From the Department of Medicine Solna
Division of Respiratory Medicine
Karolinska Institutet, Stockholm, Sweden

Studies on Neuroimmune Interactions in Allergic Inflammation

with Focus on Neurotrophins

Cecilia Kemi

Karolinska
Institutet

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It’s only love
that gets you through

ABSTRACT

Allergic asthma is a chronic airway disease characterized by an eosinophilic inflammation, bronchoconstriction, increased mucus production and bronchial hyperreactivity. The disease involves several mediators and cell types and is associated with a Th2-mediated immune response.

Stress is a factor reported to deteriorate the allergic inflammation. Stress can influence the immune system by activating the HPA axis, resulting in release of glucocorticoids which could effects functions such as leukocyte trafficking and mediator release. These functions could be important for the course of the allergic inflammation.

NGF, BDNF and NT-3 are members of the neurotrophin family, and since they have important functions in both the nervous and immune systems they have been suggested to play a role as neuroimmune modulators. The neurotrophins are essential growth factors in the nervous system, and can also be produced by and activate inflammatory cells. Elevated neurotrophin levels have been found both in blood and locally in the airways of asthmatic subjects, and the levels have been shown to be elevated further following allergen exposure. The neurotrophins have also been linked to bronchial hyperreactivity. However, it is not completely established which cells in the airways that produce the elevated levels of neurotrophins. Further, the levels of NGF have been found to be elevated in healthy humans in response to stress, but it is unknown if stress affects other neurotrophins than NGF, such as BDNF, and whether the neurotrophins are regulated differently in allergic compared to healthy subjects in response to stress.

The first major focus of this thesis was to determine if bronchial smooth muscle cells (SMC) could be a source of the neurotrophins in the human airways, and how inflammatory cytokines might influence such production. This was studied by using an *in vitro* cell culture model. The second focus was to determine how stress could influence immune regulation, allergic inflammation and neurotrophin release, and the possible involvement of glucocorticoids in stress-evoked neuroimmune interaction. This was performed by utilizing both human and *in vivo* animal stress models.

It was shown that human bronchial SMC could produce NGF, BDNF and NT-3, and that the production was diffrentially regulated by inflammatory cytokines. In the human stress modell, stress increased the proportion of regulatory T-cells in both allergic and healthy subjects, whereas a decrease in blood NK cell numbers and Th1/Th2 cytokine ratio was observed in allergic subjects only. Furthermore, PBMC from asthmatic subjects released more BDNF than PBMC from healthy controls. In response to stress, the release of BDNF from PBMC increased in healthy controls, but not in asthmatic subjects. However, the levels of BDNF from asthmatic subjects at the stress period correlated positively to the levels of IL-5. In the allergic animal model, stress aggravated airway eosinophilia. The stress-induced eosinophilia was reduced when glucocorticoid release was inhibited. In response to stress, the levels of NGF decreased in the airways of non-allergic animals, whereas higher levels of NGF were detected in the airways of allergic compared to non-allergic animals during stress. In contrast to the eosinophils, the NGF levels were elevated when glucocorticoid release was inhibited.

The results indicate that in inflammatory conditions, human bronchial SMC may be a source of neurotrophins. Also, PBMC may be a source of neurotrophins, especially in allergic inflammation. Further, atopic and non-atopic subjects shared some immune changes in response to stress. However, other stress-induced immune changes are unique to atopic individuals, indicating that some pathogenic mechanisms in atopics may be more strongly affected by stress than others. Data also supports an increased eosinophilic airway inflammation and increased neurotrophin production in response to stress, and an involvement of glucocorticoids in these responses. Altogether, it is suggested that stress could contribute to an aggravated allergic inflammation, and that neurotrophins may be suggested as messengers in this response.

LIST OF PUBLICATIONS

The present thesis is based on the following papers, which will be referred to by their Roman numerals.

I. **Kemi, C.**, Grunewald, J., Eklund, A. and Olgart Höglund, C.
Differential regulation of neurotrophin expression in human bronchial smooth muscle cells.
Respiratory Research 2006, 7, 18

II. Olgart Höglund, C., Axen, J., **Kemi, C.**, Jernelöv, S., Grunewald, J., Müller-Suur, C., Smith, Y., Grönneberg, R., Eklund, A., Stierna, P. and Lekander, M.
Changes in Immune Regulation in Response to Examination Stress in Atopic and Healthy Individuals.
Clinical and Experimental Allergy 2006 36, 982-92

III. **Kemi, C.**, Lekander, M., Grunewald, J., Axén, J., Jernelöv, S., Müller-Suur, C., Smith, Y., Grönneberg R., Eklund A., Stierna, P., and Olgart Höglund, C.
BDNF expression in response to academic stress in asthmatic and healthy students.
Manuscript

IV. **Kemi, C.**, Georén, S., Grunewald, J., Stierna, P., and Olgart Höglund, C.
Effects of acute stress on airway inflammation and NGF levels in a murine model of allergic asthma.
Manuscript

CONTENTS

1 INTRODUCTION 1

1.1 GENERAL INTRODUCTION 1
1.2 PHYSIOLOGY OF THE AIRWAYS 1
1.3 ALLERGIC INFLAMMATION 2
1.3.1 Allergic rhinitis 2
1.3.2 Allergic asthma 3
1.4 INFLAMMATORY CELLS 3
1.4.1 Mast cells 3
1.4.2 Granulocytes 3
1.4.2.1 Eosinophils 3
1.4.2.2 Neutrophils 4
1.4.2.3 Basophils 4
1.4.3 Lymphocytes 4
1.4.3.1 T-lymphocytes 4
1.4.3.2 B-lymphocytes 5
1.4.3.3 Natural killer cells 6
1.4.4 Mononucelar phagocytes - Monocytes and macrophages 6
1.5 STRUCTURAL CELLS IN THE AIRWAYS 6
1.5.1 Smooth muscle cells 6
1.5.2 Epithelial cells 7
1.5.3 Fibroblasts 7
1.6 PROTEIN MEDIATORS IN ALLERGIC INFLAMMATION 8
1.7 NERVE MEDIATED CONTROL OF THE AIRWAYS 8
1.8 THE NEUROTROPHINS 9
1.8.1 Neurotrophin receptors 9
1.8.2 Neurotrophins in the nervous system 10
1.8.3 Neurotrophins in the immune system 10
1.8.3.1 Mast cells 10
1.8.3.2 Eosinophils 10
1.8.3.3 Lymphocytes 11
1.8.3.4 Monocytes/macrophages 11
1.8.3.5 Structural cells in the airways 11
1.8.4 Role of neurotrophins in allergic inflammation 11
1.9 PSYCHONEUROIMMUNOLOGY 12
1.9.1 Hypothalamic-pituitary-adrenal axis and the sympathetic-adrenal-medullary axis 12
1.9.2 Acute versus chronic stress 12
1.9.3 Effects of stress on the inflammatory system 13
1.9.4 Stress and neurotrophins 13

2 AIMS 14

3 MATERIAL AND METHODS 15

3.1 STUDIES ON NEUROTROPHIN PRODUCTION IN HUMAN BRONCHIAL SMOOTH MUSCLE CELLS (I) 15
3.1.1 Study material 15

3.1.2 Analysis methods *15*
3.1.2.1 mRNA quantification 15
3.1.2.2 Quantification of proteins by enzyme-linked immunosorbent assay 17
3.2 HUMAN STUDIES ON IMMUNE REGULATION AND NEUROTROPHIN LEVELS IN RESPONSE TO EXAMINATION STRESS (II-III) 18
3.2.1 Study material *18*
3.2.1.1 Study design 19
3.2.2 Analysis methods *19*
3.2.2.1 Stress, anxiety and stress hormones 19
3.2.2.2 Phenotyping of inflammatory cells and analysis of mediators in blood and upper airways ... 20
3.2.2.3 Bronchial hyperreactivity 21
3.2.2.4 Lung function tests 21
3.2.2.5 Exhaled nitric oxide 21
3.3 ANIMAL STUDIES ON AIRWAY INFLAMMATION AND NGF LEVELS IN RESPONSE TO ACUTE STRESS (IV) 22
3.3.1 Study material *22*
3.3.1.1 Study design 22
3.3.2 Analysis methods *22*
3.3.2.1 Bronchoalveolar lavage and bone marrow preparations 22
3.3.2.2 Phenotyping of inflammatory cells and analysis of neurotrophins in BALF and bone marrow. 23
3.4 STATISTICAL ANALYSIS 23
4 RESULTS AND DISCUSSION 24
4.1 STUDIES ON NEUROTROPHIN PRODUCTION IN HUMAN BRONCHIAL SMOOTH MUSCLE CELLS (I) 24
4.1.1 Expression and regulation of NGF, BDNF and NT-3 in bronchial SMC *24*
4.2 STUDIES ON IMMUNE REGULATION IN RESPONSE TO EXAMINATION STRESS (II) 26
4.2.1 Effect of examination on perceived stress and anxiety *26*
4.2.2 Effect of examination on subsets of white blood cells *26*
4.2.3 Effect of examination on cytokine production from white blood cells *27*
4.2.4 Effect of examination on exhaled nitric oxide, airway hypereactivity and lung function .. *28*
4.3 STUDIES ON NEUROTROPHIN LEVELS IN RESPONSE TO EXAMINATION STRESS (III) 28
4.3.1 Effect of stress on neurotrophin release from white blood cells *28*
4.3.2 Effect of stress on levels of neurotrophins in blood and upper airways *29*
4.3.3 Correlations of BDNF release to inflammatory mediators and bronchial hyperreactivity *29*
4.4 STUDIES ON AIRWAY INFLAMMATION AND NGF LEVELS IN RESPONSE TO ACUTE STRESS (IV). 30
4.4.1 Effect of allergen airway challenge on inflammatory cells and NGF *30*
4.4.2 Effect of restraint stress on inflammatory cells and NGF *31*
4.4.3 Effect of GC-release inhibition on inflammatory cells and NGF release in response to stress *31*
5 GENERAL DISCUSSION 33
5.1 AIRWAY SMOOTH MUSCLE CELLS AS PRODUCERS OF NEUROTROPHINS 33
5.2 POTENTIAL ROLES OF SMC-DERIVED NEUROTROPHINS 34
5.3 INFLUENCE OF STRESS ON IMMUNE REGULATION AND ALLERGIC INFLAMMATION 34
5.4 STRESS AND NEUROTROPHINS 37
5.5 CONCLUDING REMARKS 37

6 CONCLUSIONS 39

7 ACKNOWLEDGEMENTS 40

8 REFERENCES 42

LIST OF ABBREVIATIONS

ACTH	Adrenocorticotropic hormone
BAL	Bronchoalveolar lavage
BALF	Bronchoalveolar lavage fluid
BDNF	Brain-derived neurotrophic factor
BHR	Bronchial hyperreactivity
BM	Bone marrow
CNS	Central nervous system
COX	Cyclooxygenase
CRH	Corticotrophin-releasing hormone
DMEM	Dulbecco's Modified Eagle Medium
dNTP	Deoxyribonucleoside triphosphate
ECP	Eosinophil cationic protein
EDN	Eosinophil-derived neurotoxin
EPO	Eosinophil peroxidase
FEV1	Forced expiratory volume in one second
FVC	Forced vital capacity
GINA	Global Initiative for Asthma
GITR	Glucocorticoid-induced TNF receptor
GM-CSF	Granulocyte-macrophage colony stimulating factor
HLA	Human leukocyte antigen
HPA	Hypothalamic-pituitary-adrenocortical
IFN	Interferon
Ig	Immunoglobulin
IHC	Immunohistochemistry
IL	Interleukin
In	Intra nasal
Ip	Intra peritoneal
LT	Leukotriene
MBP	Major basic protein
NAL	Nasal lavage
NGF	Nerve growth factor
NT-3	Neurotrophin-3
NT-4/5	Neurotrophin-4/5
OVA	Ovalbumin
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PEF	Peak expiratory flow
PG	Prostaglandin
PNS	Peripheral nervous system
RPMI	Roswell Park Memorial Institute (culture medium)
SAM	Sympathetic-adrenal-medullary
SEM	Standard error of the mean
Th	T helper
TNF	Tumor necrosis factor
VAS	Visual Analogue Scale
VC	Vital capacity

1 INTRODUCTION

1.1 GENERAL INTRODUCTION

The main purpose of the respiratory system is to supply the body with oxygen and remove carbon dioxide. Inflammatory diseases of the airways, such as asthma, may disrupt this vital function. Hence, asthma, which affects more than 10 % of the population in western countries, is characterized by brochoconstriction, increased mucus production and bronchial hyperreactivity. Mechanisms regarding the underlying inflammation are still incompletely understood. Today, most asthma treatments are based on symptom relief and are not directed towards the cause of the disease.

Apart from anecdotes, stress has been shown increase the extent and number of asthma exacerbations (Rietveld et al., 2000; Sandberg et al., 2000). This implies a neuroimmune communication in asthma of importance for disease outcome. Therefore, an increased knowledge about the interplay between the nervous and immune systems are needed

The current work is focused on allergic inflammation, mainly in the airways, the role of neuroimmune interaction, such as psychological stress, on the inflammatory processes in allergic disease, and the role of neurotrophins as mediators in this response.

1.2 PHYSIOLOGY OF THE AIRWAYS

Via the nose, pharynx and larynx the airways extends to the trachea (Weibel and Taylor, 1998). Approximately in the middle of the thorax the trachea divides into two bronchi (primary bronchi, see *Figure 1*). The bronchi are further divided into secondary and tertiary bronchi, bronchiole, terminal bronchiole, respiratory bronchiole and ends in the alveoli after 23-24 generations. Gas exchange occurs by diffusion in the respiratory bronchiole and the alveoli (*Figure 1*). To keep the trachea and bronchi stable and open,

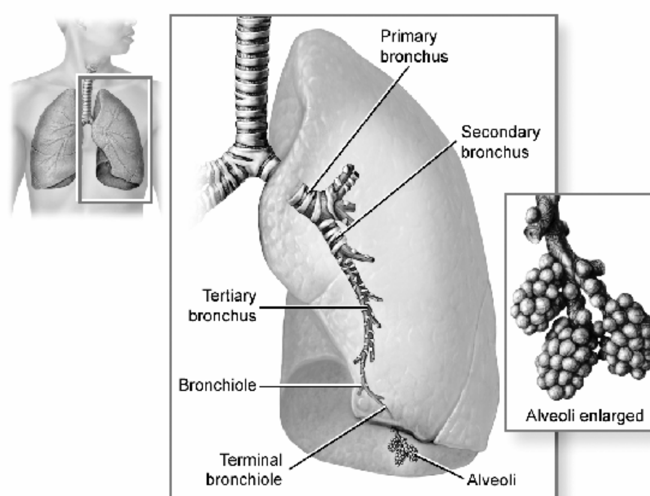

Figure 1: The human bronchial tree

cartilage is embedded in the airway wall, whereas the lower airways are kept open by negative pressure.

