered significant.

Results

Increased vascularization in human tissue sections from the lung of asthma patients

Patients with mild to moderate asthma (n = 9, 4 females/5 males, age 23-60 years) had reversible airway obstruction documented in the past, with median FEV_1 of 84.6% of the predicted value (ranging from 68.5% to 124.9%). Milligan's trichrome staining of human airway tissue cross-sections demonstrated marked differences between non-asthmatic (Figure 1A) and asthmatic (Figure 1B) patients; the airway walls of asthmatic patients exhibited hyperplastic epithelium, increased thickness of the basement membrane, increased bulk of smooth muscle bundles, as well as increased microvessel density within sub-epithelial cell layers.

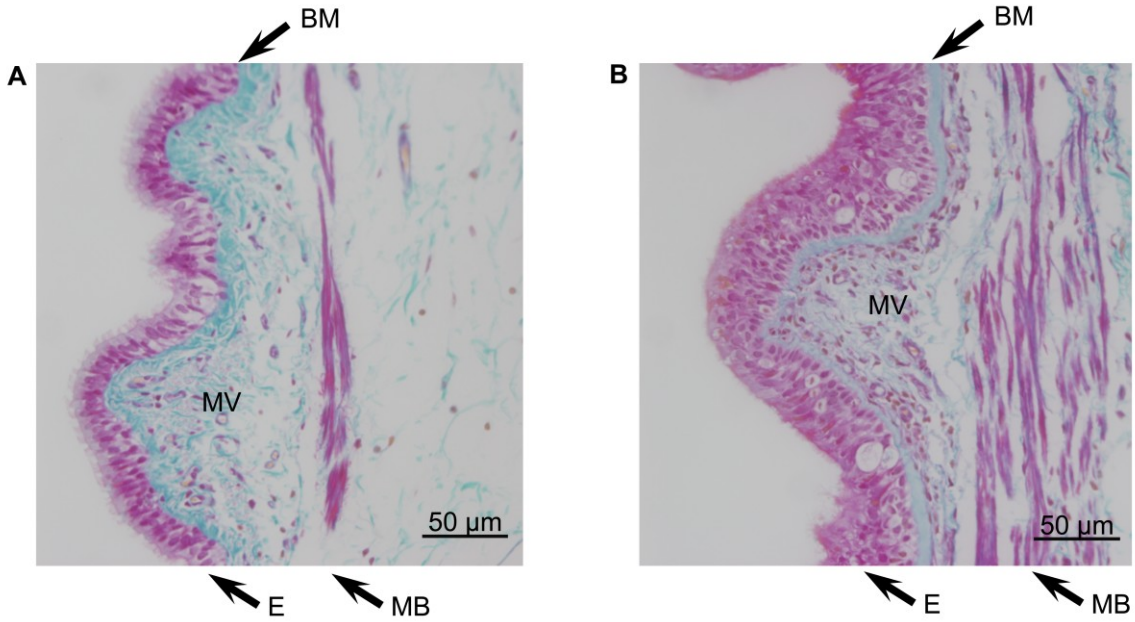


Figure 1: Milligan's trichrome stained sections of airway tissue from non-asthma (A) and asthma (B) patients. Images are representative of tissues obtained from 3 non-asthmatic and 3 asthmatic patients. Nuclei and muscle: magenta, collagen: green, RBC: orange. Note epithelial hyperplasia, thickening of muscle bundles and basement membrane, and increased microvessel density in asthmatic airways. E = epithelium, MB = muscle bundles, BM = basement membrane, MV = microvessels.

Increased angiogenic potential of BSMC obtained from asthmatics

Angiogenic potential of culture supernatants (CM) collected from normally growing BSMC of asthmatics and non-asthmatics was examined using the EC-spheroid *in vitro* angiogenesis assay. Spheroids were also cultured in unconditioned medium (i.e. with medium that had not been included with BSMC) to control for "spontaneous" sprout outgrowth. Figure 2 presents representative images of spheroids (Figure 2A) and the quantitation of sprout outgrowth into the 3D fibrin-gel matrix as mean tubule length/spheroid (Figure 2B) after a 24 h culture period.

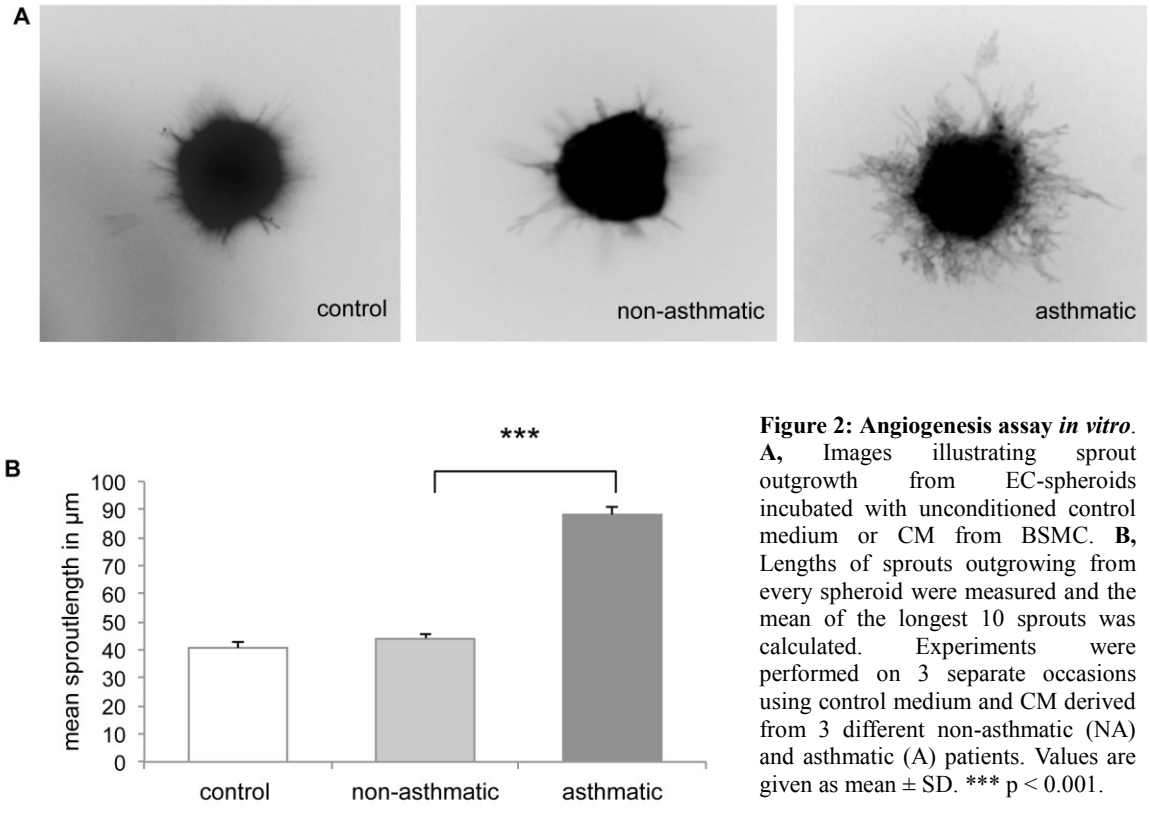


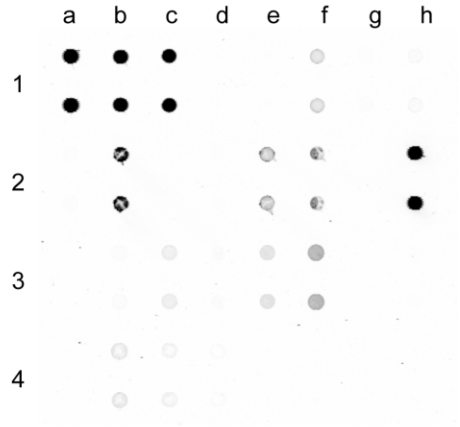
Figure 2: Angiogenesis assay *in vitro*. **A,** Images illustrating sprout outgrowth from EC-spheroids incubated with unconditioned control medium or CM from BSMC. **B,** Lengths of sprouts outgrowing from every spheroid were measured and the mean of the longest 10 sprouts was calculated. Experiments were performed on 3 separate occasions using control medium and CM derived from 3 different non-asthmatic (NA) and asthmatic (A) patients. Values are given as mean ± SD. *** $p < 0.001$.

Incubation of EC-spheroids with CM of BSMC from asthmatic patients resulted in a twofold increase in sprout outgrowth as compared with CM of BSMC from non-asthmatic patients ($p < 0.001$). In the latter, sprout outgrowth was not significantly different from "spontaneous" outgrowth ($p = 0.43$). Thus the angiogenic potential of BSMC isolates derived from asthmatic patients is likely due to their secretion of pro-angiogenic factors into culture supernatants.

Identification of differentially expressed angiogenic factors in BMSC of asthmatics *vs.* non-asthmatics

We used a Raybiotech angiogenesis antibody array to identify and compare expression levels of soluble BSMC-derived angiogenic and angiostatic factors in the CM of normally growing BSMC derived from non-asthmatic and asthmatic patients (Figure 3A-3D and S1). The array identified six factors that were present at higher levels in the CM of BSMC obtained from asthmatics patients, namely angiogenin, ENA-78, GRO-α, IL-6, IL-8 and MCP-1 (Figure 3D)**.** ENA-78, GRO-α and IL-8 share the same receptor (CXCR2), and have therefore been the focus of all further studies.

A



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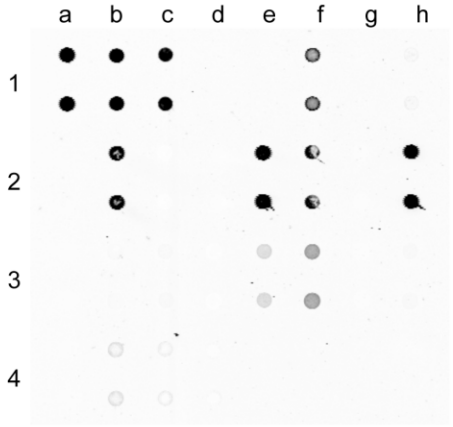


C

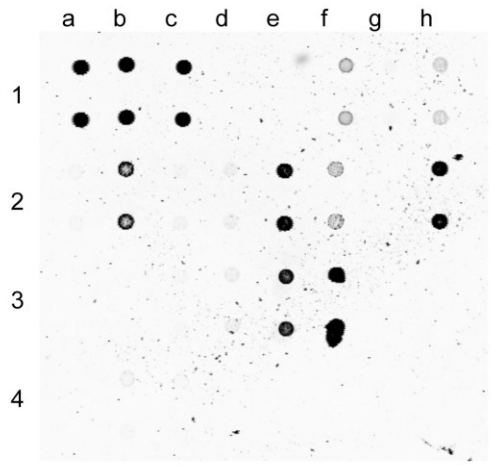
	a	b	c	d	e	f	g	h
1	Pos	Pos	Pos	Neg	Neg	Angio-genin	EGF	ENA-78
2	bFGF	GRO	IFN-g	IGF-1	IL-6	IL-8	Leptin	MCP-1
3	PFGF-BB	PIGF	RANTES	TGF-beta	TIMP-1	TIMP-2	Thrombo-poeitin	VEGF
4	VEGF-D	IC1	IC2	IC3	Neg	Neg	Neg	Neg

D

	exp.1	exp. 2	exp. 3	exp. 4		exp. 1	exp. 2	exp. 3	exp. 4	
	no FCS					5% FCS				
Angiogenin	1.2	13.4	3.8	3.8	+++	0.7	4.1	1.5	3.3	+++
ENA-78	1.2	3.4	1.6	9.7	+++	5.1	1.4	1.3	4.6	++++
GRO	2.3	1.8	1.2	1.8	+++	1.2	0.9	1.0	2.1	+
IL-6	38	2.4	7.4	nq	+++	7.1	1.2	1.3	3.5	+++
IL-8	6	2.4	1.5	3	++++	2.5	1.3	1.0	2.4	+++
MCP-1	1.9	1.6	1.0	2.5	+++	1.0	1.7	0.9	2.3	++

Figure 3: Human angiogenesis antibody array. Examples of the angiogenesis antibody array (exp. 3) comparing CM from BSMC of non-asthmatic (**A**) and asthmatic (**B**) patients. **C,** Antibody array map. Standard abbreviations for the detected proteins are used, Pos: positive control, Neg: negative control, IC1-IC3: internal controls 1-3. D, Quantitative analysis from each of the 4 independent experiments performed. Intensity ratios (A:NA) in a paired analysis are shown. Upregulation in any single experiment is indicated by a cross. nq = not quantifiable (out of range).

A



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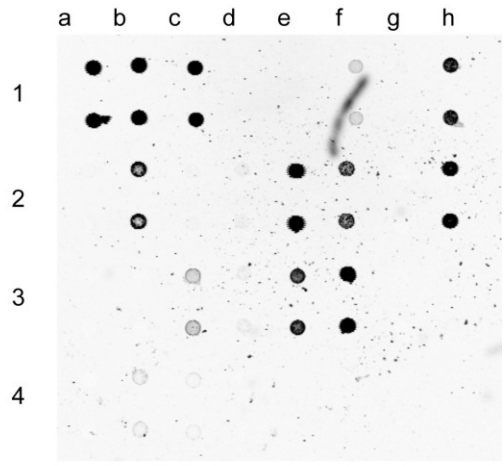

C

	a	b	c	d	e	f	g	h
1	Pos	Pos	Pos	Neg	Neg	Angio-genin	EGF	ENA-78
2	bFGF	GRO	IFN-g	IGF-1	IL-6	IL-8	Leptin	MCP-1
3	PFGF-BB	PIGF	RANTES	TGF-beta	TIMP-1	TIMP-2	Thrombo-poeitin	VEGF
4	VEGF-D	IC1	IC2	IC3	Neg	Neg	Neg	Neg

Figure S1: Human angiogenesis antibody array. Examples of the angiogenesis antibody array (exp. 4) comparing CM from BSMC of non-asthmatic (**A**) and asthmatic (**B**) patients of a longer exposure time to demonstrate differential expression levels in proteins with a low signal (e.g. ENA-78). **C**, Antibody array map. Standard abbreviations for the detected proteins are used, Pos: positive control, Neg: negative control, IC1-IC3: internal controls 1-3.

Increased CXCR2-ligands secretion by BSMC of asthmatics

To validate the array findings we elected to measure levels of GRO-α , IL-8 and ENA-78 in CM by ELISA; these all belong to the family of CXC-chemokines and signal *via* the shared receptor CXCR2 [329]. After 24 h resting culture conditions (without FCS) BSMC from asthma patients and control subjects secreted comparable amounts of GRO-α, IL-8, and ENA-78 (Table 1). After 72 h resting conditions levels of GRO-α, IL-8, and ENA-78 were significantly greater in CM of BSMC from asthmatic patients than from non-asthmatic patients ($p < 0.05$) (Table 1). Figure 4 shows levels of the measured secreted chemokines in CM from each of the tested BSMC after 24 h or 72 h periods of culture under normal growth conditions (presence of 5% FCS). Levels of GRO-α (Figure 4A), IL-8 (Figure 4B) and ENA-78 (Figure 4C) were significantly greater in CM of BSMC from asthmatic patients than from non-asthmatic patients ($p < 0.05$; all values summarized in Table 1).

Table 1: Summary of the levels (ng/ml) of CXCR2 ligands GRO-α, IL-8 and ENA-78. Concentration of the cytokines were measured in CM of BSMC from non-asthmatic (NA) and asthmatic (A) patients (n = 6 per group) after 24 h and 72 h culture under resting (serum-free) and normal growth (presence of 5% FCS) conditions. p-values were calculated using Mann Whitney U test.

	FCS (5%)	24 hours			72 hours		
		NA	A	p-value	NA	A	p-value
GRO-α	-	0.49 ± 0.30	0.60 ± 0.26	0.370	2.54 ± 1.66	3.50 ± 0.87	0.002
	+	2.24 ± 1.00	5.68 ± 2.00	>0.0001	4.40 ± 2.01	10.10 ± 3.23	0.022
IL-8	-	1.27 ± 0.47	1.69 ± 1.02	0.750	1.51 ± 0.51	5.82 ± 3.53	0.026
	+	3.80 ± 1.22	9.67 ± 2.05	0.002	5.23 ± 1.34	12.42 ± 2.08	0.002
ENA-78	-	0.11 ± 0.04	0.07 ± 0.03	0.625	0.23 ± 0.16	0.32 ± 0.12	0.016
	+	0.42 ± 0.23	0.89 ± 0.33	0.021	1.00 ± 0.60	1.62 ± 0.72	0.023

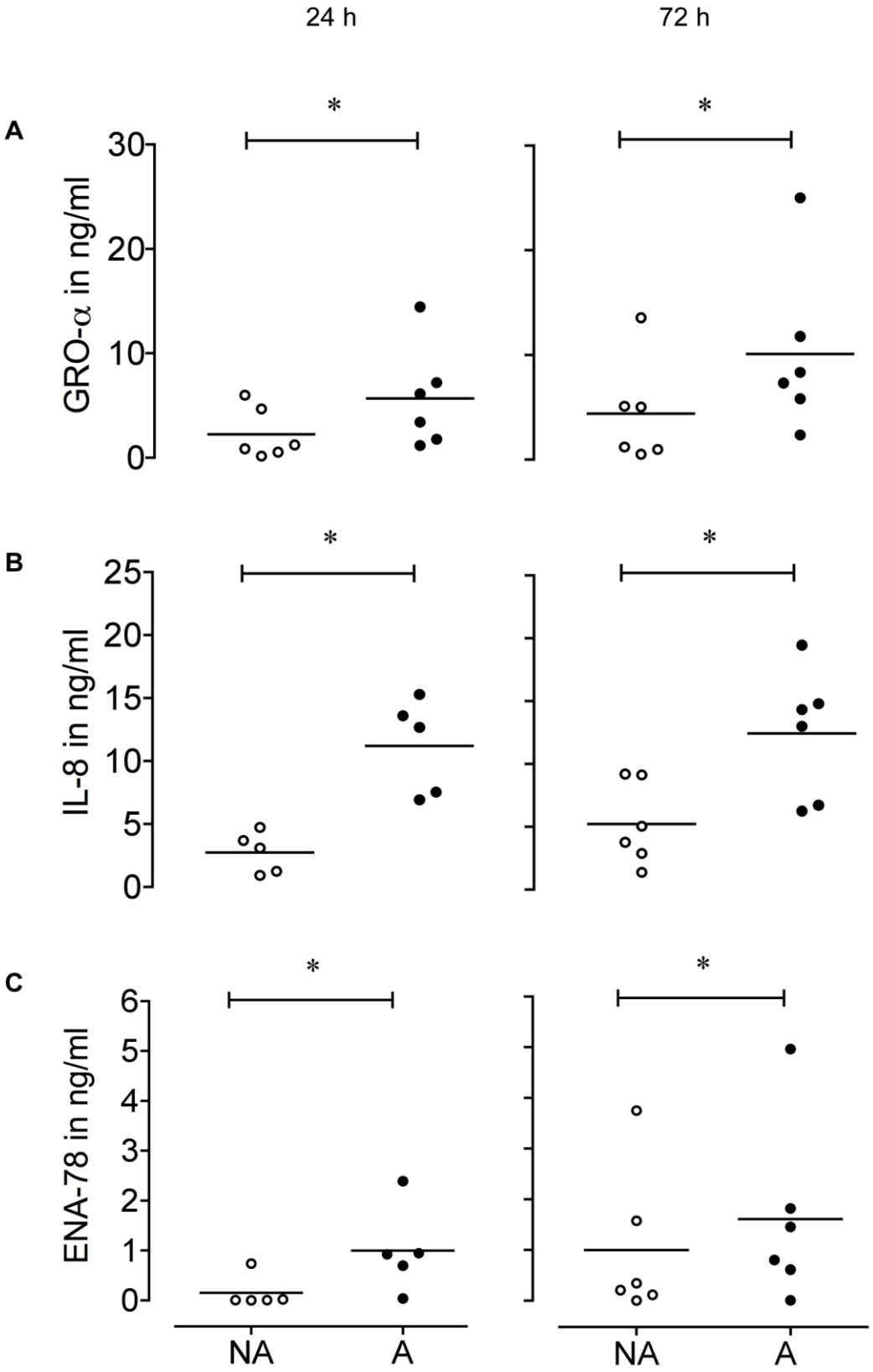

Figure 4: Chemokine release from BSMC derived from asthmatics and non-asthmatics. Concentrations of GRO-α (**A**), IL-8 (**B**) and ENA-78 (**C**) in CM were collected from BSMC of non-asthmatic (NA) and asthmatic (A) patients after 24 h and 72 h were determined by ELISA.* $p < 0.05$ ($n = 6$).

Figure S2 illustrates the high variance between cell isolates of different patients with respect to the concentrations and predominance of specific CXCR2 angiogenic factors.

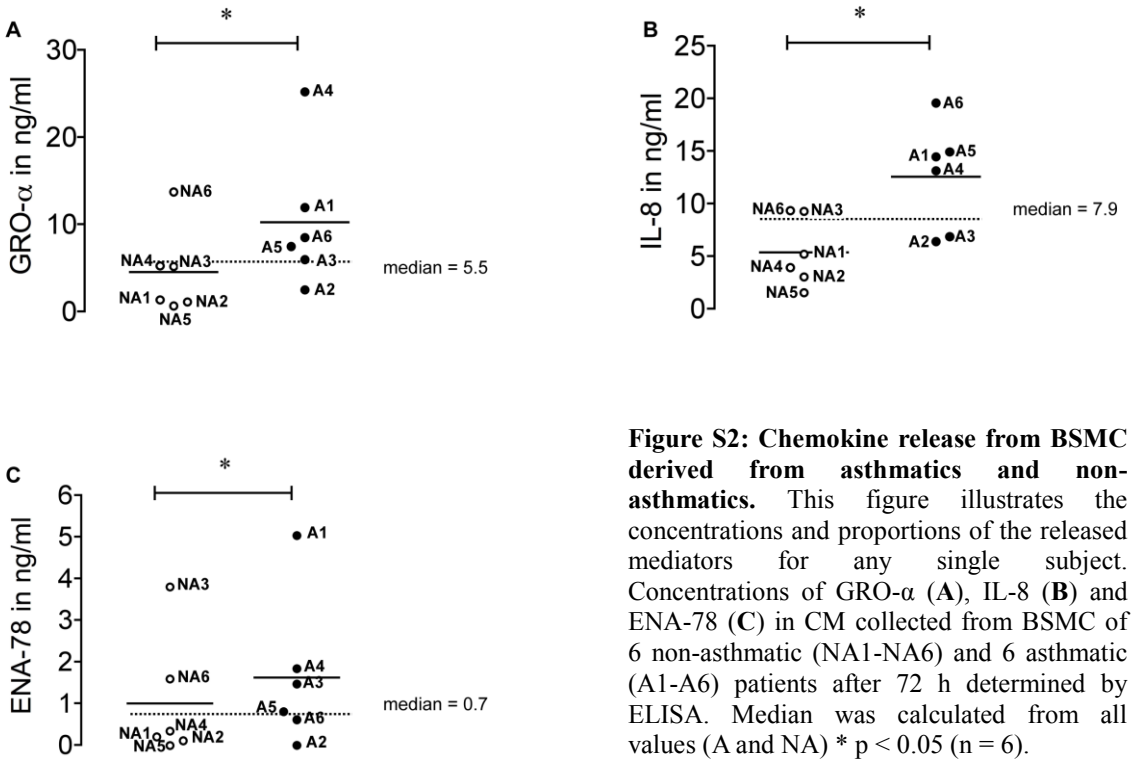

Figure S2: Chemokine release from BSMC derived from asthmatics and non-asthmatics. This figure illustrates the concentrations and proportions of the released mediators for any single subject. Concentrations of GRO-α (**A**), IL-8 (**B**) and ENA-78 (**C**) in CM collected from BSMC of 6 non-asthmatic (NA1-NA6) and 6 asthmatic (A1-A6) patients after 72 h determined by ELISA. Median was calculated from all values (A and NA) * $p < 0.05$ (n = 6).

