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INFLAMMATION AND CELL MIGRATION IN CHRONIC OBSTRUCTIVE PULMONARY DISEASE (COPD)

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Till Mamma och Pappa

Nog finns det mål och mening i vår färd -
men det är vägen, som är mödan värd.

Ur I rörelse av Karin Boye

ABSTRACT

Chronic obstructive pulmonary disease (COPD), the fourth most common cause of death worldwide, is characterised by chronic airflow obstruction and chronic inflammation which affects large and, especially, small airways. There is an accumulation of inflammatory cells in the airways in COPD, in particular neutrophils, macrophages and $CD8^+$ T-cells. Neutrophil numbers correlate with disease severity and neutrophils have been attributed a central pathophysiological role in COPD. The overall aim of this thesis was to elucidate how neutrophil function is altered by the inflammation observed in COPD. Thus, study I, II and IV were all performed on three groups of subjects, healthy non-smoking controls, smokers without COPD and smokers with COPD.

In *paper I* neutrophil release of CXCL8, MIP-1α and MCP-1 in response to different stimuli were studied. Also the role of TNF-α in regulating these responses was studied by inhibition of endogenous TNF-α with an anti-TNF-α antibody (infliximab). Neutrophil derived TNF-α contributed to the release of these chemokines after stimulation with LPS and organic dust as the response was inhibited by infliximab. In the COPD group infliximab did not inhibit the release of CXCL8 suggesting that the role of TNF-α is somehow altered in COPD.

In *paper II* chemotaxis towards CXCL8 was increased in smokers with and without COPD and migration towards LTB_4 was increased in smokers without COPD compared to healthy controls. In the smoker groups serum TNF-α and migration induced by CXCL8 and LTB_4 correlated. Thus chemotaxis of circulating neutrophils towards CXCL8, and partly towards LTB_4, is increased in smokers. Hence smoking may cause neutrophil activation and pro-inflammatory stimuli, such as TNF-α, may be central in this activation. The enhanced migration could to some degree explain the increase in neutrophil numbers observed in the COPD lung.

In *paper III* we studied the influence of a β_2-agonist (formoterol) and a glucocorticoid (budesonide) on circulating neutrophils isolated from healthy subjects. Budesonide inhibited and formoterol enhanced LPS-induced release of IL-6, CXCL1 and CXCL8. Moreover, formoterol up-regulated the chemokine receptors CXCR1 and CXCR2, while budesonide up-regulated CXCR2. However, the drugs did not affect the chemotactic response. Thus budesonide and formoterol, which are often used in the treatment of COPD, affect chemokine release and receptor expression, but the functional consequences of these findings are unclear.

In *paper IV* T-cell and alveolar macrophage (AM) interaction in COPD was examined by investigating if the production of CXCR3 binding chemokines (CXCL9, -10, -11) by AMs is enhanced in COPD. The macrophage product was also assessed for its chemotactic effects on CXCR3 expressing T-cells. No difference in chemokine release by AMs was detected and while the AM supernatant induced migration in CXCR3 expressing T-cells there was no difference between the groups. We thus conclude that the increase of CXCR3 expressing T-cells, which has been observed in the COPD lung, is not caused by the CXCR3 binding chemokines released by AMs.

Taken together these studies show an alteration in different aspects of neutrophil function in smokers with COPD but also in smokers without COPD.

LIST OF PUBLICATIONS

I. K Blidberg, L Palmberg, B Dahlén, AS Lantz, K Larsson
Chemokine release by neutrophils in chronic obstructive pulmonary disease
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II. K Blidberg, L Palmberg, B Dahlén, AS Lantz, K Larsson
Increased Neutrophil Migration in Smokers with and without Chronic Obstructive Pulmonary Disease (COPD)
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III. K Strandberg, K Blidberg, K Sahlander, L Palmberg, K Larsson
Effects on formoterol and budesonide on chemokine release, chemokine receptor expression and chemotaxis in human neutrophils
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IV. K Blidberg, L Palmberg, AS Lantz, B Billing, B Dahlén, K Larsson
No alteration of production and activity of CXCR3-binding chemokines from alveolar macrophages in smokers with and without COPD
Manuscript

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LIST OF ABBREVIATIONS

APC	Allophycocyanin
BAL	Bronchoalveolar lavage
CCL2	CC chemokine ligand 2 (Monocyte chemotactic protein-1)
CCL3	CC chemokine ligand 3 (Macrophage inflammatory protein-1α)
CD	Cluster of differentiation
CD11b	Macrophage antigen-1 (Mac-1)
CD162	P-selectin glycoprotein ligand-1 (PSGL-1)
CD62L	L-selectin
COPD	Chronic obstructive pulmonary disease
CXCL1	CXC chemokine ligand 1 (Growth regulated oncogene -α)
CXCL8	CXC chemokine ligand 8 (Interleukin-8)
CXCL9	CXC chemokine ligand 9 (Monokine induced by γ interferon, Mig)
CXCL10	CXC chemokine ligand 10 (Interferon-γ induced protein 10, IP-10)
CXCL11	CXC chemokine ligand 11 (Interferon-inducible T-cell α chemoattractant, ITAC)
EDTA	Ethylene diamine-tetra-actetic acid
ELISA	Enzyme linked immunosorbent assay
FITC	Fluorescein isothiocyanate
fMLP	*N*-formyl-methionyl-leucyl-phenylalanine
ICAM	Intercellular adhesion molecule
IFN-γ	Interferon-γ
IL-6	Interleukin-6
LPS	Lipopolysaccaride
LTB_4	Leukotriene B_4
mRNA	Messenger RNA
PCR	Polymerase chain reaction
PE	Phycoerythrin
PECAM	Platelet/endothelial cell adhesion molecule
PerCp	Peridinin chlorophyll protein
TNF-α	Tumour necrosis factor-α
VCAM	Vascular cell adhesion molecules

1 INTRODUCTION

As we breathe large amounts of air passes in and out of our lungs and with that follows a continuous exposure to particles, gases, and micro-organisms such as virus and bacteria. To ensure that the exposure does not cause injury or infection to the lungs there are several protective systems in place. These include mechanistic functions such as sneezing, cough and an up-ward transport of mucus brought about by the beating of cilia. The cells in the airways also release a series of antimicrobial products to help keep the lung free of infectious agents. Deep down in the lungs the clearance is mainly handled by phagocyting immune-cells which ingest particles, bacteria etc.

Naturally these systems have their limitations and sometimes infection and inflammation of the lungs occur as a result of different exposures. Respiratory diseases are common globally and ranges from acute infection to chronic disease such as asthma and chronic obstructive pulmonary disease (COPD).

COPD affects approximately 10% of the population world-wide and it is estimated to be the third most common cause of death in 2020 (1, 2). The primary cause of COPD is exposure to tobacco smoke, but other exposures are also of importance. COPD is a chronic disease characterised by a progressive and irreversible airflow limitation which is caused by an inflammation of small and large airways as well as emphysema. The airway inflammation is dominated by an increase in several inflammatory cell types, including neutrophils, macrophages and $CD8^+$ T-lymphocytes. While these cells are an important part of the natural defence against potential dangers, such as bacteria and virus, they can also cause damage to the own tissue.

During the last decades research has come a long way in characterising the airway inflammation observed in COPD, nonetheless many questions still remain. Therefore, the main aim of this thesis was to study possible alterations in neutrophil and alveolar macrophage function in smokers with and without COPD as compared to non-smoking controls. Moreover, the effects of two drugs (formoterol and budesonide) on neutrophils isolated from healthy non-smokers were investigated.

1.1 THE RESPIRATORY SYSTEM

The key function of the respiratory system is to enable gas exchange; it delivers oxygen and removes carbon dioxide. The air enters the respiratory system through the nose, passes through the nasal cavity and continues down the trachea and through the dividing branches of the respiratory tree until it reaches the alveoli. The alveoli are tiny air-filled sacs where the actual gas exchange takes place between the lung and the blood stream.

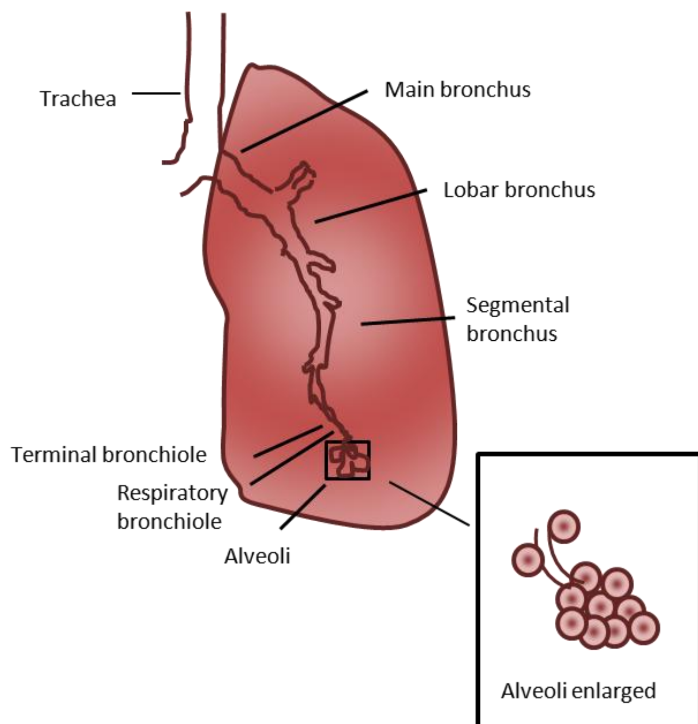


Figure 1: The anatomy of the airways

An adult person at rest breathes in approximately 7.5 litres of air every minute. This amounts to an enormous volume of air that passes through the lungs of a human being in a life-time. Naturally, this means we are also exposed to gases, particles, viruses, bacteria etc. which the body needs a strategy to cope with.

Starting from the trachea and continuing down to the terminal bronchioles, the airways are lined with ciliated epithelial cells. The cilia beat in a synchronised fashion to transport mucous, produced by cells in the airway epithelium such as goblet cells and submucosal glands, and particles out of the airways (3). The epithelial cells lining the alveoli are different from the airway epithelial cells; they are not ciliated, extremely thin and make direct contact with the capillary endothelium, thus facilitating the gas exchange (3).

The airway epithelial cells do not only function as a physical barrier but they are also active in the regulation of airway inflammation (4). Several immune cells, such as macrophages and neutrophils, T- and B-lymphocytes also help to patrol the lungs (3).

1.2 THE IMMUNE SYSTEM

In order to protect themselves from potentially dangerous bacteria, virus and parasites (pathogens) all living organisms have some sort of immune system. In humans, the skin and mucosa, including epithelial cells, provide a primary barrier against possible pathogens. However, if this first protecting wall is breached the immune system is on constant patrol, awaiting the invading pathogen with an enormous battery of protective mechanisms designed to recognise, disarm and eliminate the intruder. Normally an immune response is therefore the result of signals reporting either infection or injury. This inflammation is harmful to the pathogen, but as it also constitutes a potential harm to the host it is essential that this process is tightly controlled as not to cause unnecessary damage or become persistent.

The human immune system is traditionally divided into the innate immune system and the adaptive immune system. It is however important to bear in mind that the two cannot function as isolated entities and that there is an extensive interaction between the two.

1.2.1 The Innate Immune System

Innate immunity, or non-acquired immunity, is the primary response to invading pathogens, and as the name implies it is functional from birth. It is often described as primitive and non-specific and different forms of innate immune systems exist in all classes of living organisms. The innate immune system is also believed to be the evolutionary 'older' immune system and although it does not generate immunity in the individual it can be described as the memory of past generations. The innate immune system was long thought of as a rather crude and simple system; however, that view is gradually changing.

The innate immune cells recognise pathogen-associated molecular patterns, but also endogenous danger signals, through a multitude of different receptors, so called pattern recognition receptors (PRR's). The most well-studied group of PRR's are the Toll Like Receptors (TLR), where for example TLR2 recognise peptidoglycans typical of Gram-positive bacteria and TLR4 recognise lipopolysaccharide typical of Gram-negative bacteria (5). The cells of the innate immune system display a vast array of PPR's but they are also important in acquired immunity (6).

1.2.1.1 Cells of the innate immune system

Neutrophil granulocytes

Neutrophil granulocytes are the most abundant leucocyte in human blood and they are a central participant in the defence against invading pathogens. The group of granulocytes also includes eosinophils and basophils; however neutrophils are by far the most common constituting about 95% of the granulocyte population. The characteristic multilobular nucleus makes the neutrophil easy to recognise.