The inspiration is dependent on the diaphragm and other airway muscles, whereas expiration is spontaneous. The lung volume is measured by spirometry, which is commonly divided into static and dynamic spirometry. Static spirometry measures lung volumes, such as the vital capacity. Dynamic spirometry measures air flow, and can be used to determine peak expiratory flow (PEF), forced vital capacity (FVC) and forced expiratory flow in one second (FEV_1). Spirometry can determine if airways are obstructed or restricted. During obstructive lung disorders such as asthma, the normal passages of air is prevented, whereas restrictive airway disorders, such as emphysema, are caused by a reduced amount of respiratory tissue, or a prevented expansion of the lungs.

1.3 ALLERGIC INFLAMMATION

Allergy, from the Greek "allos" meaning changed or altered state and "ergon" meaning reaction, has become a common clinical diagnosis. Allergic diseases have strong genetic components, but do not follow simple monogenetic patterns of inheritance (Barnes et al., 2003). Also the environment can influence the allergic disease. Allergic inflammation is initiated in susceptible individuals following exposure to an allergen. Allergens are relatively small, soluble proteins (Janeway et al., 2001), and the most common allergens in Sweden include birch and grass pollen, pet dander and mite feces. Inhalation is the most common route of allergen entry, resulting in allergic rhinitis and/or allergic asthma. In susceptible individuals, the first encounter with an antigen results in a production of allergen specific immunoglobulin (Ig)E antibodies by B-cells, a production driven by interleukin (IL)-4 and IL-13 from Th2-lymphocytes. The production of Th2-associated cytokines can be inhibited by IFN-γ produced by Th1-cells. The IgE antibodies bind to the FcεRI receptor on the cell surface of cells such as mast cells and basophils, and allergic reactions are triggered by cross-linking of these IgE antibodies. An allergic reaction can be divided into an early and a late-phase response. The early reaction starts within seconds following allergen contact, and is due to the release of pre-formed histamine and other mediators from mast cells and basophils following IgE cross-linking. This release causes a rapid contraction of smooth muscle and an increased vascular permeability. The late-phase reaction takes 6-12 hours to develop, and is caused by induced synthesis of mediators, including IL-5, which attracts inflammatory cells such as eosinophils to the site of the reaction. The cells and mediators involved in the allergic inflammation are described further in section 1.4-6.

1.3.1 Allergic rhinitis

Allergic rhinitis is an allergic inflammation of the nasal passages, usually characterized by symptoms such as repetitive sneezing, rhinorrhea, post-nasal drip, nasal congestion and pruritic eyes, ears, nose or throat(Van Cauwenberge et al., 2000). The inflammation in rhinitis is related to, and often occurs with allergic asthma

1.3.2 Allergic asthma

The characteristic symptoms of asthma include wheeze, chest tightness, breathlessness, and cough. Bronchial hyperreactivity underlies much of the symptomatology of asthma, such as airway narrowing, and may be tested by bronchial provocation using aerosols of metacholine or histamine or other substances that stimulates bronchoconstriction (Barnes et al., 2003). Other pathological alterations in asthma include thickening and remodeling of the airway wall, which can reach 300% its normal thickness (Jeffery, 2003). Several factors contribute to the thickening and remodeling of the airways during asthma, such as increased smooth muscle cell mass, thickening of the reticular basement membrane and dilatation of mucosal blood vessels. Other hallmarks of asthma include increased mucus production and a damaged and shedded epithelium. Damage of the epithelium is probably an early feature of asthma, and the loss of epithelia appears to correlate to the degree of hyperresponsiveness in the airways (Jeffery et al., 1989).

1.4 INFLAMMATORY CELLS

The inflammation and remodeling observed in allergic asthma and rhinitis is orchestered by a number of different cells and mediators.

1.4.1 Mast cells

Mast cells reside in all normal tissues, where they are important for wound healing and host defense (Galli, 2000). In asthmatic airways, mast cells localize with smooth muscle cells (see section 1.5.1), submucosa, mucosal glands and epithelium (see section 1.5.2)(Bradding et al., 2006). Mast cells are one of the key cells in the early allergic response. The dominant signal for mast cell activation in allergy is cross-linking of allergen specific IgE antibodies on the mast cell surface, but mast cells can also be activated by neuropeptides, including substance P (Hart, 2001). Upon activation, the mast cells release their granule, containing histamine, prostaglandins and leukotrienes, which in the airways are capable of inducing bronchoconstriction, mucus secretion and mucosal edema (Bradding et al., 2006). Mast cells also synthesize and secrete a large number of cytokines, such as IL-4, IL-5 and IL-13.

1.4.2 Granulocytes

1.4.2.1 Eosinophils

About 0.1-7.0% of the leukocytes in human blood are eosinophils. Traditionally the eosinophils have been thought to be important in host protection against parasites, but the levels of eosinophils increase in blood and tissue also following allergy and asthma (Rothenberg and Hogan, 2006). The granule of eosinophils contains four different cytotoxic molecules, eosinophils peroxidase (EPO), major basic protein (MBP), eosinophil cationic protein (ECP) and eosinophil-derived neurotoxin (EDN), along with a variety of cytokines, chemokines and leukotrienes, including IL-4, IL-5, eotaxin and RANTES, which are released upon activation. In the asthmatic airways, this release result in bronchial hyperreactivity, which in turn can promote contraction of smooth muscle cells, mucus production and vascular permeability. The number of eosinophils in the airways are generally low, but increase in response to allergic asthma, and the

numbers of eosinophils in the airways have been associated with the clinical severity of the asthmatic disease (Bousquet et al., 1990; Vignola et al., 1998).

1.4.2.2 Neutrophils

The majority of the leukocytes in the blood, 45-74 % are neutrophils. Neutrophils are part of the innate immune system, and the essential role for neutrophils is to phagocytose foreign material (Janeway et al., 2001). Elevated numbers of neutrophils can be found at sites of infection, and the main chemoattractant for neutrophils is IL-8. Elevated levels of neutrophils can be found in patients with clinically severe asthmatic disease (Beeh and Beier, 2006).

1.4.2.3 Basophils

Less than 1 % of the leukocytes in blood are basophils. The basophils fulfill a role in the host defense against parasites (Galli et al., 2002). Like for mast cells, the activation signal for basophile is cross-linking of IgE bound to the cell surface via the high affinity FcεRI receptor. The granule basophils release upon activation contains histamine, leukotrienes and cytokines, including IL-4 and IL-13.

1.4.3 Lymphocytes

Lymphocytes help to mount the adaptive immune response against virtually any foreign antigen (Janeway et al., 2001). This is possible because each maturing T and B-lymphocyte bears a unique antigen-receptor. The adaptive immune response can also provide protection from re-infection of the same pathogen. In humans, lymphocytes constitute approximately 16-45% of the leukocytes in blood.

1.4.3.1 T-lymphocytes

T-cells start their development in the bone marrow and complete it in the thymus, whereafter they enter the blood stream. T-cells can be divided into two different groups dependent on their receptors and effects; $CD4^+$ T-helper (Th) cells and $CD8^+$ cytotoxic (killer) T-cells (Janeway et al., 2001).

1.4.3.1.1 $CD4^+$ T-helper cells

When a naïve $CD4^+$ T-cell encounter its antigen for the first time, it differentiates into either a T-helper type 1 (Th1) or T-helper type 2 (Th2) cell (Janeway et al., 2001). This differentiation is mainly regulated by cytokines and dendritic cells (Georas et al., 2005). Production of IL-12 from dendritic cells promotes differentiation of Th1 cells. When IL-12 is absent, naïve T-cells produce IL-4, promoting Th2 cell differentiation. In addition can $CD4^+$-cells differentiate into regulatory T-cells (see section 1.4.3.1.2). Th1 cells mainly produce IFN-γ, which is important for activation of macrophages and NK-cells (Janeway et al., 2001). Th2 cells produce IL-4, IL-5 and IL-13 which activates mast cells and eosinophils and induce production of IgE antibodies from B-lymphocytes (see section 1.4.3.2). In asthmatic patients, the number of $CD4^+$ T-cells increase in the airways, and the majority of these are Th2 (Georas et al., 2005). Therefore, asthma is regarded as a Th2-driven disease, and the levels of Th2-associated cytokine increase in asthmatic patients compared to healthy controls.

1.4.3.1.2 Regulatory T-cells

Regulatory T-cells were previously called suppressor T-cells, due to their ability to suppress other T-cell mediated immune responses. Both natural and induced populations of regulatory T-cells exist, and they probably have overlapping functions in the control of the immune response (Mills, 2004). Natural, or $CD4^{+}CD25^{bright}$ regulatory T-cells, develop in the thymus, while T_R1 and Th3 cells are generated from naïve T-cells in the periphery, following an antigen encounter. Most likely, the suppression by $CD4^{+}CD25^{bright}$ regulatory T-cells is mediated by cell-cell contact, whereas T_R1 and Th3 suppress immune function by secretion of IL-10 and TGF-β, respectively.

A role for natural regulatory T-cells in atopic disease has been implied, since natural regulatory T-cells have been shown to decrease the levels of eosinophils in the airways and modulate the production of cytokines (Suto et al., 2001; Curotto de Lafaille and Lafaille, 2004). In addition, natural regulatory T-cells from atopic subjects have been suggested to be dysregulated, since they have been shown to be less efficient in inhibiting Th2-associated cytokine production, than natural regulatory T-cells from healthy individuals (Grindebacke et al., 2004; Ling et al., 2004). In animal models of allergic airway disease, natural regulatory T-cells have in addition been shown to reduce bronchial hyperreactivity (Kearley et al., 2005).

Activated non-regulatory T-cells can also express CD25 on their surface, although the expression of CD25 on natural T-regulatory cells generally is higher, hence the term "$CD25^{bright}$". However, apart from the high expression of CD25 on the cell surface, natural regulatory T-cells can also be characterized by the transcription factor Foxp3, which represents a more specific marker for natural regulatory T-cells. Natural regulatory T-cells can in addition be characterized by cell-surface markers, including CTLA-4, CD45RO, CD38 and GITR (van Oosterhout and Bloksma, 2005). However, like CD25, none of these markers are able to completely discriminate between natural regulatory T-cells and conventional T-cells.

1.4.3.1.3 $CD8^{+}$-cytotoxic T-cells

$CD8^{+}$ T-cells protect the host from virus and cytoplasmic bacteria by inducing apoptosis of infected cells (Janeway et al., 2001). Cells undergoing apoptosis are rapidly digested by nearby phagocytic cells (see section 1.4.4). Apart from the secretion of perforin and granzymes which induce apoptosis, $CD8^{+}$ T-cells also release cytokines such as IFN-γ, TNF-α and TNF-β, who also contribute to host defense by inhibition of viral replication and activation of phagocytic cells. $CD8^{+}$ T-cells have been described as suppressors of airway inflammation and AHR, however, the levels of $CD8^{+}$ T-cells are elevated in cases of fatal asthma, and the expression of IL-4 is higher in $CD8^{+}$ T-cells from atopics compared to healthy donors (Gelfand and Dakhama, 2006).

1.4.3.2 B-lymphocytes

The B-lymphocyte is the only cell capable of producing antibodies, and it is therefore essential in the humoral immune response (Ganoug, 2005). For activation, B-lymphocytes are dependent on activated Th2 cells. Upon activation, the B-lymphocyte proliferates and transform into plasma cells, which secrete large number of antigen

specific antibodies into the circulation. IgE antibody production, which has a central role in the allergic response, by B-lymphocytes requires activated Th2 cells that produce IL-4 and IL-13 (Janeway et al., 2001).

1.4.3.3 Natural killer cells

Natural killer (NK)-cells lack antigen receptors and are part of the innate immune system (Janeway et al., 2001). Activated NK-cells help to control viral and intracellular bacterial infections. IFN-α, IFN-β and IL-12 activates NK-cells, which kill infected cells by inducing apoptosis. In asthmatic individuals, the role of NK-cells is far from clear, but besides the cytotoxic properties of NK-cells, activated NK-cells rapidly produce large amount of IFN-γ, IL-4 and IL-13, by which they could regulate an allergic inflammation (Dombrowicz, 2005).

1.4.4 Mononucelar phagocytes - Monocytes and macrophages

About 4.0-10% of the leukocytes in human blood are mononuclear phagocytes. When the monocytes enter extravascular tissue, they differentiate into macrophages (Janeway et al., 2001). The main purpose of the macrophage is to remove foreign material from the body, which is performed by phagocytosis. Th1 cells can activate macrophages to increase their antimicrobial effectiveness, and apart from their role as phagocytes, macrophages are also important for recruiting other inflammatory cells to the site of an infection by secretion chemokines such as IL-8. Because of their effectiveness, dysregulated macrophages can contribute to a variety of diseases, including diseases in the airways such as alveolitis, by recruitment of inflammatory cells as well as release of oxygen radicals and proteolytic enzymes (Sibille and Reynolds, 1990).

1.5 STRUCTURAL CELLS IN THE AIRWAYS

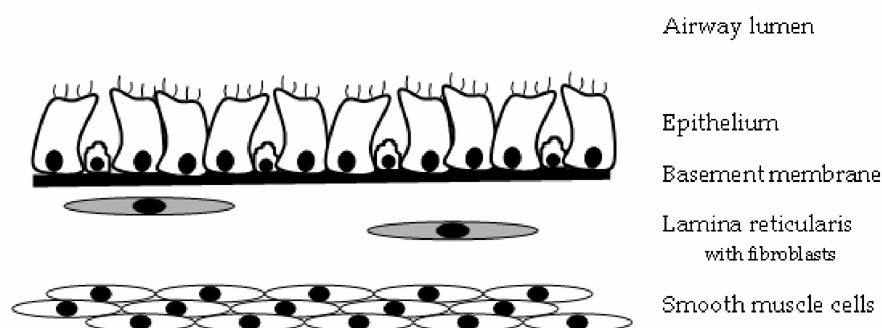

Figure 2: Structural cells in the airway wall

1.5.1 Smooth muscle cells

Since airway smooth muscle cells (SMC, *Figure 2*) mediates contraction of the airways during the asthmatic attack, they are considered as a key tissue in allergic asthma. In an individual with allergic asthma, the airway SMC layer is increased, mainly due to hyperplasia, but also hypertrophy (Ebina et al., 1993). A dysregulation in the airway

SMC from asthmatics is also illustrated in the enhanced proliferation compared to SMC from healthy controls (Johnson et al., 2001). It has been reported that SMC from asthmatics have an increased contractile response to histamine, compared to healthy controls (Bai, 1991).

Apart from bronchoconstriction, the airway SMC also participates in airway inflammation and remodeling in the asthmatic airway by production of various inflammatory mediators (Black et al., 2003; Lazaar and Panettieri, 2005). Hence, airway SMC are capable of producing a variety of chemokines, including RANTES, eotaxin and IL-8, which can recruit leukocytes, such as mast cells and lymphocytes into the airways (Lazaar and Panettieri, 2005). In addition to chemokines, airway smooth muscle cells can produce cytokines, including IL-5, and components of the extracellular matrix, including collagen type I and IV (Panettieri et al., 1998).