Previously, Simcock *et al.* demonstrated that VEGF was the predominant factor released by TGF-β1- and/or IL-13-stimulated BSMC and that BSMC of asthma patients increased endothelial cell tube formation through VEGF [260]. Therefore, we screened our samples for VEGF protein by ELISA. BSMC obtained from asthmatics secreted significantly more VEGF compared to non-asthmatics (301 ± 48 pg/ml versus 179 ± 34 pg/ml, respectively; mean ± S.E.M.; $p < 0.05$, Figure S3).

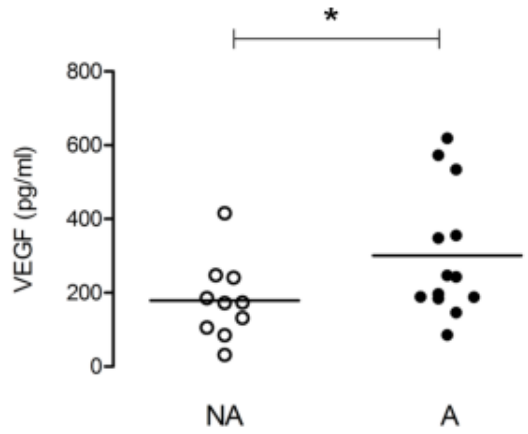

Figure S3: VEGF release by BSMC of asthmatic (A) and non-asthmatic (NA) subjects. BSMC were grown for 72 h in the presence of 5% FCS and VEGF was measured by ELISA. * $p < 0.05$, n=13 (A) and n=10 (NA).

Increased angiogenic potential of BSMC obtained from asthmatics is reversed by blocking CXCR2 with the competitive agonist SB 265610

Since GRO-α, ENA-78 and IL-8 exert their biological effects through CXCR2, their proangiogenic actions should be attenuated by the competitive CXCR2-selective antagonist SB 265610. CXCR2 expression by HMEC-1 cells was demonstrated by RT-PCR analysis of total RNA from HMEC-1 cells (Figure 5A); amplification of cDNA with CXCR2 specific primer demonstrates CXCR2 expression from HMEC-1 cells. Immunofluorescence staining protocols confirmed the presence of CXCR2 on HMEC-1 cells (Figure 5B). SB 265610 did not affect proliferation (Figure 5C) or viability (Figure 5D) of HMEC-1 monolayers (in ECGM/10% FCS). Furthermore, SB 265610 added alone did not affect basal sprout outgrowth in the EC-spheroid assay (data not shown). In contrast sprout outgrowth induced by CM of BSMC from asthma patients was markedly attenuated ($p < 0.001$) by SB 265610, even at the lowest dose (Figure 5E).

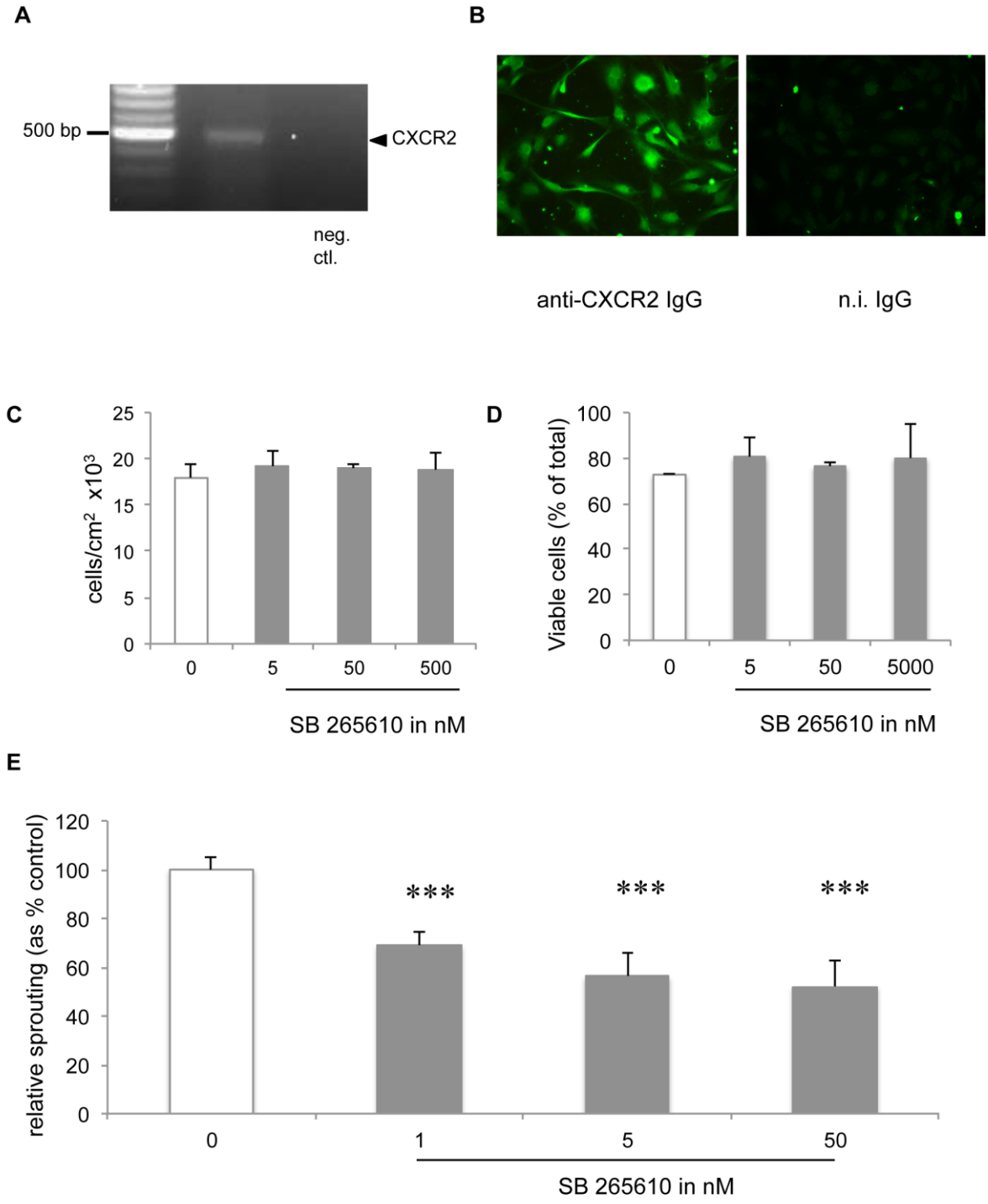

Figure 5: Involvement of CXCR2 in BSMC-induced neovascularization. **A,** RT-PCR of CXCR2 in HMEC-1. **B,** Immunofluorescence detection of CXCR2 on HMEC-1. C, D, HMEC-1 monolayers under normal EC growth conditions were cultured without or with SB 265610 for 48h (**C**) or 24h (**D**) (mean ± SD, n=3) and evaluated for proliferation (**C**) and viability (**D**). **E,** Effect of SB 265610 on sprout outgrowth induced by CM of BSMC from asthmatics. Values are mean ± S.E.M. after normalization to the control condition. *** $p < 0.001$ ($n \geq 3$).

Discussion

This study has demonstrated that BSMC from asthma patients have an increased angiogenic potential compared to BSMC from non-asthma control subjects. Angiogenesis antibody arrays revealed that CM of BSMC from asthma patients contained significantly higher levels of angiogenin, ENA-78, GRO-α, IL-6, IL-8 and MCP-1. Increased production of the CXCR2 ligands ENA-78, GRO-α and IL-8 was confirmed by ELISA and functionality of CXCR2 ligands in mediating pro-angiogenic effects of BSMC from asthma patients was demonstrated by reduction of EC sprout outgrowth in the presence of the specific CXCR2 antagonist SB 265610.

Neovascularization is increasingly recognized as an important feature of airway wall remodeling in asthma and it has become a topic of major interest. The Milligan's Trichrome staining of airway tissue sections presented in this study demonstrated that neovascularization occurs in close proximity to BSMC. Therefore, BSMC may play a more important role in the process of angiogenesis than previously considered. Several studies have examined mechanisms underlying angiogenesis in airway wall remodeling, and demonstrated roles for basic fibroblast growth factor (bFGF), angiogenin, endostatin and VEGF [20, 21, 24 34-36]. The study by Simcock *et al.* is of particular interest because they also used human airway smooth muscle cells isolated from non-asthmatic and asthmatic patients and a similar angiogenesis antibody array to that used in this study [24]. They found that BSMC from asthmatic patients produced higher levels of angiogenin, angiopoietin, VEGF, EGF, IGF-1, IFNγ, TIMP-1 and TIMP-2 in response to stimulation with either IL-13 or TGF-β [20, 24]. We found that in the presence of 5% FCS human BSMC of asthma patients released a different complement of angiogenic regulators including angiogenin, IL-6, MCP-1 and importantly three CXCR2 ligands, namely ENA-78, GRO-α and IL-8. ELISA assay revealed that BSMC of asthma patients also released significantly more VEGF than BSMC of controls. However, VEGF levels were low compared to other studies (pg/ml range rather than ng/ml) [20, 24] and below concentrations generally used to induce *in vitro* angiogenesis [24, 30, 37]. The very low concentration of VEGF in CM of FCS-cultured BSMC may explain why we could not detect VEGF with the antibody array we used. The discrepancy between the two studies regarding VEGF expression might be due to the use of different antibody array techniques (membrane [24] *versus* glass platform based array). It is also likely that production of any specific set of angiogenic regulators by BSMC is context dependent and is defined by the

microenvironmental setting, meaning that stimulation with IL-13 or TGF-β20 may induce a distinctly different set of angiogenic factors relative to stimulation with FCS. Additionally, intra-and inter-study variations with respect to specific composition and quantity of angiogenic factors produced might also reflect the heterogeneous character of asthma [38]. Nonetheless, both studies underscore the importance of enhanced release of angiogenic factors by BSMC from asthmatic patients.

CXCR2 ligands are known mediators of angiogenesis mainly in the context of tumor angiogenesis [25] and in other diseases like idiopathic pulmonary fibrosis [39, 40] where angiogenesis plays a role. To the best of our knowledge the increased release of this set of CXCR2 ligands (ENA-78, GRO-α and IL-8) from BSMC from asthmatic patients stimulated with FCS has not been reported before; neither has this release been linked to the induction of angiogenesis in the context of asthma airway remodeling. ENA-78, GRO-α, and IL-8 all mediate their angiogenic effect through CXCR2, although IL-8 has also been shown to bind the CXCR1-receptor [41]. Our findings point toward a previously unrecognized role for CXCR2 and its ligands in directing endothelial cell activation and neovascularization in asthma specifically, because slightly lower levels of these ligands present in CM of non-asthmatic controls did not significantly affect sprout outgrowth from endothelial cell spheroids. This may indicate that only BSMC obtained from asthmatics produce sufficient factors to reach the threshold required to induce sprouting.

CXCR2 is expressed in several different tissues and cell types including cells of the immune system, epithelial cells, EC and cells of the nervous system [42]. Our study showed that CXCR2 is expressed on HMEC-1 and functionally relevant since CXCR2 antagonist SB 265610 diminished sprout outgrowth induced by CM of BSMC from asthmatic patients. SB 265610 is considered a competitive antagonist and an allosteric inverse agonist of CXCR2 and has been shown to be a highly specific inhibitor for this receptor [31]. This observation may be the first step towards a new specific treatment of remodeling in the airway wall of asthma patients.

In asthma patients, increased BSMC mass [14, 17, 27] and increased number of mitochondria in BSMC [43] have been observed, which suggests increased energy consumption and an according prompt for induction of angiogenesis to supply the cells with nutrition and oxygen. Therefore, reducing neovascularization in the sub-epithelial cell layers of the airway wall of asthma patients might help to reduce airway wall remodeling. Clinical studies have shown that symptoms of severe asthma could be markedly reduced

by the use of thermoplasty of specifically the bronchial smooth muscle cell layer [44]. The heating of the airways led to a decrease in the amount/mass of BSM [45] and reduced the frequency of asthmatic exacerbations [46], thus supporting increased BSM mass as being a key feature of airway remodeling in asthma. Blocking CXCR2 and thereby inhibiting BSMC-dependent angiogenesis and associated airway wall remodeling may therefore have a similar beneficial effect.

Identification of factors that might ubiquitously regulate and/or control pathological features in the asthmatic lung remains a challenge. Our study presents CXCR2-ligands (GRO-α, ENA-78, IL-8) as candidate factors contributing toward angiogenesis and airway wall remodeling in asthma. Studies with CXCR2-blockers and ligand-neutralizing agents in the context of different diseases (such as rheumatoid arthritis, COPD) are ongoing [42, 47]. Our findings open a door to exploiting CXCR2-targeted treatments for bronchial asthma as well.

References

1. GINA Report (Internet), From the Global Strategy for Asthma Management and Prevention, Global Initiative for Asthma (GINA) (updated December 2011; cited May 2012). Available from http://www.ginasthma.org. [Internet]; Available from: http://www.ginasthma.org.

2. Masoli M, Fabian D, Holt S, Beasley R. The global burden of asthma: executive summary of the GINA Dissemination Committee report. Allergy. 2004;59(5):469-78.

3. Grainge CL, Lau LC, Ward JA, Dulay V, Lahiff G, Wilson S, et al. Effect of bronchoconstriction on airway remodeling in asthma. The New England journal of medicine. 2011;364(21):2006-15.

4. Jeffery PK. Remodeling in asthma and chronic obstructive lung disease. American journal of respiratory and critical care medicine. 2001;164(10 Pt 2):S28-38.

5. Postma DS, Timens W. Remodeling in asthma and chronic obstructive pulmonary disease. Proceedings of the American Thoracic Society. 2006;3(5):434-9.

6. Lloyd CM, Robinson DS. Allergen-induced airway remodelling. The European respiratory journal : official journal of the European Society for Clinical Respiratory Physiology. 2007;29(5):1020-32.

7. Borger P, Miglino N, Baraket M, Black JL, Tamm M, Roth M. Impaired translation of CCAAT/enhancer binding protein alpha mRNA in bronchial smooth muscle cells of asthmatic patients. The Journal of allergy and clinical immunology. 2009;123(3):639-45.

8. Hakonarson H, Grunstein MM. Autocrine regulation of airway smooth muscle responsiveness. Respiratory physiology & neurobiology. 2003;137(2-3):263-76.

9. Oliver BG, Johnston SL, Baraket M, Burgess JK, King NJ, Roth M, et al. Increased proinflammatory responses from asthmatic human airway smooth muscle cells in response to rhinovirus infection. Respiratory research. 2006;7:71.

10. Borger P, Matsumoto H, Boustany S, Gencay MM, Burgess JK, King GG, et al. Disease-specific expression and regulation of CCAAT/enhancer-binding proteins in asthma and chronic obstructive pulmonary disease. The Journal of allergy and clinical immunology. 2007;119(1):98-105.

11. Miglino N, Roth M, Tamm M, Borger P. House dust mite extract downregulates C/EBPalpha in asthmatic bronchial smooth muscle cells. The European respiratory journal : official journal of the European Society for Clinical Respiratory Physiology. 2011;38(1):50-8.

12. Roth M, Johnson PR, Borger P, Bihl MP, Rudiger JJ, King GG, et al. Dysfunctional interaction of C/EBPalpha and the glucocorticoid receptor in asthmatic bronchial smooth-muscle cells. The New England journal of medicine. 2004;351(6):560-74.

13. Barbato A, Turato G, Baraldo S, Bazzan E, Calabrese F, Panizzolo C, et al. Epithelial damage and angiogenesis in the airways of children with asthma. American journal of respiratory and critical care medicine. 2006;174(9):975-81.

14. Bentley JK, Hershenson MB. Airway smooth muscle growth in asthma: proliferation, hypertrophy, and migration. Proceedings of the American Thoracic Society. 2008;5(1):89-96.

15. Hashimoto M, Tanaka H, Abe S. Quantitative analysis of bronchial wall vascularity in the medium and small airways of patients with asthma and COPD. Chest. 2005;127(3):965-72.

16. Wilson JW, Hii S. The importance of the airway microvasculature in asthma. Current opinion in allergy and clinical immunology. 2006;6(1):51-5.

17. Woodruff PG, Dolganov GM, Ferrando RE, Donnelly S, Hays SR, Solberg OD, et al. Hyperplasia of smooth muscle in mild to moderate asthma without changes in cell size or gene expression. American journal of respiratory and critical care medicine. 2004;169(9):1001-6.

18. Tanaka H, Yamada G, Saikai T, Hashimoto M, Tanaka S, Suzuki K, et al. Increased airway vascularity in newly diagnosed asthma using a high-magnification bronchovideoscope. American journal of respiratory and critical care medicine. 2003;168(12):1495-9.

19. Abdel-Rahman AM, el-Sahrigy SA, Bakr SI. A comparative study of two angiogenic factors: vascular endothelial growth factor and angiogenin in induced sputum from asthmatic children in acute attack. Chest. 2006;129(2):266-71.

20. Simcock DE, Kanabar V, Clarke GW, O'Connor BJ, Lee TH, Hirst SJ. Proangiogenic activity in bronchoalveolar lavage fluid from patients with asthma. American journal of respiratory and critical care medicine. 2007;176(2):146-53.

21. Asai K, Kanazawa H, Kamoi H, Shiraishi S, Hirata K, Yoshikawa J. Increased levels of vascular endothelial growth factor in induced sputum in asthmatic patients. Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology. 2003;33(5):595-9.

22. Ammit AJ, Moir LM, Oliver BG, Hughes JM, Alkhouri H, Ge Q, et al. Effect of IL-6 trans-signaling on the pro-remodeling phenotype of airway smooth muscle. American journal of physiology Lung cellular and molecular physiology. 2007;292(1):L199-206.

23. Raidl M, Sibbing B, Strauch J, Muller K, Nemat A, Schneider PM, et al. Impaired TNFalpha-induced VEGF expression in human airway smooth muscle cells from smokers with COPD: role of MAPkinases and histone acetylation--effect of dexamethasone. Cell Biochem Biophys. 2007;49(2):98-110.

24. Simcock DE, Kanabar V, Clarke GW, Mahn K, Karner C, O'Connor BJ, et al. Induction of angiogenesis by airway smooth muscle from patients with asthma. American journal of respiratory and critical care medicine. 2008;178(5):460-8.

25. Strieter RM, Burdick MD, Mestas J, Gomperts B, Keane MP, Belperio JA. Cancer CXC chemokine networks and tumour angiogenesis. Eur J Cancer. 2006;42(6):768-78.

26. Johnson PR, Armour CL, Carey D, Black JL. Heparin and PGE2 inhibit DNA synthesis in human airway smooth muscle cells in culture. The American journal of physiology. 1995;269(4 Pt 1):L514-9.

27. Johnson PR, Roth M, Tamm M, Hughes M, Ge Q, King G, et al. Airway smooth muscle cell proliferation is increased in asthma. American journal of respiratory and critical care medicine. 2001;164(3):474-7.

28. Ades EW, Candal FJ, Swerlick RA, George VG, Summers S, Bosse DC, et al. HMEC-1: establishment of an immortalized human microvascular endothelial cell line. The Journal of investigative dermatology. 1992;99(6):683-90.

29. Timmins NE, Nielsen LK. Generation of multicellular tumor spheroids by the hanging-drop method. Methods Mol Med. 2007;140:141-51.

30. Philippova M, Banfi A, Ivanov D, Gianni-Barrera R, Allenspach R, Erne P, et al. Atypical GPI-anchored T-cadherin stimulates angiogenesis in vitro and in vivo. Arteriosclerosis, thrombosis, and vascular biology. 2006;26(10):2222-30.

31. Bradley ME, Bond ME, Manini J, Brown Z, Charlton SJ. SB265610 is an allosteric, inverse agonist at the human CXCR2 receptor. Br J Pharmacol. 2009;158(1):328-38.

32. Salchow K, Bond ME, Evans SC, Press NJ, Charlton SJ, Hunt PA, et al. A common intracellular allosteric binding site for antagonists of the CXCR2 receptor. Br J Pharmacol. 2010;159(7):1429-39.

33. Ahuja SK, Murphy PM. The CXC chemokines growth-regulated oncogene (GRO) alpha, GRObeta, GROgamma, neutrophil-activating peptide-2, and epithelial cell-derived neutrophil-activating peptide-78 are potent agonists for the type B, but not the type A, human interleukin-8 receptor. The Journal of biological chemistry. 1996;271(34):20545-50.

34. Asai K, Kanazawa H, Otani K, Shiraishi S, Hirata K, Yoshikawa J. Imbalance between vascular endothelial growth factor and endostatin levels in induced sputum from asthmatic subjects. The Journal of allergy and clinical immunology. 2002;110(4):571-5.

35. Hoshino M, Takahashi M, Aoike N. Expression of vascular endothelial growth factor, basic fibroblast growth factor, and angiogenin immunoreactivity in asthmatic airways and its relationship to angiogenesis. The Journal of allergy and clinical immunology. 2001;107(2):295-301.

36. Kristan SS, Malovrh MM, Silar M, Kern I, Flezar M, Kosnik M, et al. Airway angiogenesis in patients with rhinitis and controlled asthma. Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology. 2009;39(3):354-60.

37. Liu Z, Kobayashi K, van Dinther M, van Heiningen SH, Valdimarsdottir G, van Laar T, et al. VEGF and inhibitors of TGFbeta type-I receptor kinase synergistically promote blood-vessel formation by inducing alpha5-integrin expression. Journal of cell science. 2009;122(Pt 18):3294-302.

38. Borish L, Culp JA. Asthma: a syndrome composed of heterogeneous diseases. Annals of allergy, asthma & immunology : official publication of the American College of Allergy, Asthma, & Immunology. 2008;101(1):1-8; quiz -11, 50.

39. Keane MP, Arenberg DA, Lynch JP, 3rd, Whyte RI, Iannettoni MD, Burdick MD, et al. The CXC chemokines, IL-8 and IP-10, regulate angiogenic activity in idiopathic pulmonary fibrosis. J Immunol. 1997;159(3):1437-43.

40. Keane MP, Belperio JA, Burdick MD, Lynch JP, Fishbein MC, Strieter RM. ENA-78 is an important angiogenic factor in idiopathic pulmonary fibrosis. American journal of respiratory and critical care medicine. 2001;164(12):2239-42.

41. Heidemann J, Ogawa H, Dwinell MB, Rafiee P, Maaser C, Gockel HR, et al. Angiogenic effects of interleukin 8 (CXCL8) in human intestinal microvascular endothelial cells are mediated by CXCR2. The Journal of biological chemistry. 2003;278(10):8508-15.

42. Chapman RW, Phillips JE, Hipkin RW, Curran AK, Lundell D, Fine JS. CXCR2 antagonists for the treatment of pulmonary disease. Pharmacology & therapeutics. 2009;121(1):55-68.

43. Trian T, Benard G, Begueret H, Rossignol R, Girodet PO, Ghosh D, et al. Bronchial smooth muscle remodeling involves calcium-dependent enhanced mitochondrial biogenesis in asthma. The Journal of experimental medicine. 2007;204(13):3173-81.

44. Shifren A, Chen A, Castro M. Point: efficacy of bronchial thermoplasty for patients with severe asthma. Is there sufficient evidence? Yes. Chest. 2011;140(3):573-5; discussion 8.

45. Cox PG, Miller J, Mitzner W, Leff AR. Radiofrequency ablation of airway smooth muscle for sustained treatment of asthma: preliminary investigations. The European respiratory journal : official journal of the European Society for Clinical Respiratory Physiology. 2004;24(4):659-63.

46. Miller JD, Cox G, Vincic L, Lombard CM, Loomas BE, Danek CJ. A prospective feasibility study of bronchial thermoplasty in the human airway. Chest. 2005;127(6):1999-2006.

47. O'Byrne PM, Naji N, Gauvreau GM. Severe asthma: future treatments. Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology. 2012;42(5):706-11.