Neutrophils are rapidly produced at the rate of 1-2 $x10^{11}$ cells per day in a normal adult, but this production can be increased by 10-fold if required (7). Neutrophils are produced in the bone marrow, a process that takes between 12 and 14 days. During their development the neutrophil is transformed from a myeloblast into a segmented

cell packed with granules. Once the neutrophil enters the circulation it has a rather short half-life of about 6 to 8 hours, although this is significantly extended upon migration to inflammatory sites (7).

The neutrophil granules are divided into subsets based on the presence of characteristic proteins (8). For example azurophil granules contain myeloperoxidase (MPO) and defensin, while specific granules contain collagenase and lactoferrin and gelatinase granules contain gelatinase. The granules also contain receptors such as Mac-1 (CD11b) and components of the NADPH-oxidase. By separating different proteins in the different granule the neutrophil can display different properties at different time points (8).

Circulating neutrophils are recruited to sites of action by so called chemoattractants; these include bacterial fragments (e.g. N-formyl-methionyl-leucyl-phenylalanine (fMLP)), products of the complement cascade (e.g. C5a), chemokines (e.g. interleukin (IL)-8; CXCL8) and eicosanoids such as leukotriene B_4 (LTB_4). Neutrophils sense the direction of the chemoattractant gradient and migrate along it towards the target. The forward movement of the cells arises from a number of synchronised events including, polarisation of the cell, protrusions of a leading edge caused by the extension of actin filaments and an actin-myosin-based contraction (9). In neutrophils chemoattractants act through G-protein coupled receptors (GPCRs) which trigger a heterotrimeric G-protein causing the $G_{\beta\gamma}$ to be released from the inhibitory $G_{\alpha i}$ and thus inducing a series of down-stream events (figure 2) (10, 11).

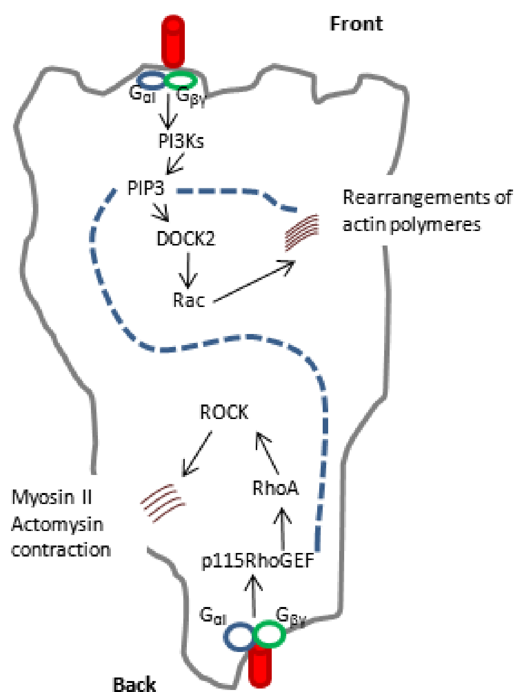


Figure 1. Schematic figure illustrating the key signalling pathways involved in neutrophil migration adopted from Stephens et al, 2008 (9). Dashed lines represent unidentified pathways.

When migrating through tissues neutrophils are often exposed to several, sometimes conflicting, chemoattractant signals. The ultimate effect of the signals is determined by timing (when they appear), intensity (how strong they are) and by the type of signal

(12). It has recently been shown that neutrophils are able to prioritise between different signals and that end target signals (e.g fMLP, C5a) can override intermediate signals (e.g CXCL8, LTB_4) (13).

CHEMOATTRACTANT	RECEPTOR
C5a	C5a receptor
CXCL1 (GRO-α)	CXCR1*, CXCR2
CXCL5 (ENA-78)	CXCR1*, CXCR2
CXCL7 (NAP-2)	CXCR1*, CXCR2
CXCL8 (IL-8)	CXCR1*, CXCR2
fMLP	FPR1
LTB_4	BLT1
CCL3 (MIP-1α)	CCR4, CCR5
PAF	PAF receptor

Table 1. Neutrophil chemoattractants and their receptors. All receptors listed above are classified as G-protein coupled receptors. *CXCR1 is believed to be less important for chemotaxis than CXCR2.

The migration of neutrophils from the circulation out into the adjacent tissue is an extremely complex and minutely regulated process initiated by chemoattractants, but it also involves a series of other components including adhesion molecules. This process has been extensively studied and is often described by five major steps; capture, slow rolling, adhesion strengthening, intraluminal crawling and finally, paracellular or transcellular migration through the endothelium (14, 15). The process involves a number of adhesion molecules both on the neutrophil and on the endothelium. There are different classes of adhesion molecules, including integrins and selectins. Both integrins and selectins are transmembrane glycoproteins. Integrins are heterodimeric and consist of two subunits, α and β, while selectins are single-chained. A schematic overview of the adhesion molecules involved in the different steps is presented in figure 3.

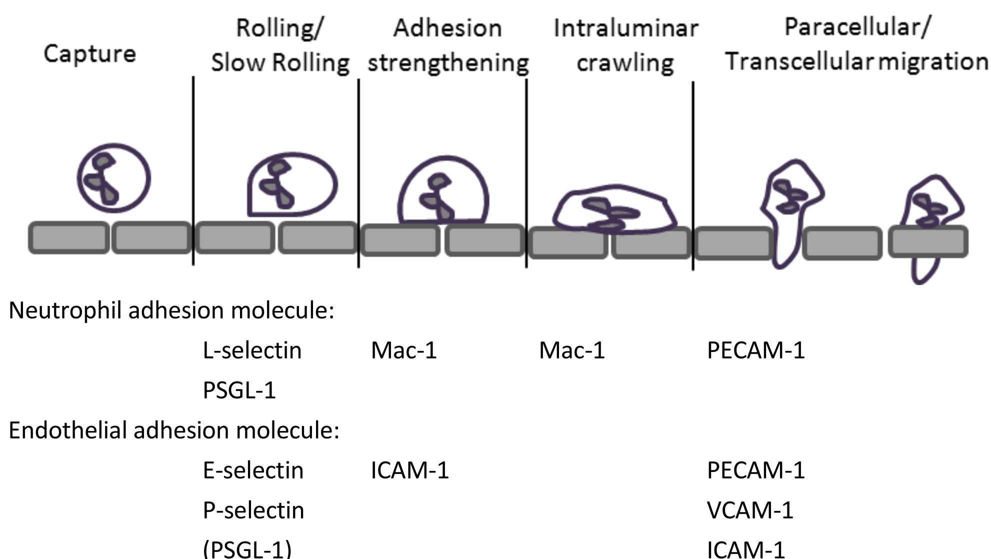


Figure 3: Schematic illustration of neutrophil migration from the blood vessel lumen. Adopted from Ley et al (14) and Gane et al (15)

Migration of neutrophils from the pulmonary circulation into the lung differs somewhat from migration into other tissues (15, 16). The alveolar capillary bed is an intricate web of interconnecting capillaries and the diameter of the vessels is often smaller than that of the neutrophil. As this causes the neutrophils to slow down or even stop, the mechanisms for rolling becomes unnecessary (16). Nonetheless, it appears that L-selectin and β_2-integrins can still be involved and act as activating stimuli on the neutrophils. Moreover, it has been suggested that substantial neutrophil sequestration does not occur in the capillary bed unless the neutrophil is activated (15).

Once the neutrophil arrives at the site of infection or inflammation its main task is to phagocytose and remove invading microorganism and cell debris. Neutrophils can engulf both opsonised and non-opsonised particles. The particles can be internalised in two different ways, firstly by being enclosed by pseudopods extending from the neutrophil and secondly by "sinking" into the cell (17). Next, the milieu in the vacuole undergoes a series of changes to become a phagosome with antimicrobial characteristics. This is brought about through the fusion of the vacuole with different granules and secretory vesicles that hold enzymes essential for the microbicidal activity.

During phagocytosis of invading of microbes, neutrophils increase their oxygen consumption, a phenomena called the respiratory burst. Central to this process is the NADPH-oxidase which generates superoxide anion (O^-_2) and hydrogen peroxidase (H_2O_2) which in turn generate other reactive oxygen species (18). Although the purpose of these reactive oxygen species is antimicrobial, they can also damage nearby tissues and immune cells and thereby worsen the inflammatory reaction.

Circulating neutrophils can be activated through a process called priming. In short, exposure of neutrophils to low levels of priming agents (e.g. Tumour Necrosis factor (TNF)-α) increase their capacity to respond to activating stimuli (19). Primed neutrophils have an increased respiratory burst activity, are less deformable, display enhanced expression of certain adhesion molecules (e.g. Mac-1/CD11b) and have a longer life-span compared to non-primed neutrophils (19). Taken together, these effects enhance the antimicrobial capacity of the neutrophils.

Traditionally, neutrophils have predominantly been recognised for their ability to capture, engulf and kill microorganisms. However, it is now generally recognised that neutrophils also regulate the immune responses executed by other immune cells (20, 21). Through the secretion of cytokines and chemokines, but also through direct cell-cell contact, the neutrophils are able to attract and activate several other types of immune cells including monocytes/macrophages, lymphocytes and dendritic cells (21, 22). Each neutrophil is capable of a relatively modest cytokine production, but this is compensated for by the high number of neutrophils present at the site of inflammation (21).

Finally, recent findings suggest that neutrophils are also involved in the resolution of inflammation via the production of lipid mediators with anti-inflammatory effects (20). One example is the production and release of lipoxin A which inhibits neutrophil recruitment (23).

Monocytes/Macrophages

Circulating monocytes differentiate into macrophages upon migration into the tissues. Macrophages are long lived, and together with neutrophils they are the only professional phagocytes. They are involved in inflammatory responses but also have central homeostatic functions like clearing erythrocytes and cells that have undergone apoptosis, processes that occur without macrophage activation. However, upon activation macrophages actively participate in the inflammatory process through the recruitment and activation of other immune cells as well as through production of pro-inflammatory cytokines and the release of toxic products (e.g oxygen radicals) (24).

Macrophages are often divided into two subgroups, M1 and M2, where M1 macrophages are considered to be classically activated, and M2 macrophages are activated by alternative mechanisms. The activation occurs on a floating scale where M1 and M2 represent the outermost alternatives (25). Macrophages of the M1 type are generated by IFN-γ stimulation alone or in combination with microbial products, they have a high antigen presenting capacity and a high production of pro-inflammatory cytokines and toxic elements (25). The M2 macrophages on the other hand, are generated by stimulation with IL-4 and IL-13 and represent a less pro-inflammatory subtype, where the ability to produce pro-inflammatory cytokines is less pronounced and also dependent on the stimulatory signals (25).

1.2.2 The Adaptive Immune System

Adaptive immunity, or acquired immunity, is characterised by high specificity and memory. It is slower to respond than the innate immune system and it takes about a week before a full response has been mounted to an invading pathogen. The two main cells of the adaptive immune system are T- and B-lymphocytes.
The high specificity is acquired by a system of receptors developed through a complex system of somatic gene rearrangements. Each B- or T-cell expresses only one type of receptor capable of recognising only one antigen. Upon activation of the cell, a clonal expansion is induced, resulting in a highly specific response. Once a pathogen has been removed the majority of the effector cells die, but a small fraction remain to form a memory. As a result of this memory, the adaptive immune system can respond faster and more efficiently next time the same pathogen is encountered.

1.2.2.1 Cells of the adaptive immune system

T-lymphocytes

The T-cells are produced in the bone marrow, but do not mature until they enter the thymus where the T-cell receptor is developed. The selection process in the thymus is uncompromising and only cells equipped with a receptor capable of recognising antigens leave the thymus. Thus less than 5% of all immature T-cells matures and re-enter the circulation (26).

Through the T-cell receptor, T-cells recognise antigen presented by the major histocompatibility complex (MHC) class I or II. T-cells are divided into several different subgroups, the first two being $CD4^+$ T helper (Th) cells which recognise antigen (e.g. extracellular/particulate peptides) presented to them by MHC class II and

$CD8^+$ T cytotoxic (Tc) cells which recognise antigen (e.g intracellular/cystolic peptides) presented by MHC class I (26).

Th-cells are further divided into subgroups. Th1-cells secrete IFN-γ and IL-2 and cause macrophage activation and inflammation. Th2-cells release IL-4 and IL-13 and increase antibody production and thus battle parasite infections. Th17-cells secrete IL-17 and are involved in neutrophil activation (27).

The main function of cytotoxic T-cells is to kill infected cells. This is achieved by the release of cytotoxins on the surface of the infected cells. In a manner analogous with the T helper cell nomenclature Tc-cells are also divided into type 1 and 2. Although Tc2-cells are associated with several chronic inflammatory conditions (e.g. COPD) they are not characterised in detail (28).