1.5.2 Epithelial cells

The epithelium lining (*Figure 2*) of the respiratory tree consists of several cell types. The upper bronchi are mainly lined with ciliated epithelial cells, mucus producing goblet cells and basal cells. In the bronchioli few goblet cells are present, whereas secretory Clara cells appear. In the most distal part of the lung, the alveoli are lined with type I and type II cells. Type I cells facilitates gas exchange, wheras type II cells produce surfactant which prevents the alveoli from collapsing, and in addition has a role in immune defense.

In addition to constituting a first barrier and protection against foreign materials, the epithelial cells can contribute to the regulation of inflammation in the lung, by releasing cytokines and chemotactic factors, including IL-5, IL-8 and granulocyte-macrophage colony stimulating factor (GM-CSF). Thereby have epithelial cells a potential to regulate which inflammatory cells that are recruited into the lungs. Epithelial cells depend on a number of adhesion molecules for their adhesion to each other as well as the basal membrane (Albelda, 1991). The shredding of the epithelium which can be observed in asthma could be due to impaired effect of this adhesion. Damage in the epithelial cell layer could expose nerv endings. Stimulation of these nerve endings by inflammatory mediators could release neuropeptides promoting smooth muscle contraction and mucus secretion. See section 1.7 for details on nerve mediated control of the airways.

1.5.3 Fibroblasts

Fibroblasts (*Figure 2*) produce extracellular matrix proteins, including collagens and fibronectin, which are important both for structure as well as tissue repair and remodeling. The fibroblasts are also able to produce inflammatory mediators, including IL-1, IL-8 and TGF-β, and are important for both the inflammatory and remodeling processes in the airways (Sime et al., 2000). A role of the fibroblast in the pathogenesis of asthma has been suggested, partly because it has been shown that fibroblast from asthmatic subjects express more matrix metalloproteinases and tenascin compared to fibroblasts from healthy subjects, which could contribute to an increased remodeling of the airways of asthmatics (Nakamura et al., 2004).

1.6 PROTEIN MEDIATORS IN ALLERGIC INFLAMMATION

Cytokines are extra cellular signaling proteins. They predominantly function in a paracrine manner, although endocrine and autocrine effects have been reported (Barnes et al., 2003). The effects of cytokines on target cells vary, not only depending on the target cell type, but also its maturity. In the allergic inflammation, cytokines are expressed both at the acute- and chronic phase of the inflammation. TNF-α, IL-1β and IL-6 are sometimes referred to as the "first wave" cytokines, due to their swift release in response to inhaled allergens or other inflammatory stimuli. They can be released from a number of cells, but macrophages are central producers. TNF-α, IL-1β and IL-6 may then act on other cells, such as epithelial cells, to release a second wave of cytokines, including IL-5 and GM-CSF, as well as the chemokines, IL-8 and RANTES. These mediators attract cells, such as T-cells and eosinophils, to the site of the inflammation. The recruited cells then play important roles in the late inflammatory response by their release of cytokines.

Cytokines of special interest in the allergic inflammation include IL-4, IL-5 and IL-13. IL-4 and IL-13 are derived from Th2 cells and mast cells, and promote B-lymphocytes to switch to IgE-production. IL-4 also increases the expression of adhesion molecules on epithelial cells, thereby aiding further recruitment of inflammatory cells. IL-5 is also produced by Th2-cells, and is important for eosinophils differentiation, activation and survival. The function of Th2-derived cytokines is counteracted by Th1 associated cytokines, such IFN-γ, which has an inhibitory effect on Th2-cells. IFN-γ may also have pro-inflammatory effects, and stimulate cytokine release from both epithelial cells and macrophages. IL-2 functions as an autocrine growth factor for Th1 cells.

1.7 NERVE MEDIATED CONTROL OF THE AIRWAYS

The peripheral nervous system (PNS) provides an interface between the central nervous system (CNS) and the periphery (Willis, 1993). The airways are innervated by neurons leading information both to and from the CNS. Sensory neurons lead information from the epithelium, lung parenchyma, SMC and submucosa to the CNS, whereas the autonomous nervous system leads information from the CNS, via autonomic ganglia to effector cells. The autonomous nervous system can be divided into to two major parts; the sympathetic and the parasympathetic nervous systems. Roughly, the sympathetic nervous system signals through release of norepinephrine, binding to α or β adrenergic receptors on the target cells, which include SMC, glands and blood vessels. The parasympathetic system releases acetylcholine, which binds to muscarinic (M) receptors on glands and SMC. In the airways, the cholinergic nerves are the dominant pathway for bronchoconstriction of airway SMC, whereas the adrenergic system mediates relaxing signals. Nerve cells can also communicate by release of neuropeptides, such as the tachykinin substance-P. Apart from acting as neurotransmittors, neuropeptides can act as neuromodulators by changing the amount of neurotransmitter released, or the response of the transmittor. In the airways, neuropeptides can stimulate increased mucus secretion, blood vessel permeability, and possibly smooth muscle contraction. In addition, there is a complex interaction between neurons and inflammatory cells in the airway, where inflammatory mediators have modulatory effects on neurotransmission and neurotransmitters, and vice versa.

In allergic asthma, the bronchial hyperreactivity might increase due to a sensitization of sensory neurons by inflammatory mediators, unprotected neurons due to damaged epithelium, increased release of neurotransmitters and/or increased neurotransmitter receptor density (Barnes et al., 2003). In addition have the number of substance-P-containing nerve fibers been found to be increased in subjects with fatal asthma (Ollerenshaw et al., 1991).

1.8 THE NEUROTROPHINS

In 1952, Levi-Montalcini showed that a potent growth of sensory and sympathetic nerves was induced when tumors from mice were transplanted to chick embryos (Levi-Montalcini, 1952). Since the outgrowth did not require direct contact, it was concluded that the tumors released a nerve growth-promoting factor which had a selective action on certain types of nerves. The substance was named nerve growth factor (NGF), and since its discovery in birds, it has been found also in mammals, reptiles, amphibians and fish (Lewin and Barde, 1996). Since the numbers of neurons depending on a growth factor for normal development excided the restricted neuronal specificity of NGF, the existence of other neuronal growth factors was early hypothesized. In 1989, brain-derived neurotrophic factor (BDNF) was cloned as the second member of the neurotrophin family (Leibrock et al., 1989). Today, the neurotrophin family in mammals also consists of neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5). Neurotrophin-6 and neurotrophin-7 have also been identified, but only in fish (Gotz et al., 1994; Lai et al., 1998). The neurotrophins are dimers of two identical subunits, linked together by non-covalent bonds, and are highly conserved through evolution. BDNF and NT-3 are more conserved than NGF, which in turn is more conserved than NT-4/5 (Gotz et al., 1992; Gotz and Schartl, 1994). In mammals the neurotrophins are all synthesized as precursors, and are processed either inside the cell by furin or pro-hormone convertases, or extracellularly by plasmin or matrix metalloproteinases, to produce mature neurotrophins (Allen and Dawbarn, 2006). The mature neurotrophins have a weight of about 25 kD.

1.8.1 Neurotrophin receptors

To exert their effects, neurotrophins use two types of receptors, the tyrosine kinase receptors (Trk) and the p75 neurotrophin receptor (p75NTR) (Kaplan and Miller, 2000). p75NTR was the first functionally described neurotrophin receptor (Johnson et al., 1986). It binds all neurotrophins (Rodriguez-Tebar et al., 1992), and belong to the tumor necrosis factor receptor gene family (Naismith and Sprang, 1995). Interestingly, while Trk receptors only transmit signals for enhanced survival, activation and growth, whereas p75NTR can transmits signals for both activation and survival, as well as apoptosis (Kaplan and Miller, 2000).

Three Trk receptors have been identified: TrkA, TrkB and TrkC. NGF binds to TrkA (Kaplan et al., 1991; Klein et al., 1991a), while BDNF and NT-4/5 bind to TrkB (Klein et al., 1991b; Soppet et al., 1991; Squinto et al., 1991; Klein et al., 1992). NT-3 can bind to all Trk receptors, but preferably to TrkC (Ryden and Ibanez, 1996). Neurotrophin binding the Trk-receptor stimulate Trk-dimerization (Jing et al., 1992) and tyrosine transphosphorylations, leading to activation of signal transduction pathways such as the Ras/Raf/Map-kinase pathway, which mediates neurite outgrowth,

and the PI-3K/Akt pathway which mediates important anti-apoptotic signals (Kaplan and Miller, 2000). The p75NTR signalling pathways include activation of NF-κB, which mediates cell survival (Khursigara et al., 1999), and the JNK-p53-Bax-pathway, which induces apoptosis (Kaplan and Miller, 2000).

The p75NTR and the neurotrophin Trk-receptors can act individually, but can also interact on several levels, for example have p75NTR been shown to enhance tyrosine phosphorylation of TrkA (Verdi et al., 1994).

1.8.2 Neurotrophins in the nervous system

As mentioned earlier, neurotrophins have an important role in neuronal survival and maintenance (Lindholm et al., 1997), but also in response to brain injury (Lindvall et al., 1994). Studies on knockout mice show that the neurotrophins are necessary for a normal development of the CNS (Crowley et al., 1994; Jones et al., 1994). The neurotrophins have been suggested to be involved in a number of diseases involving the nervous system, such as Alzheimer's disease which has been associated with a decrease in both NGF and BDNF in the CNS (Connor et al., 1997; Hellweg et al., 1998), but also in the blood (Schaub et al., 2002). Decreased levels of BDNF have also been associated with depression (Allen and Dawbarn, 2006). In addition, increased production of neurotrophins in the CNS been implied to suppress inflammation also in the neurodegenerative disorder multiple sclerosis (Villoslada and Genain, 2004).

1.8.3 Neurotrophins in the immune system

Although the neurotrophins were first described in the nervous system, it is now clear that they have important functions also in the immune system. Production of at least one of the neurotrophins, as well as the expression of various neurotrophin receptors have been described for most inflammatory cells, including mast cells, eosinophils, lymphocytes and macrophages who have important functions in inflammation, including allergic asthmatic disease. Furthermore, expression of neurotrophins and their receptors have been shown for structural cells, such as smooth muscle, epithelium and fibroblasts.

1.8.3.1 Mast cells

Mast cells are able to produce the neurotrophins NGF, BDNF and NT-3 (Leon et al., 1994; Tam et al., 1997). Mast cells express TrkA constitutively, and NGF is important for mast cell survival and differentiation (Matsuda et al., 1991; Kawamoto et al., 1995). In addition, NGF can function as a chemoattractant for mast cells (Sawada et al., 2000), and regulate mast cell degranulation (Bruni et al., 1982; Pearce and Thompson, 1986; Horigome et al., 1993).

1.8.3.2 Eosinophils

Eosinophils have been shown to produce NGF (Solomon et al., 1998) and express all neurotrophin receptors (Nassenstein et al., 2003). The neurotrophins have been shown to have anti-apoptotic effects on eosinophils from asthmatic airways (Nassenstein et al., 2003), and NGF has been shown to enhance eosinophilic cytoxicity (Hamada et al., 1996). In addition, it has been shown that more eosinophils are recruited to the airways

in models of allergic airway inflammation that over-express NGF in the airways (Path et al., 2002).

1.8.3.3 Lymphocytes

Lymphocytes are able to produce NGF, BDNF, NT-3 and NT-4/5, but the production is often dependent on cell activation (Besser and Wank, 1999; Kerschensteiner et al., 1999; Moalem et al., 2000). Neurotrophin-receptor expression has been reported in both T- and B-lymphocytes (Ehrhard et al., 1993; Brodie et al., 1996; Melamed et al., 1996; Besser and Wank, 1999), and NGF can induce both proliferation and differentiation of lymphocytes (Thorpe and Perez-Polo, 1987; Otten et al., 1989).

1.8.3.4 Monocytes/macrophages

Monocytes/macrophages have been shown to produce NGF, BDNF and NT-4/5 (Schober et al., 1998; Braun et al., 1999; Caroleo et al., 2001), and monocytes from allergic patients produce more NGF than monocytes from healthy donors (Rost et al., 2005). Immunohistochemical studies have revealed NGF expression, as well as expression of TrkA, TrkB and TrkC in alveolar macrophages (Ricci et al., 2004).

1.8.3.5 Structural cells in the airways

Apart from the inflammatory cells, neurotrophins and their receptors have been shown also on structural cells. Hence, in humans both *in vivo* and *in vitro* studies have shown that airway epithelial cells and SMC can express NGF, BDNF and NT-3 (Fox et al., 2001; Pons et al., 2001; Freund et al., 2002; Olgart Hoglund et al., 2002; Ricci et al., 2004). Airway fibroblasts have been shown to express NGF (Olgart Hoglund et al., 2002). SMC have also been shown to express the TrkA, TrkB and TrkC receptors, as well as p75NTR, and can hence be activated by neurotrophins (Ricci et al., 2004).

1.8.4 Role of neurotrophins in allergic inflammation

A pathological role for the neurotrophins has been suggested in both allergic rhinitis and allergic asthma, since affected subjects exhibit elevated levels of neurotrophins both in the blood and locally in the airways (Bonini et al., 1996; Sanico et al., 2000; Olgart Hoglund et al., 2002). In asthmatics, these levels have been shown to increase further following allergen challenge (Virchow et al., 1998; Sanico et al., 2000; Kassel et al., 2001; Nassenstein et al., 2003).

Elevated levels of neurotrophins have been suggested to contribute to the allergic pathology in several ways. NGF and BDNF may contribute to the development of bronchial hyperreactivity (BHR), a hallmark in asthma, as evident from animal studies (de Vries et al., 1999; Braun et al., 2004). This hyperreactivty has been shown to be neurokinin-1-dependent, suggesting involvement of tachykinins, such as substance-P (de Vries et al., 1999). In support of a role for NGF in the allergic response, over expression of NGF has been demonstrated to increased bronchoconstriction following allergen challenge in an animal model of allergic asthma, whereas anti-NGF treatment was shown to inhibit bronchoconstriction (Path et al., 2002). The neurotrophins may also increasing the neuropeptide content and the number of sensory neurons innervating the airways (Hoyle et al., 1998; Hunter et al., 2000).

Since the inflammatory cells invading the lung following allergen challenge have been shown to express neurotrophin receptors, they could be influenced by the elevated levels of neurotrophins. Hence, previous studies have shown that airway eosinophils from asthmatic have an increased expression of neurotrophin receptors compared to blood eosinophils (Nassenstein et al., 2003). This increased expression was associated with an enhanced survival and an activation. NGF has also been shown to increase tissue remodeling processes by inducing fibroblast migration and differentiation into myofibroblasts (Micera et al., 2001).

However, even though a number of cells outside the nervous system have been shown to be able to produce neurotrophins, it is still not fully established which cells that stands for the elevated levels of neurotrophins found in the airways of asthmatics, and how this production is regulated.

1.9 PSYCHONEUROIMMUNOLOGY

The nervous system, the endocrine system and the immune system are all complex systems that interact with each other to endorse homeostasis. The interplay among these systems can be affected stress. Stress has been defined as a process 'in which environmental demands tax or exceeds the adaptive capacity of an organism' (Cohen et al., 1995), resulting in psychological and biological changes that may place a person in risk of disease. A stressor, such as fear or environmental changes, is the stimuli activating this process.