Hypoxia exerts dualistic effects on inflammatory and proliferative responses of healthy and asthmatic primary human bronchial smooth muscle cells

Laura Keglowich, MSc[1]; Melissa Baraket, PhD[3], Michael Tamm, MD[1,2]; Peter Borger, PhD[1]

Affiliations:

1 Pulmonary Cell Research, Department of Biomedicine, University of Basel, Basel, Switzerland

2 Department of Pneumology, University Hospital Basel, Basel, Switzerland

3 Sydney Medical School, The University of Sydney, Sydney, Australia.

Running head: Dualistic effects of hypoxia on BSMC

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Abstract

Background: For oxygen supply, airway wall cells depend on diffusion though the basement membrane, as well as on delivery by microvessels. In the asthmatic lung, local hypoxic conditions may occur due to increased thickness and altered composition of the basement membrane, as well as due to edema of the inflamed airway wall.

Objective: In our study we investigated the effect of hypoxia on proliferation and pro-inflammatory and pro-angiogenic parameter production by human bronchial smooth muscle cells (BSMC). Furthermore, conditioned media of hypoxia-exposed BSMC was tested for its ability to induce sprout outgrowth from endothelial cells spheroids.

Methods: BSMC were cultured in RPMI1640 (5% FCS) under normoxic (21% O_2) and hypoxic (1% and 5% O_2) conditions. Proliferation was determined by cell count and Western blot analysis for cyclin E and Proliferating Cell Nuclear Antigen (PCNA). Secretion of IL-6, IL-8, ENA-78 and VEGF was analyzed by ELISA. BSMC conditioned medium was tested for its angiogenic capacity by endothelial cell (EC)-spheroid *in vitro* angiogenesis assay.

Results: Proliferation of BSMC obtained from asthmatic and non-asthmatic patients was significantly reduced under severe hypoxia (1% O_2), whereas moderate hypoxia (5% O_2) only reduced proliferation of asthmatic BSMC. Hypoxia induced HIF-1α expression in asthmatic and non-asthmatic BSMC, which coincided with significantly increased release of IL-6, IL-8 and VEGF, but not ENA-78. Finally, sprout outgrowth from EC spheroids was increased when exposed to hypoxia conditioned BSMC medium.

Conclusion: Hypoxia had dualistic effects on proliferative and inflammatory responses of both asthmatic and non-asthmatic BSMC. First, hypoxia reduced BSMC proliferation. Second, hypoxia induced a pro-inflammatory, pro-angiogenic response. BSMC and endothelial cells may thus be promising new targets to counteract and/or alleviate airway wall remodeling.

Introduction

Persistent airway wall remodeling is an important pathology of asthma, which is characterized by basement membrane thickening, increased bronchial smooth muscle (BSM) mass and neovascularization. Together with enhanced extracellular matrix deposition these pathologies result in thickening of the basement membrane and the underlying tissue layers. It is of interest that BSMC from asthmatic patients have a distinct "phenotype" compare to cells from non-asthma subjects [1]. This disease specific phenomenon is associated with diminished expression of the CCAAT/enhancer binding protein (C/EBP)-α in BSMC of asthma patients [2-5]. Lower C/EBP-alpha levels may explain several parameters associated with the hyper-reactive ("primed") phenotype of the asthmatic BSMC, including an increased release of pro-inflammatory factors and mediators [6, 7]. Smooth muscle changes in allergic asthma include myofibroblast differentiation, BSMC hyperplasia and hypertrophy and vascular smooth muscle thickening [8].

For oxygen supply, BSMC depend on direct diffusion though the basement membrane, as well as on oxygen delivered by microvessels present in the airway wall. In the asthmatic airway, local hypoxic conditions may occur due to increased thickness and altered composition of the basement membrane, as well as of edema, which may affect the rate of oxygen diffusion [9]. In healthy lungs, the thickness of the basement membrane measures 5-7 μm, while in asthmatics the size was found up to 5 times increased [10]. Even in young asthmatic children the basement membrane was significantly thicker compared to non-asthmatic controls [11]. The increased thickness of the basement membrane may readily induce a locally restricted hypoxic environment, which affects the properties and behavior of BSMC. In addition, increased oxygen consumption by inflammatory cells and growing BSMC may further add to locally restricted hypoxic conditions in the lung [9].

It had only recently been suggested that hypoxia may play a role in the pathogenesis of asthma. For instance, hypoxia aggravated airway inflammation, remodeling, and furthered the development of asthma in a murine model of asthma [12]. The activation of proteins of the hypoxia inducible factor (HIF)-family is a major regulator of the cellular response to hypoxia [13] and increased level of HIF-α, have been detected in endobronchial biopsies and in the broncho-alveolar lavage fluid (BALF) of asthma patients [12]. Similarly, increased levels of the HIF subunits (HIF-1α and HIF-1β) have been observed in lung

tissue, epithelial cells and BALF of asthma patients and have been correlated with the level of VEGF [14].

In order to overcome hypoxia, resident cells of the airway wall, including BSMC and fibroblasts, may induce the production of angiogenic factors leading to the formation of new microvessels in the airway wall. Indeed, VEGF one of the most potent angiogenic factors, is produced by smooth muscle cells [15-17] and is able, to stimulate the proliferation of vascular smooth muscle cells [18, 19]. These findings indicate that BSMC are able to induce angiogenesis and may direct the observed neovascularization of the airway wall.

In our present study we examined the effects of hypoxia on inflammatory and proliferative responses of BSMC obtained from the lungs of both healthy and asthmatic subjects. Our data demonstrate that hypoxia diminished the proliferation-rate of BSMC, whereas it concomitantly induced HIF-1α and the subsequent the release of VEGF, IL-6 and IL-8.

Methods

Patients

Patients with mild to moderate asthma (n = 7, 3 females/4 males, age 23-64 years) had reversible airway obstruction documented in the past, with a median FEV_1 of 70,5% of the predicted value (ranging from 45,3% to 84,6%).

Cryosectioning, staining and light microscopy of endobronchial biopsies

Superficial endobronchial biopsy specimens of the airway mucosa were immediately snap frozen in optimal cutting temperature (OCT) medium (Tissue-Tek, Sakura Finetechnical, Tokyo, Japan) on a cork disc by immersion in isopentane (2-methylbutane, HPLC grade, Sigma-Aldrich) suspended in liquid nitrogen and kept at -80ºC. Frozen biopsies were sectioned on a cryostat (Shandon Cryotome 620E) at -18ºC into sections of 7 µm and stored at -80ºC. The frozen sections were air dried at room temperature, fixed in 50% acetone/50% methanol (90 s) and stained with Harris's haematoxylin and alcoholic eosin (Fronine Laboratory Supplies, Taren Point, Australia). Light microscopic photographic images were taken at 60x (Olympus AX70 microscope, DP50 camera).

Isolation of primary bronchial smooth muscle cells

BSMC were obtained from endobronchial biopsies by flexible bronchoscopy or therapeutically resected lung tissue (Department of Internal Medicine, Pneumology, University Hospital Basel, Basel, Switzerland) with the approval of the local ethical committees and written informed consent of all patients. Isolation of BSMC was performed as described earlier [1, 20] with some modifications. BSMC bundles were dissected from the surrounding tissue using a binocular microscope. Muscle bundles were then placed in a 25 cm^2-flask in 1 ml Dulbecco`s modified Eagle`s medium (DMEM) containing GlutaMax-I 4.5 g/l glucose (Gibco®, Bioconcept, Allschwil, Switzerland), 5% FCS, 1x antibiotics-antimycotics, and 1x modified Eagle`s medium vitamin mix (Invitrogen, Lubio, Luzern, Switzerland). BSMC were characterized by immunofluorescence as described earlier [21].

BSMC culture

BSMC were grown in BSMC-growth medium (GM): RPMI 1640 supplemented with 5% fetal calf serum (FCS), 8 mM L-glutamine, 20 mM hydroxyethyl piperazine ethane sulfonic acid, 1x antiobiotics-antimycotics and 1x modified Eagle's medium vitamin mix (Invitrogen, Lubio, Luzern, Switzerland) under normoxic conditions (21% O_2, 5% CO_2, 37

°C) until they reached confluency and then subcultured. To study hypoxia, cells were cultured at oxygen concentrations of 1% and 5% (5% CO_2, 37 °C). Conditioned cell culture medium was harvested after 72 h and kept at -20 °C until analysis. All experiments were performed at least in duplicates. Conditioned media (CM) used in endothelial cell sprout outgrowth assays was obtained from BSMC grown under hypoxic (1% O_2, 5% CO_2, 37 °C, 72 hours) and normoxic conditions (21% O_2, 5% CO_2, 37 °C, 72 hours).

Proliferation experiments

To determine the effect of hypoxia on proliferation BSMC were seeded at $5x10^5$ cells/well in 12 well plates, allowed to adhere for 24 h in BSMC-GM, serum deprived for 24 h prior to stimulation and then cultured for 72 h in the presence or absence of 5% FCS under three different oxygen concentrations (21%, 5% or 1% O_2). Cell counts were performed in duplicates before stimulation and after 72 h using a particle counter (Beckman Coulter particle counter Z1, Nyon, Switzerland).

SDS-PAGE and Western blot

Total protein lysates were size-fractioned on gradient (4-20%) Tris-HEPES gels (Pierce, Thermo-Scientific, Lausanne, Switzerland) and transferred to nitrocellulose membranes as described earlier [4]. Membranes were blocked for 30 min with 3% BSA in Tris buffered saline with 0.1% Tween 20 (TBST) for detection of PCNA, cyclin E, α-tubulin, GAPDH (Santa Cruz Biotech, Lucerna-Chem, Luzern, Switzerland) or for 1 h in 5% BSA in TBST for detection of HIF-1α (Cell Signaling Technologies, Bioconcept, Allschwil, Switzerland). Primary antibodies were applied for 2 h at RT in TBST/1%BSA (PCNA, cyclin E, α-tubulin, GAPDH) or overnight at 4°C in TBST/5%BSA (HIF-1α). After 3x washing with TBST the HRP-conjugated species-specific secondary antibodies were applied for 30 min (PCNA, cyclin E, α-tubulin, GAPDH) in TBST or for 2 h in 3% milk powder in TBST (HIF-1α). After 3 washes the membrane were incubated with SuperSignal Western Pico Chemiluminescent Substrate (Pierce, Thermo-Scientific, Lausanne, Switzerland) for 5 min. Signal were detected on Fujifilm Super RX X-ray films (Lucerna-Chem, Luzern, Switzerland) and developed in a Curix60 film-processor (Agfa, Dübendorf, Switzerland).

Cytokine-ELISA

Enzyme linked immunosorbent assay (ELISA) kits for ENA-78, IL-6, IL-8 and VEGF-A were purchased from R&D (Abingdon. UK) and performed according to the manufacturer´s instructions. Cytokine levels were determined in 100 µl of CM obtained

from BSMC and were measured undiluted (VEGF A), 1:5 diluted (ENA-78, IL-6) or 1:50 diluted (IL-8).

Endothelial tube-formation assay

Human microvascular endothelial cells (EC) HMEC-1 [22] were normally maintained in EC growth medium (ECGM, Provitro, Bioconcept, Allschwil, Switzerland) supplemented with 10% FCS under normoxic conditions. Spheroids composed of 500 HMEC-1 cells were prepared using the hanging drop method [23]. The tube-forming (sprout outgrowth) assay was performed as previously described [24]. At least 10 spheroids per gel were embedded within fibrin gels in 48-well plates. Gels were overlaid with a 1:1 mixture of ECGM supplemented with 2% FCS and either BSMC medium (to determine spontaneous sprout outgrowth) or CM obtained from BSMC grown under hypoxic (1% O_2) and normoxic (21% O_2) conditions. In experiments studying the effects of the CXCR2 antagonist SB 265610 (Sigma-Aldrich, Buchs, Switzerland) the inhibitor was added to the gel 1 h prior to stimulation with CM. VEGF neutralizing antibody (R&D, Abigdon, UK) was added to CM for 1 h (37°C) prior to its addition to the gels containing the spheroids. After incubation for 24 h, spheroids were fixed in-gel, stained with TRITC-conjugated phalloidin (Sigma-Aldrich, Buchs, Switzerland) and sprout outgrowth from each spheroid was quantitated by morphometric analysis of the length of outgrowing tubules [24]. The length of the 10 longest tubules per spheroid of 7 randomly chosen spheroids/well was quantitated by morphometric analysis using AnalySIS software (Soft Imaging System GmbH, Munich, Germany) and the mean ± S.E.M. was calculated.

Statistics

Cytokine and proliferation data are presented as mean ± S.E.M., immuno-blot analysis are shown as mean ± S.E.M. after densitometric image analysis (ImageJ software, National Institute of Mental Health, Bethesda, Maryland, USA). Unpaired Student's t-test was performed and p-values < 0.05 were considered significant.

Results

Increased basement membrane thickness in human tissue sections from the lung of asthma patients

Superficial endobronchial biopsy specimens were stained using Harris's haematoxylin and alcoholic eosin and light microscopic photographic images were recorded. Figure 1 presents a typical example of a human airway tissue cross-section demonstrating the increased thickness of the basement membrane present in asthmatics (Figure 1B) relative to non-asthma (Figure 1 A) patients.

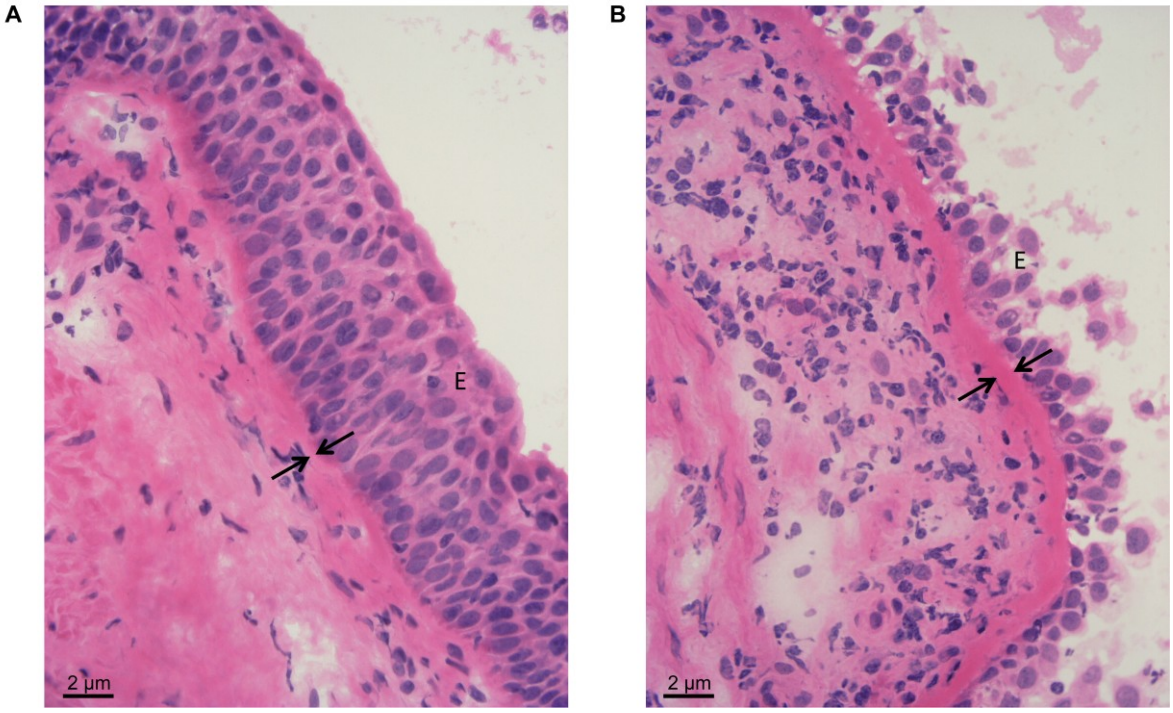

Figure 1: Light microscopic images of airway tissue sections obtained from a non-asthma (A) and an asthma (B) patient. Images (magnification 60x) are representative of tissues obtained from 3 non-asthmatic and 9 asthmatic patients stained with Haematoxylin-Eosin. Note the increased thickening of the basement membrane in the asthmatic airways. E = epithelium, arrows are indicating the basement membrane.

Hypoxia reduced proliferation of BSMC of asthmatic and non-asthmatic subjects

BSMC obtained from asthmatic (n = 4) and non-asthmatic (n = 5) subjects were cultured for 72 hours under either normoxic (21% O_2) or hypoxic conditions (5% and 1% O_2) in presence and absence of 5% FCS. As shown in Figure 2A, under normoxic conditions and in the presence of 5% FCS, BSMC numbers significantly increased in both the asthmatic group (2.08 ± 0.13-fold) and the non-asthmatic group (1.85 ± 0.13-fold). Although BSMC of asthmatics proliferated slightly faster, the fold-increase between asthma and non-asthma was not significantly different ($p = 0.21$). In the presence of FCS, moderate hypoxia (5% O_2) significantly reduced cell numbers of asthmatics only to 1.62 ± 0.12-fold ($p < 0.05$). Severe hypoxia (1% O_2) did not further reduce BSMC proliferation of asthmatics (1.65 ± 0.14-fold), but a similar significant reduction of proliferation was observed in BSMC of non-asthmatics to 1.30 ± 0.13-fold; $p < 0.05$). In the absence of FCS, BSMC did not proliferate and hypoxia (1% O_2) did not have any effect (Figure 2B). As shown in Figure 2C- 2F, under hypoxic conditions the proliferation markers PCNA and cyclin E were both significantly down regulated (PCNA: 56 ± 1% compared to normoxia, $p < 0.001$ and cyclin E: 84 ± 5% compared to normoxia, $p < 0.05$). No differences were observed between the asthmatic and non-asthmatic group.

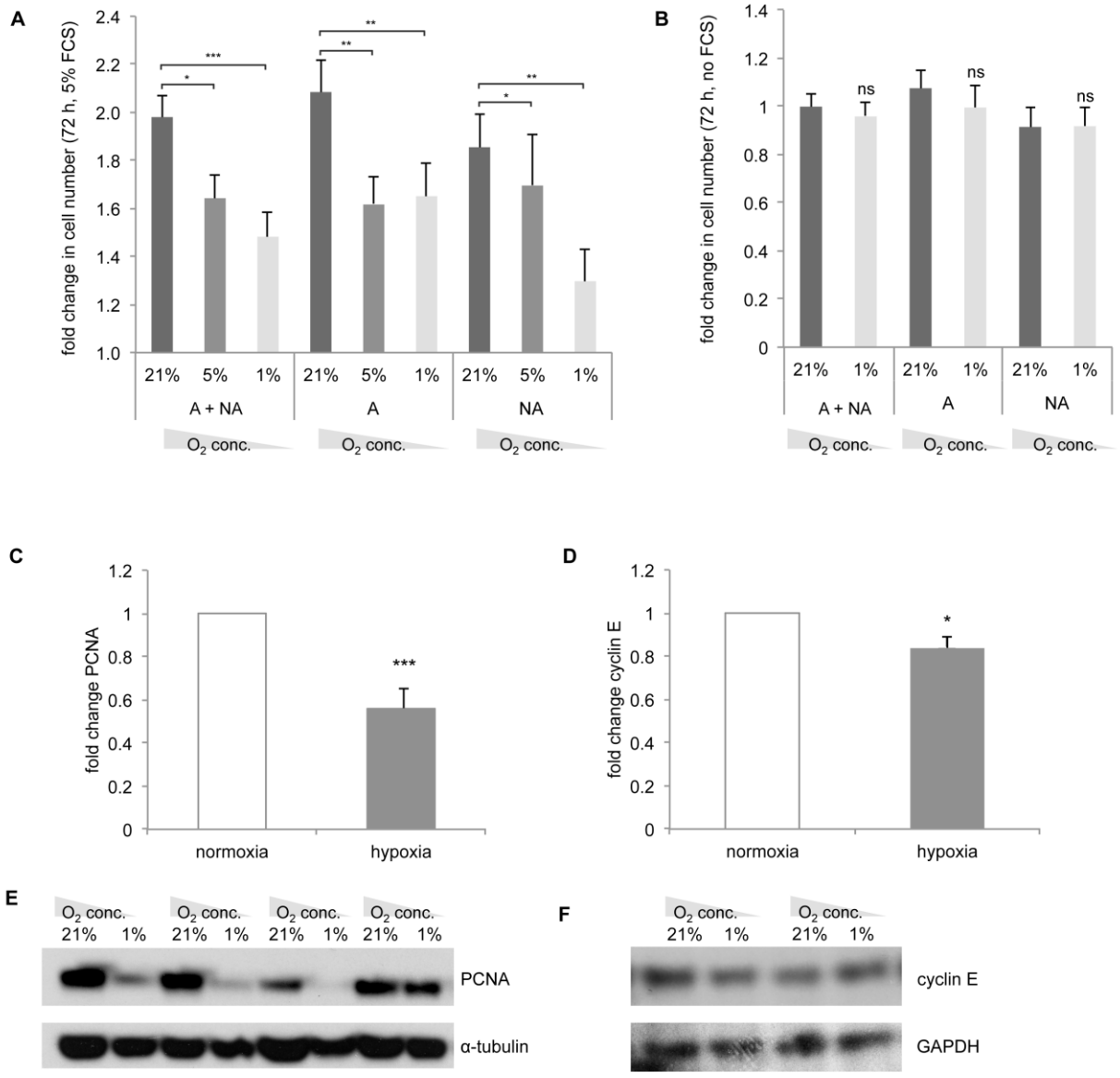

Figure 2: Proliferation characteristics of BSMC from asthmatic (A) and non-asthmatic (NA) subjects in the presence of 1%, 5% and 21% O_2 (72 h). BSMC were cultured for 72 hours in presence (**A**) and absence (**B**) of 5% FCS. n = 12 (6A/6NA) for 21% and 1% O_2 and n = 7 (4A/3NA) for 5% O_2. **C** and **D,** show the densitometric analysis of effects on hypoxia (1% O_2) relative to normoxic conditions (21% O_2) for PCNA (n = 9) and cyclin E (n = 6). Representative Western blots for PCNA and cyclin E protein expression are demonstrated in the lower panel (**E** and **F**). Values are given as mean ± S.E.M.. * : p-value ≤ 0.05, ** : p-value ≤ 0.01, *** : p-value ≤ 0.001.

Hypoxia induced HIF-1α in BSMC

To study whether hypoxia was associated with the induction of HIF-1α, BSMC (n = 5) were cultured in the presence of 5% FCS and under normoxic (21% O_2), or stringent hypoxic conditions (1% O_2). As demonstrated in Figure 3, normoxic conditions did not induce HIF-1α. In contrast, HIF1-α was transiently induced and peaked after 4 hours in BSMC under stringent hypoxic conditions. Extended exposure to hypoxic conditions (24 and 48 h) HIF-1α levels decreased, but did not return to control levels. Cobalt chloride, a chemical inhibitor of the prolyl-hydroxylase that targets HIF-1α for proteasomal degradation under normoxic conditions, was used as a positive control (50 and 100 μM).

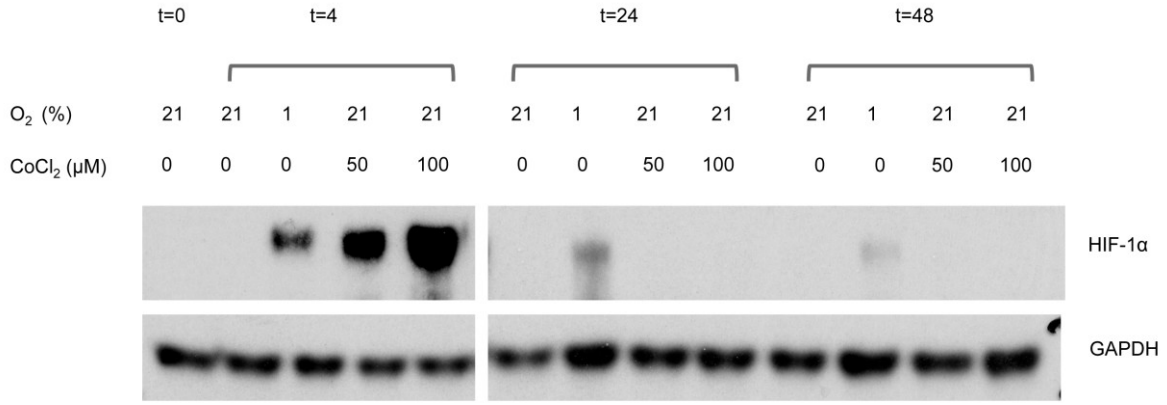

Figure 3: Western blot analysis detecting HIF-1α and GAPDH (loading control) in lysates of BSMC. Cell were incubated under 1% and 21% O_2 for 4, 24 and 48 h (indicated at top). $CoCl_2$ was used as positive control to stabilize HIF-1α protein. In contrast to $CoCl_2$, which has a transient effect only, hypoxia induced a prolonged expression of HIF-1α. Experiment shown is representative for 5 independent experiments. The right hand panel (showing the 24 h and 48 h expression levels of HIF-1α) was obtained after longer exposure of the same blot, which contained all samples.

