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Studies on Neurotrophins in
Inflammatory Pulmonary Diseases

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Stockholm 2010
Till Markus
ABSTRACT

Asthma, sarcoidosis and chronic obstructive pulmonary disease (COPD) are inflammatory pulmonary diseases, all being characterized by tissue inflammation, tissue damage (airway remodeling) and loss of lung function. In order to increase possibilities to find new biomarkers, drug targets and better treatment options for the patients, further research on the underlying mechanisms driving the inflammation and airway remodeling in these diseases are required. In the present thesis, a family of mediators, the neurotrophins, were studied to elucidate how these mediators are involved in the inflammatory and/or airway remodeling processes in asthma, sarcoidosis and COPD. The neurotrophin family consists of NGF, BDNF and NT-3, and these factors were initially characterized for their essential role as survival factors for nerve cells. However, they have been shown to display a far broader spectrum of functions, being mediators also involved in inflammation and tissue remodeling. Indeed, enhanced levels of neurotrophins have been found in several inflammatory conditions, including allergic diseases, such as asthma.

The aim of this thesis was to elucidate if neurotrophin levels are altered in inflammatory pulmonary diseases other than asthma. Therefore, we set up studies to determine the protein levels of NGF, BDNF and NT-3 in bronchoalveolar lavage fluid (BALF) from patients with pulmonary sarcoidosis, patients with COPD, and relevant control subjects, including healthy non-smokers and current smokers with normal lung function. In addition, the aim was to study human bronchial smooth muscle cells, as possible target cells for the effects of the neurotrophins in the airways.

We found that, similarly to asthma, sarcoidosis patients showed enhanced levels of both NGF and NT-3 in the airways as compared to healthy subjects. We also investigated the relation between levels of neurotrophins and clinical as well as inflammatory parameters, and found significant positive correlations between NGF and the concentration of lymphocytes and eosinophils, as well as inflammatory cytokines (IFN-γ, IL-4, IL-10, IL-12) in BALF of sarcoidosis patients. We found that the levels of NT-3 were higher in the sub-groups of patients with a higher risk of developing chronic disease and fibrosis, suggesting that NT-3 levels relate to the risk of developing chronic disease. To further elucidate cellular sources of, and possible targets for, neurotrophins, in the lungs of sarcoidosis patients, we performed immunohistochemistry on lung tissue biopsies. We found that granulomas expressed NGF and NT-3, as well as the corresponding neurotrophin receptors TrkA and TrkC, indicating that the granulomas are possible sources of the enhanced levels of neurotrophins in sarcoidosis, and that the neurotrophins may be able to mediate autocrine functions in the granuloma microenvironment. In contrast to sarcoidosis, COPD patients, as well as smokers with normal lung function, showed decreased levels of NGF and NT-3 in the airways as compared to healthy non-smokers. This indicated that cigarette-smoke exposure may impact neurotrophin content in human airways. Indeed, when exposing human lung fibroblasts in vitro to cigarette smoke extract, a down-regulation of neurotrophin expression was shown on both transcriptional- and protein levels. Studies on human bronchial smooth muscle cells revealed that they are possible target cells for the neurotrophins in the airways, as they express the neurotrophin receptors TrkA, -B, and –C, and we showed that NGF, BDNF and NT-3 enhanced the secretion of the tissue degrading enzyme MMP-9, but not MMP-2. Additionally, BDNF and NT-3, but not NGF, stimulated cell migration. These results support the concept that neurotrophins have pro-fibrotic properties and may be involved in airway tissue remodeling, such as that seen in asthma.

Taken together, the results of the present thesis contributes to a better understanding regarding the involvement of neurotrophins in inflammatory pulmonary diseases, and gives further support to the concept of neurotrophins as being mediators involved in airway inflammation and tissue remodeling.
LIST OF PUBLICATIONS

Effects of neurotrophins on human bronchial smooth muscle cell migration and matrix metalloproteinase-9 secretion.
Translational Research. 2007 Nov;150(5):303-10.

Increased levels of nerve growth factor in the airways of patients with sarcoidosis.

Neurotrophins and neurotrophin receptors in pulmonary sarcoidosis - granulomas as a source of expression.
Manuscript

IV. Dagnell C, Mikko M, Roos-Engstrand E, Löfdahl M, Blomberg A, Sköld CM, Olgart Höglund C.
Airway nerve growth factor and neurotrophin-3 are decreased in patients with COPD and in smokers.
Submitted
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<table>
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<th>Full Form</th>
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<tbody>
<tr>
<td>α-SMA</td>
<td>α-smooth muscle actin</td>
</tr>
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<td>AHR</td>
<td>Airway hyperresponsiveness</td>
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<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
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<tr>
<td>BAL</td>
<td>Bronchoalveolar lavage</td>
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<tr>
<td>BALF</td>
<td>Bronchoalveolar lavage fluid</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>BHL</td>
<td>Bilateral hilar lymphadenopathy</td>
</tr>
<tr>
<td>BrdU</td>
<td>Bromo-deoxy-uridine</td>
</tr>
<tr>
<td>CBD</td>
<td>Chronic beryllium disease</td>
</tr>
<tr>
<td>CGRP</td>
<td>Calcitonin gene-related peptide</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>COPD</td>
<td>Chronic obstructive pulmonary disease</td>
</tr>
<tr>
<td>CSE</td>
<td>Cigarette smoke extract</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
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<tr>
<td>ECP</td>
<td>Eosinophil cationic protein</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EN</td>
<td>Erythema nodosum</td>
</tr>
<tr>
<td>ERK1/2</td>
<td>Extracellularly-regulated kinase-1 and -2</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FEV₁</td>
<td>Forced expiratory volume in 1 sec</td>
</tr>
<tr>
<td>FVC</td>
<td>Forced vital capacity</td>
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<tr>
<td>HBSMC</td>
<td>Human bronchial smooth muscle cells</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
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<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IPF</td>
<td>Interstitial pulmonary fibrosis</td>
</tr>
<tr>
<td>JNK</td>
<td>Jun N-terminal kinase</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MBP</td>
<td>Major basic protein</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MMPs</td>
<td>Matrix metalloproteinases</td>
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<tr>
<td>MS</td>
<td>Multiple sclerosis</td>
</tr>
<tr>
<td>NFkB</td>
<td>Nuclear factor kB</td>
</tr>
<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>NT</td>
<td>Neurotrophin</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
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<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
</tr>
<tr>
<td>PHA</td>
<td>Phytohemagglutinin</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol-3-kinases</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
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<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<td>--------------</td>
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<tr>
<td>RA</td>
<td>Rheumatoid arthritis</td>
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<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RSV</td>
<td>Respiratory syncytial virus</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase-polymerase chain reaction</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>TIMPs</td>
<td>Tissue inhibitors of metalloproteinases</td>
</tr>
<tr>
<td>TK</td>
<td>Tackykinin</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>TRPV1</td>
<td>Transient receptor potential cation channel, subfamily V</td>
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1. INTRODUCTION

1.1 GENERAL INTRODUCTION

In the present work, the neurotrophins have been investigated in inflammatory pulmonary diseases, including asthma, sarcoidosis and chronic obstructive pulmonary disease (COPD). These three diseases affect the lungs, and share the common features of inflammation of the airways, that may lead to permanent damages with loss of lung function. Elucidating the underlying mechanisms driving the inflammation, and lung tissue changes, occurring in these diseases, opens up for possibilities of finding new biomarkers, drug targets and better treatment options for these patients. In the present thesis, a family of proteins, the neurotrophins, were investigated. The aim was to understand if, and how, these mediators may play a role in the inflammatory and tissue remodeling processes in asthma, sarcoidosis and COPD.

1.2 FUNCTIONAL ANATOMY OF THE AIRWAYS

The main function of the respiratory system is to facilitate gas exchange between the inhaled air and the blood, supplying the body with oxygen and removing carbon dioxide. The respiratory system begins in the mouth and nose and extends down to the small bronchioles and alveoli where the actual gas exchange occurs. The upper airways, which includes the nose down to pharynx, serves to filter, warm and humidify the inhaled air, thereby protecting the more delicate surfaces of the lower respiratory system. The lower airways include the larynx (the site for sound production through the vocal cords), trachea, bronchi, bronchioles, and the alveoli of the lungs. From the trachea to the alveoli, the airways divide about 23 times, giving rise to the bronchial tree. The most peripheral airway structures are the alveolar sacs that are surrounded by clusters of alveoli (see Figure 1). Each lung contains approximately 240 million alveoli (Ochs et al. 2004). The alveoli are surrounded by a network of capillaries, and gas exchange occurs through direct diffusion from the alveolar air to the blood and vice versa.

Figure 1. Schematic picture of the human lung and bronchial tree.
1.3 AIRWAY STRUCTURAL CELLS

Mesenchymal cells, including smooth muscle cells, fibroblasts and epithelial cells, are structural cells that build up the airway tissue (see Figure 2). Additionally, they have synthetic properties, and are considered to be important modulators of-, and responders to, the chronic inflammation in asthma, sarcoidosis and COPD. In the present thesis, structural cells of the airways have been studied, as they are able to produce neurotrophins and also respond to effects of neurotrophins. The main functions and properties of these cells are thus presented below.

SMOOTH MUSCLE CELLS

Constriction of the airways during an asthma attack is mediated by the contraction of the smooth muscle cells. Also, one of the main pathological findings in asthma is an increased smooth muscle mass which is suggested to contribute to airway hyperresponsiveness (Berend et al. 2008). The trachea and bronchi are supported by cartilage that holds the passage open. As the bronchi divides in the bronchial tree, the cartilage is progressively exchanged by smooth muscle and the walls of the bronchioles are dominated by smooth muscle tissue and connective tissue and lack cartilage. Through smooth muscle contraction, the diameter of the bronchioles is regulated, and thereby controls the resistance to airflow and the distribution of air within the lungs. Smooth muscle contraction is regulated by the autonomic nervous system, where sympathetic stimulation leads to bronchorelaxation and parasympathetic stimulation leads to smooth muscle contraction and a reduction in the diameter of the airway. During an allergic reaction, bronchoconstriction occurs in response to histamine release by primarily activated mast cells.

Except from the structural properties of airway smooth muscle cells and their ability to contract and proliferate, these cells are also capable of synthesizing and secreting several mediators, like pro-inflammatory cytokines, chemokines and growth factors, to the surrounding environment (Hirst 2003). These properties makes the smooth muscle cells potent players in immune regulation and highly relevant to study in terms of inflammatory pulmonary diseases. Further information on the role of smooth muscle cells in asthma is found under 1.5 Inflammatory pulmonary diseases/Asthma.

FIBROBLASTS

Fibroblasts are present in the matrix of connective tissue and are key cells in the process of wound healing in response to injury. The fibroblasts migrate into wounded areas where they proliferate and produce extracellular matrix (ECM) components, including collagens, proteoglycans and fibronectin. Acute and chronic inflammatory reactions can induce tissue damage and consequently a healing process. The healing process can be well balanced or can result in an exaggerated response, resulting in scarring and fibrosis. As such, fibrosis may occur in response to chronic airway inflammation, and is suggested to be the result of an exaggerated fibroblast proliferation and matrix deposition. In the present thesis, fibroblasts were studies because of their potential to produce neurotrophins.
EPITHELIAL CELLS

The epithelium lines the bronchial tree and act as a first line of defence against airway infections by building a physical barrier between the environment and the underlying structures of the lung. The structure of the epithelium changes along the respiratory tract, but from the nasal cavity to the bronchioles, the epithelial cells are pseudo-stratified ciliated cells containing many mucus-secreting (goblet) cells. The sticky mucus function to trap inhaled particles and the mucus is continuously transported by the cilia towards the pharynx, thus protecting the airways from potential harmful substances. The number of goblet cells and the amount of mucus secretion is increased in both asthma and COPD.

NERVES

The lungs are innervated by the autonomic nervous system, which helps to regulate airway function in many ways. Parasympathetic nerves are the dominant pathway in the airways and regulate smooth muscle tone, where activation of the pathway leads to increased bronchial tone and bronchoconstriction (van der Velden and Hulsmann 1999). The major neurotransmitter is acetylcholine which signals through muscarinic receptors on target cells, such as the smooth muscle (Barnes 1990). Release of acetylcholine from parasympathetic nerves also stimulates mucus secretion and vasodilation in the airways. The parasympathetic pathway can be activated by the central nervous system (CNS) or by sensory neurons present within the airway wall that triggers a local reflex in the tissue. Importantly, asthmatics are suggested to have an increased cholinergic bronchoconstriction, through stimulation of sensory receptors by inflammatory mediators like histamine and prostaglandins (Barnes 1992). The sensory neurons belong to the nonadrenergic noncholinergic (NANC) nervous system and release neuropeptides, including the tachykinins (TK) substance P and neurokinin A. The TK receptors are widely distributed throughout the airways, but the main target cells are suggested to be the smooth muscle cells, blood vessels and immune cells (van der Velden and Hulsmann 1999).

The airways are thought to be sparsely innervated by the sympathetic nervous system. The sympathetic pathway signals through the neurotransmitter noradrenalin via β-adrenergic receptors, which are widely distributed in the airways, predominantly on smooth muscle cells (van der Velden and Hulsmann 1999). Release of adrenalin from the adrenal glands, reach the
airways through the systemic circulation, and causes smooth muscle relaxation via the presence of β2-adrenergic receptors on these cells (Barnes 1992). Importantly, β2-agonists have very good effect in the treatment of asthma.

**NEURO-IMMUNE CROSS-TALK**

A reciprocal communication between the nervous- and immune system has been suggested to contribute to enhanced inflammation in the airways, such as in asthma and COPD (Barnes 2001). This neurogenic inflammation is mediated by neuropeptide release by sensory nerves but also by inflammatory cells. Thus, inflammatory cytokines may stimulate tachykinin (TK) release by immune cells, which in turn stimulate further vascular leakage, mucus secretion and bronchoconstriction. TK receptors are also present on mast cells and eosinophils and substance P has been shown to degranulate mast cells and to function as a chemoattractant for eosinophils (Barnes 2001). The neurotrophins are also considered to be mediators that can function as communication molecules between the nervous- and the immune system. Read more under 1.6 Neurotrophins.

**1.4 THE IMMUNE SYSTEM**

The immune system consists of an orchestra of specialized cells and molecules, that each plays important roles in the protection against the very diverse population of pathogens that we are exposed to. Since the airways are under constant exposure to inhaled pathogens, the immune system plays an important role here. Asthma, sarcoidosis and COPD are all airway diseases characterized by a chronic inflammation that may lead to adverse effects, such as tissue remodeling. To understand the different inflammatory processes in these diseases, I will here briefly introduce the major cells and components of the immune system, focusing on those that are discussed further in the present thesis with regard to inflammatory pulmonary diseases.