1.9.1 Hypothalamic-pituitary-adrenal axis and the sympathetic-adrenal-medullary axis

In response to stress, the hypothalamic-pituitary-adrenal (HPA) axis and/or the sympathetic-adrenal-medullary (SAM) axis is activated to release stress hormones, such as cortisol, epinephrine, norepinephrine and acetylcholine (Glaser and Kiecolt-Glaser, 2005). Activation of the HPA axis first leads to release of corticotrophin-releasing hormone (CRH) from the hypothalamus (Chrousos, 1998). CRH triggers the release of adrenocorticotropic hormone (ACTH) from the frontal pituitary, which in turn activates the adrenal cortex to release the glucocorticoid (GC) cortisol into the blood system. The GC receptor is predominantly located in the cytoplasm of target cells, and when cortisol bind to the receptor the construct moves into the nuclear compartment (Barnes and Adcock, 1997). The GC receptor folds in a special way with two folds, which facilitate the DNA interaction at the upstream promoter of the GC responsive genes. GC receptor binding to the DNA may both activate and suppress gene transcription.
When the SAM axis gets activated, the adrenal medulla and sympathetic neurons as quickly stimulated to release the catecholamines adrenaline and noradrenaline (Glaser and Kiecolt-Glaser, 2005).

1.9.2 Acute versus chronic stress

Even though stressors may be differentiated by, duration, intensity, frequency or quality, no clear criteria for the definition between acute and chronic stress exists in the

literature (Tewes, 1999). Hence, whether a stressor is described as acute or chronic can only be observed by the reaction of the organism. If the biological system soon is regulated back to its normal values, the stressor is acute, whereas a stressor that results in a prolonged increase of the setpoint for the biological system, the stressor could be described as chronic. It has been suggested that acute stress might enhance some aspects of immune function, such as leukocyte trafficking to peripheral organs, whereas chronic stressors may be deleterious to immune function (Glaser and Kiecolt-Glaser, 2005).

1.9.3 Effects of stress on the inflammatory system

Almost all immune cells have receptors for the stress hormones released by the activated HPA- and SAM-axes. Hence, lymphocytes, eosinophils, neutrophils and monocytes/macrophages all express receptors for both GC and catecholamines (Katz et al., 1985; Miller et al., 1998; Madden, 2001; Elenkov and Chrousos, 2002; Pujols et al., 2002). Because of the wide distribution of the GC receptors, activation of the HPA axis has profound effects in the inflammatory system. Hence, has cortisol release been suggested to alter leukocyte trafficking and function as well as decrease production of cytokines and other inflammatory mediators (Chrousos, 1995). However, the effect of GC release is complex and involves both the levels released, as well as the GC receptor distribution on the target cells (Wust et al., 2004).

Many studies have shown that GC inhibits Th1 associated cytokines, such as IL-12, and promote the production of Th2 associated cytokines, such as IL-4 (Franchimont et al., 1998; Agarwal and Marshall, 2001; Elenkov, 2004). In response to stress, a drive towards Th2 in the balance between Th1/Th2 has previously been shown in allergic subjects (Kang et al., 1997; Liu et al., 2002). In addition have stress been shown to enhance the number of exacerbations as well as elevate the levels of airway eosinophils in asthmatics (Sandberg et al., 2000; Liu et al., 2002).

1.9.4 Stress and neurotrophins

Previous studies on healthy humans and animals have shown elevated levels of NGF in plasma and serum in response to stress (Maestripieri et al., 1990; Aloe et al., 1994; Hadjiconstantinou et al., 2001). It has been suggested that NGF could have important functions in the stress-mediated immune response, since NGF has been shown to activate the HPA axis (Otten et al., 1979) in addition to the role for NGF as a survival and activation factor for both neurons and inflammatory cells. However, knowledge regarding the regulation of neurotrophins other than NGF in response to stress, particularly during allergic inflammation, is still scarce.

2 AIMS

1) To determine if human bronchial smooth muscle cells could function as a local source of the neurotrophins NGF, BDNF and NT-3 in the airways.

2) To determine how the production of neurotrophins in human bronchial smooth muscle cells could be regulated by pro-inflammatory, Th1- and Th2-associated cytokines as well as prostaglandins.

3) To determine if hematopoetic cells in the blood could be a systemic source of neurotrophins in allergic asthma.

4) To determine how stress could influence systemic immune regulation, such as cytokine and immune cell levels, in healthy and allergic subjects.

5) To determine how stress could influence local inflammatory cells associated with the asthmatic inflammation, such as eosinophils, in airways and bone marrow.

6) To determine how stress could influence the production of neurotrophins, both systemically in the blood and locally in the airways in healthy and allergic conditions.

7) To determine the role of endogenous GC release in stress-evoked changes in inflammatory cells and neurotrophins in the airways during allergic inflammation.

3 MATERIAL AND METHODS

3.1 STUDIES ON NEUROTROPHIN PRODUCTION IN HUMAN BRONCHIAL SMOOTH MUSCLE CELLS (I)

3.1.1 Study material

A commercially available primary cell line of human bronchial smooth muscle cells (HBSMC) was used to analyze NGF, BDNF and NT-3 expression and regulation. The cells were obtained from a healthy donor, and had a normal phenotype according to previously published criteria (Hirst, 1996). The HBSMC were grown in monolayer in DMEM (Sigma-Aldrich) supplemented with 10% FBS (Invitrogen) and 100 U/mL insulin (Lilly), and all experiments were conducted on cells in passage 9. At 80% confluence, cells were growth arrested for 24 h in a low-FBS (0.3%), insulin-free DMEM. Cytokines, cyclooxygenase (COX)-inhibitors and dexamethasone (10 μM) were added to the culture medium to analyze time- and dose-dependent effects. After stimulation cell supernatants were collected and centrifuged before analyzed. Cells were collected by lysis in RLT lysis buffer (Qiagen).

3.1.2 Analysis methods

3.1.2.1 mRNA quantification

3.1.2.1.1 mRNA extraction and cDNA analysis

After collecting the cells in RLT-buffer (see section3.1.1 for details), total RNA was extracted from the cells using the RNeasy extraction kit (Qiagen Inc), according to the manufacturer's protocol. Before total RNA was eluted, genomic DNA was removed by DNAse I (Qiagen Inc), according to the manufacturer's instructions. To analyze yield and purity, RNA was measured spectrophotometrically at $\lambda = 260/280$ nm.

RNA was reverse transcribed to cDNA using SUPERSCRIPT™ II RNase H^- Reverse Transcriptase (Invitrogen), random primers, dNTP-mix and RNAsin (Pharmacia Biotech) according to the protocol and with the buffers supplied by the manufacturer. To enable detection of eventual genomic DNA contamination, non-reversed transcribed total RNA was diluted 1:2 with water where after it was analyzed in the same way as cDNA.

3.1.2.1.2 Real Time PCR

The basal and stimulated NGF, BDNF and NT-3 mRNA expression was quantified following 0.5, 1, 2.5, 6, 24 and 48h of cell culture by TaqMan PCR (*Figure 3*) using the ABI Prism 7700 Sequence Detection System (Applied Biosystems) with a two-step PCR protocol (95˚C for 10 min to activate the polymerase, followed by 40 cycles of 95˚C for 15 sec and 60˚C for 1 min). cDNA was diluted 1/5 and mixed with primer and probe, Buffer A, AmpliTaq Gold (all from Applied Biosystems) and dNTP-mix (Pharmacia Biotech), according to the guidelines provided by Applied Biosystems. The primers and probes for NGF, BDNF and NT-3 were designed using the Primer Express software (Perkin Elmer) and the Perkin Elmer Biosystems Guidelines for amplifying custom target sequences for quantification (TaqMan Univeresal master mix protocol P/N 4304449 p 13-21). The guidelines make it possible to amplify all targets using the

same reaction conditions and parameters. The sequences for the housekeeping gene 18S rRNA primers and probe were commercially available. The probes were synthesized with the fluorescent reporter dye FAM (6-carboxy-fluorescein) attached to the 5'-end and a quencher dye TAMRA (6-carboxy-tetramethyl-rhodamine) to the 3'-end. Preferably, either one of the primers, or the probe, should be placed over an exon-exon boundary to avoid amplification of genomic DNA. However, because both the start- and stop codon for NGF, BDNF and NT-3 are within the same exon, neither of the primers, nor the probe could be placed over an exon-exon boundary. To avoid amplification of genomic DNA all samples were treated with DNase (see section 3.1.1 for details) and the elimination of DNA in the RNA was tested by analyzing un-transcribed RNA in the Real Time PCR.

The results were quantified using the relative standard curve method, with amplification of neurotrophins and the housekeeping gene 18S in separate tubes. Standard curves for each neurotrophin and 18S were created using five serial dilutions (1:1, 1:2, 1:10, 1:20 and 1:100) of cDNA from the human foetal fibroblast cell line HFL-1 (American Type Culture Collection, Rockville, MD, USA). All samples were run in duplicates and the mean values were used in further analysis. The relative amount of neurotrophin mRNA in each sample was calculated as the ratio between the neurotrophin mRNA and the housekeeping gene 18S rRNA before samples were compared.

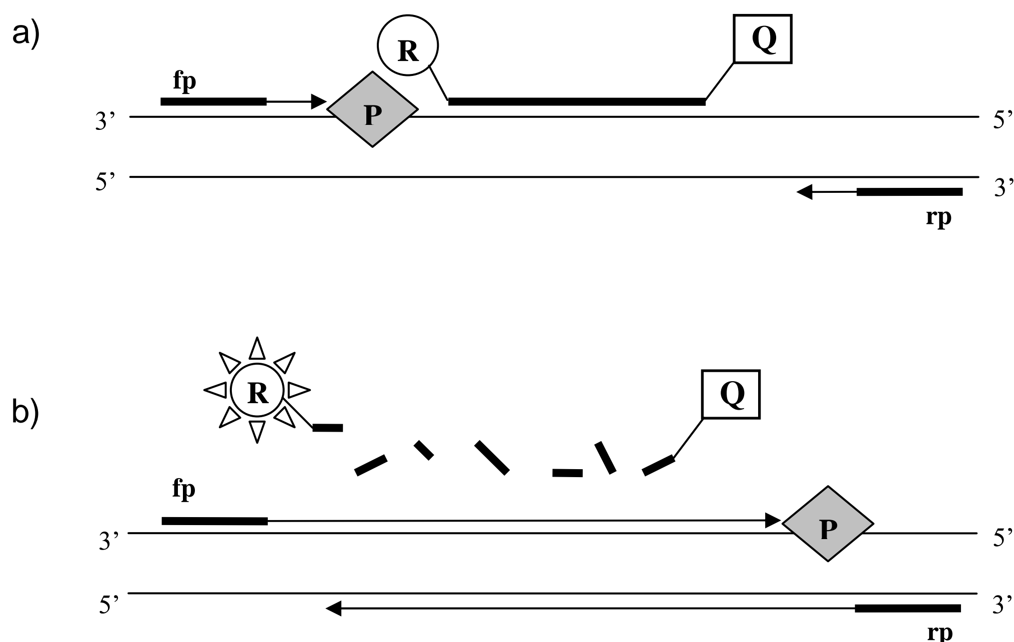

Figure 3: TaqMan PCR. ***a)*** *The two fluorescent dyes, a reporter (R) and a quencher (Q) are attached to the end of a probe, which anneals to the template between the forward (fp) and rewerse (rp) primers. The fluorescence is quenched when the probe is intact.* ***b)*** *During amplification the probe is degraded by the polymerase (P), releasing the reporter florophore. The fluorescence intensity is detected following each cycle in the reaction.*

3.1.2.1.2.1 Methodological considerations for TaqMan RT-PCR

TaqMan RT-PCR allows measurement of an accumulating PCR product in real time by utilizing a dual-labeled fluorogenic probe which hybridizes to the target sequence during the PCR. When the probe is intact the reporter fluorophore emission is suppressed by a quencher florophore (Figure 4). During the PCR, the endogenous nuclease activity of the Taq polymerase degrades the probe, separating the reporter fluorophore from the quencher, resulting in increased fluorescence. The intensity of the fluorescence is directly proportional to the amount of target in the original sample. To quantify the results obtained by TaqMan PCR two different methods are commonly used: the standard curve method and the comparative threshold method (Giulietti et al., 2001). The standard curve method can be either relative or absolute. A relative standard curve is made up by serial dilutions of a calibrator known to express the gene of interest, and can be used to determine changes in the transcription of a gene by comparing the threshold cycle of the target with the threshold cycle of the calibrator. The threshold cycle is the first cycle in which the amount of amplicon exceeds the minimal detection level. In contrast to relative quantification does absolute quantification allow precise determination of target copy number. However, it requires the construction of an absolute standard curve for each amplicon to be measured. The comparative threshold method utilizes arithmetic formulas to calculate expression levels compared to a calibrator, such as a control sample. Only relative quantification is possible with the comparative threshold method.

3.1.2.1.3 Conventional PCR

Conventional PCR was used to analyze constitutive mRNA expression of NGF, BDNF and NT-3 in HBSMC. Cells were analyzed following 0.5, 1, 2.5, 6, 24 and 48h of cell culture on a GeneAmp PCR System 9600 (Applied Biosystems). cDNA was diluted 1/5 and mixed with dNTP-mix (Pharmacia Biotech), primer (same as for Real Time RT-PCR), TaqGold-buffer II, $MgCl_2$ and AmpliTaq Gold (all Applied Biosystems). The thermal cycling conditions were: 94°C for 12 min, followed by 30 cycles for 18S and 40 cycles for the neurotrophins of 94°C for 30 sec, 60°C for 30 sec and 72°C for 1 min, before ending with 72°C for 9 min. The products were analysed on a 1.5% agarose (Invitrogen) gel complemented with 0.025‰ ethidium bromide (Pharmacia Biotech). A 1kb DNA-ladder (Invitrogen) was run in parallel to the samples. The gels were pictured using a Kodak Digital Science 1D™ analyzing system (Kodak Scientific Imaging Systems).

3.1.2.2 Quantification of proteins by enzyme-linked immunosorbent assay

To quantify the NGF, BDNF and NT-3 protein levels in cell culture supernatants, commercially available enzyme-linked immunosorbent assay (ELISA) kits (Promega) were used, according to the manufacturer's instructions. NGF, BDNF and NT-3 levels were measured in HBSMC-culture supernatant. Prior to analysis of NT-3 the supernatant was concentrated using the Amicon Ultra-15 Centrifugal Filter Units (Millipore), with a molecular weight cut off of 5,000 Da, according to the protocol obtained from the manufacturer. The detection range for NGF and BDNF was 2.0-250 pg/mL, and for NT-3 the range was 2.4-150 pg/mL. All measurements were performed in duplicates. Inter-assay variation between plates was analyzed by preparing a stock solution of 70 pg/mL recombinant NGF, BDNF or NT-3 protein (R&D Systems)

diluted in DMEM. The stock solution was frozen in aliquots, and a fresh aliquot analyzed on each plate.