Hypoxia induced IL-6, IL-8 and VEGF, but not ENA-78

Next, we studied the effect of hypoxia on the release of four pro-angiogenic/pro-inflammatory cytokines: IL-6, IL-8, VEGF and ENA-78. BSMC obtained from asthmatic ($n = 5$) and non-asthmatic ($n = 5$) subjects were cultured for 72 h under either normoxic (21% O_2) or stringent hypoxic conditions (1% O_2) in presence or absence of 5% FCS. In the absence of FCS, hypoxia significantly increased the release of VEGF protein in both asthmatic and non-asthmatic cells (8.62 ± 2.86-fold and 9.88 ± 2.79-fold; both $p < 0.05$; Figure 4A). Similarly, under the same conditions the release of IL-6 protein increased in both asthmatic and non-asthmatic cells (5.42 ± 1.29-fold and 5.34 ± 1.18-fold; both $p < 0.05$; Figure 4B). The effect of hypoxia on the release of IL-8 was less pronounced, but significant (2.72 ± 0.63-fold and 1.98 ± 0.29-fold; both $p < 0.05$; Figure 4C).

In the presence of 5% FCS, hypoxia significantly increased the release of VEGF protein by both asthmatic and non-asthmatic cells (6.37 ± 0.96-fold and 6.54 ± 1.74-fold; both $p < 0.05$; Figure 4A). Under the same conditions the release of IL-6 protein increased in both asthmatic and non-asthmatic cells (2.36 ± 0.59-fold and 3.03 ± 0.76-fold; both $p < 0.05$; Figure 4B). In the presence of FCS, hypoxia did not affect the release of IL-8 (Figure 4C). Furthermore, did hypoxia not affect the release of ENA-78 significantly (0.88 ± 0.24-fold, $p = 0.59$). No significant differences were observed between asthmatic and non-asthmatic BSMC in any condition.

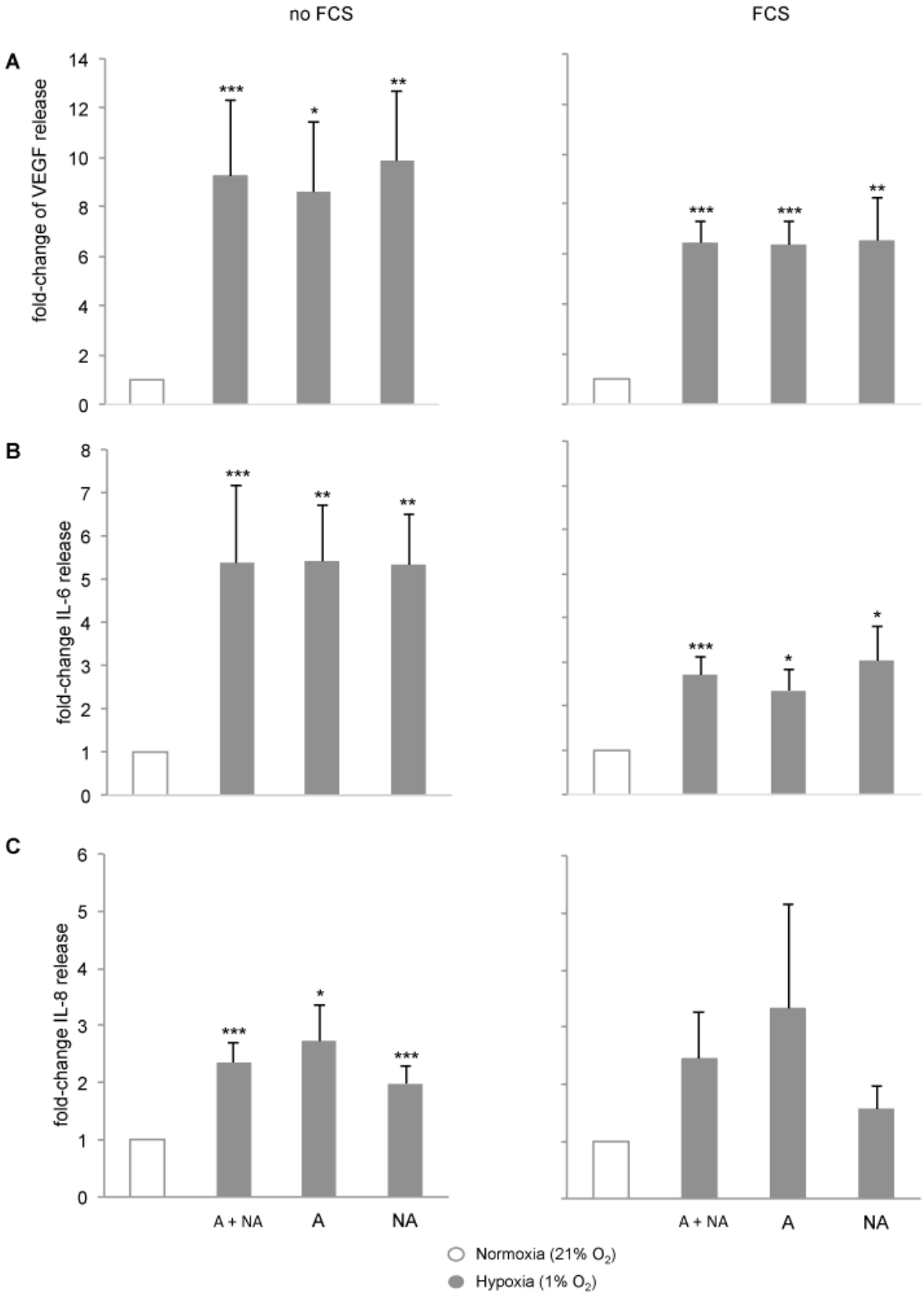

Figure 4: Chemokine release from BSMC grown under hypoxic conditions (1% O2, 72 h) compared to normoxic conditions. Concentrations of VEGF (**A**), IL-6 (**B**) and IL-8 (**C**) in CM collected from BSMC (n=10, 5A/5NA) were determined by ELISA. Values are given as mean ± S.E.M.. * : p-value ≤ 0.05, ** : p-value ≤ 0.01, *** : p-value ≤ 0.001.

Increased angiogenic potential of BSMC grown under hypoxic conditions is reversed by anti-VEGF and SB265610

The angiogenic potential of CM collected from BSMC grown under hypoxic (1% O_2) and normoxic (21% O_2) conditions was examined using the EC-spheroid *in vitro* angiogenesis assay. Spheroids were also cultured in unconditioned medium (i.e. with medium that had not been included with BSMC) to control for "spontaneous" sprout outgrowth. Figure 5 shows representative images of spheroids incubated with CM from BSMC grown under normoxic (left panel) or hypoxic (right panel) conditions (Figure 5A). The quantitation of sprout outgrowth as the mean tubule length/spheroid after a 24 h culture period is shown in Figure 5B. CM of BSMC cultured under hypoxic conditions significantly increased the sprout formation compared to CM of BSMC grown under normoxic conditions (53,7 ± 2.4 μm *vs.* 73.0 ± 4.5 μm; $p < 0.001$; $n = 3$).

Finally, we sought to define a mechanism involved in the aforementioned findings. Since IL-8 signals through the chemokine receptor CXCR2, we studied the effects of a competitive CXCR2-selective antagonist SB 265610 (10 nM) and a neutralizing anti-VEGF antibody (0.2μg/ml) in the EC-spheroid *in vitro* angiogenesis assay. We examined the effect of hypoxia on pooled CM, derived from asthmatics and non-asthma controls. CM from non-asthma controls did not show any difference whether BSMC were grown under hypoxic or normoxic conditions. In contrast, CM from asthmatics significantly increased the sprout outgrowth from EC spheroids comparing normoxia (42.83 ± 11.06 μm) and hypoxia (69.39 ± 9.22 μm; $p < 0.001$). As demonstrated in Figure 5C, SB 265610 and anti-VEGF significantly reduced CM-induced EC-sprouting (SB 265610: 51.31 ± 9.77 μm, $p < 0.05$; anti-VEGF 34.33 ± 7.09 μm, $p < 0.001$).

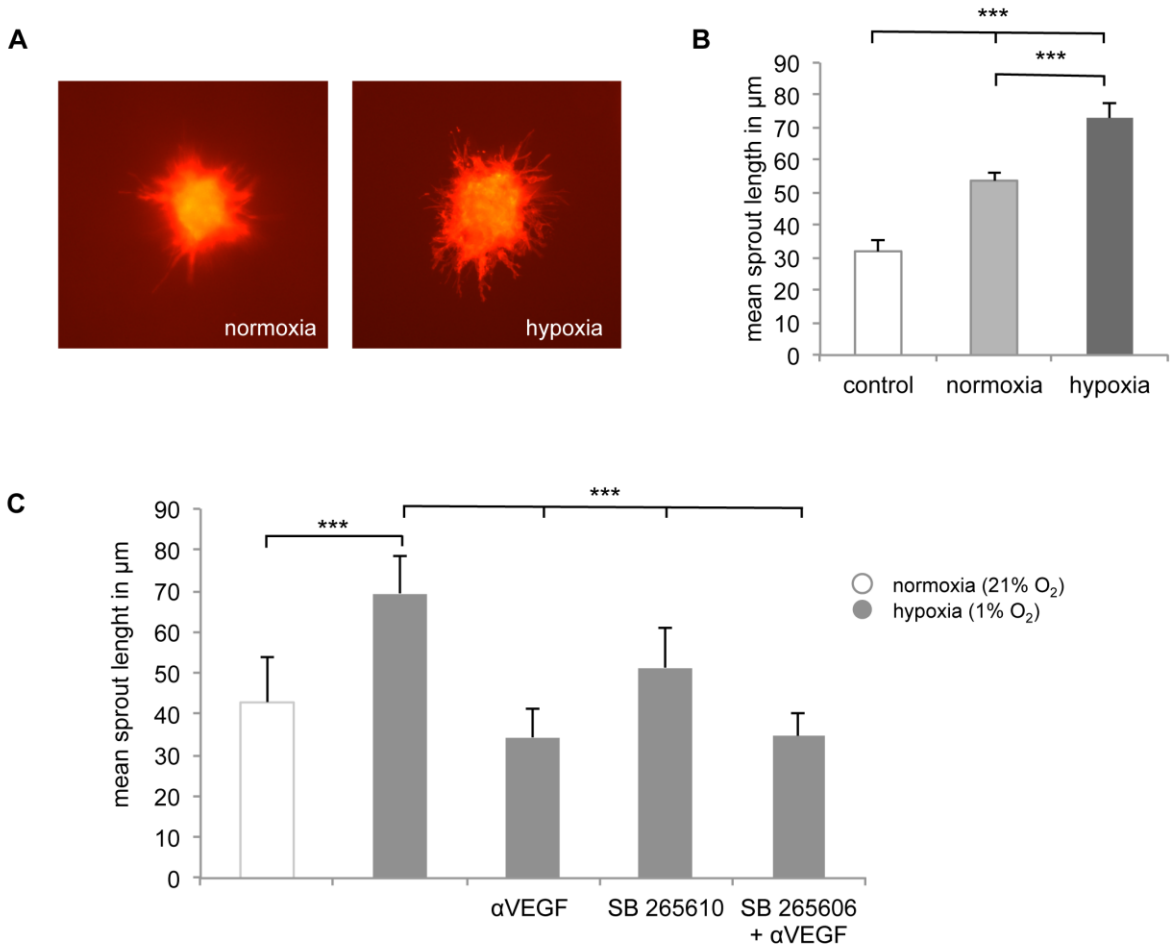

Figure 5: . *In vitro* angiogenesis assay. A, Representative images of the *in vitro* angiogenesis assay showing sprout outgrowth from EC-spheroids incubated with CM from BSMC cultured in presence of 21% O_2 (normoxia) or 1% O_2 (hypoxia). **B,** Lengths of sprouts outgrowing from every spheroid were measured and the mean of the longest 10 sprouts of 7 randomly chosen spheroids was plotted. **C**, shows the effects of the CXCR2-blocker SB 265610, anti-VEGF, and the combination of SB 265610 plus anti-VEGF on EC-sprouting using the *in vitro* angiogenesis assay. EC-sprouting was induced by pooled CM of BSMC (n=5) cultured under hypoxic conditions (1% O_2, 72 h). Values are given as mean of the mean ± S.E.M.. * : p-value ≤ 0.05, ** : p-value ≤ 0.01, *** : p-value ≤ 0.001.

Discussion

Hypoxia of the airways can be caused by a range of mechanisms, including diminished ventilation drive, airway obstruction, intra-alveolar exudates, airway wall inflammation, as well as fibrosis and basement membrane thickening [25]. The latter is one of the striking features of the asthmatic airway wall and may well contribute to locally restricted hypoxia. This may in turn affect the properties of resident cells present in the airway wall. Biopsy studies in adults and children showed a significant increase in the number of microvessels present in the airway wall of asthma patients and there is evidence that endothelial cells undergo proliferation [26-28]. In our current study, we showed that hypoxia has a profound effect on the proliferation of and angiogenic factors release by primary human BSMC of asthmatic and non-asthmatic subjects.

First, hypoxia has the capacity to significantly reduce the proliferation rate of BSMC of both asthmatic and non-asthmatic subjects. Importantly, a significantly reduced proliferation of BSMC of asthma patients was observed in the presence of 5% O_2, whereas the same conditions did not affect BSMC of control subjects. Stringent hypoxic conditions (1% O_2) significantly reduced the proliferation of BSMC from asthmatics and non-asthmatics. This indicates that BSMC of asthmatics are more prone to hypoxic (culture) conditions. The reason for this difference is unclear, but it might reflect diminished proliferation control responses as observed in cultured asthmatic BSMC [2-5, 29]. The reduced proliferation under stringent hypoxic conditions (1% O_2) was confirmed by lower levels of PCNA- and cyclin E proteins in BSMC of asthmatic and non-asthmatic subjects. Our data also indicate that the effects of hypoxia may be distinct in different model organisms, since it has been demonstrated that BSMC of rats have significantly increased proliferation rates under hypoxic conditions [30]. The reason for this discrepancy between primary human BSMC and those of rats is currently not known.

Second, our study showed that hypoxia enhanced the release of VEGF, a mediator of microvascular leakage, EC proliferation and vascular remodeling, as well as IL-6 and IL-8, which are known to be involved in angiogenesis [31-33]. The mechanisms underlying airway wall remodeling present in the lungs of asthma patients, which also involve angiogenesis of the airway wall, are complex and incompletely understood. Hypoxia may trigger the BSMC to express HIF-α, a transcription factor critically involved in the regulation of several pro-inflammatory and pro-angiogenic factors. VEGF and HIF-α are

expressed in the airways and airway lining fluids of asthma patients, suggesting that both factors may play and important role in inflammation as well as vascular remodeling in asthma [34, 35]. The observed increased number of microvessels present in the airway wall of patients with asthma [26-28], may be explained by the significant higher levels of VEGF and increased HIF expression in the sub-epithelial cell layers of asthma patients relative to healthy controls [14]. It is important to note that high expression of VEGF was reported in the airways of asthma patients [35, 36].

Our results demonstrated that hypoxia transiently induced HIF-1α protein expression in BSMC, with no differences between cells of asthmatic and non-asthmatic subjects. The expression of HIF-1α, the hypoxia-induced subunit of the hypoxia inducible factor (HIF), was transient and optimal after 4 hours, indicating that the effects on the release of VEGF, IL-6 and IL-8 might be via this transcription factor. CM isolated from BSMC cultured under hypoxic conditions demonstrated a significantly increased endothelial sprout outgrowth relative to CM of BSMC grown under normoxic conditions, confirming that BSMC cultured under hypoxic conditions induced the release of pro-angiogenic factors. Indeed, our blocking experiments using SB265610 and/or anti-VEGF demonstrated both the involvement of CXCR2 signaling and VEGF in the angiogenic process.

Previously, we showed that BSMC isolated from asthma patients exhibit increased angiogenic potential, which associated with increased productions of CXCR2 ligands (ENA78, GRO-α and IL-8) and VEGF [unpublished data, manuscript submitted]. Our present study showed that hypoxia predominantly induced VEGF and IL-6, whereas IL-8 was only 2-fold increased and ENA-78 not affected. It has been observed that hypoxia regulates VEGF activities mainly through transcriptional repression of the neutropilin-2 receptor [37]. Furthermore, increased levels of IL-6 and IL-8 have been reported in asthma patients. [38]. The involvement of HIF-1α in the increased release of IL-6 and IL-8 is largely unknown, although both genes are associated with hypoxia-inducible promoter and/or enhancer sequences [own observations after checking the annotated sequences with GENE tools]. This suggests that hypoxia may exert differential effects on different cytokines via distinctly different mechanisms.

In conclusion, hypoxia has dualistic effect on proliferative and inflammatory responses of both healthy and asthmatic primary human BSMC. Our data imply that hypoxia cannot be a direct cause for the observed increased smooth muscle mass in the airway wall of asthma patients. Rather, these cells start to elicit a range of pro-inflammatory cytokines and growth

factors that intensify airway wall inflammation and remodeling through advancing the process of neovascularization. A novel strategy to counteract airway wall remodeling may therefore be found in the development of drugs that counteract the process of neovascularization. Both, BSMC and EC, are therefore promising new targets to counteract and/or alleviate airway wall remodeling.

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References

1. Johnson PR, Roth M, Tamm M, Hughes M, Ge Q, King G, et al. Airway smooth muscle cell proliferation is increased in asthma. American journal of respiratory and critical care medicine. 2001;164(3):474-7.

2. Borger P, Matsumoto H, Boustany S, Gencay MM, Burgess JK, King GG, et al. Disease-specific expression and regulation of CCAAT/enhancer-binding proteins in asthma and chronic obstructive pulmonary disease. The Journal of allergy and clinical immunology. 2007;119(1):98-105.

3. Borger P, Miglino N, Baraket M, Black JL, Tamm M, Roth M. Impaired translation of CCAAT/enhancer binding protein alpha mRNA in bronchial smooth muscle cells of asthmatic patients. The Journal of allergy and clinical immunology. 2009;123(3):639-45.

4. Miglino N, Roth M, Tamm M, Borger P. House dust mite extract downregulates C/EBPalpha in asthmatic bronchial smooth muscle cells. The European respiratory journal : official journal of the European Society for Clinical Respiratory Physiology. 2011;38(1):50-8.

5. Roth M, Johnson PR, Borger P, Bihl MP, Rudiger JJ, King GG, et al. Dysfunctional interaction of C/EBPalpha and the glucocorticoid receptor in asthmatic bronchial smooth-muscle cells. The New England journal of medicine. 2004;351(6):560-74.

6. Hakonarson H, Grunstein MM. Autocrine regulation of airway smooth muscle responsiveness. Respiratory physiology & neurobiology. 2003;137(2-3):263-76.

7. Chambers LS, Black JL, Ge Q, Carlin SM, Au WW, Poniris M, et al. PAR-2 activation, PGE2, and COX-2 in human asthmatic and nonasthmatic airway smooth muscle cells. American journal of physiology Lung cellular and molecular physiology. 2003;285(3):L619-27.

8. Rydell-Tormanen K, Risse PA, Kanabar V, Bagchi R, Czubryt MP, Johnson JR. Smooth muscle in tissue remodeling and hyper-reactivity: airways and arteries. Pulmonary pharmacology & therapeutics. 2013;26(1):13-23.

9. Mojsilovic-Petrovic J, Callaghan D, Cui H, Dean C, Stanimirovic DB, Zhang W. Hypoxia-inducible factor-1 (HIF-1) is involved in the regulation of hypoxia-stimulated expression of monocyte chemoattractant protein-1 (MCP-1/CCL2) and MCP-5 (Ccl12) in astrocytes. Journal of neuroinflammation. 2007;4:12.

10. Kim ES, Kim SH, Kim KW, Park JW, Kim YS, Sohn MH, et al. Basement membrane thickening and clinical features of children with asthma. Allergy. 2007;62(6):635-40.

11. Saglani S, Malmstrom K, Pelkonen AS, Malmberg LP, Lindahl H, Kajosaari M, et al. Airway remodeling and inflammation in symptomatic infants with reversible airflow obstruction. American journal of respiratory and critical care medicine. 2005;171(7):722-7.

12. Huerta-Yepez S, Baay-Guzman GJ, Bebenek IG, Hernandez-Pando R, Vega MI, Chi L, et al. Hypoxia inducible factor promotes murine allergic airway inflammation and is increased in asthma and rhinitis. Allergy. 2011;66(7):909-18.

13. Safran M, Kaelin WG, Jr. HIF hydroxylation and the mammalian oxygen-sensing pathway. The Journal of clinical investigation. 2003;111(6):779-83.

14. Lee SY, Kwon S, Kim KH, Moon HS, Song JS, Park SH, et al. Expression of vascular endothelial growth factor and hypoxia-inducible factor in the airway of asthmatic patients. Annals of allergy, asthma & immunology : official publication of the American College of Allergy, Asthma, & Immunology. 2006;97(6):794-9.

15. Ammit AJ, Moir LM, Oliver BG, Hughes JM, Alkhouri H, Ge Q, et al. Effect of IL-6 trans-signaling on the pro-remodeling phenotype of airway smooth muscle. American journal of physiology Lung cellular and molecular physiology. 2007;292(1):L199-206.

16. Raidl M, Sibbing B, Strauch J, Muller K, Nemat A, Schneider PM, et al. Impaired TNFalpha-induced VEGF expression in human airway smooth muscle cells from smokers with COPD: role of MAPkinases and histone acetylation--effect of dexamethasone. Cell Biochem Biophys. 2007;49(2):98-110.

17. Simcock DE, Kanabar V, Clarke GW, Mahn K, Karner C, O'Connor BJ, et al. Induction of angiogenesis by airway smooth muscle from patients with asthma. American journal of respiratory and critical care medicine. 2008;178(5):460-8.

18. Osada-Oka M, Ikeda T, Imaoka S, Akiba S, Sato T. VEGF-enhanced proliferation under hypoxia by an autocrine mechanism in human vascular smooth muscle cells. Journal of atherosclerosis and thrombosis. 2008;15(1):26-33.

19. Ray JB, Arab S, Deng Y, Liu P, Penn L, Courtman DW, et al. Oxygen regulation of arterial smooth muscle cell proliferation and survival. American journal of physiology Heart and circulatory physiology. 2008;294(2):H839-52.

20. Johnson PR, Armour CL, Carey D, Black JL. Heparin and PGE2 inhibit DNA synthesis in human airway smooth muscle cells in culture. The American journal of physiology. 1995;269(4 Pt 1):L514-9.

21. Seidel P, Roth M, Ge Q, Merfort I, S'Ng C T, Ammit AJ. IkappaBalpha glutathionylation and reduced histone H3 phosphorylation inhibit eotaxin and RANTES. The European respiratory journal : official journal of the European Society for Clinical Respiratory Physiology. 2011;38(6):1444-52.

22. Ades EW, Candal FJ, Swerlick RA, George VG, Summers S, Bosse DC, et al. HMEC-1: establishment of an immortalized human microvascular endothelial cell line. The Journal of investigative dermatology. 1992;99(6):683-90.

23. Timmins NE, Nielsen LK. Generation of multicellular tumor spheroids by the hanging-drop method. Methods Mol Med. 2007;140:141-51.

24. Philippova M, Banfi A, Ivanov D, Gianni-Barrera R, Allenspach R, Erne P, et al. Atypical GPI-anchored T-cadherin stimulates angiogenesis in vitro and in vivo. Arteriosclerosis, thrombosis, and vascular biology. 2006;26(10):2222-30.