**MACROPHAGES**

The macrophages, together with dendritic cells, are so-called antigen-presenting cells (APCs) which main function is to capture pathogens that invade the body by passing through the epithelium of the respiratory tract, gastrointestinal tract or the skin. When APCs have digested a pathogen, or part of a pathogen (antigen), they travel to the nearest lymph node where they present these antigens to the lymphocytes and thereby trigger these cells to become activated. Macrophages present within the airways are called alveolar macrophages and are the main APC in the lung. The alveolar macrophages secrete soluble proteins called cytokines, like interleukin (IL)-1 and tumor necrosis factor (TNF). These pro-inflammatory cytokines attracts other cells of the immune system to the site of infection, thus playing an important role in the acute phase of an inflammatory response. Except from functioning as APCs, the macrophages also has the capacity to kill the engulfed pathogen through the release of intracellular enzymes and reactive oxygen species (ROS), a process called phagocytosis (Parham 2009).
LYMPHOCYTES

The lymphocytes are the white blood cells that build up the adaptive immune system and have the unique capacity to recognize distinct antigens and are capable of preserving “memory” of prior infections. The B lymphocytes are capable of producing antibodies which recognize antigens and initiate cell activation. Activated B lymphocytes are called plasma cells, and they produce and secrete large amounts of antibodies which circulate in the blood where they can bind to, and target, pathogens for destruction. The T lymphocytes are activated by the recognition of antigens that are presented to them in form of peptides, bound to molecules (MHC) on the surface of APCs. Activation of CD8+ T cells, so called cytotoxic T cells, leads to direct killing of the infected cell by the T cell. Activation of CD4+ T cells, so called helper T cells, leads to cytokine production, which in turn helps to activate other cells of the immune system, like the B cells and macrophages. There are two subtypes of helper T cells, the Th1 and the Th2 cells, and they differ in the repertoire of cytokine production. IL-12 production by dendritic cells promotes differentiation of Th1 cells, and Th1 cells produce interferon (IFN)-γ which is an important cytokine for the activation of macrophages. In contrast, when IL-12 is absent, Th2 cell differentiation is promoted. Th2 cells produce the cytokines IL-4, IL-5 and IL-13 which are important in activating mast cells, eosinophils and B lymphocytes. A disturbed balance between Th1 and Th2 responses is thought to play an important role in several immune diseases, where autoimmune diseases are dominated by Th1 cells and allergic diseases are dominated by Th2 cells.

GRANULOCYTES

The granulocytes are a group of cells with prominent cytoplasmic granules, which contain toxic mediators used to kill microorganisms. The most abundant white blood cell is the neutrophil which is specialized in phagocytosis. The neutrophils are rapidly recruited to sites of infection and are important for the fast clearance of invading pathogens. The second most common granulocyte is the eosinophil, which is important for the protection against helminth worms and other parasites, but is also involved in the allergic inflammation. The substances released by activated eosinophils are highly toxic and may cause tissue damage. The third and most rare granulocyte is the basophil, which makes up less than 1% of the white blood cells. Basophils have similar functions as eosinophils, but also have the unique capacity to secrete the cytokines IL-4 and IL-13 early in an immune response, which initiates a polarization of T-cells towards a Th2 response (Parham 2009).

MAST CELLS

Mast cells do not circulate in the blood, but reside within the mucosal and epithelial tissues lining the body surfaces, where they serve as a first line of defence. Importantly, mast cell activation has long been associated with asthma (Holgate 2008). The mast cell cytoplasm is filled with granules containing several inflammatory mediators like histamine, enzymes and cytokines. Mast cells are easily triggered, and the classical activation signal for mast cells is cross-binding of IgE molecules on their surface (Abbas and Lichtman 2004). Upon activation, mast cells degranulate and the contents are released extracellularly where they provide inflammatory signals to the milieu, but also cause tissue damage. Mast cell mediators like
Histamine, prostaglandins and cytokines are responsible for many of the reactions in the allergic response. Read more under 1.5 Inflammatory pulmonary diseases/Asthma.

1.5 INFLAMMATORY PULMONARY DISEASES

The present thesis includes studies with regards to three inflammatory pulmonary diseases; asthma, sarcoidosis and chronic obstructive pulmonary disease (COPD). These lung diseases have common features, i.e. they are all characterized by inflammation of the airways and lung parenchyma, leading to structural changes/remodeling of tissues. However, as described below, the cause of the diseases are different and the type and characteristics of the immune response and the remodeling of the lungs are specific for each disease.

ASTHMA

Epidemiology & definition

Asthma is one of the most common chronic diseases in the world and affects people of all ages. It is estimated that around 300 million people are affected worldwide, and it is becoming more common in both children and adults. In Sweden, it is estimated that the prevalence of asthma is 6.5 % in the general population, and over 10% of Swedish children have asthma symptoms (www.ginasthma.com). Both genetic and environmental factors influence the risk of developing asthma. One of the most debated and interesting hypothesis on why the prevalence of asthma is increasing in the developed countries, is the so called “hygiene hypothesis”, which suggests that exposure to infections early in life, influence the child’s immune system and protects against the development of allergy and asthma. This would explain why, for example, young children with older siblings and those who attend day care are at higher risk of infections, but seem to be more protected against developing allergic diseases (Ball et al. 2000; Illi et al. 2001).

Asthma is defined as “a chronic inflammatory airway disorder in which many cells and cellular elements play a role. The chronic inflammation is associated with airway hyperresponsiveness that leads to recurrent episodes of wheezing, breathlessness, chest tightness, and coughing, particularly at night and in the early morning. These episodes are usually associated with widespread, but variable, airway obstruction within the lung that is often reversible either spontaneously or with treatment” (Global Initiative for asthma (GINA) guidelines). The main physiological feature of asthma is a reversible airway obstruction with expiratory airflow limitation. The airway obstruction is caused by smooth muscle contraction, airway inflammation, mucus secretion and oedema, and is associated with persistent airway structural changes, so called airway remodelling.

Airway inflammation in asthma

The inflammation can be either allergic or non-allergic. Allergic (atopic) individuals react to certain allergens, like pollens, house dust mite, furry animals etc., by eliciting a specific IgE-mediated immune response against the allergen/allergens to which you have been sensitized. Non-allergic asthma is a non-IgE mediated inflammation and may be triggered by exposure to irritants such as cigarette smoke, air pollutant or infections.
Many different inflammatory cells have been shown to be involved in asthma and the inflammation in asthma can be sub-divided into acute inflammatory episodes, corresponding to asthma attacks or exacerbations, and a chronic persistent inflammation, causing long-term consequences on lung function.

In the acute phase of the inflammatory response, inhalation of allergens or other irritants cause mast cell activation and degranulation. Blockage of mast cell degranulation attenuates the acute bronchoconstriction in asthma, showing the importance of these cells in the acute inflammatory response (Brightling et al. 2003). Granulation of mast cells occurs either through cross-linking of IgE on the cell surface by the inhaled allergen, or by indirect stimuli, such as cold air or exercise. Upon degranulation, mast cells release a number of mediators, such as histamine and prostaglandins, which directly cause bronchoconstriction, vasodilation and increased vascular permeability, thus causing the main symptoms of the acute phase. Increased numbers of mast cells are found in the lungs of asthmatic patients (Wardlaw et al. 1988) and they have specifically been localised within the smooth muscle bundles and have been associated with airway hyperresponsiveness in asthma (Brightling et al. 2002).

Th2 lymphocytes are also increased in asthmatic airways together with the Th2-associated cytokines IL-4, IL-5 and IL-13, and are important regulators of the immune response in asthma, inducing IgE production by B-cells and recruiting secondary effector cells into the airways. IL-5 is considered to be a crucial chemokine for eosinophil recruitment to the airways. Eosinophil infiltration to the airways is a characteristic feature of the late phase in the allergic inflammation and they are thought to be involved in the persistence of a chronic inflammation. They release toxic proteins, such as major basic protein (MBP) and eosinophil cationic protein (ECP), which have the capacity to damage the lung tissue. Thus, eosinophils are proposed to be involved in airway tissue remodeling in asthma. A schematic illustration of the asthmatic airway versus the healthy airway is shown in Figure 3.

![Figure 3. Schematic illustration of the asthmatic airway.](image)

**Airway remodeling in asthma**

The structural changes of the airway wall in asthma are characterized by epithelial damage, basement membrane thickening, increased number of blood vessels (angiogenesis),
subepithelial fibrosis, increased deposition of extracellular matrix proteins and an increased thickness of the smooth muscle layer (see Figure 3). The increased smooth muscle mass in asthma is suggested to be caused by hyperplasia (increase in number of cells) and hypertrophy (increase in cell size) (Ebina et al. 1993) but also smooth muscle cell migration in response to inflammatory mediators (Black et al. 2001). The changes in extracellular matrix deposition in asthma, with increased amounts of collagens and fibronectin (Roberts 1995) is in part explained by an imbalanced ratio in the expression of tissue degrading enzymes, matrix metalloproteinases (MMPs), and their inhibitors, tissue inhibitors of metalloproteinases (TIMPs) (Mautino et al. 1999a). Airway remodeling is believed to play a major role in particular severe asthma, where airway obstruction and bronchial hyperresponsiveness is preserved despite full asthma treatment (Pascual and Peters 2005).

Airway hyperresponsiveness

Airway hyperresponsiveness (AHR) is one of the central features of asthma and can be defined as an excessive response of the airways to a variety of stimuli, resulting in periodic bronchoconstriction (Berend et al. 2008). Direct stimuli, such as histamine or metacholine, act directly on smooth muscle receptors, whereas indirect stimuli, such as cold air, exercise, or cigarette smoke, mediate their effects via intermediate mechanisms, such as stimulation of inflammatory cells, like the mast cell, or stimulation of neural pathways within the airways. The mechanisms of AHR asthma are still incompletely understood, but several mechanisms have been suggested, which highlights the probability of multiple contributing pathways (Berend et al. 2008). Suggested mechanisms for AHR include an abnormal response of the smooth muscle, an overall thickening of the airway wall which contributes to airway stiffness (McParland et al. 2003), increased numbers of mast cells within the smooth muscle layer that triggers smooth muscle contraction through histamine release (Brightling et al. 2002). In addition, inflammatory mediators, like prostaglandins and neurotrophins, have been shown to increase the excitability of afferent sensory nerves within the airways (Ho et al. 2000; Undem and Nassenstein 2009).

Treatment

The treatment of asthma is dependent on the severity of the disease and ranges from short- and long-acting β2-agonists to high doses of inhaled glucocorticosteroids. β2-agonists are bronchodilators and acts directly on the smooth muscle. Glucocorticosteroids have anti-inflammatory effects and are effective in reducing asthma symptoms, improving lung function, decreasing airway hyperresponsiveness and reducing asthma exacerbations. Other treatment strategies for asthma are anti-leukotrienes and anti-IgE treatment (www.ginasthma.com). Despite the broad repertoire of treatment options, asthma symptoms persist in severe asthma patients.

SARCOIDOSIS

Clinical features & epidemiology

Sarcoidosis is a systemic granulomatous inflammatory disease with an unknown aetiology. The disease primarily affects the lungs but almost any organ in the body may be involved such as the eyes, skin, heart and the nervous system. In pulmonary sarcoidosis lymphocytes,
Introduction

particularly CD4+ Th1 lymphocytes, infiltrate into the airways and an increased ratio of CD4+/CD8+ lymphocytes is commonly found in bronchoalveolar lavage fluid (Grunewald and Eklund 2007a). The affected organ/organs in sarcoidosis are manifested with noncaseating epitheloid cell granulomas. The prevalence of sarcoidosis is fairly low and varies among racial groups, with highest prevalence rates found in Scandinavians (0.05%) and Afro-Americans (Milman and Selroos 1990; Statement 1999). In Sweden, approximately 2000 persons are diagnosed with sarcoidosis every year. It is most common in young and middle-aged persons and affects both sexes. In Scandinavia, there is a second peak of incidence in women over 50 years of age (Milman and Selroos 1990). In the Scandinavian population, about 1/3 of all sarcoidosis patients have an acute disease onset with fever, bilateral hilar lymphadenopathy (BHL), erythema nodosum (EN) and/or ankle arthritis (Grunewald and Eklund 2007b). This form of the disease is known as Löfgren’s syndrome.

Diagnosis

The diagnosis of sarcoidosis is based on a clinical picture compatible with sarcoidosis together with radiologic and histological findings. Histological finding of non-caseating epithelioid cell granulomas supports the diagnosis, and exclusion of other diseases capable of producing similar histologic or clinical picture is important (Statement 1999). Bronchoalveolar lavage (BAL) and studies on the lymphocyte subpopulations are helpful. Findings of lymphocytosis and a CD4/CD8 ratio over 3.5 strongly supports the diagnosis of sarcoidosis. Pulmonary involvement, including enlarged lymph nodes, parenchymal infiltrates and fibrosis are visualized on chest radiographs and the disease has five radiologic stages (see Table 1).

<table>
<thead>
<tr>
<th>Stage</th>
<th>Finding</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>Normal chest radiograph</td>
</tr>
<tr>
<td>I</td>
<td>Bilateral hilar lymphadenopathy (BHL)</td>
</tr>
<tr>
<td>II</td>
<td>BHL plus pulmonary infiltrations</td>
</tr>
<tr>
<td>III</td>
<td>Pulmonary infiltrations (without BHL)</td>
</tr>
<tr>
<td>IV</td>
<td>Pulmonary fibrosis</td>
</tr>
</tbody>
</table>

Aetiology and genetics

The cause of sarcoidosis remains unknown. However, there is evidence suggesting that sarcoidosis results from exposure of genetically susceptible individuals to specific environmental agents. Several infectious organisms have been implicated to cause sarcoidosis, such as Mycobacterium tuberculosis or other mycobacteria, Propionibacterium acnes, and viruses. Also, other organic and inorganic agents have been suggested to cause sarcoidosis, such as pine tree pollen and aluminium (Statement 1999). Finally, sarcoidosis has been suggested to have an autoimmune component, driven by an immune response against self-antigens (Wahlstrom et al. 2009). Differences in incidence between racial groups (Rybczki et al. 1997) and familial clustering of cases (Brennan et al. 1984) suggest a genetic predisposition in the development of sarcoidosis. Gene variants seem to be important not only
for the risk of developing sarcoidosis, but also in determining the pattern of disease in terms of severity and prognosis. Indeed, in the Scandinavian population, there is a strong correlation between the human leukocyte antigen (HLA) allele HLA-DRB1*03 and good prognosis, and between HLA-DRB1*15 and a chronic form of the disease (Berlin et al. 1997).

The granuloma formation

Granuloma formation is the body’s defence reaction to protect against infectious agents or other particles that cannot be completely eliminated. The characteristic non-caseating granulomas found in sarcoidosis are suggested to be initiated by an immunological reaction against the still unknown antigen/antigens, where CD4+ lymphocytes and macrophages are activated and recruited to the site of exposure, most commonly the lungs (Agostini et al. 1997). In the initiation of the granuloma formation, CD4+ lymphocytes are considered to be crucial in attracting histiocytes and facilitate the transformation to epithelioid cells through the secretion of the cytokines IFN-γ and TNF (Gudmundsson and Hunninghake 1997; Morris et al. 2003). The granuloma is built up by an accumulation of epithelioid cells and multinucleated giant cells, either “naked” or surrounded by T-lymphocytes (see Figure 4). The epithelioid cells are cytoplasmic-rich highly differentiated macrophages that have changed their phenotype from being phagocytic to having more secretory functions. The multinucleated giant cells are the results of fusion of epithelioid cells and are characterized by the arrangement of several nuclei in a horseshoe-shaped pattern. The granuloma may resolve spontaneously or with treatment, or develops into fibrosis, which is an irreversible tissue scaring. Factors that influence the development of fibrosis are not well understood.

Figure 4. Possible mechanism for granuloma formation in sarcoidosis. (Adopted from Grunewald 2006 with minor modification.)

Treatment

Many patients with sarcoidosis recover spontaneously without the need of any treatment. This is particularly seen in patients with Löfgren’s syndrome. A chronic disease course is
more common in Non-Löfgren patients, in Afro-Americans, and in sarcoidosis with extra-
pulmonary involvement. Treatment is initiated in patients with severe pulmonary dysfunction
or if there is an involvement of the eye, heart or central nervous system. The first line of
treatment is oral corticosteroids, which sometimes is combined with other
immunosuppressants, such as methotrexate (Newman et al. 1997).