Another subgroup of T-cells are the regulatory T-cells (Treg), whose task it is to control and down-regulate the different T-cell responses (26).

B-lymphocytes

Like the T-cells, the surface of the B-cells is also covered by receptors, each cell expressing only one type of unique receptor. The B-cell becomes activated through the binding of an antigen to the B-cell receptor, in most cases the activation also requires a co-stimulatory signal from a T-cell. Upon activation, the B-cells differentiate into plasma cells or memory cells. The plasma cells produce copious amounts of antibodies directed at the pathogen. The memory cells remain in the circulation and if they encounter the same antigen again they rapidly differentiate to form new plasma and memory cells (26).

1.2.3 Interaction between the innate and adaptive immune system

Naturally the innate and the adaptive immune system cannot function as two non-communicating separate entities. One example of the crucial interaction between the two is the initiation of T-cell responses through activation by antigen presenting cells (e.g. dendritic cells and macrophages). Dendritic cells are present in all tissues and especially in the lungs and other areas in close contact with the external environment. The dendritic cells continuously sample their surroundings and present the resulting antigens on MHC class II molecules. Once activated the dendritic cells transfer to the secondary lymphoid tissues where they interact with appropriate T-cells and initiate a T-cell response (26).

Recently, it has also become apparent that there is cross-talk between neutrophils and dendritic cells through the release of chemokines and cytokines but also by direct cell-cell contact. Moreover, there is evidence that neutrophils interact with B- and T-cells. and thereby contribute in the forming of adaptive immune responses (20).

1.2.4 Inflammatory mediators

There are a vast number of cytokines and chemokines (chemotactic cytokines) with functions ranging from activation and regulation to termination of immune responses. The chemokines are characterised by their ability to induce chemotaxis and are mainly

associated with inflammation, however, several of them also has homeostatic and house-keeping functions. Listed below are a number of the cytokines and chemokines of particular importance to this thesis.

CCL2 is produced by monocytes/macrophages as well as neutrophils, and is an important chemoattractant for monocytes and dendritic cells. In addition it has an activating effect on macrophages and promotes a Th2 response (26).

CCL3 is a chemoattractant for a number of different cells including T-cells, monocytes and neutrophils. The producers include neutrophils, lymphocytes and macrophages (29).

CXCL1 (GRO-α) is produced by a number of different cells including neutrophils, macrophages and epithelial cells. CXCL1 is a chemokine mediating its effects mainly through the CXCR2 receptor, expressed primarily on neutrophils.

CXCL8 (IL-8) is produced by various cell types including neutrophils, macrophages and epithelial cells. Of all the cytokines produced by neutrophils, CXCL8 is the most abundant and also the most studied (30). Moreover, neutrophils are the primary target for CXCL8 in which the induced responses include migration, activation, degranulation and increased respiratory burst (30). The effects are mediated through CXCR1 and CXCR2 receptors.

CXCL9, **CXCL10** and **CXCL11** all bind to the CXCR3 receptor. They are produced by a variety of cells including macrophages and neutrophils (31). These chemokines are regulated by IFN-γ and have been attributed a role in the recruitment of T-cells particularly those of cytotoxic type (32).

IFN-γ is an important cytokine with both activating and regulating functions. IFN-γ has a central function in promoting cell-mediated immunity. It is produced primarily by T-cells and NK-cells but asserts its effect on several immune cells including macrophages (33). There is also evidence that some cell types (e.g. macrophages) can produce IFN-γ in self-activating purpose (33).

LTB_4 is produced by neutrophils as well as macrophages and dendritic cells. LTB_4 is an important chemoattractant for neutrophils as well as T-cells and it is considered to be one of the key chemoattractants for neutrophil migration into the lungs. Moreover, it initiates and enhances several important microbicidal activities in neutrophils. Most of its actions are mediated through interaction with the BLT_1 receptor (34, 35).

TNF-α is a powerful pro-inflammatory cytokine produced by macrophages, T-cells and many other immune cells. It is expressed locally at sites of inflammation but also systemically and has a series of different effects including recruitment of immune cells and production of pro-inflammatory cytokines (36). The effects are mediated through the interaction with TNFR1 and TNFR2 receptors (36).

Several different antibodies directed at TNF-α or its receptors are currently used successfully in the treatment of inflammatory diseases such as rheumatoid arthritis.

However, the few studies performed in COPD patients show discouraging results (36). The most promising results from infliximab trials showed a modest trend towards improvement in 6 minute walk test in one study of moderate to severe COPD patients and a minor effect on markers of systemic inflammation in cachectic COPD patients (37, 38).

1.3 CHRONIC OBSTRUCTIVE PULMONARY DISEASE (COPD)

Chronic obstructive pulmonary disease (COPD) is a growing world-wide health problem. Several projections of the global burden of COPD have been made, a study frequently referred to estimates COPD to be the third leading cause of death globally in 2020 (2).

The most common cause of COPD is tobacco smoking, but other exposures such as occupational exposures are also of importance (1). Exposure to smoke from biomass fuels is an important factor especially in the developing world where cooking over open fire together with poor indoor ventilation is a common cause of COPD in women (39). Naturally, there is a genetic element to the disease and it is well-known that persons with alpha-1-antitrypsin deficiency have an increased risk of developing emphysema with chronic airflow limitation (40). Associations with other genetic factors have been found but repeatability between studies is often low (41).

There have been, and still are, variations in the definitions of COPD. An attempt to unite the views on COPD diagnosis, treatment and intervention and also increase the awareness of COPD has resulted in the WHO sponsored Global Initiative for Chronic Obstructive Lung Disease (GOLD) (42). The GOLD classifications of COPD are used throughout this thesis (Table 2).

SPIROMETRIC CLASSIFICATION OF COPD FEV1/FVC<0.70 IS REQUIRED FOR ALL STAGES.	
Stage I: *Mild*	$FEV_1 \geq 80\%$ predicted
Stage II: *Moderate*	$50\% \leq FEV_1 < 80\%$ predicted
Stage III: *Severe*	$30\% \leq FEV_1 < 50\%$ predicted
Stage IV: *Very Severe*	$FEV_1 < 30\%$ predicted or <50% predicted and chronic respiratory failure with additional negative prognostic factors

Table 2. Classifications of COPD according to GOLD(42). All values must be measured after bronchodilation.

The historical inconsistencies in the definitions used to identify COPD, an unawareness of the disease in its early stages and cultural biases has resulted in a large variance in the estimations of the disease prevalence. However, a recent world-wide study based on the GOLD criteria suggests that the prevalence of COPD is about 10% (1). The

proportion of smokers who develop COPD increases with increasing age and although there are diverging results, COPD seems to be present in approximately 50% of smokers who have reached the age of 75 years (43, 44).

The disease is characterised by an irreversible airflow obstruction caused by a chronic inflammation of both small and large airways. The airflow limitation has several components. Firstly, contributing to the airflow limitation only marginally, there is an inflammation of the central airways, bronchitis. This is associated with an increased mucous production, malfunctioning mucociliary clearance, disruption of the epithelial barrier and a thickening of the bronchial wall (45). Secondly, airflow limitation arises from obstruction of the small peripheral airways. This is caused by mucus and a narrowing of the airway lumen which in turn is the result of the on-going inflammation (45). Thirdly, airflow limitation is caused by the emphysema. It has recently been shown that a narrowing and loss of terminal bronchioles precede the emphysema in COPD (46). The emphysema is distant to the terminal bronchiole and the destruction of the tissue has several components; loss of alveolar walls, enlargement of alveolar spaces and loss of alveolar attachments. In particular, the loss of alveolar attachments causes the elastic recoil to be reduced (45).

The most characteristic symptom of COPD is dyspnoea, but often it does not appear until the disease has reached a moderate or, more often, severe stage. Instead the first symptoms are often long-lasting cough, sputum production and wheeze, together with repeated and long-lasting infections (47).

Exacerbations are defined as periods of worsening of the disease, often triggered by viral or bacterial infections. An exacerbation is characterised by a worsening of dyspnoea, cough, sputum production or sputum purulence (48). During exacerbations the inflammation, both in the lungs and systemically, is increased and it is well-known that a high exacerbation frequency has a negative impact on not only quality of life but also disease progress (49-51). Exacerbation are also related to several other factors that are of importance for the course of the disease and mortality; these include dyspnoea, decreased exercise capacity, lung function impairment over time, and increased levels of biomarkers such as C reactive protein (CRP) and fibrinogen (49, 52, 53). The increase in circulating markers of inflammation demonstrates that the disease is not restricted to the airways and COPD is today recognised as a systemic disease (54). Subsequently, COPD is also associated with a series of comorbidities including cardiovascular disease, lung cancer, metabolic syndrome, osteoporosis, skeletal muscle dysfunction and cognitive dysfunction (55). Only in the more severe stage of the disease are respiratory problems the primary cause of death (56).

Inflammatory cells in COPD

The airway inflammation in COPD involves a number of different cell types and the number of macrophages, neutrophils and $CD8^+$ T-cells are all increased in the COPD lung. In biopsies, bronchoalveolar lavage (BAL) fluid and also sputum a series of studies have found increased numbers of neutrophils in the COPD lung (57-59). There is also a relationship between neutrophil numbers in sputum and the rate of decline in lung function, indicating that neutrophils contribute to the disease progression (60). During exacerbations, the neutrophil influx into the airways increases further and there is also an increase in the neutrophil chemoattractant CXCL8 and its receptors (61).

Many neutrophil characteristics designed to fight pathogens also have the potential to cause tissue damage and emphysema. These include the release of neutrophil elastase (NE), proteinase-3, matrix metalloproteinases (MMP)-9 and the production of reactive oxygen species (62). Neutrophil elastase can also stimulate mucus production and has also been shown to reduce the beat frequency of epithelial cilia (62).

Both circulating and sputum neutrophils from subjects with COPD exhibit an increased expression of the adhesion molecule and activation marker CD11b (Mac-1) (63, 64). Also several other markers of neutrophil activation such as myeloperoxidase (MPO) and human neutrophil lipocalin (HNL) are increased in BAL fluid, even in smokers with mild COPD (65). An increased ability of neutrophils from COPD patients to digest fibronectin *in vitro* has also been described (66), contributing further to the picture of neutrophil activation in COPD.

There are several plausible explanations for the airway neutrophilia observed in COPD; these include increased migration of neutrophils to the airways as well as prolonged survival of the neutrophils. Acute smoke exposure causes circulating neutrophils to become less deformable and it is likely that this contributes to an increased sequestration of neutrophils into the lung (67). Oxidative stress has been suggested to be one of the causes of the reduced neutrophil deformability (68). In addition, there are other mechanisms that may contribute to the increased neutrophil presence in the COPD lung. For example, important neutrophil chemoattractants (e.g. CXCL8 and LTB_4) are found in increased levels in the COPD airways and there is also a relationship between the levels of CXCL8 and the number of neutrophils (69, 70). However, data on neutrophil chemotaxis in COPD are conflicting (66, 71). An early study found increased chemotaxis towards fMLP in circulating neutrophils from subjects with emphysema (66) while a more recent study found decreased migration to CXCL8 and fMLP by circulating neutrophils from subjects with COPD (71). Circulating neutrophils from patients with COPD do not differ in apoptosis rate compared to neutrophils from healthy subjects (72).

There is an increase in macrophages and chemokines important for macrophage recruitment in the airways of COPD (57, 73). Several of the characteristic macrophage features (e.g. release of reactive oxygen species and metalloproteinases) could give rise to the tissue damage observed in the COPD lung. Macrophages from COPD patients have also been shown to release increased amounts of CXCL8, a key chemoattractant for neutrophils (74).

Alveolar macrophages and monocyte derived macrophages from smokers with COPD phagocytose bacteria less efficiently than the same cells from non-smoking healthy controls (75). It is possible that this defect could be of importance for the initiation of bacterial exacerbations. Moreover, corticosteroids are less effective at reducing airway inflammation in COPD than in asthma. As one of the reasons for this is a reduced histone deacetylase 2 (HADAC2) activity in alveolar macrophages from patients with COPD has been suggested (76). The decreased HADAC2 activity in alveolar macrophages correlates with an increased production of pro-inflammatory cytokines and a decreased response to corticosteroids (77).