25. Tuder RM, Yun JH, Bhunia A, Fijalkowska I. Hypoxia and chronic lung disease. J Mol Med (Berl). 2007;85(12):1317-24.

26. Barbato A, Turato G, Baraldo S, Bazzan E, Calabrese F, Panizzolo C, et al. Epithelial damage and angiogenesis in the airways of children with asthma. American journal of respiratory and critical care medicine. 2006;174(9):975-81.

27. Vrugt B, Wilson S, Bron A, Holgate ST, Djukanovic R, Aalbers R. Bronchial angiogenesis in severe glucocorticoid-dependent asthma. The European respiratory journal : official journal of the European Society for Clinical Respiratory Physiology. 2000;15(6):1014-21.

28. Wilson JW, Robertson CF. Angiogenesis in paediatric airway disease. Paediatric respiratory reviews. 2002;3(3):219-29.

29. Johnson PR. Role of human airway smooth muscle in altered extracellular matrix production in asthma. Clinical and experimental pharmacology & physiology. 2001;28(3):233-6.

30. Cogo A, Napolitano G, Michoud MC, Barbon DR, Ward M, Martin JG. Effects of hypoxia on rat airway smooth muscle cell proliferation. J Appl Physiol. 2003;94(4):1403-9.

31. Brat DJ, Bellail AC, Van Meir EG. The role of interleukin-8 and its receptors in gliomagenesis and tumoral angiogenesis. Neuro-oncology. 2005;7(2):122-33.

32. Heidemann J, Ogawa H, Dwinell MB, Rafiee P, Maaser C, Gockel HR, et al. Angiogenic effects of interleukin 8 (CXCL8) in human intestinal microvascular endothelial cells are mediated by CXCR2. The Journal of biological chemistry. 2003;278(10):8508-15.

33. Yao JS, Zhai W, Young WL, Yang GY. Interleukin-6 triggers human cerebral endothelial cells proliferation and migration: the role for KDR and MMP-9. Biochemical and biophysical research communications. 2006;342(4):1396-404.

34. Simcock DE, Kanabar V, Clarke GW, O'Connor BJ, Lee TH, Hirst SJ. Proangiogenic activity in bronchoalveolar lavage fluid from patients with asthma. American journal of respiratory and critical care medicine. 2007;176(2):146-53.

35. Abdel-Rahman AM, el-Sahrigy SA, Bakr SI. A comparative study of two angiogenic factors: vascular endothelial growth factor and angiogenin in induced sputum from asthmatic children in acute attack. Chest. 2006;129(2):266-71.

36. Chetta A, Zanini A, Foresi A, D'Ippolito R, Tipa A, Castagnaro A, et al. Vascular endothelial growth factor up-regulation and bronchial wall remodelling in asthma. Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology. 2005;35(11):1437-42.

37. Coma S, Shimizu A, Klagsbrun M. Hypoxia induces tumor and endothelial cell migration in a semaphorin 3F- and VEGF-dependent manner via transcriptional repression of their common receptor neuropilin 2. Cell adhesion & migration. 2011;5(3):266-75.

38. Stankiewicz W, Dabrowski MP, Chcialowski A, Plusa T. Cellular and cytokine immunoregulation in patients with chronic obstructive pulmonary disease and bronchial asthma. Mediators of inflammation. 2002;11(5):307-12.

Proteolytic activity of house-dust-mite extract degrades ENA-78 and reduces neutrophil migration

Laura Keglowich, MSc[1]; Michael Tamm, MD[1,2]; Jun Zhong, PhD[1]; Nicola Miglino, PhD[1]; Peter Borger, PhD[1]

Affiliations:

1 Pulmonary Cell Research, Department of Biomedicine, University of Basel, Basel, Switzerland

2 Department of Pneumology, University Hospital Basel, Basel, Switzerland

Running head: ENA-78 is degraded by HDM extract derived proteases

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Abstract

Background: Bronchial smooth muscle cells (BSMC) are a major source of pro-inflammatory and pro-angiogenic cytokines and chemokines, including VEGF and CXC-chemokines. CXC-chemokines act primarily on neutrophils, mediating their recruitment to and activation at the side of inflammation. In humans, house-dust mite (HDM) allergens can cause asthmatic exacerbations and trigger an inflammatory response through protease-dependent mechanisms.

Objective: We investigated the effect HDM extract on the release of pro-angiogenic and pro-inflammatory cytokines from BSMC.

Methods: Human primary BSMC were stimulated with HDM extract in the absence or presence of fetal calf serum (FCS). Twenty angiogenic cytokines were detected by a specific antibody array and modified protein levels were confirmed by ELISA. Neutrophil migration was detected using a 96-well Boyden chamber.

Results: The concentration of ENA-78 in conditioned medium collected from BSMC stimulated with HDM extract was significantly reduced, whereas ENA-78 mRNA levels were not altered. In the presence of 5% FCS the inhibitory effect of HDM on ENA-78 expression was abrogated. Furthermore, recombinant ENA-78 was degraded by incubation with HDM extract, which was prevented by addition of FCS as well as by addition of a serine protease inhibitor. Neutrophil migration towards recombinant ENA-78 was reduced in HDM extract-stimulated conditions.

Conclusion: HDM proteases degrade ENA-78. Thus exposure to HDM allergens may alter ENA-78 levels in the lungs and may affect angiogenesis and the inflammatory response in the airways of asthma patients.

Introduction

House dust mites (HDM; *Dermatophagoides pteronissinus*) allergens are among the most potent stimuli of asthma attacks [1, 2]. HDM excretions contain a plethora of biologically active compounds, which have allergenic potential that prompts IgE response in sensitized subjects. The same HDM compounds also have pro-inflammatory properties, which are independent of the IgE response. In asthma, neoangiogenesis of the airway wall is a recently recognized pathology, which contributes to airway wall thickness [3-5]. There is also evidence from animal models that HDM and other allergens can induce neovascularization of the inflamed airway [6, 7]. However, the effect of HDM allergens on asthma related angiogenesis remain incompletely understood.

HDM extracts have been shown to disrupt the tight-junctions between epithelial cells and lead to the complete desquamation of the epithelial cell layer, hence facilitating the passage of allergens across the epithelial air-tissue border [8-10]. The major HDM allergen, Der p1, is a cysteine protease [11-13] and may be responsible for the observed epithelial cell desquamation, for the release of cytokines, and facilitates the transport of allergens across cultured epithelial cell layers [9-11, 14-16]. In consequence, the desquamation of the protective epithelium may allow allergenic compounds to penetrate deeper into the airway wall, thereby facilitating airway hyper-sensitization.

Proteases present in HDM excrements also exerted a direct modulatory effect on regulatory proteins in cultured human bronchial smooth muscle cells (BSMC) that affect the cell's ability to proliferate and to secrete cytokines [17]. In particular, BSMC exposed to HDM extract increased interleukin (IL)-6 release and cell proliferation through the protease-activated receptor (PAR)-2 [17]. Hence, HDM exposure contributes to inflammation and airway wall remodeling in a mechanism independent of the immune system by direct interaction with BSMC. Furthermore, proteolytic activities present in fungal and bacterial extracts have been shown to specifically and dose-dependently degrade human interleukin (IL)-6 and IL-8 [18], as well as RANTES, monocyte chemotactic protein (MCP)-1 and epithelial-derived neutrophil attractant (ENA)-78 [19], hence reducing the bio-availability of these cytokines.

Together, these data imply that the exposure of the airways to external factors with intrinsic proteolytic activity affects the relative level of immuno-modulatory cytokines, which may affect inflammation and microvessel formation and airway remodeling. In the present

study we analyzed the effect of HDM extract on the release and degradation of a panel of cytokines that have pro-inflammatory and angiogenic activity and are implicated in airway wall remodeling. Our data show that the proteolytic activity present in HDM extract degraded ENA-78 and reduced the migration of neutrophils. These findings imply that HDM allergens may have the capacity to modify the development of an eosinophil dominated inflammatory response in the lung.

Methods

Isolation of primary bronchial smooth muscle cells

BSMC were obtained from endobronchial biopsies obtained by flexible bronchoscopy or from therapeutically resected bronchial tissue obtained from the Department of Internal Medicine, Pneumology (University Hospital Basel, Basel, Switzerland) with the approval of the local ethical committee (EK:05/06) and written informed consent of all patients. Isolation of BSMC was performed as described earlier [20, 21]. BSMC bundles were dissected from the surrounding tissue by microscopy and pure muscle bundles were plated in a 25 cm^2-flask pre-wetted with 1 ml of Dulbecco`s modified Eagle`s medium (DMEM) containing GlutaMax-I 4.5 g/l glucose (Gibco®, Bioconcept, Allschwil, Switzerland), 5% FCS, 1x antibiotics-antimycotics, and 1x modified Eagle`s medium vitamin mix (Invitrogen, Lubio, Luzern, Switzerland). BSMC were characterized by immunostaining as described earlier [22].

Generation of conditioned medium (CM)

BSMC were grown in BSMC-growth medium (GM): RPMI 1640 supplemented with 5% fetal calf serum (FCS), 8 mM L-glutamine, 20 mM hydroxyethyl piperazine ethane sulfonic acid, 1x antiobiotics-antimycotics and 1x modified Eagle's medium vitamin mix (Invitrogen, Lubio, Luzern, Switzerland) under normoxic conditions (21% O_2, 5% CO_2, 37°C) BSMC were seeded and kept in BSMC-GM for 24 h before they were serum-deprived for 24 h prior to stimulation. A stock solution (1 mg/ml) of HDM extract (ALK-Abello, Hørsholm, Denmark) was prepared in RPMI 1640 medium and diluted in RPMI 1640 to the final concentration (20, 10 and 2 μg/ml), which was then sterilized by filtration (0.22 μm) (MN Sterilizer PES; Macherey-Nagel AG, Oensingen, Switzerland). 10 ng/ml of Colistin (Roth, Arlesheim, Switzerland) was to avoid effects of lipopolysaccharids (LPS) in HDM extract. To obtain HDM-CM, BSMC were incubated for 24 and 72 h (37°C) with HDM extract (2 and 10 μg/ml) or without HDM extract in the presence or absence of 5% FCS.

Angiogenesis Antibody Array

The Human Angiogenesis Antibody Array G 1000 (Raybiotech, Lucerna-Chem, Luzern, Switzerland) was used to detect the release of 20 angiogenesis-related cytokines and growth factors in CM obtained from BSMC stimulated with HDM extract or unstimulated BSMC. 100 μl of undiluted CM collected after 72 h were applied to each array and the

angiogenesis-related factors were evaluated according to the manufacturer instructions. Cy3 fluorescence was measured by a NimbleGen MS 200 microarray Scanner (Roche, Basel, Switzerland) and signal intensity was analyzed by AIDA software (Raytest, Straubenhardt, Germany).

Cytokine-ELISA

Enzyme linked immunosorbent assay (ELISA) kits for IL-8, ENA-78 and VEGF were purchased from R&D (Abingdon. UK) and ELISA were performed according to the manufacturer´s instructions. 100 µl of CM obtained from BSMC were measured undiluted (VEGF), 1:5 diluted (ENA- 78) or 1:50 diluted (IL-8).

ENA-78 mRNA expression by RT-PCR

BSMC were grown in absence and presence of HDM extract (20 µg/ml) for 6 h. Total RNA was extracted from 10^6 cells (t = 0 h, and t = 6 h) by Trizol method (Gibco®, Bioconcept, Allschwil, Switzerland). Then, 5 µg of total RNA were converted into cDNA. Semi-quantitative PCR (94°C, 65°C, 72°C) was performed for 28 cycles using a Thermo Hybaid PCR Express (Catalys, Wallisellen, Switzerland) as described elsewhere [23]. The primers for ENA-78 were: forward: 5'-ATC TCC GCT CCT CCA CCC AGT-3' and reverse: 5'-TTC TTG TCT TCC CTG GGT-3' generating a PCR fragment of 493 bp. The primers GAPDH were: forward 5'-CCA AAG GGT CAT CAT CTC TGC-3' and GAPDH reverse: 5'-ATT TGG CAG GTT TTT CTA-3' generating a PCR fragment of 417 bp.

Polymorphonuclear cells (PMNC) isolation and neutrophil chemotaxis

Anti-coagulated blood was obtained from the local blood bank (Blutspendezentrum Basel; Switzerland) after written informed consent of all donors. PMNC were isolated using Polymorphprep™ separation medium as recommended by the manufacturer (Axis-Shield, Axonlab, Baden, Switzerland). Chemotaxis was assessed using 96-well Boyden chamber (Neuroprobe, Gaithersburg, USA) as previously described [24]. The lower compartments of the 96-wells chamber were filled with either 100 ng/ml recombinant ENA-78, or 10 ng/ml recombinant ENA-78 (stimulated for 24 h with or without 10 µg/ml HDM extract), or 10µg/ml HDM extract in RPMI 1640, or RPMI 1640 alone, and then covered with a 5 µm pore-sized polycarbonate filter (Neuroprobe, Gaithersburg, USA). Next, 200 µl of neutrophil suspension ($2x10^6$ cells/ml) was added to the upper compartments. After 2 h of incubation (37°C, 5% CO_2) the upper compartment was removed, the filter was fixed and stained with Differential Quik Staining Kit (Polyscience, Brunschwig, Basel, Switzerland).

The membrane was scanned with a desktop scanner, and the intensity of spots was analyzed using Image J software.

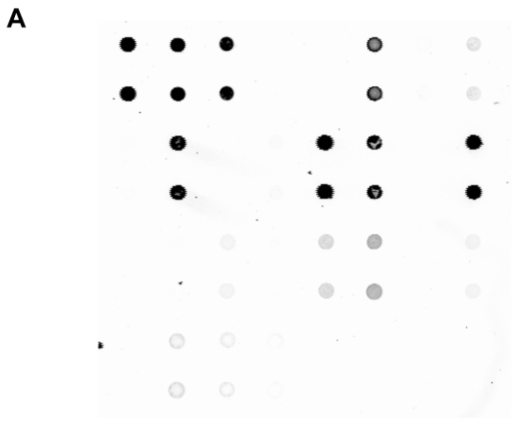
Statistics

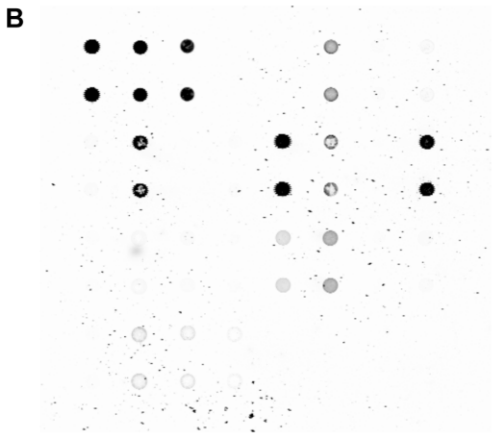
Cytokine data are presented as mean ± S.E.M. Migration analysis is shown as mean ± S.E.M. after densitometric image analysis (ImageJ software, National Institute of Mental Health, Bethesda, Maryland, USA.). Unpaired Student's t test was performed and p-values < 0.05 were considered significant.

Results

HDM extract down-regulated ENA-78 protein levels

To identify proteins that are affected by the addition of HDM extract to BSMC, CM of stimulated and unstimulated BSMC were applied to an antibody array. Comparing the protein expression pattern by the angiogenesis array the expression of ENA-78 was reduced in CM collected from BSMC that had been stimulated with HDM extract (Figure 1A and 1B). The panel of pro-angiogenic proteins of the array is shown in Figure 1C. We then confirmed this finding with an ENA-78 ELISA in CM collected from BSMC stimulated with HDM extract for 24 and 72 h. As shown in Figure 1D, HDM extract (10 µg/ml) significantly decreased the concentration of ENA-78 in CM of stimulated BSMC ($p < 0.05$; $n = 10$). In contrast, HDM extract (10 µg/ml) did not significantly alter the levels of other cytokines such as IL-8 or VEGF (data not shown).





C

	a	b	c	d	e	f	g	h
1	Pos	Pos	Pos	Neg	Neg	Angio-genin	EGF	ENA-78
2	bFGF	GRO	IFN-g	IGF-1	IL-6	IL-8	Leptin	MCP-1
3	PFGF-BB	PIGF	RANTES	TGF-beta	TIMP-1	TIMP-2	Thrombo-poeitin	VEGF
4	VEGF-D	IC1	IC2	IC3	Neg	Neg	Neg	Neg

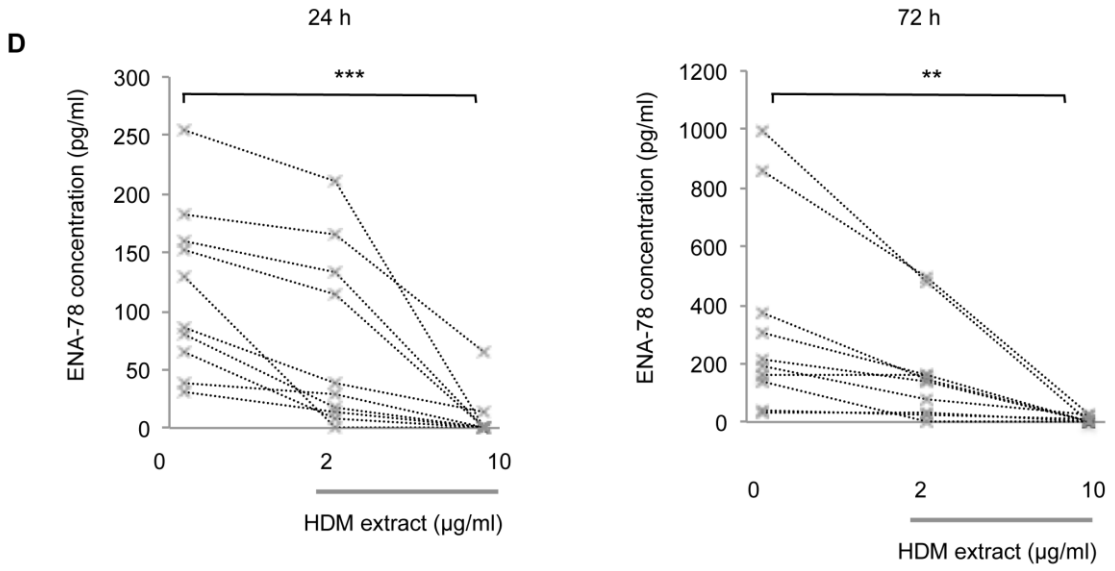


Figure 1: Effect of HDM extract on the release of angiogenic factors. A and **B**, show representative images of two angiogenesis antibody arrays incubated with CM collected from BSMC either unstimulated (**A**) or stimulated with 10 µg/ml HDM extract (**B**) for 72 h. **C**, provides the respective antibody array map for panels **A** and **B**. Standard abbreviations for the detected proteins are used, Pos: positive control, Neg: negative control, IC1-IC3: internal controls 1-3. **D**, Dose-dependency of reduced ENA-78 concentration in CM from BSMC (n = 10) stimulated with HDM extract (2 or 10 µg/ml). CM was collected after 24 and 72 h. Asterisk indicate statistic significant difference of HDM stimulated BSMC compared to unstimulated cells: **p < 0.005, *** p < 0.001.

HDM extract did not affect ENA-78 mRNA levels

To determine whether the HDM-dependent reduction of ENA-78 protein levels were associated with reduced gene transcription RT-PCR was performed using total RNA extracted after 0 and 6 h from BSMC grown in the absence and presence of HDM extract (20 µg/ml). As shown in Figure 2, exposure to HDM extract did not significantly affect RNA levels of ENA-78.

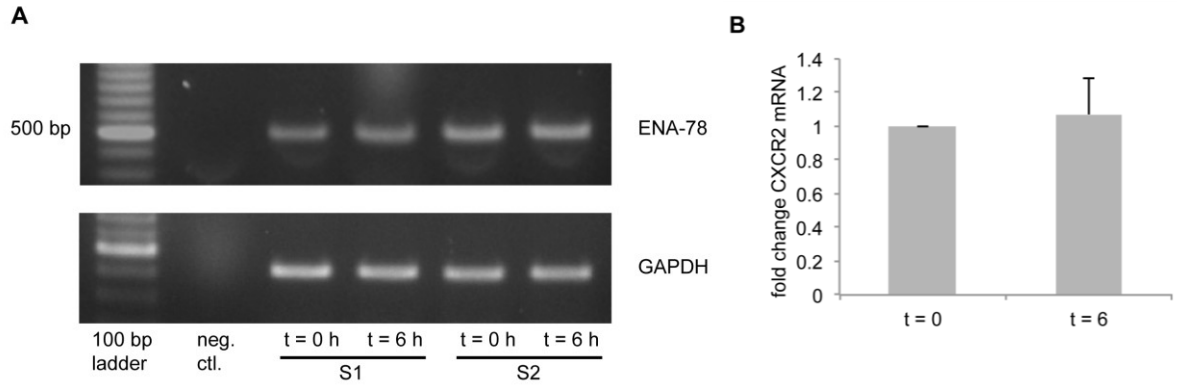


Figure 2 Detection of ENA-78 by RT-PCR. A, ENA-78 mRNA expression (fragment size: 493 bp) in an agarose-gel-electrophoresis. GAPDH (fragment size: 417 bp) served as internal control. S1/S2: subject 1/2. **B,** Densitometric analysis of RT-PCR as mean ± S.E.M (n = 2)

HDM extract specifically degraded ENA-78 protein

The observation that HDM extract stimulation decreased ENA-78 protein levels, while ENA-78 mRNA levels were unchanged indicated that the ENA-78 protein may be degraded by proteolytic activities of the HDM extract. Therefore, we incubated BSMC with 5% FCS which has strong anti-proteolytic activity in the presence of HDM extract (2 and 10 µg/ml). As shown in Figure 1D, HDM extract reduced the ENA-78 protein level in CM collected from BSMC. In the presence of 5% FCS this effect was completely abrogated (Figure 3A), suggesting a protease-dependent mechanism of ENA-78 degradation.

Next, we tested the effect of HDM extract on the basal concentration of ENA-78 in CM from BSMC (n = 7) grown in the absence of FCS for 24 and 72 h. After collection of the CM, we incubated with either HDM extract alone or a combination of HDM extract + 5% FSC. In the absence of FCS, the addition of HDM extract significantly reduced the basal concentration of ENA-78 in CM. FCS abrogated this effect (Figure 3B). We confirmed the proteolytic activity of HDM extract and the anti-proteolytic effect of FCS on ENA-78 degradation with recombinant ENA (data not shown). Furthermore, we assessed the effect of the serine protease inhibitor AEBSF and the cysteine protease inhibitor E64 on the degradation of recombinant ENA-78 by HDM extract. Incubation with E64 (0.5 µM, 5 µM, 10 µM) did not prevent ENA-78 degradation by HDM extract in any of the used concentrations (data not shown). In contrast, the addition of AEBSF led to a significant increase in ENA-78 concentration compared to control condition (without AEBSF) (Figure 3C).