CHRONIC OBSTRUCTIVE PULMONARY DISEASE

Epidemiology & risk factors

Chronic obstructive pulmonary disease (COPD) is a disease characterized by chronic
inflammation of the airways, small airway fibrosis and destruction of the alveoli
(emphysema), leading to a progressive loss of lung function. Tobacco smoking is the main
risk factor for developing COPD, making this a preventable disease. Still, COPD is a major
public health problem as it affects 5-15 % of all adults in industrialized countries and it is
estimated to become the fifth most frequent burden of disease worldwide (Lopez et al. 2006;
Mannino and Buist 2007). Estimations suggest that between 15%, but perhaps as many as
50%, of smokers develop COPD (Lundback et al. 2003). This shows that also other factors
are involved in the development of the disease, including a genetic predisposition. Also, other
environmental exposures are known risk factors for COPD, such as occupational dust and
indoor- and outdoor air pollutants. Notably, the prevalence of COPD increases with age,
making the burden of COPD increase as the world’s population ages (Mannino and Buist
2007).

Definition & clinical features

COPD is characterized by airflow limitation that is not fully reversible. The airflow
limitation is usually progressive and associated with an abnormal inflammatory response of
the lung to noxious particles or gases (Rabe et al. 2007). COPD is defined by the Global
Initiative for Chronic Obstructive Lung Disease (GOLD) as a “preventable and treatable
disease with some significant extrapulmonary effects that may contribute to the severity in
individual patients”. The primary symptoms of COPD are dyspnea (breathlessness), chronic
cough and sputum production, and the diagnosis is confirmed by lung function measurements,
i.e. spirometry, confirming an irreversible airflow limitation. Accordingly, a diagnosis of
COPD is established when spirometry measurements show a (post bronchodilator) forced
expiratory volume in the first sec (FEV$_1$)/forced vital capacity (FVC) below 0.70 (Rabe et al.
2007). With more advanced stage of disease, patients suffer from greater breathlessness,
reduced exercise capacity, fatigue and repeated exacerbations of the disease that have a great
impact on the patients’ quality of life. The severity of the disease is classified by spirometry
and includes four stages: stage I, mild; stage II, moderate; stage III, severe; stage IV, very
severe, (see Table 2) (Rabe et al. 2007).

Airway inflammation in COPD

Lung inflammation is present in everyone with a cigarette smoking habit (Hunninghake
and Crystal 1983; Thompson et al. 1989), but why only a fraction of these individuals
develops COPD with a decline in lung function, is still unknown. However, it is believed that the inflammatory response is exaggerated in susceptible individuals who develop the disease.

Table 2. Spirometric classification of COPD severity.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Description</th>
<th>FEV₁/FVC</th>
<th>FEV₁ (% predicted)</th>
</tr>
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<tbody>
<tr>
<td>I, mild</td>
<td>FEV₁/FVC &lt; 0.70</td>
<td>FEV₁ ≥ 80% predicted</td>
<td></td>
</tr>
<tr>
<td>II, moderate</td>
<td>FEV₁/FVC &lt; 0.70</td>
<td>50% ≤ FEV₁ &lt; 80% predicted</td>
<td></td>
</tr>
<tr>
<td>III, severe</td>
<td>FEV₁/FVC &lt; 0.70</td>
<td>30% ≤ FEV₁ &lt; 50% predicted</td>
<td></td>
</tr>
<tr>
<td>IV, very severe</td>
<td>FEV₁/FVC &lt; 0.70</td>
<td>FEV₁ &lt; 30% predicted</td>
<td></td>
</tr>
</tbody>
</table>

The airway inflammation is characterized by an accumulation of neutrophils, macrophages and T-lymphocytes, and the degree of inflammation increases with the severity of the disease (Hogg et al. 2004). Increased numbers of neutrophils are found in sputum and BAL in COPD, especially during exacerbations, and are associated with greater airflow obstruction (O'Donnell et al. 2004). Neutrophils are able to release oxygen radicals, elastase and cytokines, and are thought to be central players in the inflammation and induction of emphysema in COPD. Macrophages are also recruited to the lungs in COPD and are potentially very important effector cells through their release of reactive oxygen species (ROS), cytokines (TNF and IL-1β) and chemokines. Alveolar macrophages from COPD patients have been shown to release increased amounts of MMP -1 and -9 (Finlay et al. 1997), which are important enzymes involved in tissue destruction. CD8⁺ T-cells are found elevated throughout the airways and are considered as important drivers of the inflammation in COPD and are associated with a decline in lung function (O'Shaughnessy et al. 1997). The CD8⁺ T-cells are cytotoxic and are thought to contribute to emphysema development through their release of lytic substances like perforin and granzyme B. A schematic illustration of the airway in COPD is shown in Figure 5. Importantly, there is evidence of a systemic inflammation in COPD which is thought to be linked to the association of COPD with extrapulmonary effects such as weight loss, muscle weakness, cardiovascular disease and osteoporosis (Agustí 2005). Thus, elevated serum levels of IL-6 and CRP are found in severe COPD (Broekhuizen et al. 2006).

Airway remodeling in COPD

Repeated exposure to cigarette smoke, pollutants and/or infections and a persistent inflammation causes injury to the lung tissue. The structures that are mostly affected in COPD are the lung parenchyma and small airways, in contrast to asthma where the larger, more proximal airways are most affected. The airways in COPD are characterized by squamous and goblet cell metaplasia, submucosal gland hypertrophy and subepithelial fibrosis (Jeffery 2004) (see Figure 5). In contrast to asthma, increased smooth muscle mass is not found in the larger Airways, but is found in the smaller airways in COPD, however less prominent than in asthma (Bosken et al. 1990; Saetta et al. 1998). The extensive destruction of the alveolar wall (emphysema), leading to enlargement of alveolar spaces, is a specific feature of COPD, and is
an important component that contributes to the dynamic collapse of the airways. Proposed mechanisms of emphysema include protease-antiprotease imbalance resulting in destruction of lung elastin, apoptosis of alveolar cells and inhibition of alveolar repair (Chung and Adcock 2008).

![Figure 5. Schematic illustration of the airway in COPD.](image)

**Introduction**

**Treatment**

Pharmacological treatment of COPD aims to prevent and control symptoms and to reduce the frequency and severity of exacerbations. Bronchodilators, including β2-agonists and anticholinergics, are central medications in the management of COPD. Treatment with inhaled glucocorticosteroids in combination with long-acting β2 agonists is used for patients in more advanced stages of disease (stage III-IV), but the inflammation in COPD generally responds poorly to glucocorticoids as compared to asthma (Barnes 2006).

**1.6 THE NEUROTROPHINS**

The aim of the present thesis was to study the role of neurotrophins in inflammatory pulmonary diseases. Therefore, the neurotrophins are presented below and knowledge on their functions span from the nervous system to the immune system.

**DISCOVERY**

Nerve growth factor (NGF) was the first member of the family of neurotrophins to be discovered and characterized. Today the human neurotrophins consist of the structurally and functionally related polypeptides NGF, brain-derived neurotrophic factor (BDNF), neurotrophin (NT)-3 and NT-4/5. NGF was discovered in the early 1950’s by the Italian developmental biologist Rita Levi-Montalcini. She found that a mouse sarcoma tumor secreted a soluble factor that induced outgrowth of sympathetic nerve fibers when implanted close to the spinal cord of chicken embryos (Levi-Montalcini 1952). Shortly thereafter, in collaboration with the biochemist Stanley Cohen, the nerve growth–promoting activity was isolated to a protein molecule with a molecular weight of approximately 20 kDa (Cohen and Levi-Montalcini 1956). Interestingly, even richer sources of NGF were thereafter found in snake venom and in mouse salivary glands. NGF was initially characterized for its essential role in the development of the sympathetic nervous system, as injections of NGF anti-serum...
in neonatal rodents, resulted in the near total disappearance of sympathetic ganglia (Levi-Montalcini 1964). In 1986, Rita Levi-Montalcini and Stanley Cohen were awarded the Nobel Prize in Medicine for their discoveries of the growth factors NGF and epidermal growth factor (EGF) respectively. NGF and EGF were the first of many growth-regulating signal substances to be discovered and characterized.

THE NEUROTROPHIN RECEPTORS

The neurotrophins NGF, BDNF, NT-3 and -4/5 are homodimers and highly homologous in protein sequences. They mediate their effects by binding to two classes of receptors; the high-affinity receptors (receptor tyrosine kinases, Trks) and the low-affinity receptor p75 neurotrophin receptor (p75NTR). The p75NTR belong to the TNF/Fas/CD40 superfamily of death receptors and binds all neurotrophins with equal affinity. The Trk family of receptors consists of TrkA, TrkB and TrkC, each with ligand specificity for the different neurotrophins (see Figure 6). Hence, TrkA is the preferred receptor for NGF, TrkB for BDNF and NT-4/5, and TrkC for NT-3. However, some cross-talk exists, especially for NT-3, which is also described to be able to signal through TrkA and TrkB (Ryden and Ibanez 1996). Simplified, binding and activation of Trk receptors mediate survival-promoting effects, while activation of the p75NTR receptor triggers apoptosis. However, there is also an interaction between the Trk and p75NTR receptor. Thus, p75NTR can also induce survival and differentiation by forming a heteromeric co-receptor complex with the Trk receptors, which increases the affinity of TrkA for NGF (Hempstead et al. 1991).

INTRACELLULAR SIGNALING PATHWAYS

To give a bit more detail on the intracellular signaling pathways induced by the Trk’s and the p75NTR receptor, and to highlight the complexity of neurotrophin signaling, I will here give an overview of the intracellular pathways induced by the TrkA and p75NTR receptor, respectively.

TrkA signaling

Activation of the TrkA receptor by NGF leads to receptor dimerisation and induction of its kinase activity, inducing receptor autophosphorylation. In turn, it activates three main signalling pathways: 1) activation of PLCγ and PKC or 2) activation of the small G protein Ras, leads to activation of the MAPK/ERK1/2 cascade, 3) activation of the phosphoinositol signalling pathway through PI3K that induce activation of Akt and a MAPK-independent pathway or alternatively to MAPK activation via the small G protein Rac. These pathways lead to activation of transcription factors involved in cell proliferation and survival (Chao 2003).

p75NTR signaling

The p75NTR receptor is characterized by an intracellular death domain that activates the apoptotic pathways. This involves activation of the MAPK JNK, which in turn phosphorylates pro-apoptotic proteins, such as Bad, p53 and Bax. This induces cytochrome c release from mitochondria and caspase activation (Casaccia-Bonnefil et al. 1996). However, binding of
NGF to p75NTR can also induce cell survival by activation of the transcription factor NFκB, which leads to transcription of anti-apoptotic proteins (Gentry et al. 2000). In this way, NGF is suggested to be able to counterbalance the pro-apoptotic signal. Additionally, the p75NTR receptor has been shown to bind pro-neurotrophins with higher affinity than it does mature neurotrophins (Lee et al. 2001). By this means, the biological activity of neurotrophins is regulated by proteolysis, where proneurotrophins preferentially induce apoptosis by activation of p75NTR, while mature neurotrophins preferentially induce cell survival by activation of Trk’s.

![Diagram of Neurotrophin ligands and receptors]

**FUNCTIONS OF THE NEUROTROPHINS –FROM NERVES TO IMMUNE CELLS**

*Importance for neuronal development and adult brain function*

Neurotrophins play a fundamental role for the development of peripheral sympathetic sensory neurons, through their regulation of neuronal cell survival and differentiation (Levi-Montalcini et al. 1995). Thus, recombinant mice lacking either of the neurotrophins show severe losses of sympathetic and sensory neurons, and die within a couple of days after birth (Crowley et al. 1994; Farinas et al. 1994; Jones et al. 1994). In contrast to mice that completely lack neurotrophins, mice expressing reduced levels of neurotrophins survive to adulthood, and show abnormalities in adult brain function and behaviour. For example, lowering the levels of NGF (single allele disruption) leads to deficits in memory and learning (Chen et al. 1997), while BDNF deficiency leads to aggressiveness, hyperactivity and eating disorders as well as defects in memory acquisition (Korte et al. 1995; Lyons et al. 1999).
Mediators of pain

Neurotrophins play an important role as mediators of peripheral pain, in particular in response to inflammation (Pezet and McMahon 2006). Subcutaneous administration of NGF induces hyperalgesia (increased sensitivity to pain) as shown both in rodents and humans (Lewin et al. 1993; Dyck et al. 1997), and NGF is upregulated in many chronically painful conditions, particularly in inflamed tissues. Proposed mechanisms for NGF-induced pain include regulation of gene expression in sensory neurons, such as induction of the neuropeptides substance P and calcitonin gene-related peptide (CGRP), as well as enhancement of the responsiveness of the pain receptor vanilloid receptor 1 (TRPV1). Also, the mast cell has been proposed to play a role, as depletion of mast cells have been shown to reduce NGF-induced hyperalgesia in animal models (Lewin et al. 1994).

Neurotrophins in allergic inflammation

The first report on a non-neuronal cell type found to be targeted by NGF came in 1977, a cell type belonging to the immune system; the mast cell (Aloe and Levi-Montalcini 1977). This finding opened up for the hypothesis that NGF display far broader spectrum of biological activities than just acting within the nervous system. The research on neurotrophins within the immune system has expanded vigorously since then and it is now well accepted that NGF and the other neurotrophins not only act on many different immune cells, but are also produced by these cells. In support of a role of neurotrophins in the immune system, enhanced neurotrophin levels have been observed in diseases characterized by chronic or acute inflammation. Thus, enhanced levels of NGF have been found during viral infection with respiratory syncytial virus (RSV) (Hu et al. 2002). Elevated levels of NGF have also been described in the synovial fluid of patients with rheumatoid arthritis (RA) (Aloe et al. 1992) and in cerebrospinal fluid of patients with multiple sclerosis (MS) (Laudiero et al. 1992).

Given that NGF could target mast cells and that these cells are central players in allergic inflammation and asthma, it was speculated that neurotrophins might be involved in these diseases. The first report connecting neurotrophins to allergic inflammation showed increased circulating levels of NGF in patients with allergic diseases, including asthma, rhinocconjunctivities and urticaria (Bonini et al. 1996). Shortly thereafter, it became evident that not only NGF, but also BDNF, is enhanced in blood of asthmatics (Noga et al. 2001; Lommatzsch et al. 2005), and importantly, NGF was also shown to be elevated locally in the airways of patients with asthma (Olgart Hoglund et al. 2002). Furthermore, studies showed that neurotrophin levels are further enhanced in the airways of asthmatics after allergen exposure (Virchow et al. 1998; Kassel et al. 2001). Taken together, these studies promoted the neurotrophins as being possible mediators in airway inflammation, and especially in asthma.

Sources of neurotrophins in the airways

Immunohistochemical stainings of lung tissue biopsies from asthmatic subjects revealed that sources of NGF within the airways included both structural cells and infiltrating immune cells (see Figure 7) (Olgart Hoglund et al. 2002). Thus, the bronchial epithelium, smooth muscle layer as well as fibroblasts stained for NGF in both healthy and asthmatic airways and an increased number of NGF positive immune cells were identified within the airways of
asthmatics. These findings suggested that infiltrating immune cells contribute to the enhanced levels of NGF detected in the airways of asthmatic (Olgart Hoglund et al. 2002).