3.1.2.2.1.1 Methodological considerations for NGF ELISA

The NGF ELISA-kit from Promega contains an IgG antibody. Due to potential cross-reactivity, precaution should be taken when analysing NGF in samples containing high levels of IgG, such as plasma and serum with the NGF ELISA-kit from Promega. In this work, the absence of cross-reactivity in the samples was tested in four different ways. **1)** By analyzing cell culture medium alone. This did not give any detectable signal, indicting that the medium did not give any unspecific NGF signal. **2)** By analysing a known concentration of NGF in medium containing 0% and 0.3% bovine serum. The results show that the expected signal was achieved in both the 0% and the 0.3% medium. There was no difference in signal between the two test samples, indicating that no interference with unspecific binding or generation of an unspecific signal were found in the 0.3% medium due to IgG or any other molecule. **3)** By performing multiple dilutions. Here 1:2, 1:4 and 1:8 dilutions of i) experimental samples from cell culture experiments with a unknown concentration of NGF, ii) the same experimental samples as in i) but with a known concentration of NGF added, as well as medium with iii) 0% and iv) 0.3% serum with a known concentration of NGF added, were analyzed. All these samples generated an expected loss of signal of the same magnitude upon dilution, indicating that no interference with unspecific binding or generation of an unspecific signal were found in the 0.3% medium due to IgG or any other molecule. **4)** By performing calculations of the recovery of an addition of a known concentration of NGF to our experimental cell culture samples ("spiked recovery"). The calculation was as follows: Recovery (%) = ((Spiked sample – Unspiked sample)/ Spiked medium) x100, where "Unspiked sample" equals the signal from an experimental cell culture sample (0.3 % FBS) with an unknown concentration of NGF, "Spiked sample" equals the signal from an experimental cell culture sample (0.3 % FBS) with an unknown concentration of NGF, which is spiked through the addition of a known amount of NGF, and "Spiked medium" equals the signal from the cell culture medium (0.3 % FBS) spiked with the same known concentration of NGF as in "spiked sample". The recovery % should typically be between 80-120% in a successful assay. Test samples had a recovery of 98% with the NGF ELISA-kit from Promega, indicating no unspecific interference.

3.2 HUMAN STUDIES ON IMMUNE REGULATION AND NEUROTROPHIN LEVELS IN RESPONSE TO EXAMINATION STRESS (II-III)

3.2.1 Study material

In paper **II,** 41 undergraduate medical students at Karolinska Institutet were studied. Fourteen females and 8 males had a verified allergy. All allergic subjects had rhinitis, and 16 were also diagnosed with asthma. Nine females and 10 males were included as control subjects. From the subject material in paper **II**, the subjects with allergic asthma as well as the controls were studied further. Hence, in the study in paper **III**, 35 undergraduate medical students participated, of whom 10 females and 6 males had allergic asthma and 9 females and 10 males constituted a group of healthy controls. All students were of Caucasian origin, except for one Asian subject. All students were

subjected to screening for total serum IgE with Phadiatop, and when positive quantitative analysis of specific serum IgE was performed by radio-allergo-sorbent test (RAST). Atopic subjects had a history of allergic symptoms and a positive RAST. Selection criteria for asthma included a well-documented history of asthma and bronchial hyperreactivity with previous use of asthma medication. Students with rhinitis had a history of rhinoconjunctivitis with previous use of antihistamine or local steroids. Control subjects had no history of allergy and a negative Phadiatop. Subjects were not regular nicotine users. Only 2 of the asthmatic subjects were using corticoids on a regular basis, and another 6 during pollen season, prior to inclusion in the study. No subjects included in the study used any systemic or local corticoids during the study. All such medication was withheld at least 4 weeks prior to the start of the study. All subjects completed the protocol. Three cases, one control and one with rhinitis and asthma at the low-stress phase, and one with rinitis at the stress phase, were excluded due to respiratory infection.

3.2.1.1 Study design

Subjects were studied at a low-stress and stressful period of academic studies. The low-stress evaluation was conducted in mid-semester during a period where no oral or written exams were undertaken neither 2 weeks prior to, nor 2 weeks following the evaluation. The stress phase evaluation was conducted at a day between a major oral and a major written exam during a week of major final examinations. To avoid chronobiological variations, subjects were studied at the same time of day on both evaluations.

3.2.2 Analysis methods

3.2.2.1 Stress, anxiety and stress hormones

3.2.2.1.1 VAS

In paper **II** and **III**, the perceived stress levels were measured using the Visual Analogue Scale (VAS). VAS is a one-item rating scale, and the participants were asked to rate their overall level of stress on a straight horizontal line measuring 100 mm, with anchors placed at both poles (where the left pole corresponded to “no stress”, and the right pole “highest imaginable stress”). Participants were given written instructions to “Rate the level of stress you’re experiencing *at this moment*. Mark with an X on the line.” The VAS has previously been used extensively, and is a validated method to measure the magnitude of internal states such as pain and mood (Price et al., 1983; Wewers and Lowe, 1990).

3.2.2.1.2 STAI-S

In paper **II** anxiety was evaluated using the State-version of the State-Trait Anxiety Inventory (STAI-S) (Spielberger et al., 1970). The STAI-S includes 20 items, with a rating scale ranging from 1 (*almost never*) to 4 (*almost always*). Participants were asked to rate each statement of the inventory that best reflected how they felt at the time of data collection. Examples of statements includes: “I feel calm” and “I am worried”.

3.2.2.1.3 Cortisol

In paper **II**, the cortisol concentration was measured in urine, collected from 10 PM the evening before until the first urine in the morning, form all subjects. The cortisol concentrations were analyzed using an Auto Delfia Cortisol Kit (Wallac OY, PerkinElmer), with a detection range between 5 and 1600 nmol/L.

3.2.2.2 Phenotyping of inflammatory cells and analysis of mediators in blood and upper airways

3.2.2.2.1 Sub-typing of white blood cells

In paper **II** and **III**, complete blood count with leukocyte differentials was performed at Karolinska University Laboratory, Solna, Sweden, and leukocyte subsets were further characterized using direct-labeled monoclonal mouse antibodies (mab) as previously described (Muller-Suur et al., 2002), and anti-CD3, -CD4 and -CD8 mab (Dakopatts) were used to detect T-cell subsets. In paper **II**, anti-CD3, -CD56/16 and -CD45 mab (BD Biosciences) were used to delineate NK-cells, and for recognition of natural regulatory T cells, defined as $CD4^{+}CD45RO^{+}CD25^{bright}$ cells, anti-CD4 (Dakopatts), -CD25, and -CD45RO (BD Biosciences) were used. Anti-human leukocyte antigen-DR (HLA-DR, BD Biosciences) and anti-CD4 mab (Dakopatts) were used to identify activated CD4 T-cells. Cells were analyzed by flow cytometry using a FACS-Calibur flow cytometer (BD Biosciences).

3.2.2.2.2 Cell culture of peripheral blood mononuclear cells

In paper **II** and **III** peripheral blood mononuclear cells (PBMC) were cultured. To obtain PBMC, peripheral blood was collected in heparinized vacutainers. Whole blood was diluted 1:2 in PBS and PBMC were separated by gradient centrifugation using Ficoll-Paque PLUS (Pharmacia Biosciences AB). PBMC were cultured for 24 h in RPMI 1640 (Sigma-Aldrich), supplemented with 10% FBS (Invitrogen) and L-glutamine (Signa-Aldrich). Phytohemagglutinin (PHA) and phorbol myristate acetate (PMA, both Sigma-Aldrich) were added to stimulate the cells, as previously described (Leung et al., 1992; Kang et al., 1997). After stimulation cell culture supernatants were collected and centrifuged before analyzed.

3.2.2.2.3 Cytokine ELISA

In paper **II** and **III** the protein levels of IL-2, IFN-γ, IL-4 and IL-5 were determined in supernatants from PBMC, utilizing commercially available ELISA kits (OptEIATM Human sets, Pharmingen), according to the protocol obtained from the manufacturer. The kits allowed detection of, IL-2, IL-4 and IL-5 in the range of 7.8-500 pg/mL and IFN-γ in the range of 4.7-300 $pg{*}mL^{-1}$. All measurements were performed in duplicates. Inter-assay variation between plates was analyzed by preparing a stock solution of pooled supernatants from different subjects. The stock solution was frozen in aliquots, and a fresh aliquot analyzed on each plate.

3.2.2.2.4 Neurotrophin ELISA

In paper **III**, ELISA was utilized to analyze NGF, BDNF and NT-3 in PBMC culture supernatant and NAL (see section 3.2.2.2.5 for details). BDNF and NT-3 were also

analyzed in serum. Concentration of NAL-samples for BDNF analysis was performed as described in section 3.1.2.2.

3.2.2.2.5 Nasal lavage

In project **III**, NAL was performed on all subjects. Five mL PBS was drawn into a syringe fitted with a silicone tip (NASALINE), preventing the solution from leaking aside. The PBS-solution was then slowly spurted up into the nostril, and drawn back into the syringe. The same procedure was repeated for the other nostril. The solutions from both nostrils were pooled and centrifuged before the supernatant was analyzed for NGF and BDNF.

3.2.2.3 Bronchial hyperreactivity

In paper **II** and **III**, bronchial hyperreactivity was tested in both atopic and healthy subjects. Bronchial hyperreactivity to metacholine was evaluated using a dosimeter controlled jet nebulizer Spira Electro 2 (Respiratory Care Center). After recording the baseline FEV_1, the subject first inhaled saline followed by a methacholine solution. With the nose clipped, methacholine was inhaled during a specified number of breaths, in concentrations from 14.2 to a maximum cumulative dose of 7256 µg, with dose increments around every third minute. FEV_1 was measured 2.5 min after each dose of methacholine and the test stopped when FEV_1 had decreased by at least 20% from its post-saline value, or when the maximal cumulative dose was reached. The logarithmic metacholine doses were plotted against the percentage of the post-saline FEV_1. PD_{20} values were calculated from the cumulative dose-response curves by linear interpolation.

3.2.2.4 Lung function tests

In paper **II**, lung function was measured in both healthy and atopic subjects. Forced expiratory volume in one second (FEV_1), peak expiratory flow (PEF) rate, vital capacity (VC) and forced vital capacity (FVC) were determined using a MasterScope direct reading spirometer (Erich Jaeger GmbH). Percentage of predicted FEV_1 was calculated as measured FEV_1/predicted FEV_1, and percentage of predicted PEF was calculated as measured PEF/predicted PEF.

PEF-rate variability was determined using diurnal PEF and minimum morning PEF (% of recent best), according to the recommendations in GINA (GINA, 2005). PEF meters (MiniWright Standard Peak Flow Meter) were administered to all included subjects, and they were trained on how to use the flow meter correctly. The subjects were instructed to perform three peak flows in the morning and in the evening, before taking asthma medication, and to record the highest value of the three measurements each morning and evening in a diary one week prior to, and up until, both evaluations. Diurnal PEF variability was calculated from the daily amplitude of PEF (morning–evening) expressed as a % of the mean daily PEF value averaged over 7 days. Minimum morning PEF (% of recent best) was calculated as the lowest daily morning PEF, expressed as percent of recent best during the 7 day period.

3.2.2.5 Exhaled nitric oxide

In paper **II** exhaled NO was recorded as a measure on airway inflammation. NO was measured in oral single-breath exhalations using a NIOX® (Aerocrine AB, Solna,

Sweden), developed to meet the criteria in the guidelines from the American Thoracic Society (1999).

3.3 ANIMAL STUDIES ON AIRWAY INFLAMMATION AND NGF LEVELS IN RESPONSE TO ACUTE STRESS (IV)

3.3.1 Study material

Six to eight week old male BALB/c mice were obtained from B&K Universal AB, and maintained in a room with a 12 hour light-dark cycle. The animals were allowed food and water *ad libitum*. On day 0, 7 and 14 mice were sensitized to ovalbumin (OVA) adsorbed to $Al(OH)_3$ (both Sigma-Aldrich) and suspended in 0.2 mL PBS, by an intra peritoneal (i.p.) injection. Non-sensitized mice received an i.p injection of only $Al(OH)_3$ suspended in PBS. On day 27, 28 and 29, an OVA challenge was performed on both OVA-sensitized and non-sensitized animals by intra nasal (i.n.) instillation during slight CO_2 anesthesia.

3.3.1.1 Study design

Restraint stress was provoked by placing animals, without squeezing, into well-ventilated plastic tubes. The tubes allow the mice to swirl around, but prevent them from turning head-to-toe. Animals were stressed for a continuous 2 h for three constitutive days. Unstressed animals were left in their home cages during the stress procedure. To inhibit release of endogenous GC, mice were treated with metyrapone (ME) i.p. 2 h prior to restraint stress.

3.3.2 Analysis methods

3.3.2.1 Bronchoalveolar lavage and bone marrow preparations

Inflammatory cells and soluble mediators were collected from the airways and bone marrow. Animals were sacrificed 24 h following the last airway challenge. The trachea was exposed and the airways gently instilled with PBS. The bronchoalveolar lavage

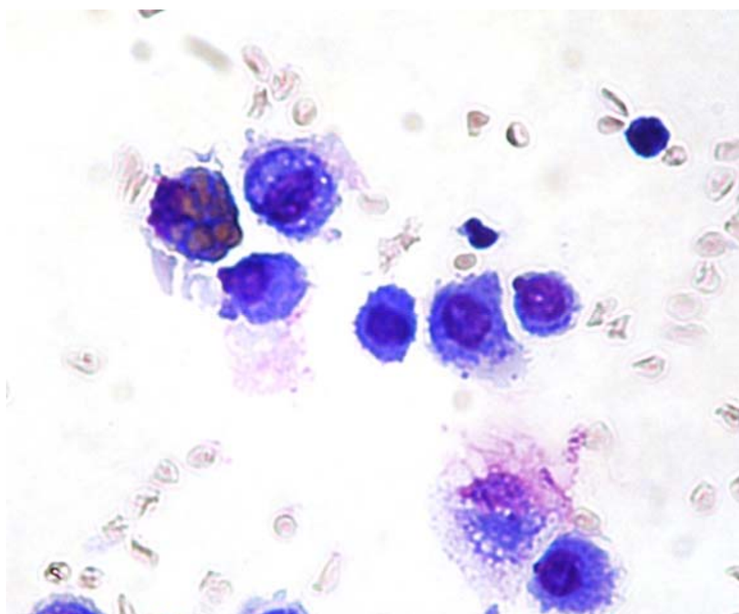

Figure 4: Inflammatory cell in mouse BALF

fluid (BALF), containing cells and soluble mediators was centrifuged and the supernatant was analyzed for NGF. The cell pellets were re-suspended in PBS and cytocentrifuged (Shandon Cytospin 2) onto glass slides for cell differentiation (*Figure 4*). The bone marrow (BM) cells were collected by flushing PBS through the right end-opened femur of each mouse. Cytocentrifugation slides were then performed in the same way as with cells from BALF.