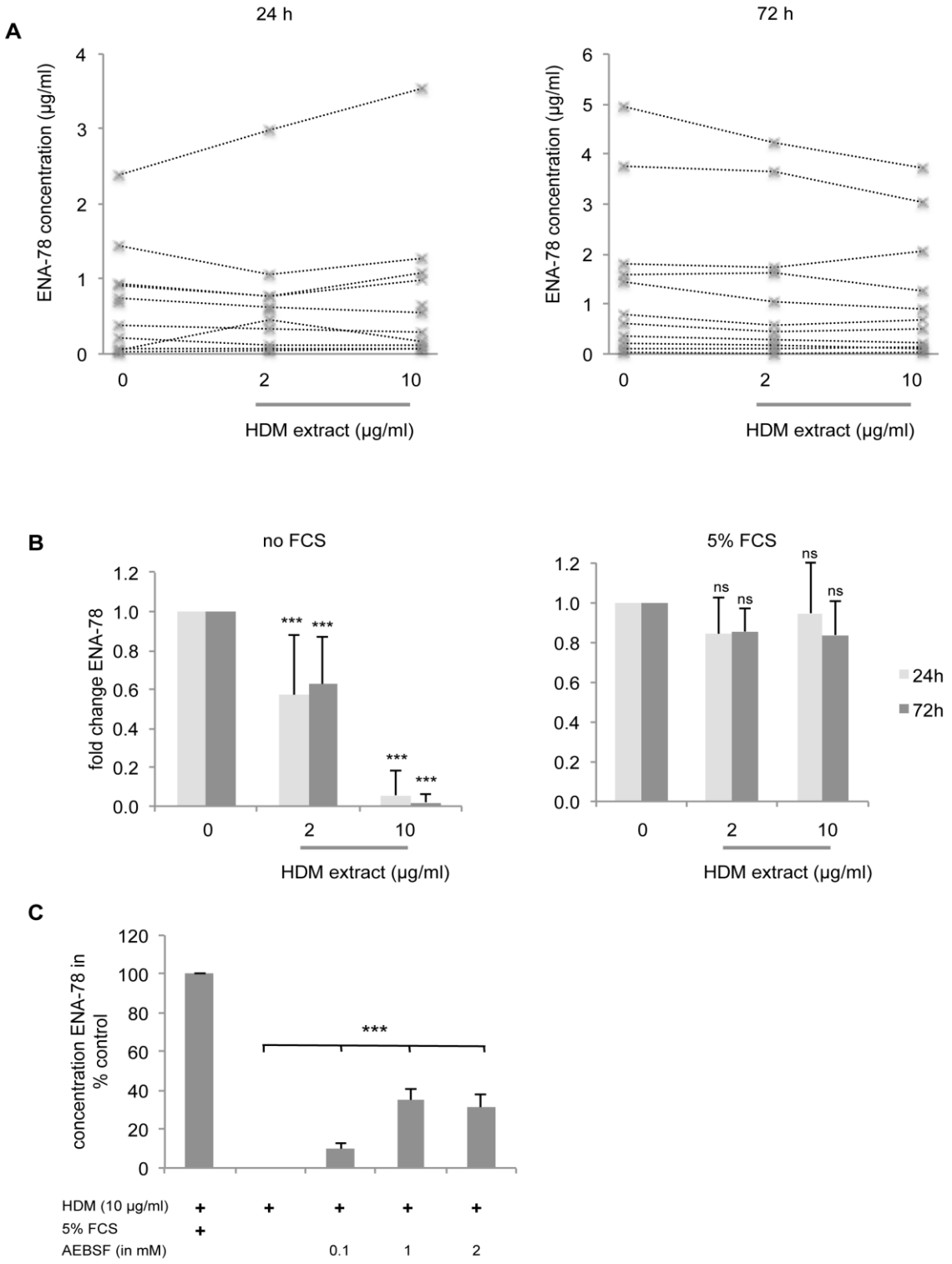


Figure 3: Effect of FCS and HDM extract (2 or 10 µg/ml) on ENA-78 concentration: A, in CM of BSMC cultured in the presence of 5% FCS after 24 and 72 h. **B**, of CM derived from BSMC cultured in the absence of FCS (n = 5) stimulated with HDM extract in the absence (left panel) or presence (right panel) of 5% FCS. *** indicate statistical significant differences with $p < 0.001$ comparing HDM extract stimulated CM to unstimulated CM. **C**, Degradation of recombinant ENA-78 by HDM extract is inhibited by the addition of the serine proteases inhibitor AEBSF or FCS. Bars represent the mean ± S.E.M, n = 3. *** indicate statistical significant differences with $p < 0.001$ comparing the rescue effect of AEBSF on HDM extract-induced ENA-78 degradation.

HDM-CM reduced chemotaxis of neutrophils

Finally, we tested the effect of HDM extract-dependent ENA-78 degradation in regard to the chemotactic capacity of ENA-78. As shown in Figure 4 ENA-78 alone dose-dependently induced neutrophil migration compared to medium only, while HDM extract alone had no significant effect on the induction of migration. When combined HDM extract reduced the neutrophil migratory effect of ENA-78.

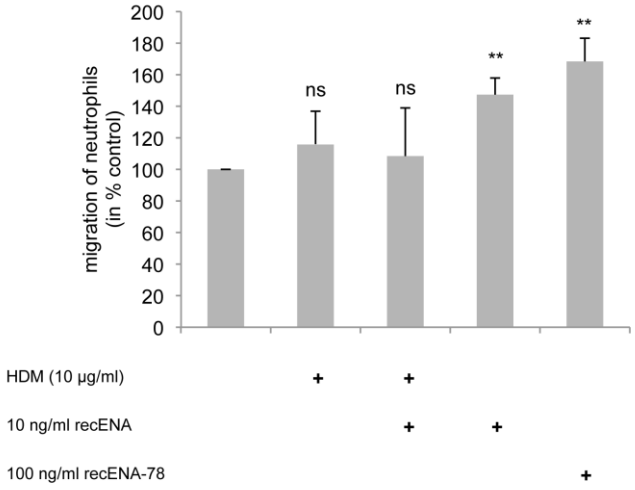


Figure 4: Densitometric analysis of neutrophil migration. Migrated cells were stained with Diff Quik staining kit, scanned with a desktop scanner and densitometric analysis was performed with Image J. Values are expressed as mean ± S.E.M., ** $p < 0.005$, $n = 3$ (neutrophils from 1 healthy subject in triplicate).

Discussion

In our study, we demonstrated for the first time that the CXCR2 ligand ENA-78 was degraded upon exposure to HDM extract, whereas a second CXCR2 ligand IL-8 was not affected. CXCR2 ligands have been suggested to play a crucial role in inflammation and angiogenesis, in particular in directing neovascularization in tumors [25].

We recently showed that angiogenic CXCR2 ligands are produced by human BSMC directing sprout outgrowth from endothelial cell spheroids [unpublished data, manuscript submitted]. In addition to its angiogenic characteristics, ENA-78 exerts strong chemotactic properties for neutrophils [26]. Several neutrophil attracting chemokines are present in the lung, including IL-8, growth related proteins (GRO-α, β, γ) and ENA-78 [27, 28]. Although ENA-78 and IL-8 share some properties, each cytokine has its own specific effects [29-33]. Comparing these studies, ENA-78 seems to be more important in chronic than in acute immunological responses.

The observation that FCS completely blocked the degradation of ENA-78 protein demonstrated that the degradation was due to the proteolytic activity present in the HDM extract and this was confirmed by the protective effect of the serine protease inhibitor AEBSF. In contrast, the cysteine protease inhibitor E64 did not prevent HDM extract-dependent ENA-78 degradation, therefore we conclude that one mechanism of ENA-78 degradation is dependent on serine proteases present in HDM extract. Proteolytic activities present in HDM extracts have been extensively studied and several HDM allergens have been identified as proteases [34]. A major effect of these proteases is the selective reduction of the bio-availability of cytokines, since not all cytokines are equally prone to proteolytic degradation [19]. Diminished bio-availability of cytokines due to proteolytic degradation has been associated with opportunistic fungi, in particular *Aspergillus fumigatus* [18], as well as with pathogenic bacteria, such as *Pseudomonas aeruginosa* [19]. Characteristic for both organisms is the secretion of a wide range of proteolytic enzymes intended for growth and existence. The cytokine-degrading properties of the proteases produced by these organisms do not only alter the relative amounts of disease relevant immunomodulatory cytokines in the airways, they may also help the organisms to invade the lung. Proteolytic modifications of ENA-78 protein have been shown to reduce and to enhance its biological activity. Cleavage of ENA-78 by MMP, MMP2, or by the aminopeptidase CD13 have been shown to enhance its activity [35, 36], whereas proteolytic modifications caused by metalloproteases from *Pseudomonas aeruginosa* was

accompanied by the loss of chemotactic activity [19]. We were able to provide evidence that proteolytic modifications by HDM extract abrogate the neutrophil-attracting properties of ENA-78.

Our data provide evidence that HDM proteases may directly affect the pro-inflammatory and pro-angiogenic cytokine response through proteolytic degradation of ENA-78 protein. Produced by epithelial cells, ENA-78 was first described as a chemo-attractant for neutrophils [31]. We have recently reported that not only epithelial cells but also BSMC release high levels of ENA-78 [unpublished data, manuscript submitted]. Continuous exposure to HDM allergens may reduce the bioavailability of ENA-78 due to proteolytic degradation, and lower the number of neutrophils that infiltrate the asthmatic airway. This may alter the immune response in favor of eosinophilic inflammation, as it is often observed in the lungs of asthmatic patients, and thought of as the main histopathology of the asthmatic lung [37-40]. It should be noted, however, that asthma sometimes is associated with neutrophilia. In a sheep model of asthma HDM extracts induced neutrophil infiltration at 6 h, followed by eosinophils and activated lymphocytes into the lung tissue and BAL, similar to the late-phase allergic response seen in human asthma [41].

It is currently unknown whether or not an association exists between exposure to HDM allergens and eosinophilia. However, selective degradation of ENA-78 by proteases present in HDM extract might provide a partial explanation.

Our experiments with the Boyden chamber showed that chemotactic migration of neutrophils towards ENA-78 is reduced in the presence of HDM extract. The microenvironment, the relative presence and balance of cytokines and chemotactic factors, ultimately determines the immunological response. Disturbance of the equilibrium between proteases and anti-proteases is a common pathological feature of inflammatory airway diseases. An external addition of foreign proteases, which may be derived from fungi, bacteria or HDM, may distort this intricate balance and lead to irreversible impairment of airway function.

Taken together, our data demonstrate that the ENA-78 protein is susceptible to degradation by proteases present in HDM extract. Although reduced bioavailability of ENA-78 could potentially cause atypical immunological characteristics *in vivo*, the biological relevance of this observation remains to be established.

References

1. Nelson RP, Jr., DiNicolo R, Fernandez-Caldas E, Seleznick MJ, Lockey RF, Good RA. Allergen-specific IgE levels and mite allergen exposure in children with acute asthma first seen in an emergency department and in non-asthmatic control subjects. The Journal of allergy and clinical immunology. 1996;98(2):258-63.

2. Thomas WR, Hales BJ, Smith WA. House dust mite allergens in asthma and allergy. Trends in molecular medicine. 2010;16(7):321-8.

3. Al-Muhsen S, Johnson JR, Hamid Q. Remodeling in asthma. The Journal of allergy and clinical immunology. 2011;128(3):451-62; quiz 63-4.

4. Detoraki A, Granata F, Staibano S, Rossi FW, Marone G, Genovese A. Angiogenesis and lymphangiogenesis in bronchial asthma. Allergy. 2010;65(8):946-58.

5. Wilson JW, Kotsimbos T. Airway vascular remodeling in asthma. Current allergy and asthma reports. 2003;3(2):153-8.

6. Van der Velden J, Barker D, Barcham G, Koumoundouros E, Snibson K. Increased vascular density is a persistent feature of airway remodeling in a sheep model of chronic asthma. Experimental lung research. 2012;38(6):307-15.

7. Avdalovic MV, Putney LF, Schelegle ES, Miller L, Usachenko JL, Tyler NK, et al. Vascular remodeling is airway generation-specific in a primate model of chronic asthma. American journal of respiratory and critical care medicine. 2006;174(10):1069-76.

8. Wan H, Winton HL, Soeller C, Gruenert DC, Thompson PJ, Cannell MB, et al. Quantitative structural and biochemical analyses of tight junction dynamics following exposure of epithelial cells to house dust mite allergen Der p 1. Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology. 2000;30(5):685-98.

9. Wan H, Winton HL, Soeller C, Tovey ER, Gruenert DC, Thompson PJ, et al. Der p 1 facilitates transepithelial allergen delivery by disruption of tight junctions. The Journal of clinical investigation. 1999;104(1):123-33.

10. Winton HL, Wan H, Cannell MB, Gruenert DC, Thompson PJ, Garrod DR, et al. Cell lines of pulmonary and non-pulmonary origin as tools to study the effects of house dust mite proteinases on the regulation of epithelial permeability. Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology. 1998;28(10):1273-85.

11. Simpson RJ, Nice EC, Moritz RL, Stewart GA. Structural studies on the allergen Der p1 from the house dust mite Dermatophagoides pteronyssinus: similarity with cysteine proteinases. Protein sequences & data analysis. 1989;2(1):17-21.

12. Chua KY, Stewart GA, Thomas WR, Simpson RJ, Dilworth RJ, Plozza TM, et al. Sequence analysis of cDNA coding for a major house dust mite allergen, Der p 1. Homology with cysteine proteases. The Journal of experimental medicine. 1988;167(1):175-82.

13. Hewitt CR, Horton H, Jones RM, Pritchard DI. Heterogeneous proteolytic specificity and activity of the house dust mite proteinase allergen Der p I. Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology. 1997;27(2):201-7.

14. King C, Brennan S, Thompson PJ, Stewart GA. Dust mite proteolytic allergens induce cytokine release from cultured airway epithelium. J Immunol. 1998;161(7):3645-51.

15. Robinson C, Kalsheker NA, Srinivasan N, King CM, Garrod DR, Thompson PJ, et al. On the potential significance of the enzymatic activity of mite allergens to immunogenicity. Clues to structure and

function revealed by molecular characterization. Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology. 1997;27(1):10-21.

16. Kauffman HF, Tamm M, Timmerman JA, Borger P. House dust mite major allergens Der p 1 and Der p 5 activate human airway-derived epithelial cells by protease-dependent and protease-independent mechanisms. Clinical and molecular allergy : CMA. 2006;4:5.

17. Miglino N, Roth M, Tamm M, Borger P. House dust mite extract downregulates C/EBPalpha in asthmatic bronchial smooth muscle cells. The European respiratory journal : official journal of the European Society for Clinical Respiratory Physiology. 2011;38(1):50-8.

18. Kauffman HF, Tomee JF, van de Riet MA, Timmerman AJ, Borger P. Protease-dependent activation of epithelial cells by fungal allergens leads to morphologic changes and cytokine production. The Journal of allergy and clinical immunology. 2000;105(6 Pt 1):1185-93.

19. Leidal KG, Munson KL, Johnson MC, Denning GM. Metalloproteases from Pseudomonas aeruginosa degrade human RANTES, MCP-1, and ENA-78. Journal of interferon & cytokine research : the official journal of the International Society for Interferon and Cytokine Research. 2003;23(6):307-18.

20. Johnson PR, Armour CL, Carey D, Black JL. Heparin and PGE2 inhibit DNA synthesis in human airway smooth muscle cells in culture. The American journal of physiology. 1995;269(4 Pt 1):L514-9.

21. Johnson PR, Roth M, Tamm M, Hughes M, Ge Q, King G, et al. Airway smooth muscle cell proliferation is increased in asthma. American journal of respiratory and critical care medicine. 2001;164(3):474-7.

22. Seidel P, Roth M, Ge Q, Merfort I, S'Ng C T, Ammit AJ. IkappaBalpha glutathionylation and reduced histone H3 phosphorylation inhibit eotaxin and RANTES. The European respiratory journal : official journal of the European Society for Clinical Respiratory Physiology. 2011;38(6):1444-52.

23. Fillmore RA, Nelson SE, Lausch RN, Oakes JE. Differential regulation of ENA-78 and GCP-2 gene expression in human corneal keratocytes and epithelial cells. Investigative ophthalmology & visual science. 2003;44(8):3432-7.

24. Henness S, van Thoor E, Ge Q, Armour CL, Hughes JM, Ammit AJ. IL-17A acts via p38 MAPK to increase stability of TNF-alpha-induced IL-8 mRNA in human ASM. American journal of physiology Lung cellular and molecular physiology. 2006;290(6):L1283-90.

25. Strieter RM, Burdick MD, Mestas J, Gomperts B, Keane MP, Belperio JA. Cancer CXC chemokine networks and tumour angiogenesis. Eur J Cancer. 2006;42(6):768-78.

26. Baggiolini M, Dewald B, Moser B. Interleukin-8 and related chemotactic cytokines--CXC and CC chemokines. Advances in immunology. 1994;55:97-179.

27. Kikuchi S, Kikuchi I, Takaku Y, Kobayashi T, Hagiwara K, Kanazawa M, et al. Neutrophilic inflammation and CXC chemokines in patients with refractory asthma. International archives of allergy and immunology. 2009;149 Suppl 1:87-93.

28. Nakagome K, Matsushita S, Nagata M. Neutrophilic inflammation in severe asthma. International archives of allergy and immunology. 2012;158 Suppl 1:96-102.

29. Strieter RM, Chensue SW, Basha MA, Standiford TJ, Lynch JP, Baggiolini M, et al. Human alveolar macrophage gene expression of interleukin-8 by tumor necrosis factor-alpha, lipopolysaccharide, and interleukin-1 beta. American journal of respiratory cell and molecular biology. 1990;2(4):321-6.

30. Walz A, Schmutz P, Mueller C, Schnyder-Candrian S. Regulation and function of the CXC chemokine ENA-78 in monocytes and its role in disease. Journal of leukocyte biology. 1997;62(5):604-11.

31. Walz A, Burgener R, Car B, Baggiolini M, Kunkel SL, Strieter RM. Structure and neutrophil-activating properties of a novel inflammatory peptide (ENA-78) with homology to interleukin 8. The Journal of experimental medicine. 1991;174(6):1355-62.

32. Imaizumi T, Albertine KH, Jicha DL, McIntyre TM, Prescott SM, Zimmerman GA. Human endothelial cells synthesize ENA-78: relationship to IL-8 and to signaling of PMN adhesion. American journal of respiratory cell and molecular biology. 1997;17(2):181-92.

33. Schnyder-Candrian S, Walz A. Neutrophil-activating protein ENA-78 and IL-8 exhibit different patterns of expression in lipopolysaccharide- and cytokine-stimulated human monocytes. J Immunol. 1997;158(8):3888-94.

34. Thomas WR, Smith WA, Hales BJ, Mills KL, O'Brien RM. Characterization and immunobiology of house dust mite allergens. International archives of allergy and immunology. 2002;129(1):1-18.

35. Song J, Wu C, Zhang X, Sorokin LM. In vivo processing of CXCL5 (LIX) by matrix metalloproteinase (MMP)-2 and MMP-9 promotes early neutrophil recruitment in IL-1beta-induced peritonitis. J Immunol. 2013;190(1):401-10.

36. Mortier A, Loos T, Gouwy M, Ronsse I, Van Damme J, Proost P. Posttranslational modification of the NH2-terminal region of CXCL5 by proteases or peptidylarginine Deiminases (PAD) differently affects its biological activity. The Journal of biological chemistry. 2010;285(39):29750-9.

37. Kay AB. The role of eosinophils in the pathogenesis of asthma. Trends in molecular medicine. 2005;11(4):148-52.

38. Kay AB, Phipps S, Robinson DS. A role for eosinophils in airway remodelling in asthma. Trends in immunology. 2004;25(9):477-82.

39. Lemiere C, Ernst P, Olivenstein R, Yamauchi Y, Govindaraju K, Ludwig MS, et al. Airway inflammation assessed by invasive and noninvasive means in severe asthma: eosinophilic and noneosinophilic phenotypes. The Journal of allergy and clinical immunology. 2006;118(5):1033-9.

40. Wenzel SE. Eosinophils in asthma--closing the loop or opening the door? The New England journal of medicine. 2009;360(10):1026-8.

41. Bischof RJ, Snibson K, Shaw R, Meeusen EN. Induction of allergic inflammation in the lungs of sensitized sheep after local challenge with house dust mite. Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology. 2003;33(3):367-75.

Key findings, conclusions and future perspectives

Key findings

In the present thesis it has been demonstrated that human primary bronchial smooth muscle cells (BSMC) release factors, which have the potential to induce angiogenesis. Therefore BSMC are able to contribute to angiogenesis in asthma-associated airway wall remodeling. Three objectives have been answered in this thesis as follows:

BSMC obtained from asthma patients exhibit a significantly increased angiogenic potential in an experimental *in vitro* inflammatory environment. The increased angiogenic potential of BSMC obtained from asthma patients was attributed to the release of ELR^+ CXC-chemokines and monitored by sprout outgrowth from endothelial cells (EC) spheroids. Blocking the corresponding receptor (CXCR2) abrogated the increased sprout outgrowth from EC spheroids induced by conditioned media (CM) of BSMC from asthma patients.

Low oxygen concentration (hypoxia) has a severe impact on the proliferative capacities of BSMC. Reduction of oxygen *in vitro* significantly reduced BSMC proliferation. Although findings in a rat model of asthma suggested a direct correlation between low oxygen concentration and increased BSM mass, where moderate hypoxia increased the proliferation in BSMC, this could not be confirmed in human BSMC. However, low oxygen concentration induced the release of pro-inflammatory and pro-angiogenic mediators by BSMC. In contrast to the angiogenic capacity of BSMC under inflammatory conditions (described above), the major regulator of hypoxia-induced angiogenesis was VEGF.

Incubation of BSMC with HDM extract did not have a direct effect on their potential to induce angiogenesis. The levels of several pro-inflammatory and pro-angiogenic proteins were unchanged in CM of HDM extract-stimulated BSMC. Nevertheless, it has been shown that ENA-78 levels in CM derived from HDM extract-stimulated BSMC were significantly decreased. Quantitative analysis of ENA-78 mRNA levels revealed that HDM extract only affected the ENA-78 protein levels. Further investigations suggested a proteasomal degradation of ENA-78 by proteases which were contained in the HDM extract. The ability of ENA-78, incubated with HDM extract, to induce neutrophil migration was reduced and might cause an altered composition of the inflammatory immune cells infiltrating the airways of asthma patients.

Conclusions

This thesis provides experimental evidence that BSMC actively participate in angiogenesis and airway wall remodeling in asthma. Furthermore, BSMC-derived factors, which can induce angiogenesis under different conditions, were identified.

Importantly, BSMC obtained from asthma patients released more of three angiogenic factors, ENA-78, GRO-α and IL-8, which all bind to the same receptor, CXCR2. Although immune cells and epithelial cells are regarded as the major sources of CXCR2-chemokines our findings showed, that BSMC are an additional source of ENA-78, GRO-α and IL-8. Elevated levels of GRO-α and IL-8 in the BALF of asthmatics [1] have been reported as well as increased ENA-78 level in sputum of asthmatic children [2]. Using different stimuli, recent studies revealed that BSMC are a potential source for IL-8 and GRO-α [3, 4]. However, the release of the CXCR2 ligands in an experimental inflammatory environment (FCS stimulation) has never been described before. The blocking of the CXCR2 and the subsequent reduction of sprout outgrowth from EC spheroids confirmed the important role of these mediators in asthma-associated angiogenesis and their cellular source – the BSMC.

The second objective was to investigate if hypoxia has a direct effect on BSMC hyperplasia. Hypoxia can be caused by decreased ventilation, airway obstruction, intra-alveolar exudate, septal thickening by edema, inflammation, fibrosis, or damage of alveolar capillaries. These pathologies or their combination can reduce proper oxygenation [5]. Our findings show that moderate and severe hypoxia on one side led to decreased proliferation of BSMC, but on the other side increased the release of pro-inflammatory and pro-angiogenic mediators.

The observation that low oxygen concentration led to reduced BSMC proliferation is contrary to an animal model. The rat model suggested hypoxia as a direct cause of BSMC hyperplasia by increasing proliferation [6]. Although we did not find evidence that hypoxia directly caused BSMC hyperplasia, we found an increased release of pro-inflammatory and pro-angiogenic mediators in CM obtained from BSMC. The effect of hypoxia on the release of other pro-inflammatory and pro-angiogenic mediators in the pathogenesis of asthma has been described before in humans and animal models [5, 7-13].

The increase in BSM mass might cause locally restricted hypoxia. It has been demonstrated that hypoxia occurs during tissue expansion and organ growth [14, 15]. Here,

the increase of the tissue mass resulted in a higher oxygen demand and insufficient delivery, which caused temporal local hypoxia. This finding suggested that the increased BSM mass could have similar effects in the airways of asthma patients. Other mechanisms how hypoxia could be induced in the airways of asthmatics are: (i) inflammation resulting in increased oxygen consumption, or (ii) edema which has an impact on oxygen diffusion through the tissue, (iii) as well as basement membrane thickening, resulting in impaired diffusion. Together these physiological alterations might lead to locally restricted hypoxia and which activate the HIF cascade, similar to that described in systemic hypoxia [10].

The connection of inflammation and angiogenesis has been noticed long ago [16], and it is now generally accepted that hypoxia and inflammation are inseparably intertwined with HIF-1 α as pivotal molecular mediator [9, 17-21]. However, to date, this interconnection has been studied mainly in the context of solid tumors [22-25].