In vitro NGF production has been demonstrated in B-lymphocytes (Santambrogio et al. 1994; Torcia et al. 1996) and activated T-lymphocytes of the Th2 type (Ehrhard et al. 1993; Lambiase et al. 1997). In addition, eosinophils, mast cells and monocytes/macrophages are reported to express and release neurotrophins, especially when activated (Tam et al. 1997; Caroleo et al. 2001; Kobayashi et al. 2002).

Neurotrophin production by human airway structural cells has been confirmed in several in vitro studies. As such, airway epithelial cells (Fox et al. 2001; Pons et al. 2001), smooth muscle cells (Freund et al. 2002; Kemi et al. 2006) and fibroblasts (Olgart and Frossard 2001) are all reported to produce and secrete neurotrophins, and importantly, the production is demonstrated to be upregulated by inflammatory stimuli, such as IL-1β and TNF. This indicates that structural cells are also able to contribute to enhanced neurotrophin levels in the airways in response to inflammation.

![Diagram of cellular sources and targets of neurotrophins](image)

**Figure 7. Cellular sources of, and targets for, the neurotrophins (NT’s).**

**Targets for neurotrophins in the airways**

Neurotrophins are believed to act in an autocrine and/or paracrine fashion within the airways, as many of the immune- and structural cells not only produce neurotrophins but also express neurotrophin receptors (see Figure 7). Human studies have demonstrated that allergic patients challenged with allergen, show an increased influx of mast cells to the airways which is accompanied by increased NGF levels, indicating a close relationship between mast cells and NGF (Kassel et al. 2001). Indeed, human mast cells express TrkA (Tam et al. 1997), and NGF has been shown to inhibit mast cell apoptosis and induce degranulation (Mazurek et al. 1986; Kanbe et al. 2000). Mice over-expressing NGF in the lungs show a higher influx of eosinophils, lymphocytes and neutrophils into the airways after allergen challenge (Path et al. 2002; Quarcoo et al. 2004). Also, the number of eosinophils recruited to the airways are reduced in anti-NGF treated mice (Path et al. 2002). In vitro studies have shown that NGF may stimulate the proliferation of T- and B-lymphocytes (Thorpe and Perez-Polo 1987; Otten
et al. 1989), as well as antibody production and survival of B-lymphocytes (Otten et al. 1989; Torcia et al. 1996). NGF, BDNF and NT-3 have also been shown to promote eosinophil survival and recruitment into the airways (Hamada et al. 1996; Nassenstein et al. 2003). Studies on effects of neurotrophins on airway structural cells are scarce, but lung fibroblasts and smooth muscle cells have been shown to express the neurotrophin receptor TrkA and respond to stimulation with NGF (Micera et al. 2001; Freund-Michel et al. 2006).

Functional role of neurotrophins in asthma -what the animal models are teaching us

There is supporting evidence that neurotrophins play a role in the inflammatory response and are involved in airway hyperresponsiveness in asthma (Nassenstein et al. 2006b) and most studies on functional roles of neurotrophins, rely on animal models of allergic asthma.

In a mouse model of asthma, addition of exogenous NGF increased the levels of the Th2 cytokines IL-4 and IL-5, whereas treatment with anti-NGF reduced IL-4 and prevented development of airway hyperresponsiveness (AHR) (Braun et al. 1998). In a similar mouse model of asthma, pretreatment with blocking anti-NGF antibodies suppressed the airway inflammation, and transgenic mice over-expressing NGF in the lung showed enhanced airway inflammation after allergen challenge as compared to wild-type mice (Path et al. 2002). Transgenic mice over-expressing NGF in the lung have also been shown to develop sensory hyperinnervation of the airways and an increased responsiveness to capsaicin as compared to normal mice (Hoyle et al. 1998). In addition, pre-treatment with NGF or BDNF has been shown to induce AHR in the guinea pig (de Vries et al. 1999; Friberg et al. 2001; Bennedich Kahn et al. 2008a; Bennedich Kahn et al. 2008b). These effects are suggested to be mediated by tachykinins, as treatment with neurokinin-1 receptor antagonist prevented the NGF-evoked hyperresponsiveness (de Vries et al. 1999). Also, the mast cell is proposed to play a role as an intermediate player. Thus, NGF-induced AHR has been shown to be inhibited by a histamine receptor antagonist (Bennedich Kahn et al. 2008b). Knock-out mice for the p75NTR receptor have been shown not to develop AHR, suggesting a role for this receptor in the mechanism of AHR mediated by NGF (Tokuoka et al. 2001; Kerzel et al. 2003). However, also the TrkA receptor has been shown to contribute to AHR. Induction of AHR by NGF has been confirmed in humans, as evident from in vitro experiments on isolated human bronchi (Frossard et al. 2005; Naline et al. 2007). Taken together, the above studies highlight the complexity of NGF-induced AHR.

NGF in wound healing and airway tissue remodeling

Since NGF has important reparative and proliferative effects in the nervous system, it was proposed that it could be involved also in tissue repair processes. It was early shown that removal of salivary glands in mice, a rich source of NGF, retarded the rate of skin wound contraction (Hutson et al. 1979). Shortly thereafter it was shown that topically applied NGF accelerated the rate of wound healing in mice, and it was suggested that one of the physiological roles of NGF in saliva is to promote wound healing by the wound-licking reflex (Li et al. 1980). Several studies have confirmed the wound-healing properties of NGF (Micera et al. 2007). For example, human clinical studies on treatment with NGF, applied as eye-drops on corneal ulcers, or as topical treatment for severe skin ulcers, have been shown to be effective (Lambiase et al. 1998; Bernabei et al. 1999). Specific mechanisms for the pro-
fibrotic effects of NGF have been linked to effects on the fibroblast. Skin-, but also lung-fibroblasts express the TrkA receptor and NGF has been shown to trigger both skin and lung fibroblast migration and their differentiation into α-smooth muscle actin (α-SMA) expressing myofibroblasts (Micer et al. 2001; Kohyama et al. 2002). NGF has also been shown to stimulate TGF-β production by fibroblasts (Micera et al. 2005).

Taken together, these studies propose an important role for neurotrophins, and especially NGF, as mediators in wound healing, and possibly also as being involved in airway tissue remodeling. Indeed, mice over-expressing NGF in the lungs have shown an increased thickening of the airway subepithelial layer (Hoyle et al. 1998). In human airways, NGF has been localised to fibrotic tissue and found to be elevated in sputum of patients with idiopathic pulmonary fibrosis (IPF) (Micer et al. 2001; Hope-Gill et al. 2003).
2. AIMS

It is well established that neurotrophins are elevated in allergic inflammation and asthma, and several roles for the neurotrophins in this disease are suggested, including enhancement of inflammation, airway hyperresponsiveness and tissue remodeling. However, few studies have been performed to elucidate the possible involvement of neurotrophins in other inflammatory pulmonary diseases, displaying inflammatory and remodeling processes. The general aim of the present thesis was to study neurotrophins in sarcoidosis and chronic obstructive pulmonary disease (COPD), and to further elucidate functional effects of neurotrophins with regard to airway tissue remodeling.

The specific aims were:

- To investigate the expression of neurotrophin receptors on human bronchial smooth muscle cells and to elucidate functional effects of the neurotrophins NGF, BDNF and NT-3, with regard to MMP secretion and migration in vitro.

- To investigate if altered levels of NGF are found within the airways, or in the circulation, of patients with pulmonary sarcoidosis as compared to healthy individuals, subjects with resolved disease and patients with chronic beryllium disease. Furthermore, to understand if NGF levels are related to the degree of inflammation or lung function in sarcoidosis patients.

- To determine if BDNF and NT-3 levels, in addition to NGF, are altered within the airways, or in the circulation, of patients with pulmonary sarcoidosis, as compared to healthy subjects, and to elucidate if neurotrohin levels differ between sub-groups of sarcoidosis patients, having different disease prognosis.

- To investigate possible cellular sources of-, and targets for-, neurotrophins within the airways of sarcoidosis patients.

- To investigate the levels of NGF, BDNF and NT-3 within the airways of patients with COPD, and compare those to the levels of asymptomatic smokers and healthy non-smokers. Furthermore, to investigate if, and how, cigarette smoke alters neurotrophin gene expression and protein secretion by human lung fibroblasts in vitro.
3. MATERIAL AND METHODS

3.1 GENERAL

The present thesis is based on studies on human cells and biological samples, including *in vitro* culture of isolated structural cells from the human airways, and analysis of human bronchoalveolar lavage fluid (BALF), serum and airway tissue biopsies. The studies were performed after approval by local ethics committees and all subjects included in the studies gave their written informed consent to participate.

3.2 STUDIES ON AIRWAY STRUCTURAL CELLS *IN VITRO* (paper I & IV)

**CELL CULTURE**

*Human bronchial smooth muscle cells (HBSMC) (Paper I)*

Two commercially available cell lines of human bronchial smooth muscle cells (HBSMC) (purchased from PromoCell, Germany and Cambrex Bio Science Walkersville, USA) were used to study functional effects of neurotrophins, with regard to MMP-production, migration and proliferation. Both cell lines were obtained from healthy donors. The cells displayed growth characteristics typical of smooth muscle cells, including a flattened and spindle-shaped appearance, parallel growth (Hirst 1996) and importantly positive immunostaining for α-SMA. The cells were grown in monolayer in DMEM supplemented with 10% fetal bovine serum (FBS) and insulin (0.12 IU/mL), and all experiments were performed in serum-free/0.3% FBS media at passages 8-10 to minimize biological variation in terms of phenotypic changes of the smooth muscle cells in later passages.

*Human fetal lung fibroblasts (HFL-1) (Paper IV)*

A commercially available cell line of human fetal lung fibroblasts (HFL-1) (purchased from ATCC, USA) were used to study effects of cigarette smoke extract exposure on neurotrophin expression. Cells were cultured in monolayer in DMEM supplemented with 10% FBS. The cells were then cultured in serum-free media containing a cytomix of TNF-α (5ng/ml), IFN-γ (5ng/ml) and IL-1β in order to study inflammation-induced stimulation of NGF and NT-3 production. In addition to the cytomix, cells were exposed to cigarette smoke extract (CSE). Cells were harvested with RNABee after 6h for neurotrophin mRNA analysis. Parallel sets of cell flasks were cultured for 24h before collection of cell supernatant for neurotrophin protein analysis by ELISA.

**IMMUNOCYTOCHEMISTRY (Paper I)**

HBSMC were cultured on glass slides and were analyzed for the presence of the neurotrophin receptors TrkA, TrkB, TrkC and p75NTR by immunocytochemistry, and this method is explained further under 3.4 Analysis of neurotrophins and neurotrophin receptors in human samples.
In order to study the production and secretion of matrix metalloproteinases (MMPs) from HBSMC, we used gelatin zymography, a method which analyses the enzymatic activity (gelatine degradation) of MMPs in the sample. MMP-2 and MMP-9 are able to digest gelatine, and this substrate is therefore casted into a standard polyacrylamide gel. Cell culture supernatant, collected after 24 h of stimulation with different doses of NGF, BDNF and NT-3 respectively, was concentrated using the Amicon® Ultra-4 Centrifugal Filter Units (Millipore), and thereafter loaded on the gelatine gels and electrophoresed. The proteins in the gel, originating from the cell culture supernatant, are thereby separated according to molecular size in the gels. The gels are then bathed in a detergent to remove the denaturating SDS, and are then incubated at 37°C in a buffer containing Zn\(^{+}\) and Ca\(^{+}\) ions, required for the enzymatic activity of the MMPs. This incubation will allow MMP-2 and -9 in the gel to digest the gelatine, and when the gels thereafter are stained with Coomassie brilliant blue, the MMPs can be identified as clear bands against a blue-stained background (see Figure 3.1). As a positive control, we ran cell medium from a cell line (HT-1080) known to secrete large amounts of MMP-2 and MMP-9. The advantages with this method is that it is very sensitive, detecting MMPs in picogram concentrations and the area of digestion can be quantified using a densitometer and associated software. In addition, identification of the MMPs according to their size and enzymatic activity can be done, making the method preferable in comparison with a regular Western blot where antibodies used to detect the proteins can be more or less specific. Also, both the pro- and active forms of the MMPs can be detected and distinguished on the basis of their molecular size, since the pro-form is also active within the gel and is 10 kDa larger than the corresponding active form.

![Figure 3.1](image1.png)  
**Figure 3.1.** Example of a gelatine zymography gel showing the detection of pro- and active forms of MMP-2 and MMP-9 as clear bands against a blue-stained background. Sample 1 & 2 (HBSMC), Sample 3 (HT-1080 cell line, used as a positive control for MMP-2 and -9 secretion).

![Figure 3.2](image2.png)  
**Figure 3.2** Schematic illustration of the Boyden chamber. Cells placed in the upper well are separated from the lower well by a porous filter. Chemoattractant is placed in the lower well and cells are allowed to migrate through the filter towards the chemoattractant.
MIGRATION ASSAY (Paper I)

To explore the hypothesis that neurotrophins may be able to stimulate migration of bronchial smooth muscle cells, we used the 48-well Boyden chamber technique (Neuro Probe Inc.). In this protocol, cells (HBSMC) in suspension are placed in the upper wells of the Boyden chamber, on top of a porous filter, and medium containing a chemoattractant is placed below the filter in the lower well (see Figure 3.2). The filter used has a pore size of 8 µm, small enough not letting cells spontaneously fall through the pores and large enough for the cells to squeeze through the pores actively. The filter was coated with collagen type 1 to allow the cells to actively attach to the filter. After an incubation time of about 5-6 h, the filter is removed and fixed in ice-cold methanol, where after cells are stained with Giemsa. Cells on the upper side of the membrane are scraped off. Cell migration is quantified by counting of the number of cells that have migrated through the pores in the filter and are attached to the bottom side. We counted cells in 3 randomized fields in a light microscope at a magnification of 20x. As a negative control we used cell medium only in the lower well, and as a positive control we added PDGF-BB in the lower well, which is a known chemoattractant for smooth muscle cells.

PROLIFERATION/VIABILITY ASSAYS

BrdU incorporation (Paper I)

To study cell proliferation of HBSMC in response to neurotrophin treatment, we applied the BrdU cell proliferation enzyme-linked immunosorbent assay (ELISA) (Roche). The principle of this assay is to quantify cell proliferation based on the measurement of Bromodeoxy-uridine (BrdU) incorporation into newly synthesised DNA in replicating cells. Cells are cultured in 96-well plates and treated with the substances of interest (in our case neurotrophins). BrdU is then added to the cells and the cells are reincubated for a couple of hours, depending on the rate of proliferation of the cells studied. We used an incorporation time of 24h for the smooth muscle cells. Thereafter cells are fixed and DNA is denatured before the addition of an anti-BrdU antibody conjugated with peroxidase. The immune complex is then detected by the addition of a substrate solution (tetramethyl-benzidine) which will result in a reaction product (blue color), that can be quantified by measuring the absorbance using a spectrophotometer. The developed color directly correlates to the amount of DNA synthesis and to the number of proliferating cells in each well.