3.3.2.2 Phenotyping of inflammatory cells and analysis of neurotrophins in BALF and bone marrow

The BALF and BM slides were air-dried and stained with May-Grünwald Giemsa. A differential cell count was performed by counting 300 leukocytes in a blinded manner under a direct light microscopy (Nikon Corporation) with 100 x magnification. In the BALF, cells were classified as eosinophils, macrophages, lymphocytes or neutrophils using standard morphological criteria. In the BM, both mature and immature eosinophils were counted. Mature eosinophils were defined as cells containing eosinophilic granules and mature nuclear morphology, and immature eosinophils as cells containing eosinophilic granules and large, non-segmented nuclei.

3.3.2.2.1 Neurotrophin ELISA

NGF ELISA was performed on BALF. ELISA was performed as described in section 3.1.2.2.

3.4 STATISTICAL ANALYSIS

Experimental data was expressed as mean ± standard error of the mean (SEM), or medians with 25-75th percentiles in parenthesis. Statistical analyses were preformed by post-hoc analysis of variance, or by unpaired or paired tests as indicated in each paper. Differences were considered significant at $p \leq 0.05$. All analyses were performed using GraphPad InStat (Graph Pad Software).

4 RESULTS AND DISCUSSION

4.1 STUDIES ON NEUROTROPHIN PRODUCTION IN HUMAN BRONCHIAL SMOOTH MUSCLE CELLS (I)

It has been proposed that airway smooth muscle cells (SMC), apart from a role as contractile cells, participate in the airway inflammatory processes by secreting soluble mediators. For instance, SMC can secreate cytokines and growth factors that could influence inflammatory cells in close contact with the airway SMC, such as mast cells. Asthmatics have elevated levels of neurotrophins in the airways, and the focus of the study was to evaluate whether bronchial SMC could be a source of the elevated levels of neurotrophins in the airways, and how this production could be regulated.

4.1.1 Expression and regulation of NGF, BDNF and NT-3 in bronchial SMC

Using conventional RT-PCR technique and ELISA, a constitutive mRNA expression of NGF, BDNF and NT-3, as well as secretion of the corresponding protein, was observed. ELISA analyses indicated that BDNF was secreted in the highest amounts, followed by NGF and NT-3.

The NGF expression from airway SMC has previously been shown to be stimulated by the pro-inflammatory cytokine IL-1β in a transient fashion (Freund et al., 2002). In paper **I**, this transient up-regulation was confirmed, however with a slightly different kinetics. Hence, a maximal up-regulation following 6 h of stimulation was observed, whereas Freund et al. showed a maximal up-regulation already following 2.5 h of stimulation. It can be speculated that this difference is dependent on the origin of the smooth muscle cells. Furthermore, the present work also showed an up-regulation of BDNF following IL-1β stimulation. The stimulated up-regulation of BDNF was markedly different than the stimulated expression of NGF. Hence, compared to NGF, the up-regulation of BDNF was slower, and reached maximal up-regulation following 24 h of stimulation. This up-regulated expression was sustained for 48 to 72 h. The effects of IL-1β on NGF and BDNF-expression were dose-dependent.

The slower induction of BDNF compared to NGF suggested a possible involvement of an intermediate mediator. Previous studies have identified a critical role for cyclooxygenase (COX) in the production of BDNF (Shaw et al., 2003). In paper **I**, IL-1β-stimulated BDNF secretion from bronchial SMC was markedly repressed when the COX enzymes were blocked utilizing the unselective COX-inhibitor indomethacin or the selective COX-2 inhibitor NS-398. These results suggest a role for prostaglandins (PG) as mediators in the formation of BDNF. The IL-1β stimulated NGF secretion was not affected by the COX-inhibitors. However, both IL-1β-stimulated NGF and BDNF expression was reduced by the glucocorticoid (GC) dexamethasone (see figure 5), indicating that GC could down-regulate neurotrophin production.

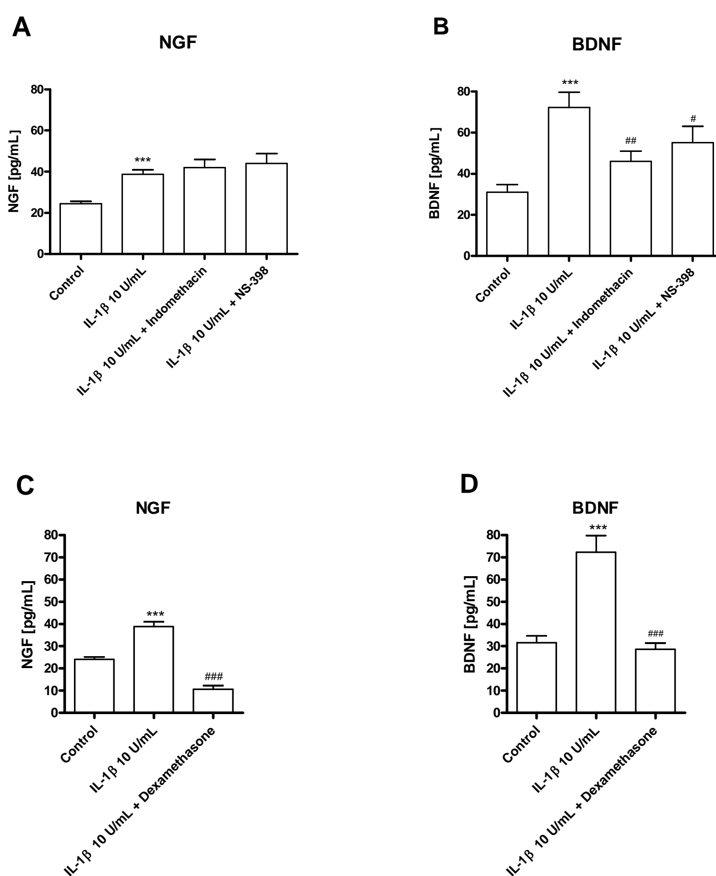

*Figure 5: Effect on COX-inhibitors and dexamethasone (all 10 μM) on IL-1β (10 U/mL)-stimulated NGF (figure A and C) and BDNF (figure B and D) secretion from bronchial SMC. Data presented as mean ± SEM of 6 independent experiments. ***: p<0.001 versus control, and #: p<0.05, ##: p<0.01, ###: p<0.001 versus IL-1β alone (figures A and B are reproduced from paper **I**).*

Also IFN-γ was observed to stimulate NGF expression in a dose-dependent manner, but with a different kinetics compared to IL-1β. Hence, IFN-γ-stimulated NGF expression was slower than IL-1β, reaching a maximal up-regulation following 48 h of stimulation. In contrast to NGF, BDNF expression was not stimulated by IFN-γ. If anything, a small decrease in BDNF expression was observed following 6 h of stimulation with IFN-γ. When the cells were stimulated with IFN-γ in combination of IL-1β a synergistic and dose-dependent up-regulation in NGF was observed. The observation that IFN-γ up-regulated the expression of NGF was somewhat surprising in an asthmatic perspective. However, this might reflect a more complex role of the Th1/Th2 cytokines in allergic asthma, or a role for neurotrophins in Th1-mediated airway inflammations. Hence, IFN-γ has been shown to enhance the development of allergen-specific asthma in mice (Dahl et al., 2004). Further, patients with allergic asthma, and especially those with severe asthma, may have elevated levels of IFN-γ (Corrigan and Kay, 1990; ten Hacken et al., 1998), and IFN-γ has furthermore been suggested to contribute to the severity of the asthmatic disease . In addition to IFN-γ, other secreted mediators, such as RANTES, has been shown to be elevated by IFN-γ in human bronchial SMC (John et al., 1997). Recent studies also suggest an involvement

of neurotrophins also in Th1-associated airway inflammations, such as sarcoidosis (Ricci et al., 2005) and our own unpublished observations).

IL-4 alone did not affect any studied neurotrophin at any tested dose or time. However, IL-4 was shown to down-regulate the IL-1β-stimulated NGF expression. NT-3 expression was unaltered by any tested cytokine at any tested dose or time.

4.2 STUDIES ON IMMUNE REGULATION IN RESPONSE TO EXAMINATION STRESS (II)

Stress has been shown to enhance the number of exacerbations and elevate the levels of airway eosinophils in asthmatics (Sandberg et al., 2000; Liu et al., 2002). Stress has also been shown to induce a drive towards Th2 in the balance between Th1/Th2 cytokines in allergic subjects (Kang et al., 1997; Liu et al., 2002). However, the underlying inflammatory and airway responses to stress are largely unknown.

The focus of the study in paper **II** was to determine systemic immunological, local inflammatory and functional airway responses to stress in healthy and atopic individuals. The atopic subjects included in the study had allergic asthma and rhinitis, or allergic rhinitis only. Subjects were examined at a low-stress and stressful (examination) period of academic studies. Atopic subjects had a higher proportions of lymphocytes and a tendency towards elevated numbers of eosinophils in blood, compared to controls. In addition, the proportion of NK-cells was lower in atopic subjects compared to controls.

4.2.1 Effect of examination on perceived stress and anxiety

The academic examination resulted in higher VAS and STAI-S scores in both atopic and control subject, indicating enhanced perceived stress and anxiety at the examination period, compared to a low-stress study period. In response to stress, cortisol levels were increased in atopic subjects only. Interestingly, this increase was accompanied by a numerically larger increase in VAS in the group of atopics, suggesting that the increase in cortisol was dependent on the enhanced perceived stress.

4.2.2 Effect of examination on subsets of white blood cells

In response to stress, the $CD4^+/CD8^+$ T-cell ratio was increased in atopic subjects, with a tendency to be increased also in controls. The increase was dependent on a slight decrease in the $CD8^+$ T-cells, as well as a slight increase in the $CD4^+$ T-cells. The decrease in $CD8^+$ T-cells might be explained by a redistribution to more peripheral compartments of the body. The increase in $CD4^+$ T-cells could partly be explained by an increase in natural regulatory T-cells, defined as $CD4^+CD45RO^+CD25^{bright}$, which increased in both atopic and control subjects in response to stress. The proportion of $CD4^+/HLA\text{-}DR^+$ cells did not increase in any of the groups in response to stress, indicating that stress did not result in a general activativation of $CD4^+$ T-cells, suggesting that the increase in $CD4^+CD45RO^+CD25^{bright}$ cells was a true increase in natural regulatory T-cells in response to stress.

4.2.3 Effect of examination on cytokine production from white blood cells

Previous studies have suggested that academic stress is accompanied by a decrease in the Th1/Th2 balance, which could influence the atopic disease (Kang et al., 1997; Liu et al., 2002). In paper **II**, the levels of IFN-γ, IL-2 and IL-5 were reduced in atopic subjects at the stress period, with a reduction, although non-significant, also in controls, whereas IL-4 was reduced in controls only. Since atopy has been proposed to be a Th2-cytokine driven disease, the Th1/Th2 ratio was examined. Already at the low-stress period atopic subjects had a lower IL-2/IL-5 ratio compared to controls. In response to stress the IL-2/IL-5, IL-2/IL-4 and IFN-γ/IL-4 ratios were decreased in atopics, whereas no changes in Th1/Th2 ratios were observed in controls. Taken together, the results may indicate that atopic subjects had a shift in the Th1/Th2 balance towards Th2, which might be aggravated in response to stress.

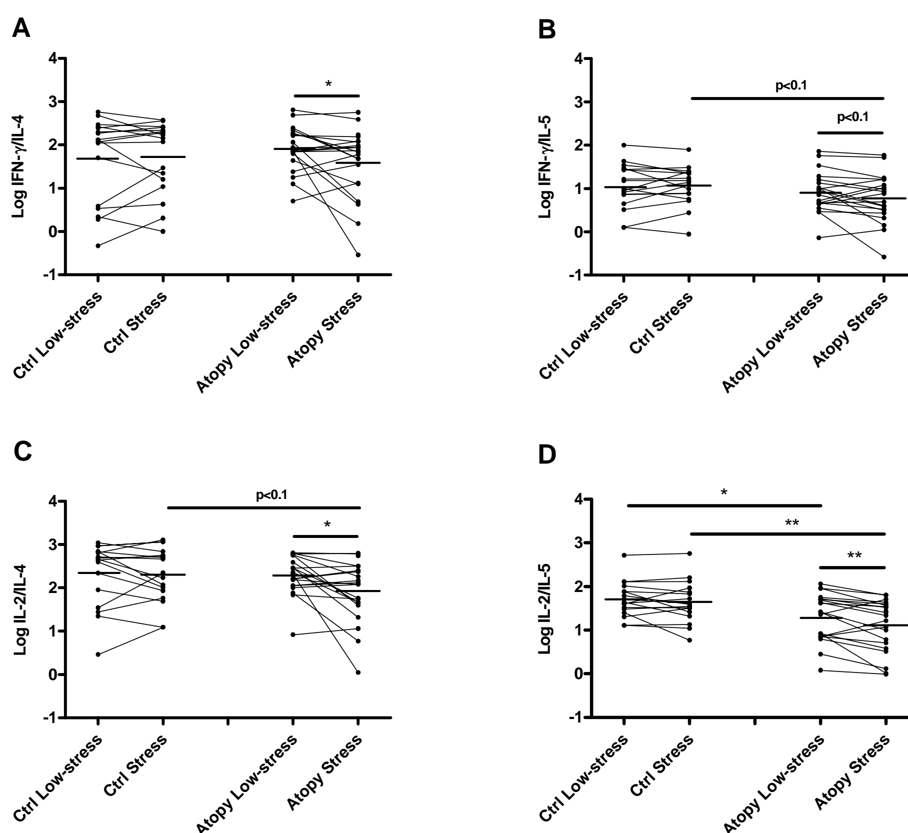

*Figure 6: Effect of cytokine production from PBMC Means are indicated in the graphs. *: p<0.05, **: p<0.01. Figure reproduced from paper **II**.*

NK-cells are major producers of IFN-γ *in vivo*. The proportion of NK-cells was lower in atopic subjects compared to controls at both periods, and in addition did the proportion of NK-cells decreased in atopic subjects in response to stress. One might therefore speculate that the decreased proportion of NK-cells could contribute to the decrease in IFN-γ and the selected shift in cytokine balance in atopics in response to stress.

4.2.4 Effect of examination on exhaled nitric oxide, airway hypereactivity and lung function

Exhaled NO could function as a local marker for airway inflammation. The main producer of the NO in exhaled air is the epithelial cells, but also inflammatory cells have been shown to produce NO (Lane et al., 2004). In paper **II**, the atopic subjects with asthma were shown to exhale more NO than controls. In response to stress, control subjects had a significant reduction in exhaled NO. A reduction of exhaled NO was not observed in the group of asthmatics in response to stress, which might indicate a sustained airway inflammation in asthmatics in response to stress.

As expected, asthmatic subjects had a significantly increased bronchial hyperreactivity, as indicated by a lower log PD_{20} for metacholine compared to controls at both periods, however the bronchial hyperreactivity was not observed to be changed in neither group in response to stress.