Another cell occurring in increased numbers in the airways of patients with COPD is the T-cell and $CD8^+$ T-cells are often increased to a larger extent than $CD4^+$ T-cells (57, 78, 79). There is a negative relationship between $CD8^+$ T-cell numbers and FEV_1 suggesting that $CD8^+$ T-cell might be of importance for disease progression (78). The cytokine profile of the T-cells in the COPD airway indicates that they mainly are of the Tc1 type releasing for example IFN-γ (80). Studies of bronchial biopsies have shown an increased expression of CXCR3, co-localised with CD8 and IFN-γ, in subjects with COPD (81). Production of the CXCR3 receptor ligands is induced by IFN-γ. It has been suggested that a self-perpetuating loop, created by the CXCR3 expressing T-cells which release IFN-γ and thereby cause production of more CXCR3 ligands and renewed T-cell recruitment, might be of pathophysiological importance (81). Moreover, CXCR3 and its ligands are of importance for the formation of lymphoid follicles in COPD (82).

Treatment

The destruction of the lungs observed in COPD is irreversible and treatment of COPD is currently targeted at slowing the disease progression, mitigating symptoms, increase the physical capacity and preventing exacerbations (83). The treatment is based on smoking cessation, pharmacological treatment, physiotherapy and rehabilitation. Of these, smoking cessation is the only alternative that has a certain impact on the rate of lung function decline (84). Physical rehabilitation has been shown to be an important component of the COPD treatment (85, 86). Physiotherapy is often a part of the rehabilitation and its aim is to improve, maintain or compensate physical problems caused by the disease.

The pharmacological treatment is based on inhaled bronchodilators and corticosteroids. The long-acting anticholinergic bronchodilator tiotropium is the primary choice and has been shown to improve lung function and quality of life and to reduce exacerbations (87). Tiotropium is a muscarinic receptor antagonist which causes relaxation of the airway smooth muscle through its binding to muscarinic receptors on the smooth muscle cell in the airways (88).

A second type of bronchodilator used in COPD treatment is the β_2-adrenoceptor agonists. The agonist binds to the β_2-receptors on the airway smooth muscle, this leads to an activation of stimulatory G-protein (G_s) which triggers a cascade of down-stream events resulting in relaxation of the smooth muscle (88). With the aim to prevent exacerbations, the β_2-agonists are used in combination with inhaled corticosteroids in patients with moderate to severe COPD with recurring exacerbations (83). Patients with severe COPD have been shown to benefit from a combination of tiotropium, a long-acting β_2-agonist (formoterol) and an inhaled corticosteroid (budesonide) compared to treatment with tiotropium alone (89).

The corticosteroids mediate their effects by switching off pro-inflammatory genes that have been activated by the inflammation (90). In short, the steroid binds to the intracellular glucocorticoid receptor (GR) and forms an active complex which translocates to the nucleus where it binds to specific DNA sequences in the promoter region of the target genes (88). As an alternative route the active steroid-receptor complex can interact directly with transcription factors such as NF-κB (88). The

combination of β_2-agonists and inhaled corticosteroids is beneficial as steroids appear to potentiate the effects of the β_2-agonists on bronchial smooth muscle, and also prevent and reverse β_2-receptor desensitisation in the airways (88). Steroids stimulate transcription of the β_2-receptor protein by binding to the glucocorticoid responsive element in the promoter region of the β_2-receptor gene. Conversely, β_2-agonists promote the localisation of GR's to the nucleus and augment the binding of GR to its specific target DNA sequences (91, 92). While β_2-agonists and inhaled corticosteroids have effects when given separately, their combination is more effective in reducing exacerbation rate and improving health status (83, 93-95).

2 AIMS

The overall aim of this thesis was to elucidate how the inflammation observed in COPD and treatment with steroids and β_2-adrenoceptor agonists alters neutrophil function. The following specific hypotheses were investigated:

- Chemokine release by circulating neutrophils is altered in COPD

- Circulating neutrophils release chemokines upon activation by LPS, organic dust and TNF-α. This release is partly mediated by neutrophil derived TNF-α. The TNF-α mediation of chemokine release is altered in smokers with COPD.

- The neutrophil chemotactic response to common chemoattractants is increased in smokers with COPD. This is part of the mechanism underlying the neutrophilia observed in the lungs of patients with COPD

- Stimulation of neutrophils with glucocorticosteroids and β_2-adrenoceptor agonists alters neutrophil chemotaxis, receptor expression and chemokine release

- Increased production of CXCR3 binding chemokines by alveolar macrophages cause the increased presence of CXCR3 expressing $CD8^+$ T-cells observed in the COPD airways

- As neutrophils migrate from the circulation into the lungs they may undergo changes in expression of adhesion molecules. These changes differ between smokers and non-smokers and between smokers with and without COPD.

3 MATERIAL AND METHODS

Material and methods are briefly summarised in the following section. More detailed information is provided in the publications and manuscripts.

3.1 STUDY POPULATION

Non-smokers with normal lung function were recruited as controls.
Smokers without COPD had a post-bronchodilator $FEV_1/FVC > 0.7$ and were matched with regard to age and cumulative exposure to tobacco smoke (assessed as pack-years) to smokers with COPD who had a post-bronchodilator $FEV_1/FVC < 0.7$ and $FEV_1 > 40\%$ predicted. Spirometry was performed according to the current ATS guidelines (96) and ERS reference values were used (97). Combivent® (ipratropium and salbutamol) was used as bronchodilator.

Smoking was not allowed on the day of the examination and all subjects had been free of respiratory infections 4 weeks prior to the visits. Furthermore, no one in the study population had a history of asthma, allergy or other chronic disease.

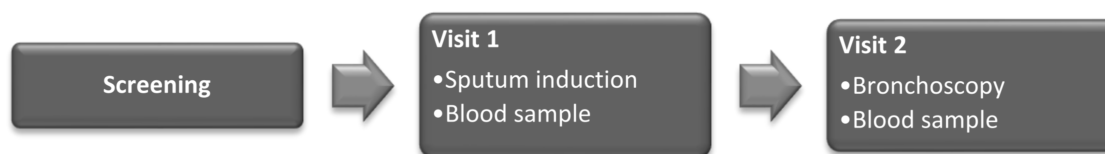


Figure 4. Study design

3.2 SAMPLE COLLECTION

Blood sampling

Blood samples for flow cytometric analysis of surface markers were collected in EDTA vacutainer tubes, while samples for isolation of different leukocyte populations were collected in heparinised tubes and samples for serum were collected in supplement-free tubes.

Bronchoalveolar lavage

After premedication with morphine or pethidine and scopolamine broncoscopy was performed with local anaesthesia with xylocain. The bronchoscope was wedged into a middle lobe segmental bronchus and isotonic saline was instilled into the airway tree and carefully sucked back. Bronchial mucosal biopsies were taken from subcarinas of an upper lobe segment.
The lavage fluid was pooled and centrifuged and alveolar macrophages were immediately isolated from the cell pellet. Prior to isolation of macrophages slides were prepared by cytocentrifugation for May-Grünwald Giemsa staining and differential cell counts. The lavage fluid was divided into aliquots and stored at -70°C until analysis.

Sputum induction and sputum processing

Following inhalation of salbutamol (0.4 mg) sputum was induced by inhalation of hypertonic saline in increasing concentrations (3%, 4% and 5%) using an ultrasonic nebuliser. Lung function (FEV_1) was measured after each concentration and the subject also made an attempt to expectorate sputum. Samples macroscopically free of saliva and >1g were accepted. The sputum sample was then treated with dithiothreitol, passed through a filter and centrifuged. The isolated cell pellet was immediately processed for analysis of surface markers. Slides were also prepared by cytocentrifugation for May-Grünwald Giemsa staining and differential cell counts.

3.3 ISOLATION OF CELLS

Isolation of neutrophils from blood

Whole blood was mixed with D-PBS containing dextran (2%) and allowed to sediment for 40 minutes to minimise the presence of erythrocytes in the subsequent steps. Next, the leukocyte containing fraction was separated over a density gradient (Lymphoprep™) and the neutrophil containing pellet was collected. From this step onwards all work was performed on ice. Contaminating erythrocytes were removed by hypotonic lysis. The neutrophils were then washed twice in D-PBS and resuspended in supplemented RPMI.

Isolation of lymphocytes from blood and preparation for chemotaxis

Similar to the protocol for neutrophil isolation dextran sedimentation of whole blood was followed by density gradient separation and hypotonic lysis. Next, the lymphocyte containing fraction was collected and incubated with CD14-labelled magnetic beads. The CD14-negative cells were isolated using a separation column and a magnet according to the instructions of the manufacturer (MACS®). The CD14-negative cells were washed and resuspended in RPMI culture media. To induce expression of CXCR3 the media contained IL-2 and PMA. After two weeks culture the cells were analysed for CXCR3 expression and used for chemotaxis.

Isolation of alveolar macrophages and lymphocytes from BAL fluid

The cell pellet obtained from the BAL fluid was resuspended and seeded into plates. The cells were allowed to adhere for two hours after which the alveolar macrophages were adherent. Non-adherent cells were collected and alveolar macrophages were left to rest over night before stimulation experiments were performed. The non-adherent cells were taken for flow cytometric analysis for phenotyping of lymphocytes subsets.

3.4 CHEMOTAXIS

Chemotaxis was performed as described by Frevert et al (98) with minor modifications. In short, a filter assay system (ChemoTx) with 5µm pores was used. Isolated cells labelled with Calcein AM were carefully placed on the top of the filter and allowed to migrate for 60 minutes at 37°C. Cells that had moved through the filter were detected using a multi well fluorescent plate reader and migration was quantified as percentage of the maximum migration corrected for spontaneous migration.

In study III neutrophils were incubated with formoterol, budesonide, formoterol + budesonide, anti-CXCR1, anti-CXCR2 or anti-CXCR1 + anti-CXCR2 for 20 minutes prior to migration.

3.5 STIMULATION OF ISOLATED CELLS

Neutrophils
In Study I neutrophils isolated from blood were incubated for 4 or 16 hours with LPS, TNF-α, organic dust alone or in combination with infliximab (anti-TNF-α antibody). The supernatants were collected and stored at -70°C until analysis.
In study III neutrophils isolated from blood were stimulated with LPS for 8 hours in the presence of formoterol and/or budesonide

Alveolar macrophages
Alveolar macrophages were stimulated with IFN-γ for 6 hours. The supernatants were collected and stored at -70°C until analysis.

3.6 MEASUREMENT OF SOLUBLE ADHESION MOLECULES AND CYTOKINES

ELISA
The following cytokines and chemokines were all measured in serum using purchased DuoSet ELISA kits: TNF-alpha (Study II), CXCL9, CXCL10, CXCL11 (Study IV). Moreover, CCL3 was measured in supernatants from stimulated neutrophils also using a DuoSet ELISA kit (Study I).

Flow cytometry
Chemokines and cytokines (CCL2, CXCL8, TNF-alpha and IL-1β) were measured on a FACS Calibur cytometer using cytometric bead array (Study I). Also using cytometric bead arrays soluble adhesion molecules were analysed in serum, sputum and BAL fluid using Adhesion 6-plex FlowCytomix™ Multiplex kit (preliminary data).

3.7 MEASUREMENT OF CELL SURFACE MARKERS

T-lymphocyte subsets
In study IV T-cell subsets (blood) were determined using a four-colour antibody mixture (CD3 (FITC)/CD8 (PE)/CD45 (PerCp)/CD4 (APC) from BD Bioscience) together with TruCOUNT™ tubes which contain a specified number of beads. Samples were analysed using MultiSet™ (BD Bioscience) to determine absolute numbers of white blood cells and T-cell subsets
BAL lymphocytes were also labelled using the four-colour antibody mixture but analysed using CELLQuest™ software (BD Bioscience). To selectively gate for lymphocytes, side scatter and CD45 were used.

CXCR1 and CXCR2
In study III cell surface expression of CXCR1 and 2 was measured on neutrophils using flow cytometry. PE-labelled antibodies for CXCR1 and 2 were used together with an

anti-CD45 (PerCp). An isotype matched control was also used. Results were expressed as relative mean fluorescence intensity (rMFI=monoclonal antibody/matched isotype control).

Adhesion molecules
Whole blood was stained with titrated amounts of anti-CD11b PE, anti-CD62L PE or anti-CD162 PE together with anti-CD45 PerCp. Isotype matched anti-bodies were used as negative controls. Results were expressed as mean fluorescence intensity (MFI=monoclonal antibody-matched isotype control).

Bronchial biopsies
Biopsy specimens were embedded in glycol methacrylate and processed as previously described with minor modifications (99). Sequential biopsy sections (2µm) were cut from the resin blocks with a microtome and floated onto 0.2% ammonia solution prior to adherence to glass microscope slides coated in poly-L-lysine.
Biopsies were double-stained for neutrophil elastase and one of the following adhesion molecules: CD11b, CD62L or CD162.