WST-1 metabolism (Paper IV)

An alternative method to study cell proliferation is based on determination of the overall metabolic activity of the cells. This method was used to study cell viability of human lung fibroblasts (HFL-1) in response to exposure to cigarette smoke extract. Tetrazolium salts, like WST-1, are metabolized by a mitochondrial enzyme, dehydrogenase, and forms a coloured reaction product directly that can be measured using a photospectrometer. The amount of dye formed correlates to the number of viable cells in the well.
PREPARATION OF CIGARETTE SMOKE EXTRACT (Paper IV)

To elucidate if cigarette smoke exposure alters neurotrophin production (mRNA and protein expression) by lung fibroblasts, we used an in vitro model where cigarette smoke is bubbled through cell culture medium to which the cells are then exposed. Using a vacuum pump (see Figure 3.3), cigarette smoke from two research cigarettes (2F4E, University of Kentucky) is bubbled through 50mL of DMEM during 4-5 minutes per cigarette and the solution is then filtered through a 0.20 µm filter to exclude large particles and microorganisms. This medium represents 100% cigarette smoke extract (CSE) and is then diluted to desired concentrations and applied to the cells within 30 minutes from preparation. This is a protocol that is widely used within medical research where one studies effects of cigarette smoke on different cell types. The concentrations of CSE used in the experiments depend on the studied cell type, as different cell types tolerate different concentrations of CSE. It is therefore of great important to perform dose-response experiments and evaluate possible toxic effects (viability tests) for different concentrations of CSE to determine which range of concentrations are appropriate for your cell type. CSE contains thousands of different compounds that may be responsible for the effects on the cells (Brunnemann and Hoffmann 1991). By preparing CSE in cell culture medium, one selects for water-soluble substances, like nicotine, and excludes lipid-soluble compounds, like aromatic hydrocarbons and aldehydes (van der Toorn et al. 2009). One should also be aware that one loses volatile and rapidly reactive components. One strategy to try to standardize the CSE solution between experiments and to avoid loss of substances and changes in chemical composition is to add the CSE immediately after preparation and not store the solution.

Figure 3.3 To prepare cigarette smoke extract, smoke from research cigarettes, is bubbled through cell culture medium using a vacuum pump.

REAL-TIME PCR (Paper IV)

The expression of mRNA levels of the neurotrophins NGF and NT-3 in HFL-1 cells were quantified with real-time RT-PCR using the ABI Prism 7700 Sequence detection System (Applied Biosystems, USA). Taqman® gene expression assays NGF, NT-3 and the house-
keeping gene GAPDH, as well as the Taqman® gene expression master mix were purchased from Applied Biosystems. The relative change of target mRNA expression was calculated using the comparative C_T method ($\Delta\Delta C_T$), using the arithmetic formula: $2^{-\Delta\Delta C_T}$ (Perkin-Elmer 1997). Accordingly, the amount of target gene was normalized to GADPH expression ($\Delta C_T$), and relative change in gene expression was calculated in relation to the mean value of gene expression in the control sample (unstimulated cells) ($\Delta\Delta C_T$).

**ELISA (Paper IV)**

Cell culture supernatants from HFL-1 cells were analysed for the presence of the neurotrophins NGF and NT-3 using commercially available ELISA kits (Promega, USA), and this method is discussed further under 3.4 Analysis of neurotrophins and neurotrophin receptors in human samples.

**3.3 STUDY SUBJECTS AND SAMPLE COLLECTION (paper II, III & IV)**

**STUDY SUBJECTS**

*Sarcoidosis patients* (paper II & III)

The patients included in the studies were all referred to the outpatient sarcoidosis clinic at Karolinska University Hospital Solna, Sweden, on suspicion on sarcoidosis, and bronchoscopy, including BAL and biopsy sampling, was done for diagnostic purpose. All subjects had a typical clinical and radiographic picture compatible with sarcoidosis, and diagnosis was set using defined criteria set up by the World Association of Sarcoidosis and other Granulomatous Disorders (WASOG) (Statement. 1999), which includes exclusion of other diseases capable of producing similar clinical or histological picture. The patients were sub-grouped into patients with Löfgren’s syndrome and Non-Löfgren sarcoidosis. Löfgren’s syndrome is defined as an acute onset of the disease with bilateral hilar lymphadenopathy (BHL), fever, erythema nodosum (EN) and/or ankle arthritis (Grunewald and Eklund 2007b). No subject received corticosteroids at the time of bronchoscopy and a minority of the patients were smokers (no smokers in paper II and 4 smokers in paper III). At the time of bronchoscopy, all subjects had an active disease, and the median time from symptom debut to performance of bronchoscopy was 5 months in paper II and 3 months in paper III. Paper II included 56 patients with sarcoidosis, as well as nine patients with clinically resolved sarcoidosis, previously displaying Löfgren’s syndrome. In addition, six patients diagnosed with chronic beryllium disease (CBD) were included as an additional control group, and these BALF samples were obtained through collaboration with a research group in USA, where bronchoscopy of these patients was performed. CBD is a granulomatous lung disease, similar to sarcoidosis, but where the cause of the disease is known, i.e. exposure to beryllium. Paper III included 41 sarcoidosis patients for BALF analysis, and additionally 19 patients, from where lung biopsies were obtained for immunohistochemical analysis.

*COPD patients* (paper IV)

25 patients with moderate to severe COPD were included. Recruitment of patients and bronchoscopy was performed at Umeå University Hospital, Sweden. Inclusion criteria included age between 50-75 years, FEV₁ between 30-80 % of predicted, FEV₁/FVC below
70% (diagnostic criteria for COPD), as well as a smoking history of more than 10 pack years (either current or ex-smokers). Patients with asthma, allergy or other chronic disease were excluded as well as patients under treatment with corticosteroids or having had symptoms of airway infection during the past 4 weeks before bronchoscopy.

**Healthy subjects (paper II, III and IV)**

The healthy control subjects included in the studies were life-long never-smokers and had a normal chest radiograph and/or normal lung function. Paper II included 31 subjects, paper III included 27 subjects, and paper IV included 12 healthy subjects. An addition healthy control group (n=16) was included in paper IV and consisted of age-matched current smokers with a smoking history of more than 10 pack years, and normal lung function and chest radiograph. Subjects with allergies and/or asthma or other chronic diseases, as well as subjects with airway infection during the past 4-6 weeks, were excluded.

**BRONCHOSCOPY AND SAMPLE COLLECTION**

Paper II, III and IV are based on analysis of human biological samples obtained through bronchoscopy and blood sampling. Before the bronchoscopy procedure, participants received morphine, injected intramuscularly. Also topical anesthesia was sprayed into the nose and throat before the flexible fiberoptic bronchoscope was inserted nasally. Bronchoalveolar lavage (BAL) was performed by wedging the bronchoscope into middle lobe bronchus. Five aliquots of 50 mL of room-tempered sterile PBS were instilled and gently re-aspirated using a negative pressure. The recovered BAL fluid was collected in a siliconised bottle and kept on ice until processed further at the laboratory. There, the BAL fluid was filtered to remove debris and mucus and the recovered volume was measured. The cells were separated from the fluid by centrifugation. The cells were counted in a Bürker chamber to determine cell viability (by trypan blue exclusion) and total cell concentration. Differential cell counts were performed by preparation of cytopsins that were stained with May-Grünwald-Giemsa after which cells were counted and classified according to staining pattern and morphology. The cell- and debris-free BAL fluid (BALF) was aliquoted and frozen at -80ºC until further analyses. Transbronchial lung biopsies were sampled from the distal airways through the bronchoscope and were fixed in buffered 10% formalin solution for 24 h and embedded in paraffin.

**3.4 ANALYSIS OF NEUROTROPHINS AND NEUROTROPHIN RECEPTORS IN HUMAN SAMPLES** *(paper II, III & IV)*

**ELISA** *(Paper II, III, IV)*

Serum and BALF from patients and controls, as well as cell culture supernatants from HFL-1 cells, were analyzed for the protein concentrations of the neurotrophins NGF, BDNF and NT-3 using commercial available enzyme-linked immunosorbent assay (ELISA) kits from Promega, USA. However, the NGF ELISA from Promega uses an anti-rat IgG conjugate that may demonstrate cross-reactivity with samples containing high concentrations of human
IgG, and is therefore not recommended to be used on samples such as serum. Therefore we choose to analyze the NGF concentration in serum samples using a separate ELISA kit, purchased from R&D Systems, UK (detection range 7.8-1000 pg/ml). The detection ranges for the ELISA kits from Promega were 7.8-500 pg/ml for NGF & BDNF and 4.7-300 pg/ml for NT-3 (defined as linearity within a standard curve of known concentrations). Serum samples were diluted in sample buffer (1:100-1:500) prior to analyses for BDNF and NT-3, and BALF fluid samples from sarcoidosis patients were diluted 1:10 before analysis of NGF. All samples were analyzed in duplicates, and inter-assay variation between plates was controlled by running an internal standard sample with each plate. The internal standards consisted of either recombinant NGF, BDNF or NT-3 protein (R&D System) diluted in a stock solution, or a patient sample, that was run with each ELISA plate. The internal standards had been frozen in aliquots.

IMMUNOHISTOCHEMISTRY (Paper III)

In paper III, lung biopsy sections from sarcoidosis patients were analyzed for the presence of the neurotrophins NGF and NT-3 and the corresponding neurotrophin receptors TrkA and TrkC. In paper I, bronchial smooth muscle cells cultured on glass slides were analyzed by immunocytochemistry for the presence of the neurotrophin receptors TrkA, -B, -C and p75NTR. All antibodies were purchased from Santa Cruz Biotechnology. The rabbit anti-NGF polyclonal antibody (sc-548) is raised against a peptide mapping the N-terminus of the mature chain of human NGF, the rabbit anti-NT-3 polyclonal antibody (sc-547) is raised against a peptide mapping within an internal region of human NT-3, the rabbit anti-TrkA polyclonal antibody (sc-118) is raised against a peptide mapping near the C-terminus of human TrkA, the rabbit anti-TrkB polyclonal antibody (sc-12) is raised against a peptide mapping near the C-terminus of mouse TrkB (differs from human sequence by two amino acids), the rabbit anti-TrkC polyclonal antibody (sc-117) is raised against a peptide mapping near the C-terminus of porcine TrkC (identical to human sequence), and goat anti-p75NTR polyclonal antibody (sc-6188) is raised against a peptide mapping near the C-terminus of human p75NTR. Corresponding blocking peptides, against which the antibodies had been raised, were obtained from Santa Cruz Biotechnology and were used to assess non-specific binding of each antibody to the tissue/cell slides. This was done by incubating slides with the antibody pre-absorbed with the respective blocking peptide. Background of immune staining was also evaluated by omitting the primary antibody. Optimal dilution of each antibody was assessed in preliminary experiments. The product of immune reaction was revealed using Vectastain® Elite® ABC Kit (Vector laboratories) followed by SIGMA FAST™ 3,3 diaminobenzidine (DAB) tablets (Sigma-Aldrich) for the lung biopsy specimens (paper III), and using ExtrAvidin-alkaline phosphatase conjugate followed by SIGMA FAST™ Fast Red TR/Naphthol AS-MX tablets for the cell slides (paper I).

IN VITRO STIMULATION OF BAL CELLS & ANALYSIS OF CYTOKINE PRODUCTION (preliminary data, not included in any paper)

Freshly isolated total BAL cells (1x10^6/mL) from sarcoidosis patients were cultured for 24-48 h in cell culture media alone (RPMI-1640 media supplemented with 1% penicillin
In the presence of the cell mitogen Phytohemagglutinin (PHA) (10 µg/ml), NGF or NT-3 treatment (50-200 ng/ml) was added to media alone or in combination with PHA stimulation. After treatment, cell culture supernatant was collected and frozen. BAL samples from a total of 12 sarcoidosis patients were collected. The BD™ cytometric bead array (CBA) human Flex Set (BD Biosciences, USA) was used for detection of secreted cytokines (IL-1, IL-2, IL-6, IL-10, IFN-γ, TNF) in BAL cell supernatant using FACSCanto™ flow cytometer. This method is a particle-based immunoassay, allowing simultaneous detection of multiple cytokines in a single sample. The detection range of the cytokines was 10-2500 pg/ml.

### 3.5 STATISTICAL ANALYSIS

In paper I, data are presented as mean ± standard errors of the mean (SEMs) and differences between groups were evaluated by parametric analysis of variance (ANOVA) followed by Dunnett’s multiple comparison test. In paper II, III and IV, data were not normally distributed and are presented as median (interquartile range). Differences between groups were then evaluated with non-parametric statistics; using Kruskal-Wallis test followed by Dunn’s post-test for comparisons between three or more groups and Mann-Whitney test for comparisons between two groups. Correlation analyses were performed using Spearman’s rank correlation test. For all tests a p-value <0.05 was considered significant. Analysis was performed with GraphPad Prism 4.03 (Graphpad Software Inc.).
4. RESULTS AND DISCUSSION

4.1 STUDIES ON EFFECTS OF NEUROTROPHINS ON HUMAN BRONCHIAL SMOOTH MUSCLE CELLS (HBSMC) IN VITRO (paper I)

Since neurotrophins are known to be elevated in the airways of asthmatics, and smooth muscle cells play a central role in airway tissue remodeling in asthma, we aimed to elucidate whether bronchial smooth muscle cells are one possible cellular target for the neurotrophins. Cell migration of bronchial smooth muscle is suggested to contribute to the increased muscle mass in asthma, and increased amounts of MMPs are found in the airways of asthmatics, which are suggested to contribute to tissue remodeling. The aim of the present study was to investigate bronchial smooth muscle cell migration, proliferation and synthesis of MMP-2 and MMP-9 in response to neurotrophins.

EXPRESSION OF NEUROTROPHIN RECEPTORS

Using immunocytochemistry, expression of the neurotrophin receptors TrkA, TrkB and TrkC, but not p75NTR, was found on HBSMC in vitro. The expression profile of neurotrophin receptors in vitro corresponds well with immunostainings on human bronchial tissue where high-affinity neurotrophin receptor expression was found, while p75NTR expression was absent in smooth muscle (Ricci et al. 2004). Our results indicated that the cells may be expected to respond functionally to NGF, BDNF and NT-3, and that the possible observed effects would be mediated through the high-affinity (Trk) receptors.

EFFECTS OF NEUROTROPHINS ON MMP PRODUCTION

Stimulation of BDNF, NT-3 or NGF for 24 h resulted in a dose-dependent increase of MMP-9 secretion, whereas MMP-2 was unaltered. Importantly, we showed that the enhanced secretion of MMP-9 in the cell culture media was not due to an increased proliferation of the cells in response to neurotrophin-stimulation, as evaluated by cell counting as well as by BrdU incorporation assay. Our results of an induced MMP-9 expression, and a high constitutive expression of MMP-2, unaltered upon neurotrophin stimulation, correspond well with previous studies (Chakrabarti and Patel 2005). Thus, MMP-2 is produced by a wide range of cell types, including endothelial cells and macrophages, whereas constitutive expression of MMP-9 is restricted to neutrophils and eosinophils. However, inflammatory stimuli, such as IL-1β and TNF, has been shown to increase MMP-9 expression in many cell types, including macrophages, fibroblasts and smooth muscle cells (Atkinson and Senior 2003; Chakrabarti and Patel 2005). As such, NGF has previously been shown to induce MMP-9 secretion by vascular smooth muscle cells (Khan et al. 2002).

MMP-2 and -9 belong to a large family of tissue-degrading enzymes, with capacity to degrade extracellular matrix (ECM) components. MMP-2 and -9 are collagenases and degrades basement membrane components, such as type IV & V collagens, elastin and proteoglycans (Shapiro and Senior 1999). All MMPs are secreted as inactive pro-forms, which are activated in the extracellular space. MMP activity is also regulated by TIMPs,
which binds the MMPs and keeps them inactivated. Among the family of MMP’s, particularly MMP-2 and -9 have been implicated in airway remodeling (Chakrabarti and Patel 2005). As such, elevated levels of MMP-9 have been found in sputum and BAL of asthmatics, both during acute exacerbations as well as in stable asthma (Mautino et al. 1997; Tonnel et al. 2001). In asthmatic airways, MMP-9 expression has been localised to the extracellular matrix, and to infiltrating neutrophils (Dahlen et al. 1999). The increased proteolytic activity in asthma is thought to contribute to epithelial cell damage and increased mucus production. Indeed, inhibition of MMPs has been shown to prevent goblet cell metaplasia (Kim et al. 2004). Importantly, also TIMP-1, the predominant inhibitor of MMP-9, has been reported to be elevated in asthma, especially in untreated patients (Mautino et al. 1999b), and an excess of TIMP-1 over MMP-9 has been proposed to favour airway fibrosis.