In this thesis it was demonstrated that hypoxic conditions significantly increased the release of VEGF by BSMC whereas CXCR2 ligands were the major mediators for inflammation-induced angiogenesis. VEGF is a crucial factor for BSMC induced angiogenesis under IL-1β and IL-13 stimulation [26]. Our finding that CXCR2 are important mediators in BSMC-mediated induction of angiogenesis provided an additional mechanism how angiogenesis in the asthmatic airway wall might be induced independently of VEGF. Furthermore this thesis provides evidence that the cellular microenvironment plays an important role in the induction of angiogenesis and the release of pro-angiogenic mediators.

The third objective of the thesis was to study the effect of HDM extract on the angiogenic properties of BSMC. Studies in several model organisms (mice, sheep, primates) showed that HDM allergens increased vascularity of the lungs [27-30]. Studies from our group demonstrated that HDM extracts elicit several changes in BSMC. Proliferation and release of pro-inflammatory mediators were increased in HDM-stimulated BSMC [31]. HDM allergens therefore directly contribute to airway wall remodeling via a non-immune cell mediated mechanism. These data support earlier findings of Plopper et al. that HDM exerted its effect independently of the immune system via resident tissue-forming cells [32].

Since we did not find a direct effect of HDM extract on the angiogenic potential of BSMC we speculate that the pro-angiogenic effects of these allergens might be mediated by

immune or endothelial cells, which have been shown to release angiogenic mediators upon stimulation with allergens [33], rather than by an altered release of angiogenic mediators from BSMC. One interesting finding of our studies was the down-regulation of the pro-angiogenic chemokine ENA-78, which is involved in neutrophil recruitment, with unchanged mRNA levels. We showed that the down-regulation of ENA-78 was a secondary effect caused by proteases present in HDM extract. Data on neutrophil transmigration suggest a diminished migration of neutrophils towards a gradient of HDM extract-incubated ENA-78 compared to untreated ENA-78. In a physiological context the degradation of ENA-78 might influence the composition of inflammatory cells in the airways.

The findings of my thesis add further evidence to the recently recognized role of tissue-forming cells and in particular of BSMC to the pathogenesis of asthma. They elucidate the role of BSMC in inflammation-induced and hypoxia-induced angiogenesis in the asthmatic lung.

Future perspectives

Changes in the microvasculature have various deleterious effects: the increased vasculature provides blood and oxygen supply to the excess number of tissue-forming cells and to the inflammatory infiltrates in the bronchial wall of asthma patients. Thereby, increased vascularization may promote airway wall remodeling. The thickening of the airway wall sub-epithelial cell layers, together with the edematous effects of increased numbers and size of blood vessels results is airway narrowing, the major long term complication of asthma [34]. Blood vessels are the primary portal for immune cells to infiltrate tissues and the increased number of blood vessels in the airway wall of asthma patients might lead to exaggerated inflammation. [35]. There is evidence that badly controlled asthma results in an increase in inflammatory cells, which are entering the airway tissue via ancillary blood vessels [36]. Therefore, endothelial cells as well as BSMC might be promising new targets to reduce airway wall remodeling and asthma symptoms.

A very important aim for future research is the better understanding of the triggers that cause the release of angiogenic mediators from BSMC and a deeper knowledge on the physiological microenvironment of BSMC in the lungs of asthma patients. We clearly showed that different stimuli lead to altered release of angiogenic mediators by BSMC. In experiments mimicking inflammation chemokines of the CXC-family were the main inducers of angiogenic effects.

BSMC under hypoxic conditions induced angiogenesis mainly by the release of VEGF. Increased VEGF level in the airways of patients with asthma and in patients with eosinophilic bronchitis have been reported [37]. This suggests that VEGF is an important, but not sufficient factor in regulation angiogenesis in airway wall remodeling in asthma. It is of interest to further assess how the different pathways might be linked and if there is a common target for future therapeutic interventions. This is of course a very ambitious aim, as the process of angiogenesis is very complex and involves multiple factors with complementary and coordinated roles.

Another important question might arise from the source of endothelial cells building the new vessels. In a mouse model of asthma, it has been shown that endothelial precursor cells (EPC) from the bone marrow are recruited to the lungs via CXCR2 ligands [38]. Furthermore, it has been shown that in sputum from asthmatics the number of EPC is

increased. It would be of interest to know, if BSMC are involved in this process, because they are an important source of CXCR2 as shown in the present thesis.

The beneficial effect of bronchial thermoplasty in reducing asthma symptoms [39-43], together with the finding that BSMC produce factors that aggravate the airway wall remodeling process [44, 45], suggest a pivotal role of BSMC in asthma pathogenesis. Therefore, the development of (inhaled) anti-angiogenic agents might be an interesting new strategy to reduce BSM mass.

In conclusion, there is a fundamental demand to develop new treatment options or curative drugs to improve the quality of life of asthma patients. In cancer, several anti-angiogenic drugs have been developed and are currently in use or are tested in clinical trials to treat tumors. Angiogenesis in asthma has only recently come into the focus of researchers and a better understanding of the mechanisms contributing to asthma pathology as well as elucidation of the interconnection of the multiple processes involved may provide the basis for the use of such treatments in asthma therapy.

References

1. Goleva E, Hauk PJ, Hall CF, Liu AH, Riches DW, Martin RJ, et al. Corticosteroid-resistant asthma is associated with classical antimicrobial activation of airway macrophages. The Journal of allergy and clinical immunology. 2008;122(3):550-9 e3.

2. Heshmat NM, Mostafa GA, Abd Al-Aziz MM, Abd El-Bary EM. Sputum Epithelial Cell-Derived Neutrophil-Activating Peptide-78 (ENA-78/CXCL5) in Asthmatic Children. The Journal of allergy and clinical immunology. 2007;119(1):S136.

3. Pepe C, Foley S, Shannon J, Lemiere C, Olivenstein R, Ernst P, et al. Differences in airway remodeling between subjects with severe and moderate asthma. The Journal of allergy and clinical immunology. 2005;116(3):544-9.

4. Al-Alwan LA, Chang Y, Baglole CJ, Risse PA, Halayko AJ, Martin JG, et al. Autocrine-regulated airway smooth muscle cell migration is dependent on IL-17-induced growth-related oncogenes. The Journal of allergy and clinical immunology. 2012;130(4):977-85 e6.

5. Tuder RM, Yun JH, Bhunia A, Fijalkowska I. Hypoxia and chronic lung disease. J Mol Med (Berl). 2007;85(12):1317-24.

6. Cogo A, Napolitano G, Michoud MC, Barbon DR, Ward M, Martin JG. Effects of hypoxia on rat airway smooth muscle cell proliferation. J Appl Physiol. 2003;94(4):1403-9.

7. Sun Y, Wang JR, Han XZ, Li HB, Sun LF, Chen X, et al. [Effects of budesonide on HIF-1alpha and VEGF expression and airway remodeling in an asthmatic mouse model]. Zhongguo dang dai er ke za zhi = Chinese journal of contemporary pediatrics. 2012;14(8):622-7.

8. Ahmad T, Kumar M, Mabalirajan U, Pattnaik B, Aggarwal S, Singh R, et al. Hypoxia response in asthma: differential modulation on inflammation and epithelial injury. American journal of respiratory cell and molecular biology. 2012;47(1):1-10.

9. Baay-Guzman GJ, Bebenek IG, Zeidler M, Hernandez-Pando R, Vega MI, Garcia-Zepeda EA, et al. HIF-1 expression is associated with CCL2 chemokine expression in airway inflammatory cells: implications in allergic airway inflammation. Respiratory research. 2012;13:60.

10. Baek KJ, Cho JY, Rosenthal P, Alexander LE, Nizet V, Broide DH. Hypoxia potentiates allergen induction of HIF-1alpha, chemokines, airway inflammation, TGF-beta1, and airway remodeling in a mouse model. Clin Immunol. 2013;147(1):27-37.

11. Fajardo I, Svensson L, Bucht A, Pejler G. Increased levels of hypoxia-sensitive proteins in allergic airway inflammation. American journal of respiratory and critical care medicine. 2004;170(5):477-84.

12. Huerta-Yepez S, Baay-Guzman GJ, Bebenek IG, Hernandez-Pando R, Vega MI, Chi L, et al. Hypoxia inducible factor promotes murine allergic airway inflammation and is increased in asthma and rhinitis. Allergy. 2011;66(7):909-18.

13. Lee SY, Kwon S, Kim KH, Moon HS, Song JS, Park SH, et al. Expression of vascular endothelial growth factor and hypoxia-inducible factor in the airway of asthmatic patients. Annals of allergy, asthma & immunology : official publication of the American College of Allergy, Asthma, & Immunology. 2006;97(6):794-9.

14. Dor Y, Porat R, Keshet E. Vascular endothelial growth factor and vascular adjustments to perturbations in oxygen homeostasis. American journal of physiology Cell physiology. 2001;280(6):C1367-74.

15. Goossens GH, Blaak EE. Adipose tissue oxygen tension: implications for chronic metabolic and inflammatory diseases. Current opinion in clinical nutrition and metabolic care. 2012;15(6):539-46.

16. Zanini A, Chetta A, Imperatori AS, Spanevello A, Olivieri D. The role of the bronchial microvasculature in the airway remodelling in asthma and COPD. Respiratory research. 2010;11:132.

17. Frohlich S, Boylan J, McLoughlin P. Hypoxia-induced inflammation in the lung: a potential therapeutic target in acute lung injury? American journal of respiratory cell and molecular biology. 2013;48(3):271-9.

18. Dehne N, Brune B. HIF-1 in the inflammatory microenvironment. Experimental cell research. 2009;315(11):1791-7.

19. Nizet V, Johnson RS. Interdependence of hypoxic and innate immune responses. Nature reviews Immunology. 2009;9(9):609-17.

20. Dehne N, Hintereder G, Brune B. High glucose concentrations attenuate hypoxia-inducible factor-1alpha expression and signaling in non-tumor cells. Experimental cell research. 2010;316(7):1179-89.

21. Rius J, Guma M, Schachtrup C, Akassoglou K, Zinkernagel AS, Nizet V, et al. NF-kappaB links innate immunity to the hypoxic response through transcriptional regulation of HIF-1alpha. Nature. 2008;453(7196):807-11.

22. Hellwig-Burgel T, Stiehl DP, Wagner AE, Metzen E, Jelkmann W. Review: hypoxia-inducible factor-1 (HIF-1): a novel transcription factor in immune reactions. Journal of interferon & cytokine research : the official journal of the International Society for Interferon and Cytokine Research. 2005;25(6):297-310.

23. Jung YJ, Isaacs JS, Lee S, Trepel J, Neckers L. IL-1beta-mediated up-regulation of HIF-1alpha via an NFkappaB/COX-2 pathway identifies HIF-1 as a critical link between inflammation and oncogenesis. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2003;17(14):2115-7.

24. Lukashev D, Klebanov B, Kojima H, Grinberg A, Ohta A, Berenfeld L, et al. Cutting edge: hypoxia-inducible factor 1alpha and its activation-inducible short isoform I.1 negatively regulate functions of CD4+ and CD8+ T lymphocytes. J Immunol. 2006;177(8):4962-5.

25. Neumann AK, Yang J, Biju MP, Joseph SK, Johnson RS, Haase VH, et al. Hypoxia inducible factor 1 alpha regulates T cell receptor signal transduction. Proceedings of the National Academy of Sciences of the United States of America. 2005;102(47):17071-6.

26. Simcock DE, Kanabar V, Clarke GW, Mahn K, Karner C, O'Connor BJ, et al. Induction of angiogenesis by airway smooth muscle from patients with asthma. American journal of respiratory and critical care medicine. 2008;178(5):460-8.

27. Rydell-Tormanen K, Johnson JR, Fattouh R, Jordana M, Erjefalt JS. Induction of vascular remodeling in the lung by chronic house dust mite exposure. American journal of respiratory cell and molecular biology. 2008;39(1):61-7.

28. Snibson KJ, Bischof RJ, Slocombe RF, Meeusen EN. Airway remodelling and inflammation in sheep lungs after chronic airway challenge with house dust mite. Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology. 2005;35(2):146-52.

29. Avdalovic MV, Putney LF, Schelegle ES, Miller L, Usachenko JL, Tyler NK, et al. Vascular remodeling is airway generation-specific in a primate model of chronic asthma. American journal of respiratory and critical care medicine. 2006;174(10):1069-76.

30. Gregory LG, Jones CP, Walker SA, Sawant D, Gowers KH, Campbell GA, et al. IL-25 drives remodelling in allergic airways disease induced by house dust mite. Thorax. 2013;68(1):82-90.

31. Miglino N, Roth M, Tamm M, Borger P. House dust mite extract downregulates C/EBPalpha in asthmatic bronchial smooth muscle cells. The European respiratory journal : official journal of the European Society for Clinical Respiratory Physiology. 2011;38(1):50-8.

32. Plopper CG, Smiley-Jewell SM, Miller LA, Fanucchi MV, Evans MJ, Buckpitt AR, et al. Asthma/allergic airways disease: does postnatal exposure to environmental toxicants promote airway pathobiology? Toxicologic pathology. 2007;35(1):97-110.

33. Walters EH, Soltani A, Reid DW, Ward C. Vascular remodelling in asthma. Current opinion in allergy and clinical immunology. 2008;8(1):39-43.

34. Hashimoto M, Tanaka H, Abe S. Quantitative analysis of bronchial wall vascularity in the medium and small airways of patients with asthma and COPD. Chest. 2005;127(3):965-72.

35. Wilson JW, Kotsimbos T. Airway vascular remodeling in asthma. Current allergy and asthma reports. 2003;3(2):153-8.

36. Khor YH, Teoh AK, Lam SM, Mo DC, Weston S, Reid DW, et al. Increased vascular permeability precedes cellular inflammation as asthma control deteriorates. Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology. 2009;39(11):1659-67.

37. Siddiqui S, Sutcliffe A, Shikotra A, Woodman L, Doe C, McKenna S, et al. Vascular remodeling is a feature of asthma and nonasthmatic eosinophilic bronchitis. The Journal of allergy and clinical immunology. 2007;120(4):813-9.

38. Jones CP, Pitchford SC, Lloyd CM, Rankin SM. CXCR2 mediates the recruitment of endothelial progenitor cells during allergic airways remodeling. Stem Cells. 2009;27(12):3074-81.

39. Cox PG, Miller J, Mitzner W, Leff AR. Radiofrequency ablation of airway smooth muscle for sustained treatment of asthma: preliminary investigations. The European respiratory journal : official journal of the European Society for Clinical Respiratory Physiology. 2004;24(4):659-63.

40. Miller JD, Cox G, Vincic L, Lombard CM, Loomas BE, Danek CJ. A prospective feasibility study of bronchial thermoplasty in the human airway. Chest. 2005;127(6):1999-2006.

41. O'Byrne PM, Naji N, Gauvreau GM. Severe asthma: future treatments. Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology. 2012;42(5):706-11.

42. Shifren A, Chen A, Castro M. Point: efficacy of bronchial thermoplasty for patients with severe asthma. Is there sufficient evidence? Yes. Chest. 2011;140(3):573-5; discussion 8.

43. Thomson NC, Rubin AS, Niven RM, Corris PA, Siersted HC, Olivenstein R, et al. Long-term (5 year) safety of bronchial thermoplasty: Asthma Intervention Research (AIR) trial. BMC pulmonary medicine. 2011;11:8.

44. Xia YC, Redhu NS, Moir LM, Koziol-White C, Ammit AJ, Al-Alwan L, et al. Pro-inflammatory and immunomodulatory functions of airway smooth muscle: emerging concepts. Pulmonary pharmacology & therapeutics. 2013;26(1):64-74.

45. Doeing DC, Solway J. Airway smooth muscle in the pathophysiology and treatment of asthma. J Appl Physiol. 2013;114(7):834-43.

Extended Methods

Cell Biology

Primary bronchial smooth muscle cells - Cryopreservation

BSMC were washed with Dulbecco's phosphate-buffered saline (DPBS) and trypsinzed (3 min, 37°C). After centrifugation (5 min, 1000 rpm) $1x10^6$ BSMC per cryostock were resuspended in 0.8 ml CyroMaxx S (PAA, GE Healthcare, Glattbrugg, Switzerland) and cooled down in a "Mr. Frosty" freezing container (Nalgene, Thermo-Scientific, Lausanne, Switzerland) in a -80°C freezer overnight. For long-term storage cells were transferred to liquid nitrogen.

Primary bronchial smooth muscle cells - Isolation and Cultivation

Human BSMC were obtained from endobronchial biopsies of asthmatic subjects. BSMC from control subjects were obtained from endobronchial biopsies from non-asthmatic patients or from macroscopically normal/healthy tissue parts of lungs from patients undergoing therapeutic partial lung resection. The study protocol was approved by the local ethical committee (Ethikkommission beider Basel, EK 05/06) and all patients gave written informed consent.

Isolation of BSMC was performed as described earlier [1, 2] with some modifications. Muscle bundles from endobronchial biopsies or from bronchi (diameter 5-10 mm) dissected from tissue sections were uncovered from the overlying epithelial cell layer under a binocular microscope. Smooth muscle bundles were dissected from the parenchyma, detached in smaller pieces and placed into 25 cm^2 tissue culture flasks (BD Biosciences, Allschwil, Switzerland) in Dulbecco`s modified Eagle`s medium (DMEM) containing GlutaMax-I 4.5 g/l glucose (Gibco®, Bioconcept, Allschwil, Switzerland), 5% FCS, 1x antibiotics-antimycotics, and 1x modified Eagle`s medium vitamin mix (Invitrogen, Lubio, Luzern, Switzerland) and grown under normoxic conditions (20% O_2, 5% CO_2, 37 °C). Cells reached confluency after 3-6 weeks and were then passaged to 75 cm^2 cell culture flasks. To confirm the cell type immunostaining against smooth muscle myosin, smooth muscle actin and fibronectin were performed [3]. Cells were cultured in Roswell Park Memorial Institute medium (RPMI) 1640 (Gibco®, Bioconcept, Allschwil, Switzerland) supplemented with 5% FCS, 1x antibiotics-antimycotics, and 1x modified

Eagle`s medium vitamin mix (BSMC-GM) and subcultured in ratios 1:2 – 1:4 upon confluency.

Primary bronchial smooth muscle cells – Stimulation and treatment

Prior to stimulation all cells were growth arrested for 24 h, in BSMC basal medium (BSMC-BM): RPMI 1640 containing 0.1% FCS, 1x antibiotics-antimycotics and 1x modified Eagle`s medium vitamin mix.

To determine the angiogenic potential of BSMC and to mimic an inflammatory environment BSMC were stimulated with 5% FCS, or kept in serum-free medium as a control. These media were used in the angiogenesis array, in the 3D *in vitro* angiogenesis assay, and in ELISA to measure cytokine secretion.

To determine the effect of house dust mite (HDM) extract (ALK-Abelló, Hørsholm, Danmark) on cytokine release and angiogenic properties HDM extract was dissolved in BSMC-BM and passed through a sterile filter (0.22 µm). BSMC were treated with either 2 µg/ml, or 10 µg/ml, or no HDM extract for 24 h and 72 h in the presence and absence of 5% FCS. Colistin (Roth, Arlesheim, Switzerland) in a concentration of 10 ng/ml was added to block possible effects of LPS-contaminations in the HDM extract.

Hypoxia conditioned medium (CM) was obtained by using a Heracell 150i CO_2 incubator (Thermo-Scientific, Lausanne, Switzerland) set to hypoxic conditions (1% O_2 or 5% O_2, 5% CO_2, 37 °C). Cells were incubated for 72 h.

All CM were harvested, spun down to remove cell fragments and kept at -80°C.

HMEC-1 cells – Cultivation

The human cell line HMEC-1 (human microvascular endothelial cell line-1) is an immortalized cell line. It was generated from the transfection of human dermal microvascular cells with a PBR-322-based plasmid coding for the large T antigen of the simian virus 40 [4]. In angiogenesis related to asthma microvessels are formed first. Therefore, it is reasonable to use microvascular cells preferably to endothelial cell of large vessel. Another advantage of the HMEC-1 cell line is their capacity to divide and maintain the EC phenotype [4]. The HMEC-1 cells used in the experiments described were a generous gift from Prof. Dr. Thérèse Resink.

HMEC-1 cells were cryopreserved (analogous to cryopreservation protocol for BSMC) and thawed upon requirement. They were cultured in EC growth medium (ECGM)/10%

FCS (Provitro, Bioconcept, Allschwil, Switzerland) and subcultured in ratios 1:5 – 1:10 as required.

The 3D in vitro angiogenesis assay

The 3D *in vitro* angiogenesis assay was used to determine the angiogenic potential of CM derived from BSMC. The EC sprouting assay is a standard procedure to study angiogenesis [5-8] and has been performed as previously described [8] with some modifications.

– Preparation of methylcellulose:

6 mg of methylcellulose with a viscosity of 4,000 cP (Sigma-Aldrich, Buchs, Switzerland) were dissolved in 250 ml pre-warmed (60°C) endothelial cell basal medium (ECBM) (Provitro, Bioconcept, Allschwil, Switzerland) without FCS or supplement (1 h, magnetic stirrer). Another 150 ml of ECBM were added and the solution was stirred for 15 min. The solution was centrifuged for 20 min at 3000 rpm and the clear, homogenous supernatant was transferred into new tubes and store at 4°C until used for spheroid formation.

– Preparation of fibrinogen solution

Fibrinogen (Sigma-Aldrich, Buchs, Switzerland) was vacuum dried in a vacuum concentrator (20 min, RT) prior to weighing. 8 mg/ml of fibrinogen have been dissolved in ECBM (no FCS, no supplements) and passed through a sterile filter (0.22 µm). Fibrinogen solution was kept on ice to avoid polymerization.

– Experimental procedure:

To prepare EC spheroids, droplets of 25 µl of HMEC-1 cell suspension (c = 20000 cells/ml, in ECGM/10% FCS containing 20% methylcellulose) were seeded in the lid of a 15 cm cell culture dish. Droplets were incubated inverted (hanging drops), overnight in a humidified incubator, as described elsewhere [9]. This procedure forces the HMEC-1 cells to form coherent 3 D-aggregates, called “spheroids”.. After the incubation droplets were harvested and centrifuged (800 rpm, 10 min), the supernatant was removed and 175 µl of fibrinogen solution and 150 µl of ECGM/10% FCS containing 20% methylcellulose were added. To induce polymerization of fibrinogen 18 µl of thrombin solution (c=10 U/ml in DPBS; Sigma-Aldrich, Buchs, Switzerland) was added, and HMEC-1 spheroid suspension was transferred into a pre-warmed (37°C) 48-well plate.

One gel contains of at least 10 spheroids. After polymerization (30 min – 1 h), the gels were overlaid with 400 µl of the to-be-tested medium. For all experiments with CM gels were incubated with a 1:1 mixture of CM and ECGM/2% FCS. In experiments with chemical inhibitors and neutralizing antibodies these were added to the gels and to the medium mixture in the indicated concentrations. In experiments using a VEGF neutralizing antibody (R&D, Abingdon, UK) CM were pre-treated with 0.2 µg/ml VEGF antibody for 1 h before adding the CM to the gels. In experiments using SB 265610 gels were overlaid with medium containing SB 265610 at a concentration of 1, 5, 50 nM for 45 min prior to stimulation with CM. After 24-32h of experimental incubation HMEC-1 spheroids were fixed in-gel with 4% formaldehyde in DPBS (1 h, RT). Gels were washed (3x DPBS, 1 h) and stained overnight with 0.5 µg/ml TRITC phalloidin (Sigma-Aldrich, Buchs, Switzerland) in DPBS. For analysis images of each spheroid were taken (Olympus IX50 inverted microscope equipped with Color View II camera, Olympus Schweiz GmbH, Le Mont-sur-Lausanne, Switzerland) and the length of the 10 longest outgrowing capillary-like structures from each spheroid was measured (analySis software, Soft Imaging Systems GmbH) and expressed as the total length of sprouts per spheroid.