3.8 STATISTICS

Data are presented as median values with 25th-75th percentiles and mean values with 95% confidence intervals as indicated in figure and table legends. Data considered to be normally distributed were analysed using analysis of variance (ANOVA).
Data not normally distributed were analysed using nonparametric tests. Kruskal Wallis and Mann Whitney were used for between group comparisons and Friedman test followed by Wilcoxon Signed Rank test were used for within group comparisons. Correlations were assessed using Spearman's rank correlation. A p-value <0.05 was considered significant in all studies.

4 RESULTS

4.1 PAPER I

The aim of study I was to investigate whether chemokine release by neutrophils in smokers with and without COPD is altered compared to non-smoking healthy controls. It has been suggested that LPS-induced release of CXCL8 occurs in two phases. The initial response is caused by LPS directly and includes the release of TNF-α, the second phase of the response is then partly mediated neutrophil derived of TNF-α (100). We thus hypothesised that this TNF-α loop is altered in neutrophils from subjects with COPD as compared to healthy subjects.

Both CXCL8 and CCL3 were spontaneously released by neutrophils. This spontaneous release could be inhibited by the addition of infliximab, indicating that there is a spontaneous TNF-α release which affects chemokine release.

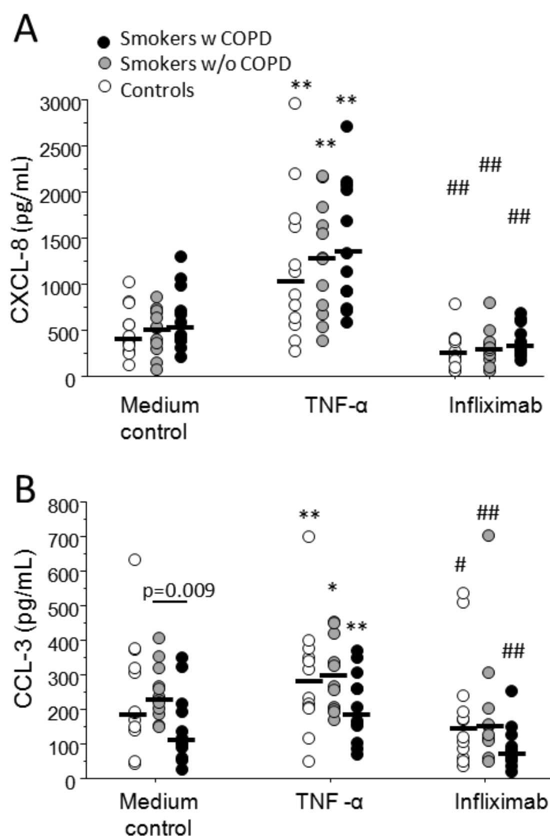


Figure 5. Release of a) CXCL-8 b) CCL-3 from unstimulated cells and cells treated with TNF (5 ng/mL) or infliximab (5µg/mL).
*p≤0.05, **p≤0.01 indicate the effect of TNF compared to unstimulated control at the same time point.
p≤0.05, ## p≤0.01 indicate the effect of infliximab compared to unstimulated control at the same time point

Stimulation with LPS caused an increase in chemokine release in all groups. The LPS induced chemokine release was inhibited by the presence of infliximab in all groups except for the CXCL8 release in the COPD group.

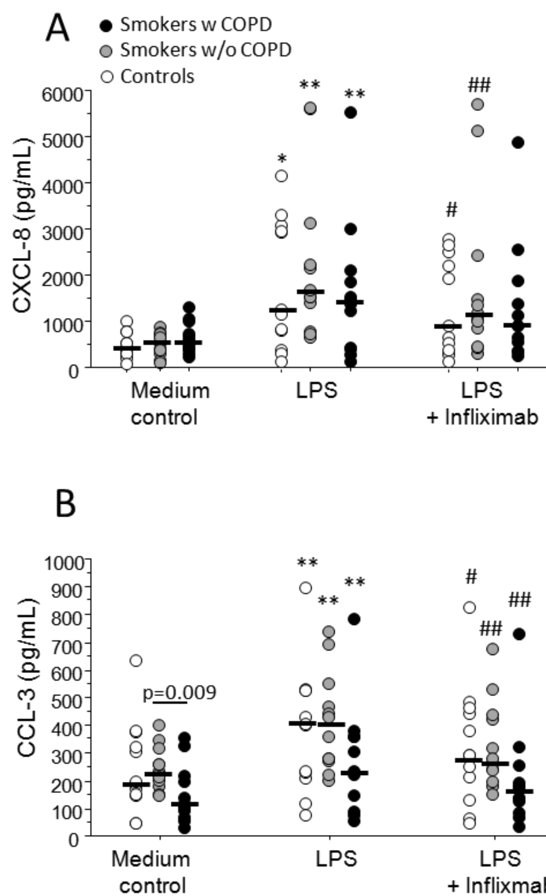


Figure 6. Release of a) CXCL-8 b) CCL-3 from LPS (1 µg/mL) stimulated cells and cells treated with LPS (1 µg/mL) and infliximab (5µg/mL). *p≤0.05, **p≤0.01 indicate comparison with unstimulated control at the same time point. # p≤0.05, ## p≤0.01 indicate comparison with LPS stimulated cells.

Stimulation with organic dust resulted in a chemokine release pattern similar to that caused by TNF-α. In addition to CXCL8 and CCL3, the release of TNF-α, IL-1β and CCL2 was also measured. The levels were generally low, with no significant differences between the groups and therefore pooled data are presented.

	Medium	**LPS**		**TNF-α**		**Organic dust**	
			p value		p value		p value
IL-1β	94.4 (46.2-149.3)	85.6 (41.7-129.9)	0.08	88.9 (58.8-130.4)	0.5	100.3 (55.5-141.0)	**0.02**
CXCL8	496.8 (351.6-715.0)	1512 (750.7-2986)	**<0.0001**	1181 (728.1-1784)	**<0.0001**	2189 (929.3-2189)	**<0.0001**
CCL2	11.9 (0.6-30.5)	15.38 (0.6-35.0)	0.9	6.0 (0.6-30.7)	0.5	13.4 (0.6-32.0)	0.8
CCL3	197.5 (132.2–314.7)	333.1 (226.0–460.1)	**<0.0001**	262.1 (184.1-346.6)	**<0.0001**	299.3 (221.9-432.4)	**<0.0001**
TNF-α	102.7 (101.2-106.8)	104.4 (102.2-106.7)	**0.05**	-	-	105.5 (103.4-108.4)	**0.006**

Table 3. Comparisons between medium control and stimuli at 16 hours (pooled data from three groups (n=36)). Results are expressed as pg/mL. Data are presented as median (25th -75th percentiles). P-values indicate comparison with medium.

4.2 PAPER II

The aim of this study was to investigate whether neutrophil chemotaxis is enhanced in smokers and in particular smokers with COPD. The results show increased chemotaxis towards CXCL8 in smokers irrespective of airway obstruction. Moreover, chemotaxis towards LTB_4 was increased in smokers without COPD, while there was no difference between groups in migration towards fMLP.

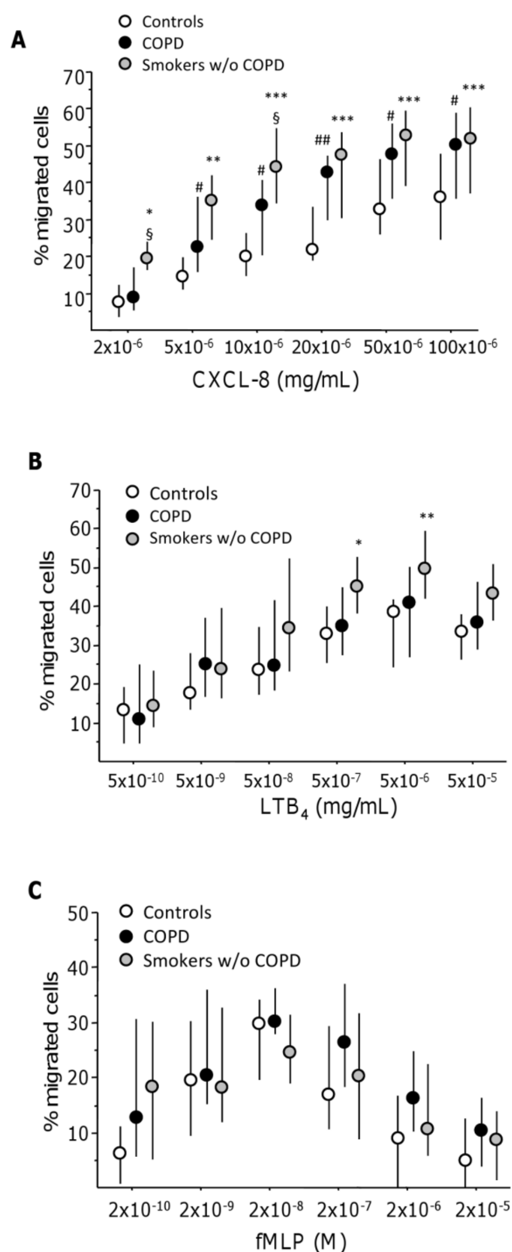


Figure 7: Neutrophil migration Data are presented as median and 25th-75th percentile**.**

A. Neutrophil migration (% migrated cells) induced by CXCL-8.
# p<0.05, ## p<0.01 represents non-smokers *vs.* COPD.
*p<0.05, **p<0.01, ***p<0.001 represents non-smokers *vs*. smokers without COPD.
§ p<0.05 represents smokers with COPD *vs*. without COPD

B. Neutrophil migration (% migrated cells) induced by LTB_4.
*p<0.05, **p<0.01 represents non-smokers vs. smokers without COPD

C. Neutrophil migration (% migrated cells) induced by fMLP.

Tumour necrosis factor-α can function as a priming agent for neutrophils and thereby increase their ability to respond to chemotactic stimuli. Thus serum TNF-α was measured to study the potential relationship with chemotactic response. Although there was no difference between groups, there was a correlation between the chemotactic response and serum TNF-α in the two smoker groups.

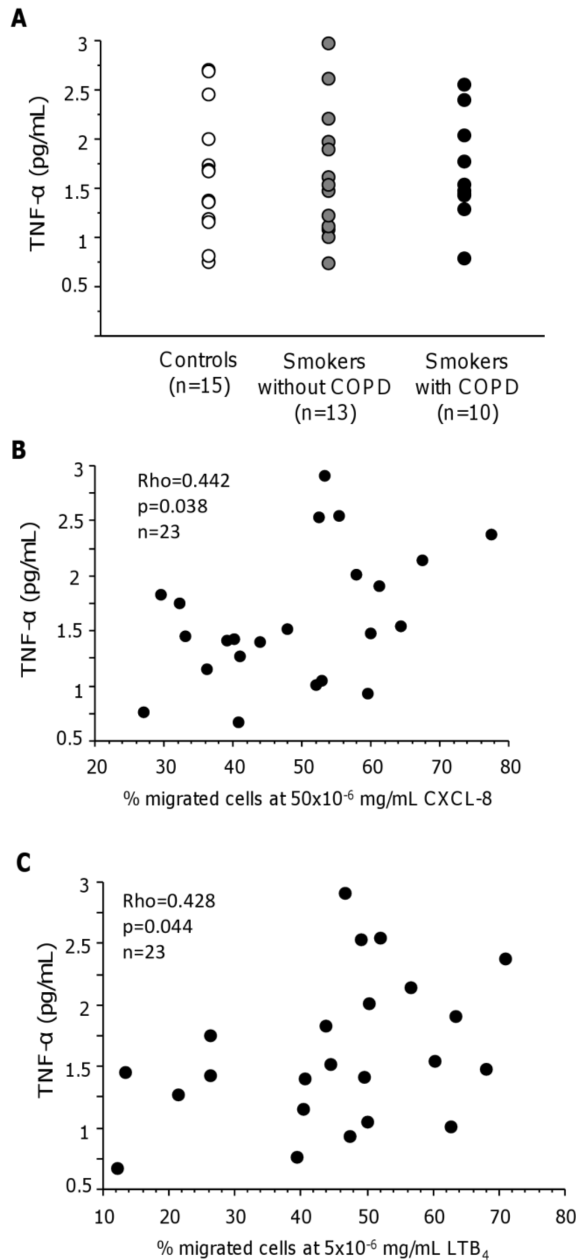


Figure 8: Serum TNF-α and neutrophil migration.
A. Serum concentration of TNF-α in smokers with and without COPD and in non-smokers. Due to technical problems with blood sampling (haemolysis and damage of test tubes) serum TNF-α was not analysed in two smokers without COPD and five smokers with COPD.
B. Correlation between serum levels of TNF-α and neutrophil migration (% migrated cells) towards CXCL-8 at a concentration of 50 $x10^{-6}$ mg/mL in the two groups of smokers (n=23).
C. Correlation between serum levels of TNF-α and neutrophil migration (% migrated cells) towards LTB_4 at a concentration of $5x10^{-6}$ mg/mL in the two groups of smokers (n=23).