In addition to its ECM-degrading capacity, MMP-9 also has several chemokines and cytokines as substrates. Thus, MMP-9 is able to cleave inactive membrane-bound TNF and TGF-β, to generate their corresponding active forms (Yu and Stamenkovic 2000). Similarly, IL-1β can be activated by MMP-9 (Schonbeck et al. 1998). As such, MMP-9 may also modulate airway inflammation, and promote tissue remodeling through activation of TGF-β.

The enhanced levels of MMP-9, reported in inflammatory pulmonary diseases, such as asthma and COPD, are suggested to origin mainly from infiltrating neutrophils, eosinophils and macrophages. However, airway smooth muscle cells also have the capacity to synthesise and secrete MMP’s (Johnson and Knox 1997; Elshaw et al. 2004), and an autocrine production of MMP’s has been shown to be required for airway smooth muscle proliferation (Johnson and Knox 1999). Thus, an increased secretion of MMP-9 in response to neurotrophin stimulation may facilitate degradation of the ECM and subsequent migration and proliferation of the smooth muscle cells in the inflammatory airway tissue.

EFFECTS ON CELL MIGRATION

Several studies have shown bronchial smooth muscle migration in vitro in response to a number of stimuli, including PDGF, IL-1β and TGF-β (Hedges et al. 1999; Goncharova et al. 2003). We found that both BDNF and NT-3 significantly induced migration of airway smooth muscle cells, whereas no effect was seen for NGF. To our knowledge, this is the first report showing migratory properties of BDNF and NT-3 on airway structural cells, whereas NGF has previously been shown to stimulate lung fibroblast as well as vascular smooth muscle cell migration (Donovan et al. 1995; Micera et al. 2001; Kohyama et al. 2002). It may therefore be suggested that the response to NGF varies between cells from different sources i.e. vascular versus bronchial origin. In addition to its capacity to induce migration, NGF has been shown to stimulate TGF-β production by fibroblasts (Micera et al. 2005) as well as inducting α-SMA expression (Micera et al. 2001), which supports that NGF may stimulate an induction of myofibroblasts. Thus, neurotrophins have been shown to have pro-fibrotic properties, acting on structural cells, including bronchial smooth muscle cells, which supports that neurotrophins may be involved in airway remodeling.
4.2 STUDIES ON NEUROTROPHINS IN PULMONARY SARCOIDOSIS (paper II & III)

Before these studies were conducted, knowledge on neurotrophins levels in the airways of patients with sarcoidosis was lacking, and the aim of these studies was therefore to determine the protein concentrations of NGF, BDNF and NT-3 in BALF, and serum, of sarcoidosis patients, including sub-groups of patients with different disease prognosis. These levels were compared to those of healthy subjects, subjects with resolved sarcoidosis and patients with a similar disease as sarcoidosis, chronic beryllium disease (CBD). Also, we aimed to relate neurotrophin levels in the airways to inflammatory- and lung function parameters. Moreover, the cellular sources of neurotrophins and the distribution of neurotrophin receptors in the airways of sarcoidosis patients were investigated.

NEUROTROPHIN LEVELS IN BALF AND SERUM (paper II & III)

In BALF, we found markedly elevated levels of NGF as well as NT-3 in patients with sarcoidosis as compared to healthy controls, whereas BDNF was found to be below the detection limit (<8 pg/ml) in all samples. In serum, we found that NGF levels were below the detection limit (<2 pg/ml) in both patients and controls, whereas BDNF and NT-3 were found in detectable concentrations (in the range of 10-80 ng/ml), however no differences in levels were found between healthy controls and patients. These studies show that enhanced levels of neurotrophins are found locally in the airways of patients with pulmonary sarcoidosis, indicating that neurotrophin induction in response to airway inflammation is not specific for asthma only, but may play a role also in pulmonary sarcoidosis. In sarcoidosis patients with resolved disease, we found the same low levels of NGF as in healthy controls, implying that neurotrophin levels return to baseline levels as the inflammation resolves. Interestingly, patients with CBD displayed the same low levels as the healthy controls, indicating a disease-specific induction of neurotrophins in sarcoidosis. CBD is an inflammatory granulomatous pulmonary disease with similar clinical and pathological features as sarcoidosis, but where the etiology is known, i.e. it is caused by exposure to beryllium dust. Our findings propose that NGF is differently regulated in CBD as compared to sarcoidosis, and it may be suggested that usage of NGF as a biomarker could be a tool in parallel to other tests, such as beryllium lymphocyte-proliferation test, to distinguish between these two clinically similar diseases (Newman 2000). Although CBD is a very rare disease in Sweden, the prevalence is higher in America and is expected to rise in the future, since beryllium-dependent industries, such as electronic and computer industries, are growing (Willis and Florig 2002).

Sub-groups of sarcoidosis patients

One specific sub-group of sarcoidosis patients has Löfgren’s syndrome. In contrast to patients with Non-Löfgren sarcoidosis, these patients have an acute disease onset and a more favorable prognosis with high rate of spontaneous disease resolution. Intriguingly, we found that Non-Löfgren patients had significantly higher levels of NT-3 in BALF as compared to Löfgren’s syndrome patients. The same trend was seen for NGF, but did not reach statistical significance. Since Non-Löfgren patients have a higher risk of developing chronic disease and develop fibrosis, we speculate that the increased levels of neurotrophins in this sub-group of
patients may reflect an association between neurotrophins with disease severity. In support of this, when dividing the sarcoidosis patients according to disease stage, significant elevated levels of NT-3 were found in patients with more advanced disease stage (stage II) as compared to healthy subjects.

Correlation to inflammatory parameters

In paper II, cytokines (IL-1β, -2, -4, -5, -6, -10, -12p70, TNF and IFN-γ) in BALF were analyzed in parallel to NGF in 32 of the 56 patients and eight of the 31 healthy controls included in that study. Significant higher levels of TNF, IL-1β, IL-2 and IL-6 were found in sarcoidosis patients as compared to healthy controls, which supports the concept of sarcoidosis as a Th1 mediated inflammatory disease. In this study material, we found that in patients, the concentration of NGF correlated positively to the concentration of the Th1 cytokines IFN-γ and IL-12, as well as the Th2 cytokines IL-4 and IL-10. Also a positive correlation was found to the pro-inflammatory cytokine IL-1β in the healthy control group. Several studies have shown that neurotrophins are able to modulate expression of both pro-inflammatory, Th1- and Th2 cytokines, and vice versa, that cytokines may regulate neurotrophin secretion. For example, in a mouse model of allergic asthma, isolated mononuclear cells have been shown to increase the IL-4 and IL-5 production in response to exogenous NGF. In the same study, treatment of the allergic mice with anti-NGF resulted in decreased IL-4 levels in the airways (Braun et al. 1998). Experiments on human peripheral blood mononuclear cells cultured in vitro showed a high correlation with BDNF and NGF administration and IL-4 protein secretion, but also, these neurotrophins enhanced IFN-γ gene expression by human T-lymphocytes (Bayas et al. 2003). In addition, epithelial cells have been shown to up-regulate IL-10 production in response to NGF, an effect that was shown to be reciprocal in that IL-10 also induced NGF synthesis (Ma et al. 2003). Furthermore, IL-1β have been shown to be a potent inducer of NGF and BDNF secretion by several airway structural cells, including fibroblasts and smooth muscle cells (Olgart and Frossard 2001; Kemi et al. 2006).

In addition to correlations with cytokines, we found a positive correlation between the concentration of NGF and lymphocytes as well as eosinophils in BALF of patients. One interpretation may be that these cells are either sources of, and/or possible responders to the effects of neurotrophins. Indeed, T-lymphocytes, especially of the Th2-type, and eosinophils are able to produce neurotrophins (Ehrhard et al. 1993; Lambiase et al. 1997; Kobayashi et al. 2002). Effects of neurotrophins on lymphocytes and eosinophils have also been studied extensively. For example, NGF has been shown to induce proliferation and survival of both T- and B- lymphocytes (Otten et al. 1989; Torcia et al. 1996). Incubation with NGF, BDNF, NT-3, or NT-4 have been shown to cause a significant increase in the viability and expression of the activation marker CD69 on airway eosinophils of allergen-challenged asthmatics (Nassenstein et al. 2003). In an additional study on peripheral blood eosinophils from allergic patients, NGF, BDNF and NT-3 were shown to inhibit apoptosis and BDNF and NT-3 could stimulate migration of eosinophils (Raap et al. 2008). Interestingly, in parallel to enhanced NGF levels, the group of sarcoidosis patients in paper II had a significantly higher concentration of eosinophils in BAL as compared to the healthy controls. In this context it is also noteworthy that higher concentrations of eosinophils in BAL of sarcoidosis patients have
been suggested to be associated with a progressive disease and the need for steroid treatment (Ziegenhagen et al. 2003). Further studies on the possible functional link between neurotrophins and eosinophils in sarcoidosis are therefore encouraged.

Taken together, the results from this study strengthen the postulated relationship between neurotrophins and airway inflammation and highlight their possible involvement in both Th1- and Th2-driven airway inflammation.

EFFECTS OF NEUROTROPHINS ON CYTOKINE PRODUCTION BY BAL CELLS (preliminary data, not included in any paper)

Since a positive correlation between NGF and several inflammatory cytokines in BALF of sarcoidosis patients was found, and previous studies have indicated that neurotrophins may regulate cytokine production by immune cells, we hypothesised that neurotrophins may stimulate cytokine production by BAL cells. To elucidate this, freshly isolated BAL cells from sarcoidosis patients were cultured in vitro in the presence of PHA only, or in combination with increasing doses of either NGF or NT-3. After stimulation, cell culture supernatants were recovered and analysed for the presence of the cytokines IL-6, TNF, IL-1β, IL-2, IL-10 and IFN-γ.

As expected, we found that PHA alone was a potent inducer of cytokine production by BAL cells. However, neurotrophins alone did not stimulate cytokine secretion, and did not alter the PHA-induced cytokine production. This suggests that neurotrophins do not act by direct stimulation of cytokine secretion by airway immune cells, obtained by BAL from sarcoidosis patients. However, it should be noted that a neurotrophin-dependent cytokine production by immune cells has been suggested to be dependent on the activation state of the cells (Ehrhard et al. 1993; Lambiase et al. 1997) and therefore, further trials with different stimulation protocols should be performed before any major conclusions from these preliminary studies can be drawn.

DISTRIBUTION OF NEUROTROPHINS AND NEUROTROPHIN RECEPTORS IN SARCOID LUNG TISSUE (paper III)

Based on our findings that NGF and NT-3 are elevated in the airways of patients with sarcoidosis, we aimed to further investigate the cellular sources of these neurotrophins. Our aim was also to identify possible target cells, i.e. cells expressing the receptors for NGF and NT-3 respectively, in the lung tissue. Based on previous studies, we know that both structural cells, as well as infiltrating inflammatory cells, are expected to display immunoreactivity for neurotrophins as well as their receptors (Olgart Hoglund et al. 2002; Ricci et al. 2004). Our hypothesis was that the granulomas, present in the lungs of sarcoidosis patients, may be a specific source of the enhanced levels of neurotrophins in the airways of these patients. To elucidate this, we performed immunohistochemical analysis on lung tissue sections from patients presenting with sarcoidosis-specific noncaseating granulomas.

Distribution of neurotrophins and neurotrophin receptors in granulomas

A marked NGF and NT-3 immunoreactivity was found in the sarcoid granulomas and was localized to epithelioid cells and multinucleated giant cells within the granulomas. In addition, the granulomas displayed marked TrkA and weaker TrkC immunoreactivity. Neurotrophin
and neurotrophin receptor immunoreactivity was not evident within fibrotic tissue, or within inflammatory cells surrounding the granulomas. Similarly to the lung tissue granulomas, granulomas present in a mediastinal lymph node, displayed NGF and NT-3 immunoreactivity, as well as immunoreactivity for NGF in the surrounding lymphoid tissue, indicating that both intra- and extra-pulmonary granulomas contain NGF. TrkA and TrkC immunoreactivity showed similar staining pattern as that found in lung granulomas. To our knowledge, this is the first report on neurotrophin expression in sarcoid granulomas, and we suggest that these pathological structures are possible cellular sources of the enhanced NGF and NT-3 levels detected in bronchoalveolar lavage fluid of sarcoidosis patients. The immunoreactivity for neurotrophin receptors found within the granulomas, suggests that neurotrophins are able to function in an autocrine manner, possibly affecting survival and/or differentiation of the epithelioid and giant cells present in the granulomas. This hypothesis needs further investigation.

**Distribution of neurotrophins and neurotrophin receptors in other lung tissue cells**
A marked NGF, NT-3 and TrkA, and weak TrkC immunoreactivity was found in ciliated bronchial epithelium and smooth muscle in the submucosa in sarcoid lung tissue. Also, alveolar macrophages present in the lung parenchyma showed strong immunoreactivity for both neurotrophins and neurotrophin receptors. NGF and NT-3 have previously been shown to be expressed in airway epithelium and smooth muscle of both healthy and asthmatic subjects (Olgart Hoglund et al. 2002; Ricci et al. 2004). *In vitro* studies on airway structural cells also confirm a constitutive expression of neurotrophins, which is upregulated in response to inflammatory mediators, such as IL-1β (Olgart and Frossard 2001; Pons et al. 2001; Kemi et al. 2006). Inflammatory cells have also previously been shown to express NGF, both in healthy and asthmatic lungs (Olgart Hoglund et al. 2002). In addition, Ricci and co-workers have demonstrated that alveolar macrophages and T-cells, retrieved from BAL from sarcoidosis patients, express neurotrophins and neurotrophin receptors to a larger degree than cells from healthy subjects (Ricci et al. 2005), suggesting an involvement of neurotrophins in airway inflammation in sarcoidosis. Thus, we confirm their results of neurotrophin and neurotrophin receptor expression by macrophages, and extend them by showing positive staining for neurotrophins and their receptors in alveolar macrophages present in the lung parenchyma of sarcoidosis patients. Since it is well known that macrophages and T-cells accumulate in the lungs in sarcoidosis, we do not exclude the possibility that these cells, besides the granulomas, contribute to the enhanced levels of neurotrophins found in BALF of these patients.
4.3 STUDIES ON NEUROTROPHINS IN PATIENTS WITH COPD, SMOKERS AND NON-SMOKERS (paper IV)

To further understand the possible involvement of neurotrophins in different inflammatory pulmonary diseases, we set up a study to investigate if neurotrophins were elevated in COPD, similarly to asthma and sarcoidosis.