Support for a stress-induced airway obstruction are weak (Rietveld et al., 2000). In paper **II**, an increase in FEV_1 was observed for controls, but not for atopics with asthma, in response to stress. Stress has previously been reported to increase FEV_1 in asthmatic subjects (Laube et al., 2003). However, this was shown using an acute stress model, suggesting activation of the sympathetic nervous system to release epinephrine. Epinephrine binds to β_2-receptors on the SMC surface, thereby relaxing the SMC, resulting in dilatation of the airways, which could be a possible explanation to the increase in FEV_1.

4.3 STUDIES ON NEUROTROPHIN LEVELS IN RESPONSE TO EXAMINATION STRESS (III)

The neurotrophins have been suggested as candidates able to influence the neuroimmune-interactions in response to stress, since they are recognized as important factors in both the nervous and inflammatory system (Aloe et al., 2002). In line with this, stress has been shown to elevate the levels of neurotrophins in the blood of healthy humans (Aloe et al., 1994; Hadjiconstantinou et al., 2001). However, knowledge regarding regulation of other neurotrophins than NGF, such as BDNF, in response to stress are scarce.

The focus of paper **III** was to determine the regulation of BDNF secretion, in parallel to studies of inflammatory parameters and airway reactivity, in response to stress in both asthmatic and healthy subjects. From paper **II**, the subjects with allergic asthma, as well as the control subjects, were studied further. The influence of stress on neurotrophin levels were studies, and correlations of neurotrophin expression to inflammatory mediators were evaluated.

4.3.1 Effect of stress on neurotrophin release from white blood cells

The levels of BDNF in supernatant from peripheral blood mononuclear cells (PBMC) were higher in asthmatic subjects compared to controls, indicating a role of BDNF in the asthmatic inflammation. The levels of BDNF increased in response to stress in controls, but not in asthmatic subjects. Although the levels of BDNF were not elevated

in asthmatics, the results suggest a role of BDNF in stress-induced neuroimmune regulation.

Apart from BDNF, the levels of NGF and NT-3 were also analyzed in supernatans from PBMC, but they were to low to be detected.

4.3.2 Effect of stress on levels of neurotrophins in blood and upper airways

The levels of BDNF in serum have previously been shown to be elevated in asthmatic subjects compared to controls (Noga et al., 2001). Although it was possible to detect BDNF in serum, no difference was observed between asthmatic subjects and controls in this study and the levels of BDNF did not change in response to stress in neither of the groups. The observation of elevated BDNF levels in PBMC, but not serum, in asthmatic subjects compared to controls suggests that PBMC constitutes a more sensitive compartment to measure BDNF during an allergic inflammation. The levels of NT-3 were also measured and detected in serum. However, no difference was observed between groups or in response to stress.

Nasal lavage (NAL) is suitable for measuring soluble mediators in the upper airways, and can be handled by the subjects themselves (Greiff et al., 1990), and an attempt to measure the levels of BDNF in the upper airways utilizing nasal lavage fluid (NALF) was performed. However, the levels of BDNF were too low to be detected even following 10 x concentration of the lavage fluid. It is nevertheless interesting to note that both NGF and NT-3 were measurable in NALF. The levels of NGF were approximately 10 x higher than the levels of NT-3 (*Table 1*), indicating differential neurotrophin levels not only in the bronchial SMC (paper **I**) and blood (see 4.3.1), but also in the upper airways.

Table 1:Levels of neurotrophins in NALF

	NGF		NT-3	
	Low-stress	Stress	Low-stress	Stress
Healthy	310 (208-643)	276 (179-800)	18.6 (12.5-23.6)	18.3 (15.3-26.0)
Asthmatic	390 (194-479)	222 (141-333)	21.7 (14.5-33.9)	18.6 (11.6-25.8)

Levels of neurotrophins in pg/mL. Values are presented as medians with 25-75th percentile in parenthesis.

4.3.3 Correlations of BDNF release to inflammatory mediators and bronchial hyperreactivity

The proportion of $CD4^+$ T-cells in blood did not differ between asthmatic and control subjects at any tested period. Nevertheless, the proportion of $CD4^+$ T-cells increased in asthmatics in response to stress, with a tendency to increase also in controls. Asthmatics had a tendency towards elevated levels of IL-5 secreted from PBMC compared to the controls at the low-stress period. In response to stress, the levels of IFN-γ, IL-2 and IL-5 decreased in asthmatics, and the levels of IL-4 decreased in controls. At the stress period, the IFN-γ/IL-5, IL-2/IL-4 and IL-2/IL-5 ratios were reduced in asthmatics, but not in healthy controls. A reduction also in IFN-γ/IL-4 was observed, although the reduction was not statistically significant ($p = 0.06$). As mentioned above (see section

4.2.4) asthmatic subjects had a significantly lower log PD_{20} for metacholine compared to controls at both periods.

When the release of BDNF from PBMC was correlated to $CD4^+$ and $CD8^+$ T-cells, as well as cytokine levels and bronchial hyperreactivity, a positive correlation was observed between the change (between low-stress and stress) in BDNF release from PBMC and $CD4^+$ T-cells, illustrating that the asthmatics with the larges increase in $CD4^+$ T-cells also hade the largest increase in BDNF. $CD4^+$ T-cells have previously been shown to both produce BDNF and express the specific BDNF receptor TrkB (Besser and Wank, 1999; Kerschensteiner et al., 1999), and may hence act as both a source and target for BDNF. In asthmatics at the stress period, a positive correlation was observed between BDNF and IL-5. Since IL-5 is essential for eosinophils survival and activation, it could be speculated that BDNF could function as an indirect mediator of eosinophils survival. In addition, a tendency towards a negative correlation was observed between BDNF and the PD_{20} for metacholine. Data in the literature suggests that the levels of BDNF in blood can be correlated to bronchial hyperreactivity, as shown for platelet BDNF (Lommatzsch et al., 2005) Future studies may reveal weather BDNF might mediate bronchial hyperreactivity in human asthma.

4.4 STUDIES ON AIRWAY INFLAMMATION AND NGF LEVELS IN RESPONSE TO ACUTE STRESS (IV)

Several murine models have been developed to study airway inflammation, and they reproduce many of the human manifestations of allergic rhinitis and asthma, including an increased eosinophilic airway inflammation and airway hyperresponsiveness (Leong and Huston, 2001). Stress has been suggested to contribute to exacerbations of allergic asthma, possibly by enhancing the recruitment of inflammatory cells into the airways. The levels of NGF have been shown to be elevated in asthma as well as in response to stress, as described in sections 1.8.4 and 1.9.4. However, data describing how stress could modulate the allergic airway inflammation as well as the levels of NGF are scarce.

The focus of paper **IV** was to study the levels of inflammatory cells and NGF on allergic inflammation in response to stress, as well as the influence of GC on these parameters. An animal model was developed in which an allergic airway inflammation and stress exposure were combined.

4.4.1 Effect of allergen airway challenge on inflammatory cells and NGF

In response to allergen challenge, the proportion of eosinophils increased (*Figure 7*), whereas the proportion of neutrophils decreased, in the airways of allergic (allergen-sensitized), compared to non-allergic (non-sensitized) animals, suggesting that allergic sensitization and airway provocation induce an eosinophilic airway inflammation.

Previous studies have shown elevated levels of NGF in the airways of both humans and animal models of allergic asthma (Bonini et al., 1996; Braun et al., 1998; Virchow et al., 1998; Olgart Hoglund et al., 2002; Path et al., 2002), whereas no elevated levels of NGF were observed in the current model. This observation is possibly due to a milder

allergic airway inflammation in our model, as assessed by the airway eosinophilia compared to previously published data (Braun et al., 1998).

4.4.2 Effect of restraint stress on inflammatory cells and NGF

In response to restraint stress, the proportion of eosinophils increased in the airways of both allergen provoked allergic and non-allergic animals (*Figure 7*). The proportion of eosinophils decreased in the BM of allergen provoked allergic animals only, suggesting a possible redistribution of eosinophils from the BM to the airways in response to stress in allergen provoked allergic animals. In non-allergic animals in response to stress, also the proportion of macrophages increased, whereas the proportion of neutrophils decreased.

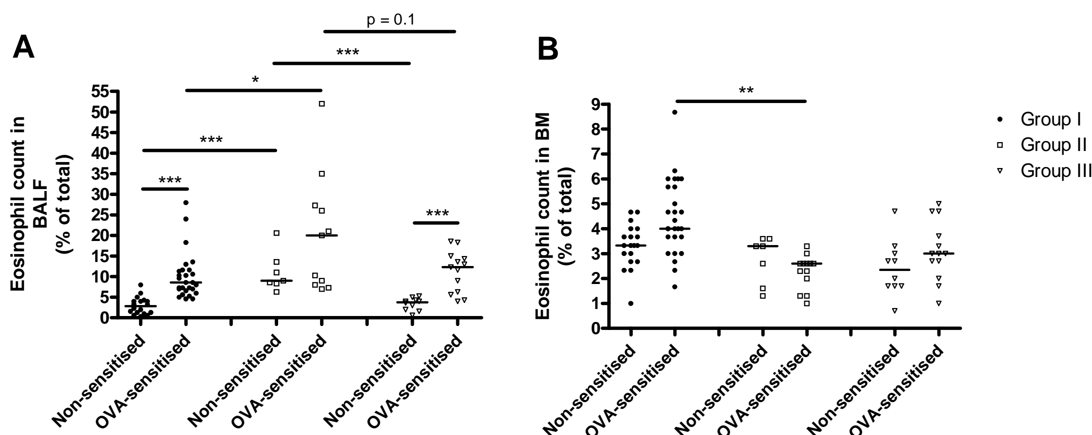

Figure 7: Proportion of eosinophils in BALF(A) and bone marrow (B) of OVA-challenged BALB/c mice. Medians are indicated. Group I: Controls, Group II: Stressed, Group III: Stressed and metyrapone-treated (figure reproduced from paper ***IV****).*

In contrast to unstressed animals, the levels of NGF increased in the airways of allergen provoked allergic animals, compared to non-allergic animals subjected to restraint stress (*Figure 8*), indicating that stress altered the NGF production, enabling an allergen-dependent increase in NGF. However, in response to stress, the levels of NGF decreased in the airways of non-allergic animals. Stress has previously been shown to elevate the levels of NGF in the blood in both humans and animals (Maestripieri et al., 1990; Aloe et al., 1994; Hadjiconstantinou et al., 2001), suggesting that stress affects NGF levels differently in local and systemic compartments, possibly due to the differentiated expression of stress-hormone receptors.

4.4.3 Effect of GC-release inhibition on inflammatory cells and NGF release in response to stress

In stressed animals, inhibition of GC-release by metyrapone resulted in a decreased proportion of eosinophils in non-allergic animals (*Figure 7*), with a tendency to decrease also in allergic animals, compared to stressed animals who did not receive ME. The result indicates that the eosinophilic airway inflammation in response to restraint stress in part might be mediated by GC. In non-allergic and stressed animals treated with ME the proportion of macrophages decreased, whereas the proportion of

neutrophils increased, compared to non-allergic and stressed animals who did not receive ME, suggesting that the stress-induced increase in macrophages and decrease in neutrophils in part could be mediated by endogenous release of GC.

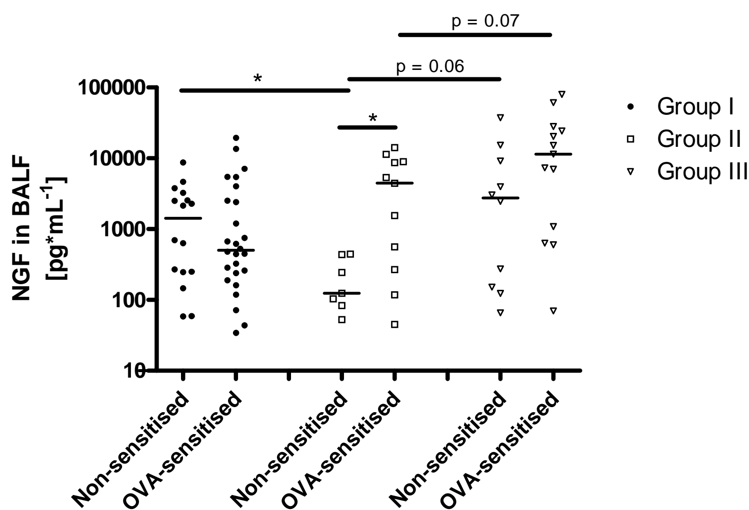

*Figure 8: Levels of NGF in BALF of OVA-challenged BALB/c mice. Medians are indicated. Group I: Controls, Group II: Stressed, Group III: Stressed and metyrapone-treated (figure reproduced from paper **IV**).*

There was a near-significant increase in the levels of NGF in the airways of both allergic and non-allergic and stressed animals treated with ME (*Figure 8*), compared to stressed animals who did not receive ME, indicating that endogenous release of GC may function as an inhibitor for NGF release in the airways.

5 GENERAL DISCUSSION

The nervous system can affect several components of the immune response, from controlling the metabolic processes that underlie immune cell functions to more complex mechanisms, such as the stress response (Besedovsky and Del Rey, 2001). The messengers of the nervous system controlling the immune system are hormones, steroids, neurotransmittors and neuropeptides, for example glucocorticoids (GC) and cathecolamines. The neuroimmune interaction is bidirectional, and hence can the nervous system also receive information from the immune system by the release of immune-derived mediators, such as cytokines and neurotrophins. Also structural cells may be involved in neuroimmune regulation by producing mediators capable of interacting with both neurons and immune cells.

In the current work, neuroimmune interactions in allergic inflammation have been investigated both locally and systemically by focusing on the neurotrophins. Neurotrophins are interesting messenger molecules due to their ability to influence both the nervous and immune systems. Moreover, the neurotrophins have been suggested to have a role in the allergic inflammation, based on the findings that elevated levels of neurotrophins have been detected both in the blood and locally in the airways (Bonini et al., 1996; Sanico et al., 2000; Olgart Hoglund et al., 2002) and the levels have been shown to increase further following allergen challenge (Virchow et al., 1998; Sanico et al., 2000; Kassel et al., 2001; Nassenstein et al., 2003). The neurotrophins NGF and BDNF may also contribute to the development of bronchial hyperreactivity (BHR) as evident from animal studies (Braun et al., 1999; de Vries et al., 1999), and most likely also have the potential to prolong allergic inflammation by, for instance, increase the survival of airway eosinophils (Nassenstein et al., 2003). Taken together, this implies a role for neurotrophins in the pathogenesis of asthma.