4.3 PAPER III

In Study III the aim was to investigate the effects of formoterol (β_2-agonist) and budesonide (corticosteroid) on neutrophil function. Formoterol and budesonide are often used as a combination therapy in COPD and asthma and here we study their effects on chemokine release, expression of chemokine receptors and chemotaxis in neutrophils. The study was performed on isolated blood neutrophils from 10 healthy subjects.

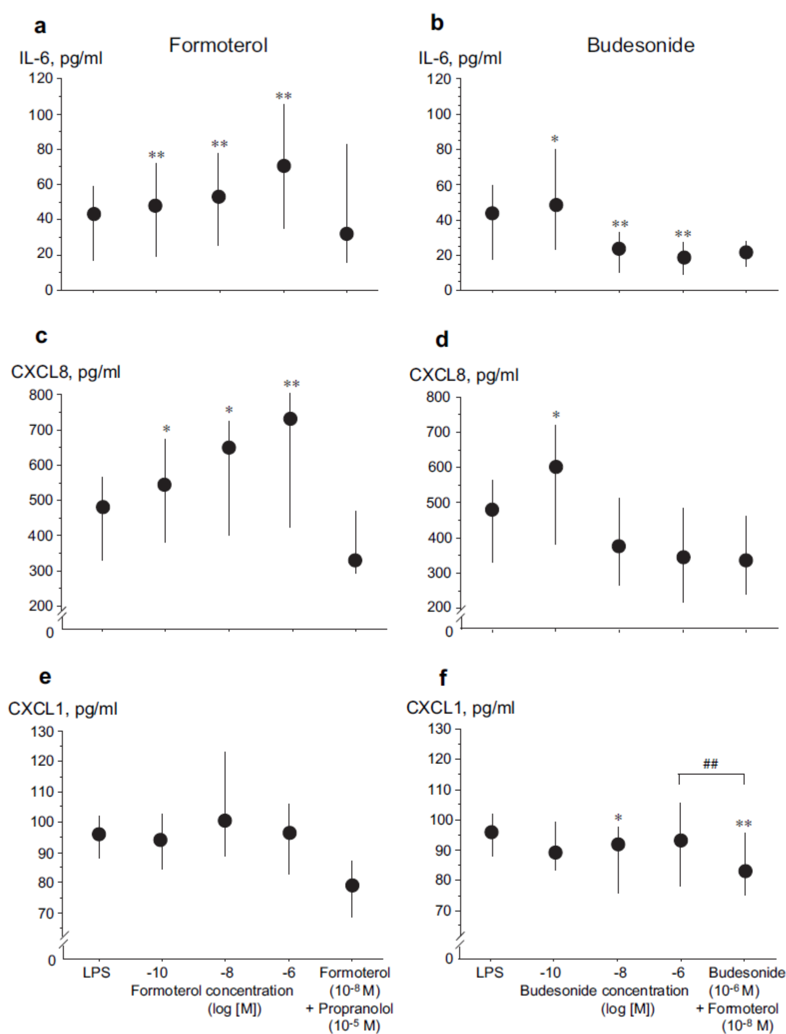


Figure 9.
Effect of formoterol and budesonide on IL-6, CXCL8 and CXCL1 release from LPS-stimulated (8h) neutrophils (n= 10). Propranolol was used for blocking of the formoterol effects. Data are presented as median and 25th -75th percentiles. *$P < 0.05$, **$P < 0.01$ indicate comparison with LPS-stimulated neutrophils

Neutrophil release of IL-6 and the two chemokines CXCL1 and CXCL8 was measured after 8 hours stimulation with LPS (1 μg/mL). The LPS induced release of IL-6 and CXCL8 was enhanced by formoterol, while no effects on CXCL1 were detected. The effects of formoterol were abolished by propranolol. Moreover, budesonide inhibited release of IL-6 and CXCL1 and the pattern for CXCL8 was similar but did not reach significance.
Budesonide and formoterol also had a synergistic effect on CXCL1 release. However, neither budesonide nor formoterol had any measurable effect on IL-6, CXCL1 and CXCL8 release in resting (not LPS stimulated) neutrophils (data not shown).

Formoterol increased the expression of CXCR1 and CXCR2 at all concentrations and the effect was blocked by propranolol. Budesonide increased the expression of CXCR2 but had no effect on CXCR1 expression; the addition of formoterol had no further effect (figure 10).

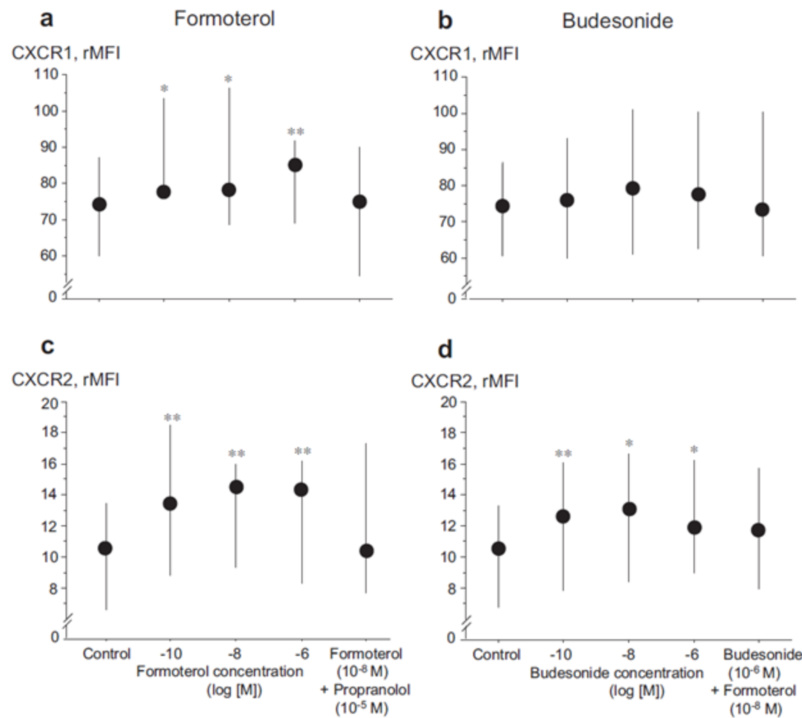


Figure 10. Effect on 30 minute incubation with formoterol and budesonide on CXCR1 and CXCR2 expression on neutrophils (n=10). Propranolol was used to block the formoterol effects. Results are expressed as rMFI and presented as median 25th-75th percentile.
*$P < 0.05$, **$P < 0.01$ indicate comparison with control values.

Incubation with CXCL8 decreased the expression of both CXCR1 and CXCR2; the addition of formoterol and/or budesonide had no effect on this receptor down-regulation. Incubation with CXCL1 decreased CXCR1 expression and this was unaffected by the addition of formoterol and/or budesonide. There was a tendency towards up-regulation of CXCR1 by CXCL1 and this effect was inhibited by the incubation with formoterol and/or budesonide.

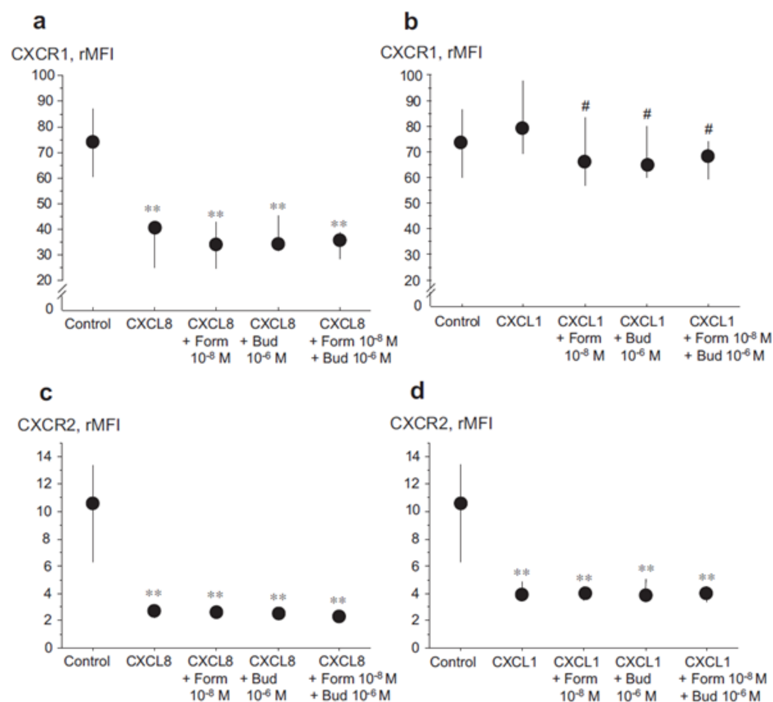


Figure 11.
Effect on 30 minute incubation with formoterol and budesonide CXCR1 and CXCR2 expression on CXCL1 and CXCL8 treated neutrophils (n=10). Results are expressed as rMFI and presented as median 25th-75th percentile.
*P < 0.05, **P < 0.01 indicate comparison with control values.

There were no significant effects of the treatments with formoterol or budesonide on neutrophil migration. The combination of anti-CXCR1 and anti-CXCR2 antibodies reduced migration towards CXCL8, but had no significant effects separately. The antibodies against CXCR1 and CXCR2 had no effect on CXCL1 induced migration.

4.4 PAPER IV

In paper IV the aim was to investigate release of CXCR3 binding chemokines by alveolar macrophages and to study their effects on lymphocyte migration. Alveolar macrophages from all three groups were stimulated with IFN-γ and the supernatants were analysed for chemokine content and ability to induce lymphocyte. Moreover, CXCR3 binding chemokines were analysed in BAL fluid.

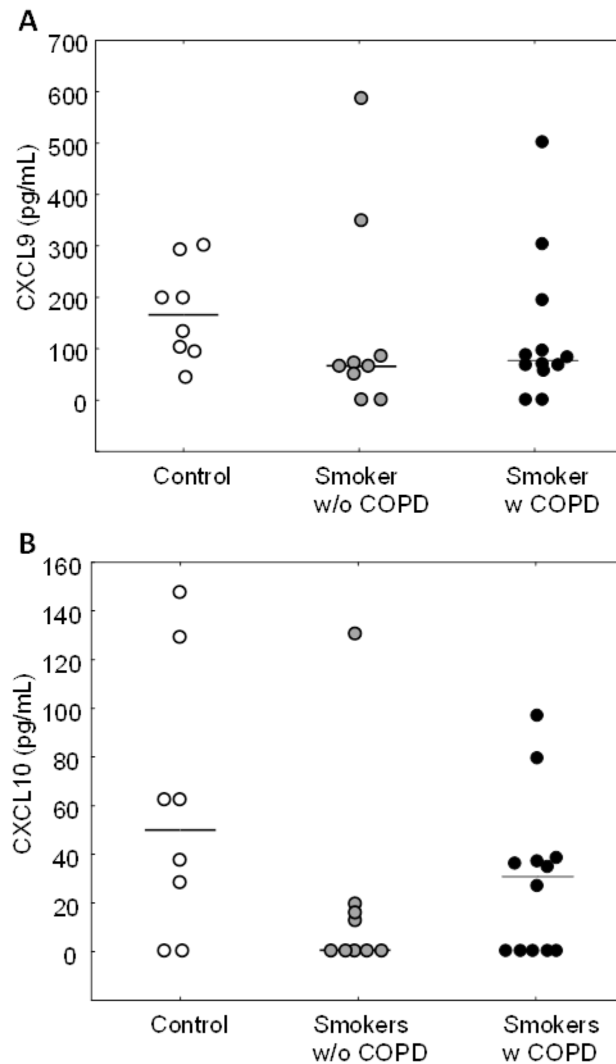
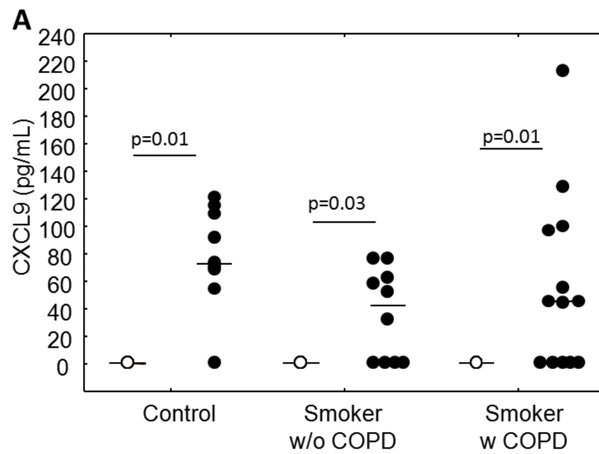


Figure 12:
Content of CXCL9 (A) and CXCL10 (B) in BAL fluid in non-smokers, smokers without COPD and smokers with COPD. Results are expressed as pg/mL. Individual values are presented and horizontal lines indicate median values.