NEUROTROPHIN LEVELS IN BALF

Surprisingly, and in contrast to asthma and sarcoidosis, we found significantly lower levels of both NGF and NT-3 in BALF from COPD patients as well as from smokers in comparison with BALF from healthy non-smokers. Similar to our previous results, BDNF levels in BALF were below the detection limit of the ELISA kit. During our work, one study by Groneberg and co-workers reported decreased gene expression of NT-3 in lung tissue of patients with COPD as compared to healthy non-smokers (Groneberg, 2005). However, whether the effect was attributed to smoking was not investigated in that study. With our results, it became evident that cigarette smoking per se affected the neurotrophin content in the airways, since the current smokers with a normal lung function had similarly low levels of neurotrophins in the airways as the COPD patients. Also, when dividing the COPD patients into current smokers and ex-smokers, we found that the median NGF concentration in the ex-smokers group was higher than in the group of current smokers, a difference that however did not reach statistical significance (p=0.07). Despite so, this may indicate that the effect of smoking is reversible, a hypothesis that needs to be tested in a prospective study. Indeed, decreased BDNF levels have been found in blood from smokers, levels which were shown to be normalized after smoking cessation (Kim et al. 2007). Additional studies have also shown a down-regulatory mechanism of tobacco smoke on neurotrophin expression. A recent study reported that, primates who had been exposed to environmental cigarette smoke in utero, had decreased concentration of NGF in the airways as infants (Yu et al. 2008).

An additional finding in our study was that the levels of NGF and NT-3 in BALF showed a weak, but significant positive correlation to lung function, measured as FEV\textsubscript{1} (% of pred), in smokers (with or without COPD). This may reflect the loss of tissue protective factors of importance for maintaining lung function. In the context of airway inflammation and remodelling in COPD, we speculate that the decreased levels of neurotrophins may contribute to an imbalanced tissue repair, since neurotrophins, and especially NGF, has been shown to promote wound healing and to have pro-fibrotic properties (Micera et al. 2007). Interestingly, decreased numbers of mast cells in the airway tissue of COPD patients has recently been reported in two separate studies (Gosman et al. 2008; Andersson et al. 2009). It is well established that NGF acts as a survival factor for mast cells (Kanbe et al. 2000), and although speculative, it may be proposed that lower levels of NGF in the airways could influence mast cell survival. Future studies are therefore encouraged to determine the role of mast cells in the pathogenesis of COPD and if lower amount of NGF in the airways could be related to the lower numbers of mast cells in airway tissue.
EFFECTS OF CIGARETTE SMOKE EXTRACT (CSE) ON NEUROTROPHIN EXPRESSION IN LUNG FIBROBLASTS

To further elucidate if cigarette smoke exposure inhibits neurotrophin production, and to investigate if the target for this possible regulation may be structural cells of the airways, we performed *in vitro* studies with human lung fibroblasts.

In culture, these cells secreted detectable protein levels of NT-3, whereas constitutive NGF production was absent, as protein levels in cell culture supernatant was below the detection limit. Inflammatory stimuli (IL-1β, TNF, and IFN-γ), enhanced the production of both NT-3 and NGF. Exposure to cigarette smoke extract (CSE) caused a dose-dependent inhibition of cytokine-induced NGF and NT-3 protein secretion as well as mRNA expression. These results indicate that the down-regulation of NGF and NT-3 secretion, by CSE, is mediated through transcriptional regulation. Importantly, the viability and proliferation of the cells in response to CSE was unaltered under the tested conditions.

In addition to being a potential source of neurotrophins in the human airways (Olgart and Frossard 2001; Olgart Hoglund et al. 2002; Ricci et al. 2007), fibroblasts are also one of the key cells involved in tissue repair and airway remodeling through their capacity to migrate into wounded areas, proliferate and produce ECM components. Importantly, NGF has been shown to stimulate lung fibroblast migration and TGF-β secretion (Micera et al. 2001; Micera et al. 2005), suggesting that neurotrophins may act on the fibroblast through an autocrine and/or paracrine mechanism. It may therefore be postulated that an impaired production of neurotrophins in response to cigarette smoke exposure, may lead to an altered functional phenotype of the fibroblast which could contribute to an imbalanced tissue repair and possibly promote tissue destruction.
5. GENERAL DISCUSSION

Asthma, sarcoidosis and COPD are inflammatory pulmonary diseases which share many aspects of inflammation and airway remodeling, but importantly, each disease is characterized by individual etiologies and pathological characteristics. Asthma and COPD are both common diseases with a growing prevalence worldwide. Asthma prevalence is suggested to increase in industrialized countries because of our modern lifestyle, which includes less exposure to infections, changed diet and fewer siblings, each component proposed to have an impact on the maturation of our immune response toward more allergy-prone, a theory postulated by the so called hygiene hypothesis. COPD prevalence is closely connected to smoking habits in the population, but also to the usage of biomass fuels for heating and cooking in undeveloped countries. Increased air pollution is one additional factor that seems to play a role for both asthma and COPD prevalence. Despite the vast knowledge on how to prevent COPD, it is a disease that is growing to become one of the most common chronic diseases worldwide. Sarcoidosis is a less common disease, with an etiology that is still unknown. Since sarcoidosis affects young people, and a proportion of patients will suffer or die from chronic complications of the disease, it is of great importance to further elucidate the cause of the disease and to find better treatment options, as well as biomarkers that may predict disease outcome and prognosis.

Our research approach

In this thesis, we investigated a family of mediators, the neurotrophins, which are considered to play a role in several inflammatory diseases. The neurotrophins have broad effects in both the nervous- and the immune system, as well as in maintaining tissue integrity after injury. The aim of the current work was to increase the knowledge on neurotrophins in pulmonary inflammation, from knowing that they are involved in the pathogenesis of asthma, to include studies on other important inflammatory pulmonary diseases, like sarcoidosis and COPD. Our research approach was to take advantage of human clinical samples, like lung tissue biopsies, BAL and blood samples to reach this goal. A close connection with the lung clinic at the Karolinska University Hospital, where sarcoidosis patients and COPD patients come for clinical investigations, gave us access to samples from well characterized patients and healthy controls. Sarcoidosis patients are routinely investigated by bronchoscopy, and BAL and biopsy sampling is performed for diagnostic purposes. Clinical studies involving COPD patients are performed at the lung clinic at Karolinska, and additionally, we gained access to a well-defined study population of COPD patients and matched healthy controls through collaboration with a research group at Umeå University Hospital, Sweden. The limitation with this approach is that it’s descriptive, in that clinical samples mirror the environment of the human airways, without giving information on the cause and effect of what we observe. By complementing with in vitro studies on human bronchial smooth muscle cells and lung fibroblasts, we were able to further test our hypotheses of an involvement of neurotrophins in airway remodeling, and study possible mechanisms of neurotrophin regulation by cigarette smoke.
Neurotrophins in inflammatory pulmonary diseases

Before these studies were performed, it was well established that neurotrophins are enhanced in the human airways of asthmatics (Virchow et al. 1998; Kassel et al. 2001; Olgart Hoglund et al. 2002), as measured in BALF. Also, elevated levels of NGF had been reported in sputum of patients with IPF, an inflammatory pulmonary disease with major fibrotic changes (Hope-Gill et al. 2003). With our studies, it became evident that the neurotrophins NGF and NT-3 are also elevated in the airways of patients with pulmonary sarcoidosis. In contrast, patients with CBD seem to have similarly low levels of neurotrophins as the healthy non-smoking controls. Quite surprisingly, we found that patients with COPD have significantly decreased levels of NGF and NT-3 in BALF as compared to healthy non-smokers. Similar pattern had been reported in one previous study, where a transcriptional down-regulation of NT-3 was found in lung tissue biopsies of COPD patients as compared to healthy non-smokers (Groneberg et al. 2005). Taken together these studies suggest that neurotrophins are elevated in the airways in response to chronic inflammation, such as that seen in asthma and sarcoidosis, but not in all inflammatory pulmonary diseases. We have shown that cigarette smoke may be important in the regulation of neurotrophin production in the airways. In our study on neurotrophin levels in patients with COPD, we found decreased levels of neurotrophins in both COPD patients as well as in current smokers with normal lung function. Since airway inflammation is present in all smokers, both in those with COPD and in those with normal lung function, and neurotrophin levels are found decreased, instead of enhanced, in these inflammatory conditions, it may be speculated that the expected enhancement of neurotrophins is lost due to a stronger down-regulatory capacity of cigarette smoke. Indeed, our in vitro studies on lung fibroblasts confirmed a cigarette smoke-dependent down-regulation of neurophin expression from these cells. Lung fibroblasts are one potent source of neurotrophins in human airways during inflammation (Olgart and Frossard 2001), but we do not exclude that also other structural cells, like epithelial cells and smooth muscle cells, as well as immune cells, may be targets for a cigarette-smoke dependent down-regulation of neurotrophin production.

While the inflammation in asthma is driven by cytokines of mainly the Th2 type, such as IL-4, -5 and -13, sarcoidosis is considered to be a Th1 driven disease with enhanced levels of cytokines such as IFN-γ and IL-12. Looking outside the airways, neurotrophins are also found elevated in other inflammatory conditions such as Th1- associated autoimmune disorders, including multiple sclerosis (MS) and rheumatoid arthritis (RA) (Aloe et al. 1992; Laudiero et al. 1992). Taken together, these studies suggest that the induction of neurotrophins is not coupled to either Th1 or Th2 driven diseases. Rather, neurotrophins seem to be up-regulated in pro-inflammatory milieus, with capabilities of regulating inflammatory responses in a broader perspective. Results from the present thesis, further support a relationship between NGF and a broad cytokine expression, in that a positive correlation between NGF levels and the concentration of both pro-inflammatory, Th1 and Th2 cytokines in sarcoidosis patients was found. Additionally, NGF levels were shown to be normalized in the airways of subjects with a resolved sarcoidosis disease, which further supports a relationship between NGF and the inflammatory status.
Studies on Neurotrophins in Inflammatory Pulmonary Diseases; a summary on findings in human airways.

<table>
<thead>
<tr>
<th>Disease/state:</th>
<th>NGF</th>
<th>NT-3</th>
<th>BDNF</th>
<th>References:</th>
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<td>IPF</td>
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<td>Hope-Gill 2003, Ricci, 2007</td>
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<td>Sarcoidosis</td>
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IPF: interstitial pulmonary fibrosis, CBD: chronic beryllium disease, COPD: chronic obstructive pulmonary disease

Importantly to consider when studying neurotrophins in relation to inflammation, is that these mediators have been proposed to be either “good” or “bad” players, depending on the context of which they have been studied. In relation to allergy and asthma, neurotrophins are thought to contribute to enhancement of inflammation, to cause airway hyperresponsiveness (AHR) and contribute to tissue remodeling. Therefore a possible therapeutic strategy would be to block the actions of the neurotrophins to prevent or dampen these adverse effects in the lungs of asthma patients. Indeed, anti-NGF and anti-BDNF antibodies have shown beneficial effects in murine models of asthma, reducing AHR to capsaicin, an exogenous agonist of the TRPV1 receptor present on sensory nociceptive nerves (Braun et al. 1998) (Braun et al. 2004). Similarly, local treatment with a soluble Trk receptor fragment, capable of binding all neurotrophins with high affinity, reduced hyperreactivity of sensory nerves in response to capsaicin, and reduced IL-4 and IL-5 levels in the airways in a mouse model of allergic asthma (Nassenstein et al. 2006a). In these studies, blockage of neurotrophins did not however affect allergen-evoked increase in responsiveness to metacholine. Thus, it has been suggested that neurotrophins act by enhancing the excitability of sensory nerves, for example by potentiating the response to TRPV1 agonists. These results also indicate that neurotrophin signaling in the development of AHR is comparable to that seen in models of hyperalgesia, where NGF induce rapid sensitization of nociceptive sensory nerves to painful thermal stimuli (Bomminston and McNaughton 2003).

In contrast to asthma, a protective role for neurotrophins in chronic inflammation in the gastro-intestinal tract has been proposed. There, NGF has been shown to protect against the tissue damage caused by the inflammation (Reinshagen et al. 2000), and seems to be mediated by upregulation of the neuropeptide CGRP in the inflamed tissue. Additionally, in the context of inflammation within the CNS, such as that seen in MS, neurotrophins are proposed to have a protective role as factors of importance for survival and regeneration of injured nerve cells (Gielen et al. 2003; Hohlfeld et al. 2006). Taken together, both neurotrophin antagonists and agonists may be considered in the treatment of inflammatory conditions, depending on the nature- and the site of inflammation. Additionally, there are also concerns about the use of neurotrophin antagonists or agonists in human disease, since these factors are involved in many essential physiological responses, especially in the CNS.
Sources of neurotrophins in human airways

A key question with regard to neurotrophin elevation in pulmonary inflammation has been the cellular sources of the neurotrophins. Neurotrophin production by inflammatory- as well as persistent lung cells has been confirmed in both *in vitro* and *in vivo* studies. Immunostainings on human lung tissue biopsies have confirmed neurotrophin expression by the lung epithelium, smooth muscle cells as well as fibroblasts in both healthy and asthmatic airways (Kassel et al. 2001; Olgart Hoglund et al. 2002; Ricci et al. 2004). Additionally, immune cells infiltrating the airways, such as alveolar macrophages, have been shown to express NGF (Ricci et al. 2000), and a higher number of NGF-positive immune cells have been identified in asthmatic as compared to healthy airways (Olgart Hoglund et al. 2002). During our work, one study was published which showed an enhanced neurotrophin expression by alveolar macrophages and T-lymphocytes, retrieved from BAL, from sarcoidosis patients as compared to healthy subjects (Ricci et al. 2005), suggesting that the neurotrophin system is activated in immune cells in sarcoidosis. We detected neurotrophin expression in airway epithelium and smooth muscle cells, as well as alveolar macrophages in lung tissue sections from sarcoidosis patients. Additionally, we identified the granuloma as an additional source of neurotrophins in the sarcoid airways. Since they are pathological structures, absent in the healthy lung, we propose that neurotrophin production by granulomas may contribute to the enhanced levels of neurotrophins found in the airways of these patients.

A role of neurotrophins in sarcoid granulomas

Granuloma formation in sarcoidosis is a remodeling process where infiltrating immune cells, in particular T-lymphocytes and macrophages accumulate in a defence reaction to protect against infectious or other harmful agents and particles. The macrophages differentiate to epithelioid cells and multinucleated giant cells, which forms noncaseating granulomas. Importantly, the mechanisms leading to the persistence of granulomas and the development to fibrosis are still poorly understood. We found that sarcoid granulomas express the neurotrophins NGF and NT-3 in addition to the corresponding receptors TrkA and TrkC respectively. This suggests that neurotrophins may have a functional role as an autocrine and/or paracrine mediator in the granuloma microenvironment. Previous reports have shown that granulomas in the brain and liver, induced by parasite infection, produce NGF, and that enhanced levels of NGF are detected in granulomatous tissue (Aloe et al. 1994; Aloe et al. 1996). There, NGF is proposed to participate in the repair of injured nerves or in the modulation of inflammation. Interestingly, NGF has been reported to protect monocytes from apoptosis (la Sala et al. 2000) and to induce macrophage migration (Kobayashi and Mizisin 2001). Whether neurotrophin expression in sarcoid granulomas participates in recruitment and possibly persistent survival of macrophages or epithelioid cells in the granuloma microenvironment, is a hypothesis requiring further investigation.

Neurotrophins in airway remodeling

Previous work by our research group and by others had identified smooth muscle cells as a potent source of neurotrophins in the human airways (Olgart Hoglund et al. 2002; Ricci et al. 2004). Interestingly, their secretion of neurotrophins was shown to be markedly enhanced in response to inflammatory stimuli, in particular IL-1β treatment (Freund et al. 2002; Kemi et
The smooth muscle-derived neurotrophins may participate in modulation of immune cell function. For example, it has been shown that mast cells reside within the smooth muscle layer in asthmatics (Brightling et al. 2002), and therefore, neurotrophin secretion by the smooth muscle may potentiate mast cell survival. Indeed, NGF has been shown to prevent apoptosis of mast cells (Kanbe et al. 2000). Based on the knowledge that airway smooth muscle cells secrete NGF, BDNF and NT-3, and express neurotrophin receptors, we hypothesized that the neurotrophins may function in an autocrine manner, thus influencing the smooth muscle itself.