5.1 AIRWAY SMOOTH MUSCLE CELLS AS PRODUCERS OF NEUROTROPHINS

When this work was initiated, immunohistochemical (IHC) studies had recently shown that structural cells of the airways, such as the smooth muscle cells (SMC) expressed NGF (Olgart Hoglund et al., 2002). Later IHC studies also confirmed expression of BDNF and NT-3 in airway SMC (Ricci et al., 2004), suggestig a role of these cells in the production of several neurotrophins in the airways. However, IHC is a less sutible technique to study regulation of protein production. Therefore, an *in vitro* model of human bronchial SMC was established, and in paper **I** of the current work the expression and regulation of neurotrophins in bronchial SMC were studied in detail. A constitutive expression of NGF, BDNF and NT-3 was observed, confirming the previous IHC studies. In addition, NGF and BDNF expression was shown to be regulated by inflammatory cytokines, indicating a role for SMC as producers of neurotrophins during inflammation in the airways. The neurotrophin production was also shown to be differentiated both over time, and in response to pro-inflammatory, Th1- and Th2-associated cytokines, which could imply different roles for the neurotrophins at different stages and types of an inflammatory response. For example, IL-1β evoked a rapid induction of NGF, whereas upregulation of BDNF had a slower

onset. This lead us to hypothezise that an intermediary messenger might be involved in the IL-1β-stimulated BDNF expression. Based on previous data from mouse astrocytes, the prostaglandins were likely candidates as such intermediate messengers (Toyomoto et al., 2004). Hence, inhibition of COX-synthesis was observed to markedly reduce IL-1β-stimulated BDNF, but not NGF release, suggesting a role for prostaglandins as intermediary mediators in IL-1β-stimulated BDNF, but not NGF formation in bronchial SMC. As shown previously, COX-inhibitors also counteracts BDNF-mediated effects in the nervous system of a murine *in vivo* model of spatial learning (Shaw et al., 2003). Little is known about how prostaglandins regulate BDNF expression, but in human keratinocytes, a cell type with a central role in atopic dermatitis, prostaglandin E_2 has been shown to stimulate expression of the neurotrophin NT-4/5 by binding to the PGE_2-receptor EP3 and activate the ERK signaling pathway (Kanda et al., 2005).

The levels of prostaglandins have been shown to be elevated in asthmatic airways (Liu et al., 1990), and in line with a differential response of NGF and BDNF to COX-inhibitors, it is interesting to note that these neurotrophins have been shown to have different roles in allergen induced airway obstruction. Hence, NGF has been shown to be involved in the early allergic response, since anti-NGF has been illustrated to attenuated the early allergen-induced bronchoconstriction (Path et al., 2002). This is in line with the notion that NGF has been demonstrated to facilitate histamine release from mast cells and basophils (Bruni et al., 1982; Pearce and Thompson, 1986; Horigome et al., 1993). In contrast to NGF has BDNF been suggested to abrogate the chronic allergic airway obstruction. It can also be speculated whether NGF indirectly can stimulate BDNF production, since NGF has been demonstrated to induce prostaglandin production, as evident from studies on mast cells (Marshall et al., 1999).

5.2 POTENTIAL ROLES OF SMC-DERIVED NEUROTROPHINS

In asthmatic subjects, it has been shown that mast cells reside within the smooth muscle cells layer (Brightling et al., 2002) and that bronchial mast cells express the neurotrophin receptor TrkA (Kassel et al., 2001). Since NGF has been demonstrated to enhance mast cell proliferation and survival (Matsuda et al., 1991; Kawamoto et al., 1995), as well as facilitate histamine release (Pearce and Thompson, 1986), neurotrophin release from bronchial SMC have the potential to influence asthmatic inflammation in the airways. In addition, NGF, BDNF and NT-3 have also been discribed as survival and activation factors for airway eosinophils in subjects with allergic asthma (Nassenstein et al., 2003), suggesting a sensitivity of airway eosinophils to neurotrophins which probably is mediated through the increased expression of neurotrophin receptors on the cell surfaces. Further, NGF and BDNF have been demonstrated to evoke BHR through tachykinins since inhibition of the tachykinin receptor neurokinin-1 abolished NGF-induced BHR (Braun et al., 1998; de Vries et al., 1999; Braun et al., 2004). Taken together, this suggest a potential role of neurotrophins in inflammatory airway disorders.

5.3 INFLUENCE OF STRESS ON IMMUNE REGULATION AND ALLERGIC INFLAMMATION

Stress is a factor suggested to influence the allergic inflammation by regulating the neuroimmune homeostasis (Aloe et al., 2002). Allergic inflammation is regarded as a

Th2-cyokine mediated disease, and stress has been suggested to enhance this already unbalanced immune response by shifting the cytokine balance towards Th2 even further. In paper **II**, systemic effects of stress were studied, and a shift in the Th1/Th2 cytokine balance towards Th2 was observed in atopic subjects in response to stress. A shift in Th1/Th2 cytokine balance could depend on multiple factors, such as GC release and changes in immune regulatory cells. Th1 cells have previously been described to have an increased sensitivity of to GC compared to Th2 cells (Franchimont et al., 1998; Agarwal and Marshall, 2001; Elenkov, 2004). In paper **II**, the more pronounced decrease in Th1 cytokines in atopics compared to healthy could therefore result from the larger cortisol increase in the group of atopics. The shift in Th1/Th2 balance could also be due to a change in immune regulatory cells, such as NK cells and regulatory T-cells. In fact, in atopics the skewed Th1/Th2 balance in response to stress was accompanied by decreased proportion of NK cells. The activation of NK cells in response to a changed homeostasis, such as stress, is quick, thus allowing NK cells to early regulate immune functions (O'Connor et al., 2006). One way in which NK cells regulate the immune response is by the production of the Th1-associated cytokine IFN-γ (Lanier, 2005). However, GC may reduce the ability of NK cells to respond to IL-12 (Elenkov and Chrousos, 2002), resulting in decreased levels of IFN-γ. In our model, decreased levels of IFN-γ was observed in atopic subjects, but not healthy controls in response to stress. Hence, one may suggest that the reduced levels of IFN-γ observed in atopic but not healthy subjects in paper **II** resulted from the reduced numbers of NK cells in atopic subjects in response to stress, which induced a shift in the Th1/Th2 cytokine balance towards Th2.

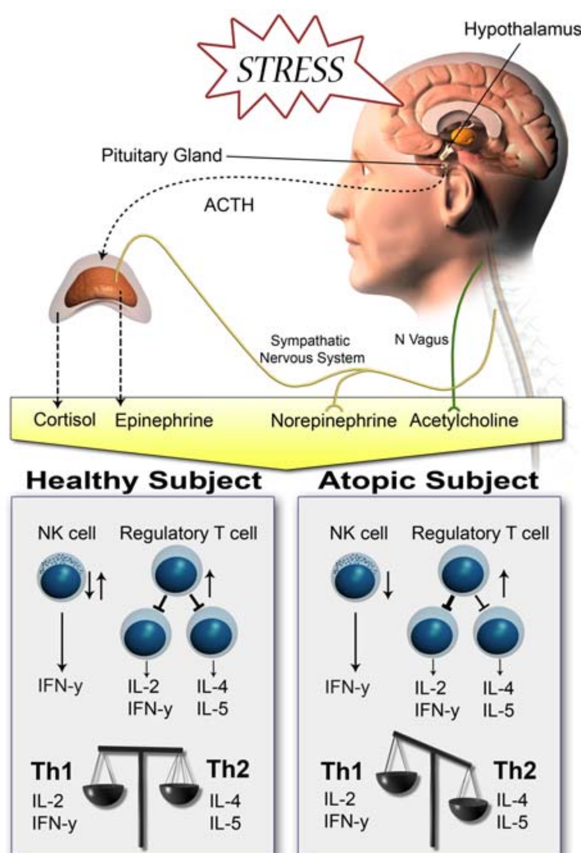

Figure 9: The impact of stress on immune cells and inflammatory mediators in healthy and atopic subjects.

Interestingly, elevated numbers of natural regulatory T-cells were observed in both atopic subjects and healthy controls in response to stress. Regulatory T-cells are important for the control of immune responses and cytokine release, and the elevated amounts of regulatory T-cells could therefore account for the decreased cytokine levels observed in both atopic and control subjects in response to stress in paper **II**. However, this does not explain the differential shift in Th1/Th2 cytokine balance in atopic subjects in response to stress. It is therefore interesting to note that regulatory T-cells have been suggested to be dysregulated in atopic disease. Hence, regulatory T-cells from atopic subjects have been shown to reduce Th2-cytokines less efficient than regulatory T-cells from controls (Grindebacke et al., 2004; Ling et al., 2004). A summary of the impact of stress on immune and inflammatory mediators is summarised in Figure 9.

Mechanistic studies of how stress affects neuroimmune regulation and allergic inflammation in general and in the lower airways in particular, were not feasible in our human stress study based on an examination stress model. Therefore, in paper **IV**, an animal model with an allergic airway inflammation was used to study stress responses. Restraint stress was chosen as a stress model. The restraint stress is intrinsically stressful for burrow-dwelling animals such as mice (Cavigelli and McClintock, 2003). The restraint stress procedure is thought to result in a stress that is largely psychological in nature because of the perception of confinement (Glavin et al., 1994). This stressor has previously been shown to activate both the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic-adrenal-medullary (SAM) axis, and results in the activation of adrenal steroid receptors in tissues throughout the body (Dhabhar et al., 1995).

In response to stress the eosinophilic airway inflammation increased in both allergen provoked allergic and non-allergic animals, suggesting that stress either inhibits apoptosis of eosinophils already in the airways, or recruit eosinophils from the blood and bone marrow. In allergen provoked allergic animals, the levels of eosinophils decreased in the bone marrow in response to stress, making it tempting to suggest a recruitment of eosinophils from the bone marrow via the blood to the airways in response to stress. A redistribution of inflammatory cells from the bone marrow to other peripheral compartments of the body, such as the skin, has previously been described (Dhabhar, 2002). Further, inhibition of endogenous GC release in response to stress decreased the proportion of eosinophils in the airways of allergen provoked non-allergic animals, with a tendency to decrease also in allergen provoked allergic animals, indicating that the potential redistribution of eosinophils to the airway in response to stress partly is dependent on GC. However, even though the present results are interesting, further mechanistic studies are warranted to confirm that the eosinophils produced in the bone marrow are redistributed to the airways in response to stress. Also, the possibility of a decreased apoptosis of the eosinophils in the airways in response to stress can not be neglected, but is maybe the less likely scenario, since GC have been shown to induce apoptosis in blood and airway eosinophils (Meagher et al., 1996; Uller et al., 2006).

5.4 STRESS AND NEUROTROPHINS

In paper **III**, elevated production of BDNF was observed in PBMC from controls in response to stress, suggesting a role for neurotrophins in the systemic neuroimmune interactions. An elevated production of NGF has previously been shown in response to both acute and chronic stress (Hadjiconstantinou et al., 2001; Aloe et al., 2002). Even though elevated levels of BDNF were not observed in asthmatic subjects in response to stress, PBMC from asthmatic subjects were shown to produce elevated levels of BDNF compared to controls at both the stress and low-stress periods. One may speculate that PBMC from asthmatics are already activated to produce BDNF, thereby masking a potential stress-related increase in BDNF secretion caused by an academic examination. Hence, the chosen stressor, an academic examination, might not be powerful enough to trigger a further enhancement in BDNF secretion from PBMC in asthmatic subjects. Alternatively, it may be speculated that absence of an elevated BDNF production in asthmatic subjects is due to inhibition by stress-hormones, since an increase in GC release observed in the group of asthmatic subjects, but not in controls and that neurotrophins, such as BDNF, are negatively regulated by glucocorticoids. Even though the levels of BDNF were not elevated in the group of asthmatic subjects in response to stress, the levels of BDNF in asthmatic subjects in response to stress correlated to inflammatory mediators, such as IL-5, which is associated with the allergic inflammation. Hence, the results indicate a possible role for BDNF as a mediator in neuroimmune regulation in response to stress.

The influence of stress on neurotrophin release was in paper **IV** further studied in the animal model with an allergic airway inflammation. The levels of NGF in the airways were not observed to be elevated in response to an allergic airway inflammation, which was an unexpected finding, as an allergy-induced increase in NGF has previously been shown (Braun et al., 1998). However, this could be explained by the mild eosinophilia in our model, which implies a milder allergic inflammation in the model, compared to previously published studies (Braun et al., 1998). Nonetheless, stress was shown to modulate the NGF allergy-dependent NGF release in a way that elevated levels of NGF were observed in allergen provoked allergic animals compared to non-allergic litter mates in response to stress. It might be speculated that endogenous GC can inhibit NGF release, since the stress-induce decrease in NGF in non-allergic animals in response to stress was reversed, and the stress-induced increase in NGF levels in the allergic animals were increased further following treatment with metyrapone. However, some precaution needs to be taken with this interpretation, since the effects of metyrapone on NGF release were tendencies (*Figure 8*).

5.5 CONCLUDING REMARKS

Taken together, the results suggest that atopic and non-atopic subjects share some inflammatory changes in response to stress. However, other stress-induced inflammatory changes are unique to atopic individuals, indicating that in atopics, some mechanisms which could be pathogenic for the atopic inflammation may be more strongly affected by stress than others. This observation could be of clinical importance, and the knowledge could in the future be used by physicians to advice atopic patients on effects of life style and stress on immune regulation and symptoms, which may reduce suffering, sickness absence and need of medications.

The present work also revealed information on how neurotrophins are regulated in the airways and by stress, even though the function of the increased NGF levels during stress in allergic inflammation needs further investigation. The elevated levels of neurotrophins in inflammatory conditions and during allergic inflammation also raise the question whether inhibition of local neurotrophin production in the airways would be beneficial for controlling the underlying inflammation in pulmonary diseases, such as allergic asthma.

6 CONCLUSIONS

1) Human bronchial smooth muscle cells could function as a local source of NGF, BDNF and NT-3 in the airways.

2) NGF, BDNF and NT-3 expression in human bronchial smooth muscle cells could be differentially regulated by pro-inflammatory, Th1- and Th2-associated cytokines, as well as prostaglandins, suggesting a dynamic interplay between inflammatory mediators and the neurotrophins, which could be important in different stages and types of airway inflammation.

3) Human peripheral blood mononuclear cells could function as a systemic source of BDNF especially in allergic asthma.

4) Allergic and healthy humans shared some immunological responses to stress, such as an increased number of regulatory T-cells and a decline in cytokine levels, but differed in others, such as a skewed Th1/Th2 ratio and reduced NK cell numbers in atopics. This indicates that some pathogenic mechanisms in the allergic inflammation are more strongly affected by stress in allergic subjects than in healthy controls.

5) The levels of eosinophils increased in the airways and decreased in the bone marrow in allergen provoked allergic animals in response to stress, suggesting that stress may aggrevate an eosinophilic inflammation and possibly through modification of leukocyte trafficking.

6) Stress enhanced the levels of BDNF systemically in the blood of healthy, but not asthmatic subjects. In the allergic animal model, stress had dual effects on NGF levels locally in the airways. Hence, in absence of an underlying allergic inflammation, NGF levels decreased in response to stress, whereas an underlying allergic airway inflammation elevated NGF levels in response to stress. The results suggest a possible role for the neurotrophins in the stress response.

7) The stress-induced increase in airway eosinophilia was reduced, whereas the levels of NGF in the airways were enhanced when glucocorticoid-release was inhibited in the allergic animal model. In summary, the results indicate that recruitment of inflammatory cells to the airways in response to stress in part could be mediated by glucocorticoids, while glucocorticoids could function as an inhibitor of stress-induced elevation of neurotrophin release.

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