There was a tendency towards lower levels of CXCL9 (p=0.2) and CXCL10 (p=0.1) in the two groups of smokers. CXCL11 could not be detected in most samples (not shown).

When stimulated with IFN-γ, alveolar macrophages increased the release of CXCL9 and CXCL10. There was no difference between the groups, although CXCL9 levels tended to be lower in the smoker groups compared to non-smoking controls (p=0.2).



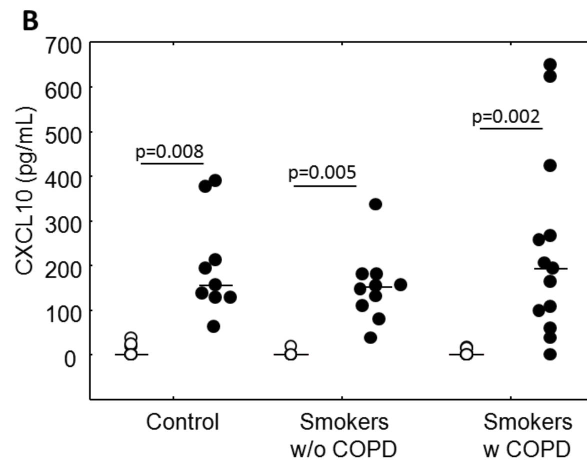


Figure 13:
Content of CXCL9 (**A**) and CXCL10 (**B**) in supernatants from alveolar unstimulated macrophages (empty circles ○) and macrophages stimulated with IFN-γ (1 µg/mL; filled circles ●). Individual values are presented and horizontal lines indicate median values.

When the supernatants from the alveolar macrophages were tested for their ability to induce migration in CXCR3 expressing lymphocytes (from healthy subjects), it was clear that the product from IFN- γ stimulated cells caused a higher chemotactic response than the product from unstimulated cells. However, we found no differences between the groups.

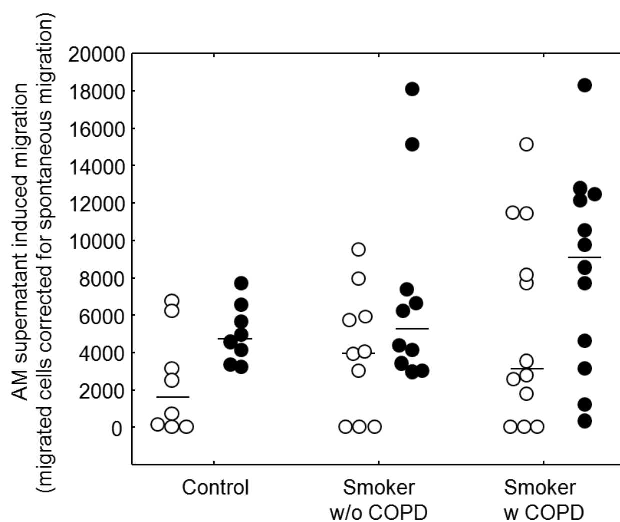


Figure 14:
Lymphocyte migration towards supernatants from unstimulated alveolar macrophages (empty circles ○) and IFN-γ stimulated alveolar macrophages (1 µg/mL; filled circles ●). Migration is expressed as the number of migrated cells corrected for spontaneous migration. Individual values are presented and horizontal lines indicate median values.

4.5 PRELIMINARY DATA

In order to assess modifications of neutrophils during transfer from the circulation to the airway lumen expression of adhesion molecules was investigated on neutrophils from three different locations, blood, bronchial biopsies and sputum. Also soluble adhesion molecules were measured in serum, BAL fluid and sputum supernatants.

Analysis of the cell numbers in the three compartments showed increased circulating neutrophils in the COPD group, compared to both smokers without COPD and controls. In BAL fluid, macrophages and neutrophils were increased in the smokers with and without COPD compared to the control group. Eosinophils were higher in the COPD group compared to controls, while lymphocytes were higher in the group of smokers without COPD, as compared to the controls. Neither the cell numbers, nor the cell distribution, in sputum differed between the groups (table 4).

		Controls	Smokers without COPD	Controls *vs* Smokers without COPD (p-value)	Smokers with COPD	Controls *vs* Smokers with COPD (p-value)
Blood (cells x10^9/L)	**Monocytes**	0.51 0.36-0.74	0.59 0.48-0.67	ns	0.61 0.52-0.79	ns
	Neutrophils	2.91 2.12-4.19	3.63 2.94-3.89	0.2	4.07 3.46-5.39	**0.01** ***0.04**
	Lymphocytes	1.83 1.67-2.75	2.44 1.86-3.15	ns	2.19 1.94-2.62	ns
	Eosinophils	0.18 0.12-0.25	0.25 0.14-0.32	ns	0.27 0.24-0.33	ns
BAL (cells x10^6/L)	**Cells x10^6/L**	114 83.2-129	290 259-523	**0.0001**	281 156-351	**0.002**
	Macrophages	91.3 79.1-115.7	264 225-498	**0.0001**	242 143-305	**0.001**
	Neutrophils	3.8 0.4-5.1	12.4 3.5-21.5	**0.03**	8.4 3.6-21.7	**0.04**
	Lymphocytes	4.7 3.6-8.9	13.3 11.2-23.5	**0.003**	10.2 3.3-19.8	0.2
	Eosinophils	0 0-0.2	0 0-1.1	0.2	2.4 0.3-4.5	**0.01**
Sputum (cells/mg)	**Cells/mg**	715 514-908	603 284-866	ns	652 409-1370	ns
	Macrophages	250 155-603	218 53.5-444	ns	234 136-424	ns
	Neutrophils	396 157-490	415 132-591	ns	501 343-786	ns
	Lymphocytes	23.2 18.2-31.8	9.3 7.2-11.3	ns	14.9 4.5-55.6	ns
	Eosinophils	0 0-5.0	1.3 0-2.6	ns	8.2 0.6-16.8	ns

Table 4. Differential cell counts of blood, sputum and BAL. Data are presented as median and 25th –75th percentile. Comparisons between groups were made using Kruskal-Wallis followed by Mann-Whitney test when appropriate. * indicate p-value for comparison between smokers with and without COPD.

Surface expression of CD11b was increased on blood neutrophils from smokers with COPD compared to non-smoking controls. On sputum neutrophils CD11b expression was increased in smokers without COPD compared to subjects with COPD, there was also a trend for higher CD11b expression in the smokers without COPD compared to non-smoking controls (p=0.051, Figure 15). Moreover, CD11b expression was increased on sputum neutrophils compared to blood neutrophils in both smoker groups but not in the control group.

Surface expression of CD62L and CD162 was decreased on sputum neutrophils compared to blood neutrophils in all groups.

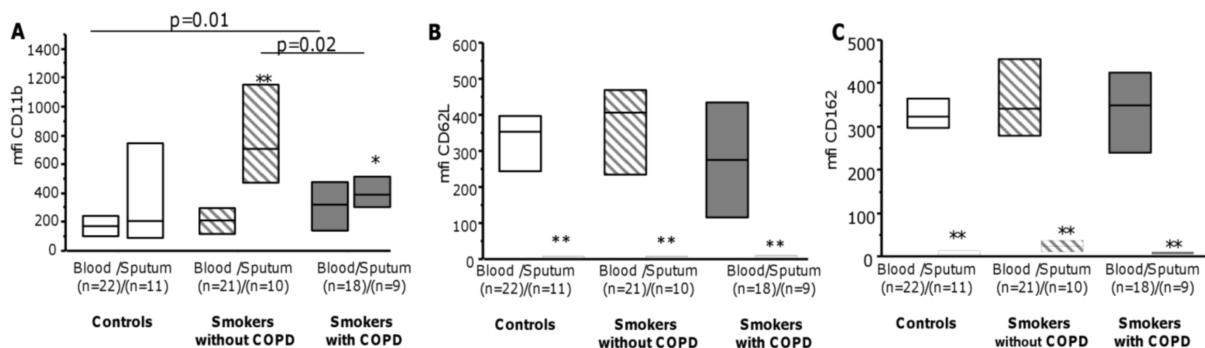


Figure 15: Surface expression of A) CD11b, B) CD62L and C) CD162 on blood neutrophils and sputum neutrophils measured by flow cytometry. Results are presented as mean florescence intensity (mfi) and data are presented as median and 25th- 75th percentile. P-values indicate comparisons between groups within the same compartment. *indicate p-value < 0.05 comparisons between compartments within the same group.**indicate p-value < 0.01 comparisons between compartments within the same group. Cell numbers were not sufficient for flow cytometric analysis in all sputum samples, the analysed numbers are indicated in the figure.

The presence of neutrophils expressing CD11b, CD62L and CD162 in bronchial biopsies was confirmed by immunohistochemical staining.

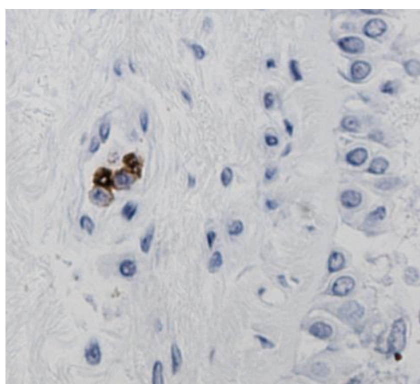


Figure 16: Co-localisation of neutrophil elastase and CD11b, in a bronchial biopsy from a patient with COPD. Neutrophil elastase positive cells are stained in brown; adhesion molecule positive cells are stained in red. Sections are counterstained with haematoxylin.

Soluble adhesion molecules were measured in serum, sputum supernatants and BAL fluid. Levels of ICAM-1 and ICAM-3 were higher in the COPD group compared to the control group and PECAM-1 was lower in smokers without COPD compared to both controls and subjects with COPD (Figure 17).

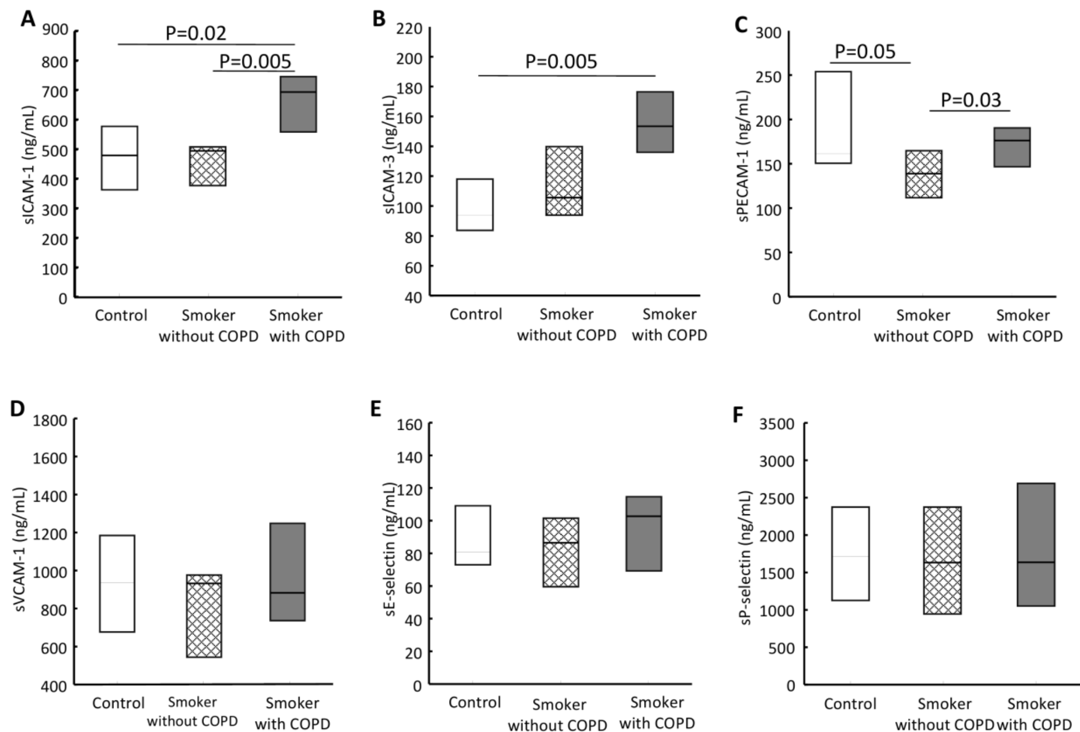


Figure 17: Levels of soluble adhesion molecules **A)** ICAM-1, **B)** ICAM-3, **C)** PECAM-1, **D)** VCAM-1, **E)** E-selectin and **F)** P-selectin measured in serum from controls (n=12), smokers without COPD (n=12) and smokers with COPD (n=12). Soluble adhesion molecules were measured in subjects where samples from all compartments (blood, sputum and BAL) were available. Results are presented as ng/mL and data are presented as median and 25th- 75th percentile. P-values indicate comparisons between groups.