The smooth muscle is involved in airway remodeling, especially in asthma, where an increased smooth muscle mass is one of the specific pathological findings in the airways. Migration is a rather newly discovered function of airway smooth muscle cells, which has gained interest because of its possible importance for airway remodelling (Madison 2003). More established is the migratory capacity by vascular smooth muscle cells, which is suggested to contribute to intima thickening in the vessel wall in atherosclerosis (Newby and Zaltsman 2000). Accordingly, it has been proposed, that inflammation in response to injury of the vessel wall, activates enzymes, such as MMPs, which degrade the basement membrane, thus allowing vascular smooth muscle cells to migrate into the intima (Newby and Zaltsman 2000). Many of the findings on vascular smooth muscle may also be relevant for smooth muscle migration in the airways. Although direct evidence for smooth muscle migration in vivo is currently lacking, it is proposed that it may contribute to smooth muscle hyperplasia, and possibly to the appearance of myofibroblasts beneath the epithelial basement membrane in asthma (Madison 2003) (Brewster et al. 1990). Given that NGF may modulate lung fibroblast and vascular smooth muscle cell migration (Kraemer et al. 1999; Micera et al. 2001), we chose to investigate airway smooth muscle cell migration in response to neurotrophin stimulation. Indeed, we found that BDNF and NT-3 stimulated cell migration. Thus, we propose that enhanced levels of neurotrophins in airway inflammation may enhance migration of both fibroblasts and smooth muscle cells and thereby contribute to airway tissue remodeling. In addition to the chemotactic activity of neurotrophins on airway smooth muscle cells, we showed that NGF, BDNF and NT-3-stimulated MMP-9 secretion by these cells, and we suggest that an induced secretion of MMP-9 by bronchial smooth muscle cells may facilitate ECM degradation and smooth muscle migration. Taken together, results by us and others, propose that neurotrophins play a role in airway tissue remodeling through their effects on airway structural cells, including fibroblasts and smooth muscle cells.

The consequence of decreased levels of neurotrophins in the airways of smokers and patients with COPD are currently unknown. Airway remodeling in COPD is characterized by small airway fibrosis and emphysema; two opposing events postulated to be the result of an imbalanced tissue repair. As such, elevated levels of MMP-9 are suggested to contribute to tissue degradation, whereas an excess of mediators with pro-fibrotic properties, such as TGF-β, is thought to contribute to fibrosis (Laurent et al. 2007). NGF has been shown to be a potent inducer of fibroblast migration and TGF-β production (Micera et al. 2001; Micera et al. 2005), and results from the present thesis showed that cigarette smoke exposure down-regulate neurotrophin production by lung fibroblasts. Therefore, it may be speculated that a loss of NGF in the airways in response to cigarette smoke exposure, results in decreased ability of fibroblast to migrate and secrete TGF-β, effects which could promote parenchymal
destruction. However, the functional role of neurotrophins in COPD pathogenesis needs to be clarified in additional studies.

Concluding remarks
The results in the present thesis broaden our understanding of neurotrophin activation in pulmonary inflammation and give support for the concept that neurotrophins have tissue remodeling properties. The results encourage further mechanistic studies on the role of neurotrophins in sarcoidosis and COPD.
6. CONCLUSIONS

From the observations in the present thesis it is suggested that:

- Human bronchial smooth muscle cells are possible targets for neurotrophins in the airways through their expression of the high-affinity neurotrophin receptors TrkA, TrkB and TrkC.

- The neurotrophins NGF, BDNF and NT-3 stimulate human bronchial smooth muscle cells to enhance their secretion of MMP-9, but not MMP-2. In addition, BDNF and NT-3, but not NGF, stimulate migration of the cells. This indicates that neurotrophins are able to modulate smooth muscle functions, connected to airway tissue remodeling processes.

- NGF proteins levels are enhanced in the airways of patients with pulmonary sarcoidosis as compared to healthy subjects, subjects with resolved sarcoidosis and patients with a clinically similar granulomatous disease; chronic beryllium disease. Also, NGF levels correlate to inflammatory cytokines and immune cells in the airways, supporting a relation between NGF and pulmonary inflammation in sarcoidosis.

- NT-3 protein levels are enhanced in the airways of patients with pulmonary sarcoidosis, and are found in higher concentrations in the group of sarcoidosis patients with a higher risk of developing chronic disease and fibrosis, indicating that neurotrophin levels may relate to disease prognosis.

- Serum levels of neurotrophins do not differ between sarcoidosis patients and healthy subjects, supporting a possible local enhanced production within the airways of patients.

- The disease-specific granulomas found in the lungs of sarcoidosis patients express NGF and NT-3, as well as the corresponding receptors TrkA and TrkC. This suggests that the granulomas are one possible local source of the enhanced levels of neurotrophins found in the airways of sarcoidosis patients, and that they may act in an autocrine manner in the granuloma microenvironment.

- Apart from the granulomas, also structural cells of the airway, such as the bronchial epithelium and smooth muscle, as well as alveolar macrophages, express neurotrophins and neurotrophin receptors. This suggests a broad neurotrophin activity within the lungs, which may impact several cellular processes connected to both inflammation and tissue remodeling.

- Decreased levels of NGF and NT-3 are found in the airways of current smokers and patients with chronic obstructive pulmonary disease (COPD) as compared to healthy non-smokers.

- Cigarette smoke exposure down-regulates neurotrophin gene expression and protein secretion by lung fibroblasts in vitro, which may reflect one mechanism by which neurotrophin levels are reduced in human airways in response to smoke.
7. POPULÄRVETENSKAPLIG SAMMANFATTNING

Astma, sarkoidos och kronisk obstruktiv lungsjukdom (KOL) är tre lungsjukdomar, som har gemensamt, att de alla är av inflammatorisk karaktär. Kroppens immunceller har till uppgift att bekämpa infektioner, men de kan också, vid vissa sjukdomstillstånd, reagera mot ”ofarliga” substanser, vilket är fallet vid allergisk astma. Vid sarkoidos ses en inflammation i lungorna, som tros bero på exponering för ett ännu okänt ämne, som i flera studier föreslagits vara någon typ av mykobakterie. Inflammationen som ses vid KOL, orsakas i de flesta fall av cigarettrökning. Långdragen, s.k. kronisk, inflammation i luftvägarna, resulterar i skador av lungvävnaden. Detta tros orsakas av att substanser som utsöndras för att bekämpa infektioner, även skadar den friska lungvävnaden. För att fäka och återbilda den skadade vävnaden, initieras en läkningsprocess, som vid kronisk inflammation kan komma i obalans, vilket resulterar i ärrbildningar eller andra permanenta förändringar i vävnaden. Vid astma, KOL och ibland även sarkoidos, ses fibros och andra typer av vävnadsförändringar, vilket leder till att lungorna blir stelare, och lungfunktionen försvinner. Vid KOL förstärker dessutom de små alveolerna där gasutbytet sker, vilket leder till en mycket försämrad syreupptagningsförmåga.

Forskning bedrivs i syfte att bättre förstå mekanismer, som driver inflammation och vävnadsförändringar i astma, sarkoidos och KOL, och på så vis öka möjligheten till bättre behandlingsalternativ för patienter med dessa lungsjukdomar.

I denna avhandling studeredes en familj av tillväxtfaktorer, s.k. neurotrofiner, som har visats kunna påverka både nervceller, immunceller och andra vävnadsceller. Till familjen av neurotrofiner hör nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) och neurotrophin-3 (NT-3). Tidigare studier har visat att astmatiker har ökade halter av neurotrofiner i lungorna jämfört med friska personer, och man tror att detta kan bidra till 1) förvärra inflammationen, 2) göra luftvägarna mer lättretliga och 3) gynna fibrosbildning i luftvägarna.

Syftet med studierna i denna avhandling var att öka förståelsen kring neurotrofinernas betydelse vid inflammatoriska lungsjukdomar. Vi studerade huruvida förändrade nivåer av neurotrofiner ses i luftvägarna vid sarkoidos och KOL. Dessutom var syftet med studierna i denna avhandling att vidare förstå på vilket sätt neurotrofiner kan bidra till fibros i luftvägarna i samband med kronisk inflammation.

För att bestämma halter av neurotrofiner i luftvägarna hos patienter och friska individer analyserades s.k. bronksköljvätska. Denna vätska fås genom en undersökningsmetod som heter bronkoskopi, och som används på patienter i syfte att ställa sjukdomsdiagnos. Vid en bronkoskopipundersökning förs ett fiberoptiskt instrument ned i luftvägarna via näsan. Med bronkoskopet kan man visuellt undersöka, och ta prover från luftvägarna. Genom att skölja luftvägarna med koksaltlösning och sedan ta tillbaka denna bronksköljvätska (BAL-vätska) kan immunceller och andra lösliga substanser, däribland neurotrofiner, analyseras.

Resultaten i denna avhandling visade, att patienter med sarkoidos, i likhet med astmatiker, hade klart förhöjda halter av neurotrofinerna NGF och NT-3 i luftvägarna jämfört med friska individer. Vi visade också, att halterna av neurotrofiner var som högst i den patientgrupp, som löper högre risk att utveckla en kronisk sjukdom och fibros. Genom att studera små vävnadsprover tagna från luftvägarna på sarkoidospatienter kunde vi visa att granulomen...

Vidare visade resultaten i denna avhandling, att patienter med KOL hade markant lägre nivåer av NGF och NT-3 i luftvägarna jämfört med friska rökfria individer. Detta var ett överraskande fynd, då neurotrofiner generellt visats öka vid inflammation. Då också sänkta nivåer av NGF och NT-3 sägs hos friska rökare, gavs en indikation på att rökning i sig påverkade neurotrofinhalterna i luftvägarna. För att undersöka hypotesen, att neurotrofinproduktion av vävnadsceller i lungorna påverkas av cigarettröksexponering, studerades fibroblaster. Dessa vävnadsceller har tidigare visats kunna vara en potentiell källa till neurotrofiner i lungorna. På laboratoriet odlades lungfibroblaster, och de exponerades för cigarettröksextrakt, och vi kunde visa att denna behandling sänkte cellernas produktion av NGF och NT-3. Sammantaget tyder dessa resultat på att långvarig exponering för cigarettrök sänker neurotrofinhalterna i luftvägarna, och att detta kan ske via effekter på fibroblaster. En avsaknad av neurotrofiner i luftvägarna skulle kunna bidra till en obalanserad läkningsprocess, och därmed bidra till vävnadsförändringar som ses vid KOL.

8. ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to all colleagues, collaborators, friends and family, who have supported me with throughout the work of this thesis;

My supervisor Caroline Olgart Höglund. Thank you for believing in me from the start, and taking me on as your PhD student. You have taught me how to make good science; from planning of projects and experiments, to critical analysis and writing of manuscripts. I’m very grateful for the freedom you have given me and for letting me take great part in every phase of a research project. Your everlasting enthusiasm and ambition is a true inspiration!

My co-supervisors Johan Grunewald and Magnus Sköld. It has been a great pleasure to have you both as my supervisors. Thank you for all your professional support and engagement throughout my work. You have contributed with your vast clinical knowledge, which has had a great impact on my projects.

Anders Eklund. Thank you for your personal support throughout my years at the lung lab, and for contributing with your great clinical knowledge.

To all my collaborators and co-authors who have supported me with clinical samples, methodological expertise and important lab work; Anders Blomberg, Ester Roos-Engstrand, Anders Eklund, Magnus Löfdahl, Anders Planck, Göran Elmberger, Lee Newman, Julius Kломinek, Per Eriksson, Cecilia Kemi, Farah Idali, Mikael Mikko, Maria Wikén, Marija Kramar, and Helga Haugom-Olsen.

To all colleagues at the lung clinic; Emma Karlsson, Margitha Dahl, Gunnel de Forest, Heléne Blomqvist, Stéphanie Mindus, Helga Haugom-Olsen and all other doctors and nurses working with the patients we do research for. Without you all, this work wouldn’t be possible!

All former and present members of the lung research lab: Especially thanks to my room-mate Maria for your friendship, for pep talks, support, and for sharing good times on conferences! Micke; thanks for your great friendship and for always spreading a positive attitude! Farah; thanks for sharing many years in the writing-room with me, I really appreciate your friendship. Marija; for fun times in the lab (snillena spekulerar!) and great friendship (see you in Bergshamra!). My PhD-friends Abraham, Betti, Annsofi, Lukas & Ernesto; for fun times together; in the lab, lunch-room and on conference-trips, I will miss it all! Benita D., Benita E., Janne, Åsa, Magnus, Cecilia (for setting the stage for me and for teaching me how to do a perfect ELISA), Ulrika and Malin (for showing me that it’s possible to be a mum and a PhD-student at the same time 😊), Karin, Kia, Fariba, Lotta, Marianne, Tove, Mahyar, Muntasir, Pernilla, Kie, Luca, Adrian & Longping. Thank you all for creating the great work place and positive atmosphere that we have in the lab!
To my dear friends outside the lab; **Marina & Rina** (house, husband, children, car, doctor’s hat... are we grown up now?), **Helena, Maria, Catarina, Jessica** (La la laaa!), **Lars** (writing your thesis in “Sörös” isn’t so bad!) & **Fredrik** (va’ bli re?). Together with your wonderful families, you all contribute into making my life so joyful!

My “Research-school buddies” **Sandra, Aurelija, Sofia, Sara & Anna**. Thanks for great times together at brunches, weddings, dissertation-parties (finally my turn!) and much more, let them continue!

To my Australian “parents” **Darlene & Paul**; This is what I’ve been doing during the last 5 years (and it’s written in English especially for you 😊). Thank you for always keeping your home open for me and my family, I hope we can come by again soon. **I love you!**

Ett stort tack till min kära svärmor **Valéria**; din hjälp i vardagen har varit ovärderlig det senaste året! Tack också till **Lászlo och Andreas**.

Till min familj som fyller mitt liv med så mycket glädje och kärlek. **Mamma och Pappa**; Tack för all er kärlek, och för ert innerliga stöd, som jag alltid känner från er. **Jag älskar er!** Till mina storebröder och förebilder **Peter, Thomas och Stefan**; titta vad lillasyster kan! **Maria, Catrin, Sandra** och alla mina underbara syskonbarn **Erika, Kajsa Hanna, Wilma, Jonna, Sofia, Sasha och Sebastian**.

Min största tacksamhet går till min älskade man **Markus**. Tack för att du alltid finns vid min sida. Ditt stöd har gjort att jag kommit dit jag är idag, och för det är jag evigt tacksam. Tack för att du gett mig det finaste vi har, vår dotter **Matilda**; mitt hjärtas solsken! Jag älskar er båda innerligt!

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I am very grateful for the generous support, in forms of doctoral- and travel grants, from the Swedish Heart-Lung Foundation. The work in the present thesis was supported by additional grants from the Swedish Research Council, Swedish Heart-Lung Foundation, King Oscar II Jubilee Foundation, Swedish Society of Medicine, Swedish Asthma and Allergy Association, Stockholm County Council, Osher Center for Integrative Medicine at Karolinska Institutet, Magnus Bergvall’s Foundation, Tore Nilson’s Foundation, Torsten and Ragnar Söderberg’s Foundations, Åke Wiberg’s Foundation, Mats Kleberg Foundation, and Karolinska Institutet.
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