Viability and proliferation assays

BSMC were seeded at a density of $5x10^5$ cells/well in a 12-well plate in BSMC-GM and grown for 24 h. After 24 h of serum-deprivation (BSMC-BM) cells were stimulated with 5% FCS and grown for 72h in humidified atmosphere incubators with 1%, 5% or 21% O_2. Wells were washed with DPBS, trypsinized and counted in a mechanical cell counter (Beckman Coulter particle counter Z1, Nyon, Switzerland). Viability was assessed by Trypan blue staining and manual cell counting (Neubauer chamber).

HMEC-1 cells were seeded at $7.5x10^4$ cells/well in 48-well plates, grown for 24 h in ECGM/10% FCS, serum-deprived for 24 h (ECBM) and then further cultured in ECGM/10% FCS without or with inclusion of SB 265610. Viability was examined after 24 h by Trypan blue staining and manual cell counting using a Neubauer chamber. Cell numbers were determined after 48 h by enzymatic disaggregation using a Beckman Coulter particle counter Z1 (Nyon, Switzerland). Experiments were performed in duplicates and the cell suspension of each well was at least counted twice.

Isolation of neutrophils

Fresh anticoagulated blood from healthy donors was used to purify neutrophils by discontinuous density gradient centrifugation Polymorphprep™ medium (Axis-Shield, Axon Lab, Baden, Switzerland). 5 ml of separation medium was carefully overlaid with 5 ml of citrate-blood and centrifuged at 500 rcf for 35 min. The layer containing polymorphonuclear cells was harvest into a 15 ml tube. One washing step (10 ml, DPBS (w/o Ca^{2+}, Mg^{2+}), 500 rcf, 10 min) was performed and then remaining erythrocytes were lysed using 2 ml of RBC Lysis medium (AppliChem, Axon Lab, Baden, Switzerland). Following a second washing step (10 ml, DPBS (w/o Ca^{2+}, Mg^{2+}), 300 rcf, 5 min) the cell pellet was resuspended in 1 ml of DPBS, cell number was determined, and 30 µl of cell suspension were used for Differential Quik staining to confirm purity and identity of isolated cells.

Chemotaxis assay

Chemotaxis was assessed using 96-well Boyden chamber (Neuroprobe, Gaithersburg, USA) The lower compartment of the 96-wells chambers were filled with 100 ng/ml recombinant ENA-78, 10 ng/ml recombinant ENA-78 in RPMI (treated for 24 with and without 10 µg/ml HDM extract), with 100 ng/ml recombinant ENA-78, with 10µg/ml HDM extract in RPMI or RPMI alone and then covered with a 5 µm pore-sized polycarbonate filter (Neuroprobe, Gaithersburg, USA). Next, 200 µl of neutrophil suspension ($2x10^6$ cells/ml) was added to the upper compartments. After 2 h of incubation (37°C, 5% CO_2) the upper compartment was removed, cells on the upper side of the filter were wiped with a tissue and the filter was fixed and stained with Differential Quik Staining Kit (Brunschwig, Basel, Switzerland). The membrane was scanned with a desktop scanner, and the intensity of spots was analyzed using Image J software.

Tissue sectioning and staining

Milligan's Trichrome Staining

Airway tissue were fixed in 4% phosphate-buffered formalin (pH 7.2) and embedded in paraffin. Sections of 3 µm thickness were cut using a Shandon Finesse 325 (Thermo Scientific, Victoria, Australia) microtome and mounted on Superfrost Plus microscope slides (Lomb Scientific, Taren Point, Australia) to be used for immunohistochemical analysis.

Tissue sections were de-paraffinized in xylene (MP Biomedicals, Santa Ana, CA) (2x, 10 min) and rehydrated through graded alcohol (2x 100%, 2x 95% and 70% Ethanol; 2 min each) and washed in ddH_2O for at least 2 min.

Staining procedure:

- Weigert's haematoxylin for 5 min, rinsed with tab water
- Scott's solution, rinsed with tab water
- Mordant solution (74.6 mM Potassium dichromate, 250 mM Hydrochloric acid in 23% Ethanol) for 5-7 min, rinsed with ddH_2O
- 0.1% acid fuschin solution for 5-8 min, rinsed with ddH_2O
- 1% phosphomolybdic acid solution for 1-5 min
- 2% Orange G solution for 5-10 min, rinsed with ddH_2O
- 1% acetic acid solution for 2 min
- 1% fast green solution for 5-10 min
- 1% acetic acid solution for 3 min.

The sections were then dehydrated through graded alcohol up to 95% ethanol and coverslipped with organic mounting media DPX (Asia Pacific Specialty Chemicals, Australia). Images were taken with an Olympus BX60 microscope equipped with an attached DP71 camera (Olympus, Hamburg, Germany) with manual light exposure and 'one push' white balance on a background region. Images were kindly provided by Mr. Gavin Tjin, Woolcock Institute of Medical Research, Australia.

Haematoxylin-Eosin Staining

Endobronchial biopsies of the airway mucosa were snap frozen in optimal cutting

temperature (OCT) medium (Tissue-Tek, Sakura Finetechnical, Tokyo, Japan) on a cork disc by immersion in a beaker of isopentane (2-methylbutane, HPLC grade, Sigma-Aldrich, Castle Hill, Australia) suspended in a flask of liquid nitrogen and stored at -80ºC for later sectioning. Sections of 7 µm thickness were cut with a cryostat (Shandon Cryotome 620E, Thermo Scientific, Victoria, Australia) at a temperature of -18ºC. The frozen sections were air dried at room temperature, fixed in 50% acetone/50% methanol for 90 s and stained with Harris's haematoxylin (5 min) and alcoholic eosin (2 min) (Fronine Laboratory Supplies, Taren Point, Australia). Light microscopic photographic images (Olympus AX70 microscope, DP50 camera) at 60x power were recorded. Cryosectioning, fixation, staining and photography were performed by Dr. Jane Radford (with the assistance of Barbara Hernandez) in the Histopathology Laboratory, Faculty of Medicine, The University of Sydney.

Differential Quik staining

The Differential Quik Stain Kit is a modified Giemsa staining and was used to confirm purity of neutrophil isolations and to stain the migrated neutrophils in the chemotaxis assay. For differential staining cell suspension smears on glass slides were used. The staining procedure was performed as recommended by the manufacturer. After airdrying the glass slides, cells were fixed for 10 s in solution A, followed by 5 dips in solution B and 5 dips in solution C and rinsing the slide with tab water. After airdrying the glass slide were dipped in Xylene and cover slipped. The staining of the polycarbonate-filters of the chemotaxis assay have been stained as follows:

To remove non-migrated neutrophils from the upper side of the filter the filter was gently wiped with a tissue. The filter was then transferred to the fixative solution (Solution A) for 2 min, incubated for 45 s in Solution B followed by 45 s incubation in solution C. The filter was washed with ddH_2O and airdried.

Immunocytochemistry

HMEC-1 cells were grown to 70% confluency, washed with DPBS, and fixed in 4% formaldehyde for 20 min. Cells were permeabelized using 0.5% Triton X-100/1% BSA in DPBS. After blocking with 5% BSA in DPBS for 1 h cells were incubated with mouse anti- CXCR2 (Abcam, Lucerna-Chem, Luzern, Switzerland) or mouse IgG (DAKO, Baar, Switzerland) as a negative control, for 2 h at RT. Cells were washed 3x with DPBS/0.05%Triton X-100 (5 min) and incubated with goat anti-mouse IgG FITC-

conjugated (Southern Biotech, Lucerna-Chem, Luzern, Switzerland) for 1 h. Cells were washed 4x with DPBS (5 min) and addition of 200 ng/ml Hoechst 33342 in the second washing step. Images were taken with an Olympus IX50 inverted microscope equipped with Color View II camera (Olympus Schweiz GmbH, Le Mont-sur-Lausanne, Switzerland).

Molecular Biology and Biochemistry

SDS-PAGE and Western Blotting

Cells were lysed using 5x loading buffer (Table 2). All analyzed proteins were size-fractioned on precast 4-20% Tris-HEPES gels (Pierce, Thermo-Scientific, Lausanne, Switzerland). Gelelectrophoresis was performed in a Mini Protean Tetra Cell® (BioRad, Cressier, Switzerland) in HEPES-running buffer (Pierce, Thermo-Scientific, Lausanne, Switzerland) for 50 min at 100 V. Gels were the subjected to an overnight transfer at 50°C to 0.45 µm pore size nitrocellulose membranes (BioRad, Cressier, Switzerland). This transfer uses diffusion of the proteins into the membrane and therefore two membranes are used, one on each side of the Tris-HEPES-Gel. Detection of α-tubulin, Cyclin E, Cyclin D, CXCR2, HIF1-α, and PCNA was performed as follows:

- Detection of α-tubulin, Cyclin E, Cyclin D, PCNA

The membrane was blocked with 3% BSA in Tris buffered saline containing 1% Tween-20 (TBST) for 30 min. Primary antibodies were applied in TBST with 1% BSA for 2 h (1 h for α-tubulin) at RT. Membranes were washed 3x in TBST for 10 min. Secondary HRP-coupled antibodies against the corresponding species of the primary antibody were applied in TBST for 1 h (30 min for α-tubulin) at RT.

Antibodies were purchased from Santa Cruz, Lucerna-Chem, Luzern, Switzerland.

- Detection of HIF1-α

The membrane was blocked for 1h with 5% BSA in TBST at RT and incubation with primary antibody was in 5% BSA in TBST at 4°C overnight. After 3x wash (10 min) TBST secondary antibodies were applied for 2 h at RT in 5% milk powder (BioRad, Cressier, Switzerland) in TBST.

Antibodies were purchased from Cell Signaling Technologies, Bioconcept, Allschwil, Switzerland.

- Detection of CXCR2

The membrane was blocked for 1 h with 4% BSA in TBST at RT and incubation with primary antibody was in 1% BSA in TBST at 4°C overnight. After 3x wash (10 min) TBST secondary antibodies were applied for 1 h at RT in TBST.

Antibodies were purchased from Abcam (CXCR2) and Santa Cruz (HRP-conjugated secondary antibody)(Luzerna-Chem, Luzern, Switzerland).

After 3x washing for 10 min in TBST membranes were incubated with SuperSignal® Western Pico Chemiluminescent Substrate (Pierce, Thermo-Scientific, Lausanne, Switzerland) for 5 min. Signal was detected on Fujifilm Super RX X-ray films (Lucerna-Chem, Luzern, Switzerland) and developed in a Curix60 film-processor (Agfa, Dübendorf, Switzerland)

Table 2: Buffers used in SDS-PAGE and Western Blotting. All chemicals were purchased from Sigma Aldrich, Buchs, Switzerland.

Buffer	*chemical*	*Volume/amount*
5x Loading buffer	0.5 M Tris pH 6.8	2 ml
	Glycerol	3.2 ml
	20% SDS	1.6 ml
	β-mercaptoethanol	0.8 ml
	1% bromphenolblue	1.6 ml
	ddH_2O	6.8 ml
TBST	0.5 M Tris pH 8	20 ml
	5 M NaCl	30 ml
	10% Tween 20	10 ml
	add ddH_2O to 1 l	
Transfer buffer	1 M Tris pH 7.5	20 ml
	0.5 M EDTA	8 ml
	5 M NaCl	20 ml
	1 M DDT	200 µl
	add ddH_2O to 2 l	

RT-PCR

CXCR2

HMEC-1 cells were plated in a 25 cm^2 cell culture flask and grown to confluency. Cells were washed 2x with DPBS and total RNA was isolated using RNeasy Mini kit (Qiagen, Hombrechtikon, Switzerland) according to the manufacturer`s instructions. RNA concentration was determined by spectroscopy (NanoDrop, Witec, Luzern, Switzerland). First strand DNA was synthesized with m-MLV Reverse Transcriptase (from 5 µg of total RNA). Following reverse transcription cDNA was subjected to amplification with HotStarTaq Plus DNA polymerase (Qiagen, Hombrechtikon, Switzerland) using the following primers: forward 5`- CAG TTA CAG CTC TAC CCT GCC – 3, reverse 5` - CCA GGA GCA AGG ACA GAC CCC – 3 generating a 451 bp spanning fragment. PCR conditions were: 5 min 95°C; 30x: 30 sec 98°C, 30 sec 58°C, 1 min 72°C; 10 min 71°C. PCR products were size-fractioned on a 1% agarose gel, stained with ethidium bromide and visualized under UV light.

ENA-78 & GAPDH

BSMC were grown to confluency in 75 cm^2 cell culture flasks, serum deprived for 24 h and stimulated with 20 µg/ml HDM extract for 6 h. Total RNA was isolated at t = 0 h and t = 6 h using Trizol reagent according to the manufacturer`s instructions. RNA concentration was determined by spectroscopy (NanoDrop, Witec, Luzern, Switzerland). First strand DNA was synthesized with m-MLV Reverse Transcriptase (from 5 µg of total RNA). Following reverse transcription cDNA was subjected to amplification with HotStarTaq Plus DNA polymerase (Qiagen, Hombrechtikon, Switzerland) using the following primers: ENA-78 forward 5'-ATC TCC GCT CCT CCA CCC AGT-3' and ENA-78 reverse 5'-TTC TTG TCT TCC CTG GGT-3' generated a PCR fragment spanning 493 bp and the primers GAPDH forward 5'-CCA AAG GGT CAT CAT CTC TGC-3' and GAPDH reverse 5'-ATT TGG CAG GTT TTT CTA-3' generating a PCR fragment spanning 417 bp. PCR conditions were: 5 min 95°C; 25x: 30 sec 98°C, 30 sec 58°C, 1 min 72°C; 10 min 71°C. PCR products were size-fractioned on a 1% agarose gel, stained with ethidium bromide and visualized under UV light. Densitometric analysis of mRNA expression was performed using Image J software and normalized to GAPDH.

Enzyme-linked Immunosorbent assay

The presence ad concentration of angiogenic proteins in CM of BSMC were analyzed in duplicates by ELISA according to the manufacturer´s protocol. For each experiment a new standard curve was generated according to the manufacturer`s recommendations. Detection range and used dilutions of the CM are displayed in **Table 3**:

Table 3: Standard curves and dilutions for commercial available ELISA kits

	Detection range and standard curve	*Dilution factor*
ENA-78	15.6-1000 pg/ml, 2-fold dilution	5
GRO-α	31.3-2000 pg/ml, 2-fold dilution	10
IL-6	4.7-600 pg/ml, 2-fold dilution	30 to 50
IL-8 (Anibiotech)	3.9-125 pg/ml, 2-fold dilution	25
IL-8 (R&D)	31.3-2000 pg/ml, 2-fold dilution	8
VEGF	31.3-2000 pg/ml, 2-fold dilution	1

.

IL-8 ELISA kits were purchased from Anibiotech (Vantaa, Finland) and from R&D (Abingdon, UK). ENA-78, GRO-α, IL-6 and VEGF ELISA kits were purchased from R&D (Abingdon, UK).

Angiogenesis array

The commercially available angiogenesis array G1 (Raybiotech, Lucerna-Chem, Luzern, Switzerland) was performed according to the manufacturer`s instructions. Undiluted CM from BSMC cells was applied to the array. The incubation with CM and with the secondary antibody cocktail (Cy3-labled) were performed at 4°C overnight. The manufacturer`s did not recommend to use FCS containing CM. Control experiments were performed to test the influence of FCS on the results by using RPMI 1640 without or with 5% FCS as negative controls. Experiments revealed no interference of FCS with the array.

Cy3-fluoresence was measures with a NimbleGen MS 200 microarray Scanner (Roche, Basel, Switzerland) at a resolution of 10 µm and intensity was analyzed with AIDA software (Raytest, Straubenhardt, Germany). Afterwards slides were kept at -20°C.

References

1. Johnson PR. Role of human airway smooth muscle in altered extracellular matrix production in asthma. Clinical and experimental pharmacology & physiology. 2001;28(3):233-6.

2. Johnson PR, Armour CL, Carey D, Black JL. Heparin and PGE2 inhibit DNA synthesis in human airway smooth muscle cells in culture. The American journal of physiology. 1995;269(4 Pt 1):L514-9.

3. Seidel P, Hostettler KE, Hughes JM, Tamm M, Roth M. Dimethylfumarate inhibits CXCL10 via haem oxygenase-1 in airway smooth muscle. The European respiratory journal : official journal of the European Society for Clinical Respiratory Physiology. 2013;41(1):195-202.

4. Ades EW, Candal FJ, Swerlick RA, George VG, Summers S, Bosse DC, et al. HMEC-1: establishment of an immortalized human microvascular endothelial cell line. The Journal of investigative dermatology. 1992;99(6):683-90.

5. Ellis L, Shah P, Hammers H, Lehet K, Sotomayor P, Azabdaftari G, et al. Vascular disruption in combination with mTOR inhibition in renal cell carcinoma. Molecular cancer therapeutics. 2012;11(2):383-92.

6. Hewing B, Stangl V, Stangl K, Enke-Melzer K, Baumann G, Ludwig A. Circulating angiogenic factors in patients with thromboangiitis obliterans. PloS one. 2012;7(4):e34717.

7. Liu Z, Kobayashi K, van Dinther M, van Heiningen SH, Valdimarsdottir G, van Laar T, et al. VEGF and inhibitors of TGFbeta type-I receptor kinase synergistically promote blood-vessel formation by inducing alpha5-integrin expression. Journal of cell science. 2009;122(Pt 18):3294-302.

8. Philippova M, Banfi A, Ivanov D, Gianni-Barrera R, Allenspach R, Erne P, et al. Atypical GPI-anchored T-cadherin stimulates angiogenesis in vitro and in vivo. Arteriosclerosis, thrombosis, and vascular biology. 2006;26(10):2222-30.

9. Timmins NE, Nielsen LK. Generation of multicellular tumor spheroids by the hanging-drop method. Methods Mol Med. 2007;140:141-51.

Curriculum Vitae

Personal Details Laura Franziska Keglowich

email: l.keglowich@gmx.ch

Born on 23rd of September 1981 in Lampertheim (D)

Nationality: German

Education

09/2009 – 09/2013 **Doctoral studies at the Biozentrum, University of Basel**

Pulmonary Cell Research, Department of Biomedicine, University Hospital Basel

Title: Angiogenesis in asthma: Novel mechanisms of human bronchial smooth muscle cells in the induction of angiogenesis

08/2008 - 07/2009 **Diploma Thesis at the Institute of Anatomy and Cellbiology, University of Freiburg**

Title: Zellbiologische und biochemische Charakterisierung humanpathologischer L1-CAM Mutationen.

Award: Diplombiologin (Diploma in Biology)

Grade: 1.1

03/2005 – 07/2009 **Advanced Studies of Biology at Albert-Ludwig-Universität Freiburg**

Subjects: Genetics and Molecular Biology
Molecular Immunology
Cell Biology
Business Management

10/2002 - 3/2005 **Undergraduate studies of Biology at Albert-Ludwigs-Universität Freiburg**

Work Experience

Since 10/2013	**Clinical Research Associate, IBCSG Coordinating Center, Bern**
09/2009 – 09/2013	**PhD student, Department of Biomedicine, University Hospital Basel**

- Tasks & Skills

Planning and accomplishment of research projects independently

Experienced in writing of protocols, scientific progress reports, and scientific articles

Experienced in searching scientific databases (Pubmed/ScienceDirect/Web of Science)

Presentation of project work at (inter-)national congresses

Establishment and/or maintenance of (inter-)national collaborations

General laboratory organization

- Hands-on Experience

Molecular Techniques:

Transformation, Real-time PCR, PCR, cloning

Immunological/biochemical Techniques:

Phagemid-Display, ELISA, Protein purification, SDS-PAGE, Western Blot, Subcellular fractioning

Cell Biology Techniques:

Establishment and maintenance of primary human cell culture, Transfection

Histology/ Immuncytochemistry:

In-situ-Hybridisation, Immuncytochemistry, Confocal Microscopy (LSM 510)

Additional Skills

Certificates: Good Clinical Practice Basiskurs

GCP Aufbaukurs Study Manager

Languages: German (mothertongue)
Englisch (fluent)
French (basic knowledge)
Latein (basic knowledge)

Publications & manuscripts

Keglowich L, Roth M, Philippova M, Resink TJ, Gavin Tjin G, Oliver B, Lardinois D, Dessus-Babus S, Gosens R, Hostettler Haack K, Tamm M, Borger P. *Bronchial smooth muscle cells of asthmatics promote angiogenesis through elevated secretion of CXC-chemokines).* (PLoS One. 2013 Dec 5;8(12):e81494)

Keglowich L, Baraket M, Lardinois D, Tamm M, Borger P. *Hypoxia exerts dualistic effects on inflammatory and proliferative responses of healthy and asthmatic primary human bronchial smooth muscle cells.* (PLOS One, 2014 Feb 24;9(2):e89875)

Keglowich L, Baraket M, Lardinois D, Tamm M, Borger P. *Proteolytic activity present in house-dust-mite extracts degrades ENA-78 and reduces neutrophil migration.* (J Allergy (Cairo). 2014 May 4; 2014: 673673)

Costa L, Roth M, Miglino N, Keglowich L, Zhong J, Lardinois D, Tamm M, Borger P. *Tiotropium sustains the anti-inflammatory action of olodaterol via the cyclic AMP pathway.* (Pulm Pharmacol Ther. 2014 Feb;27(1):29-37)

Seidel P, Costa L, Keglowich L, Lardinois D, Tamm M, Roth M. *The MNK1/ eIF4E pathway as a new therapeutic pathway to target inflammation and remodeling in asthma.*(submitted)

Marx M, Diestel S, Bozon M, Keglowich L, Drouot N, Bouché E, Frebourg T, Minz M, Saugier-Veber P, Castellani V, Schäfer MK. *Pathomechanistic characterization of two exonic L1CAM variants located in trans in an obligate carrier of X-linked hydrocephalus.* Neurogenetics. 2012 Feb;13(1):49-59

Schäfer MK, Nam YC, Moumen A, Keglowich L, Bouché E, Küffner M, Bock HH, Rathjen FG, Raoul C, Frotscher M. *L1 syndrome mutations impair neuronal L1 function at different levels by divergent mechanisms.* Neurobiol Dis. 2010 Oct;40(1):222-37

International Communications

2013

Keglowich L, Roth M, Tamm M, Borger P. *Effect of hypoxia on proliferation and VEGF-release of human bronchial smooth muscle cells.* Poster presentation, European Respiratory Society Annual Congress, Barcelona, Spain

Khan P, Keglowich L, Tamm M, Roth M. *MNK-1 inhibition reduces proliferation and CXCL10 in airway smooth muscle cells*. Poster presentation, European Respiratory Society Annual Congress, Barcelona, Spain

2012

Keglowich L, Roth M, Philippova M, Resink TJ, Tjin, G, Oliver B, Dessus-Babus S, Tamm M, Borger P.*Angiogenesis in asthma: Altered angiogenic potential of bronchial smooth muscle cells of asthmatic patients* Poster presentation, Chronic Inflammatory Disorders of the Lung Symposium, Freiburg, Germany

Keglowich L, Roth M, Philippova M, Resink TJ, Tamm M, Borger P *Angiogenesis in asthma: Altered angiogenic potential of bronchial smooth muscle cells of asthmatic patients* Poster presentation, European Respiratory Society Annual Congress, Vienna, Austria

Keglowich L, Kyriakakis E, Philippova M, Resink TJ, Tamm M, Roth M, Borger P. *Altered angiogenic potential of bronchial smooth muscle cells of asthmatic patients.* Poster presentation, European Respiratory Society Lung Science Conference, Estoril, Portugal

2011

Keglowich L, Roth M, Tamm M, Borger P. *Neovascularization in asthma: Altered angiogenic potential of ASM cells from asthmatic patients. propria* Poster presentation, European Respiratory Society Annual Congress, Amsterdam, The Netherlands

2010

Keglowich L, Kyriakakis E, Philippova M, Resink TJ, Tamm M, Roth M, Borger P. *A novel strategy to counteract airway wall remodelling in asthma: Blocking the neovascularization of the lamina propria* Poster presentation, European Respiratory Society Annual Congress, Barcelona, Spain

Costa L, Roth M, Tamm M, Gencay M, Miglino N, Keglowich L, Bodmer H, Hostettler K, Borger P. *Crosstalk between long acting b_2-adrenergic receptor agonists and tiotropium: Crosstalk of b_2-adrenergic and muscarinic receptors.* Poster presentation, European Respiratory Society Annual Congress, Barcelona, Spain