In BAL fluid the pattern for ICAM-1 was reversed and the levels were lower in the COPD group compared to both controls and smokers without COPD. There were no other significant differences between the groups in BAL fluid or sputum supernatants. Generally the levels of adhesion molecules were low in BAL fluid and most samples were under the detection limit for ICAM-3, E-selectin and P-selectin.

5 GENERAL DISCUSSION

Chronic obstructive pulmonary disease has been described as a world-spanning and growing epidemic, but concerns that this fact is not receiving sufficient attention are often raised (101). Although research on COPD has intensified there are still many white spots on the COPD map and many of the pathophysiological mechanisms remain unexplored. What is more, none of the treatments available today, except smoking cessation, are able to halt the progress of the disease and treatments are directed at symptom relief and to prevent exacerbations.

The neutrophil was long regarded as a simple cell focused only on its primary task, to pacify and eliminate potential treats to the organism. During the last decades it has, however, become clear that the neutrophil plays an active in part of the intricate immunological network and it has received increasing attention for its role in inflammation and disease. Neutrophils have together with macrophages and $CD8^+$ T-cells, been attributed a central role in the airway inflammation in COPD. Therefore, the main objective of this thesis was to study inflammation and cell migration in COPD with a special focus on neutrophil function.

In the study presented under preliminary data we found an increase in neutrophil numbers in blood and BAL fluid as well as a trend towards higher numbers also in sputum from COPD patients compared to non-smoking controls. This is in agreement with previous studies where neutrophils have been shown to be increased both in the circulation and in the airways of COPD patients (58, 69, 102). Several studies have also reported signs of neutrophil activation both in the circulation and in the airways (63, 103, 104). Moreover, there was an increase of BAL fluid eosinophils and a trend towards higher eosinophil numbers in sputum in the COPD group compared to non-smoking controls. Increased eosinophil numbers in the airways of COPD patients has been reported previously, particularly during exacerbations (58, 105, 106). Interestingly, increased sputum eosinophils appear to predict an increased responsiveness to treatment with corticosteroids (107, 108). Also, in agreement with previous reports alveolar macrophages were increased in BAL fluid from subjects with COPD compared to non-smoking controls (58). Taken together the cell distribution in blood, BAL fluid and sputum in our study was similar to earlier studies.

In Paper I attention was directed at neutrophil function, more specifically, chemokine production. In this paper it was shown that chemokine release induced by LPS and organic dust is partly regulated by neutrophil derived TNF-α, and that the TNF-α regulation of CXCL8 is somehow altered in subjects with COPD. The role of neutrophil derived TNF-α in the regulation of chemokine production was confirmed by the reduction in chemokine levels caused by the addition of infliximab. Infliximab had similar effects on CCL3 release in all groups, while infliximab failed to inhibit LPS induced release of CXCL8 in the COPD group.

One possible explanation for this finding is that the circulating neutrophils in COPD are primed (64, 104). Priming increase the neutrophils ability to respond to activating stimuli and it is conceivable that the neutrophils in the COPD group are primed and

therefore respond to LPS with an increased TNF-α production. However, measurements of released TNF-α were generally low and no differences between groups were detected. Nonetheless, the hypothesis is supported by the preliminary data where increased CD11b expression on circulating neutrophils was shown in the COPD group, indicating that the neutrophils in the COPD group are primed.

Finally, it cannot be ruled out that the LPS induced release of CXCL8 in the COPD group could have been inhibited by an increased dose of infliximab. Pilot experiments did, however, not show an increased reduction in chemokine levels when the infliximab concentration was increased above that used in the study.

The data in Paper I also confirm the neutrophil's role as a significant producer of chemokines. Both CCL2 and CCL3 are chemokines that attract monocytes and several recent reviews have emphasised the importance of neutrophils as initiators of macrophage recruitment into inflamed areas (20, 22). It has also been implied that CCL2 is involved in the COPD inflammation as increased levels have been observed in BAL fluid from subjects with COPD (73). Although a spontaneous release of CCL2 was detected none of the used stimuli induced any increased release suggesting that exogenous stimuli are of lesser importance than endogenous stimuli. Judging from our results the increased CCL2 observed in BAL fluid from COPD subjects originates from a source other than neutrophils.

Neutrophil release of CCL3 followed the same pattern as CXCL8 to a large extent; with the exception that infliximab successfully reduced the release of CCL3 in all groups after both LPS and organic dust stimulation. Interestingly, at the basal level CCL3 was decreased in the COPD group compared to smokers without COPD. CCL3 is known to attract T-cells in a concentration dependent manner, with low concentrations resulting mainly in attraction of $CD8^+$ T-cells and high levels leading to the recruitment of $CD4^+$ T-cells (109). It could thus be speculated that even small differences in chemokine levels could be of clinical relevance.

In paper III we studied the effects of formoterol and budesonide on neutrophil release of IL-6, CXCL1 and CXCL8 in healthy subjects. The results showed no effect of these drugs on chemokine release by unstimulated neutrophils. However, when neutrophils were simultaneously stimulated with LPS, formoterol enhanced the release of IL-6 and CXCL8, whereas budesonide tended to decrease the release of IL-6, CXCL8 and CXCL1. This is in line with previous results where budesonide reduced the release of IL-6, CXCL8 and TNF-α in LPS stimulated alveolar macrophages and epithelial cells and formoterol induced the release of IL-6 and CXCL8 in epithelial cells (110-112). Even if formoterol had an enhancing effect on cytokine release the combination of budesonide and formoterol did not abolish the inhibitory effects of budesonide alone. Thus the residual effect of the two drugs in the present concentrations is still inhibition of cytokine release. There are studies that show a reduction of sputum neutrophils in COPD after treatment with both formoterol and corticosteroids (113, 114). Although a decreased CXCL8 release by neutrophils could potentially reduce neutrophil migration into the lungs it is difficult to speculate on the clinical relevance of our finding, especially considering that neutrophils from healthy donors were used and our findings in Paper I indicate that chemokine release by neutrophils from COPD patients might be somewhat altered.

To elucidate whether increased neutrophil chemotaxis could be one of the mechanisms underlying the airway neutrophilia observed in COPD, neutrophil migration induced by CXCL8, LTB_4 and fMLP were studied in paper II. Both CXCL8 and LTB_4 are increased in the COPD lung and the levels of CXCL8 also correlate with neutrophil numbers (58, 69, 70). Studies of the neutrophil migration inducing properties of sputum from COPD subjects have shown that both CXCL8 and LTB_4 contribute substantially (115, 116). Previous studies of neutrophil migration are conflicting and show both enhanced and reduced migration in neutrophils from subjects with COPD (66, 71). Explanations for the divergent results could be alternative methods, differences in disease severity and smoking habits.

Our data show enhanced neutrophil migration towards CXCL8 in smokers both with and without COPD compared to controls, while chemotaxis to LTB_4 was increased only in smokers without COPD. When fMLP was used as a chemoattractant there were no differences between the groups. Several mechanisms may partly explain the increased migration observed in smokers with and without COPD. Firstly, priming enhances several neutrophil responses and as mentioned previously, circulating neutrophils in COPD show signs of priming. One important priming agent is TNF-α, which has been shown to be increased in serum in smokers (117). Another priming agent, LPS, is present in cigarette smoke and it has also been shown that nicotine itself can enhance neutrophil migration *in vitro* (118, 119). There are thus several agents which could enhance migration, either separately or in combination. Measurement of serum TNF-α did not, however, show any differences between the groups, possibly due to the relatively small sample size. However, in smokers (with or without COPD) there was a positive relationship between serum TNF-α levels and migration to CXCL8 and LTB_4 respectively. This suggests that even a small increase in TNF-α could affect neutrophil activation and migration.

There are other plausible explanations for the increased chemotaxis in the smokers. Difference in expression of the receptors for CXCL8 and LTB_4 between smokers and non-smokers is one conceivable reason. There is no clear trend for the studies of CXCR2 in COPD and previous results show both up- and down-regulation (61, 120, 121). The BLT_1 receptor is sparsely studied in COPD but has been shown to be up-regulated on the alveolar wall in COPD (122). It is also worth noting that down-regulation of CXCR2 expression does not always seem to relate to decreased migration, while up-regulation of BLT_1 on neutrophils enhances the chemotactic response to LTB_4 (123, 124). Consequently, it is difficult to make firm extrapolations from these divergent results to our findings of enhanced chemotaxis in smokers.

Studies concerning the neutrophils' ability to differentiate between diverse chemoattractants, when exposed to them simultaneously, have classified fMLP as an end-point chemoattractant while CXCL8 and LTB_4 are intermediary chemoattractants (13). This differentiation is achieved by the activation of separate signalling pathways rather than desensitisation of receptors or differences in the concentration of chemoattractants (13). It is possible that the explanation to the lack of differences between the groups in migration towards fMLP can be sought in this finding.

In a model of transendothelial migration (human pulmonary endothelial cells), neutrophils migrate in response to fMLP mainly in a CD18 dependent fashion, whereas migration to CXCL8 and LTB_4 was largely CD18 independent (125). Although migration in the filter assay occurs without interactions between neutrophil and adhesion molecules expressed on endothelium, this provides further support to the notion that chemoattractants may function differently. The filter assay used in our study is a vast simplification of the enormously complex situation under which migration occurs *in vivo* and it is clear that further studies are needed to elucidate the cellular mechanisms which cause the increased migration observed in smokers irrespective of airway obstruction. Excitingly, a recent study shows that neutrophils from subjects with COPD have a changed migratory pattern; they migrate faster but with less accuracy (126). These findings could in part explain the increased neutrophil migration we found in smokers with and without COPD.

In paper III we found neutrophil expression of both CXCR1 and CXCR2 in healthy subjects to be up-regulated by formoterol, while budesonide only enhanced CXCR2 expression. While CXCR1 and CXCR2 were up-regulated by formoterol and partly by budesonide, the effect did not extend to the chemotaxis experiments as no formoterol-induced increase in migration could be detected. This adds further strength to the previous discussion of the relationship between CXCR2 expression and migratory response. It also indicates that there is no absolute relationship between receptor expression and chemotaxis and that mechanisms other than receptor density are of importance for the migratory response. It is noteworthy that steroids (dexamethasone) also up-regulate BLT_1 expression on neutrophils (124).

Our results also confirm autologous desensitisation of CXCR1 and CXCR2 by CXCL8, while CXCL1 only down-regulated CXCR2. Although CXCL1 alone did not down-regulate CXCR1, its expression was still decreased when CXCL1 was combined with budesonide and/or formoterol. One explanation for the lack of CXCR1 down-regulation by CXCL1 might be that CXCR1 binds CXCL8 with high affinity, whereas CXCL1 is only weakly bound (127). There is also an element of cross-desensitisation between the two receptors and it is therefore feasible that the effects of the drug-CXCL1 combination are a consequence of the pronounced down-regulation of CXCR2. It has been suggested that CXCR1 is more important for cell functions other than chemotaxis (e.g. respiratory burst) (127). As CXCL1 had a down-regulating effect only when combined with formoterol and/or budesonide, it is interesting that both salmeterol and budesonide have been shown to reduce the respiratory burst in neutrophils (128, 129).

While our results show no impact of formoterol and budesonide on chemotaxis other studies have shown divergent results. Thus a short-acting β_2-agonists (terbutaline) and aminophylline inhibited neutrophil migration in therapeutic doses but enhanced it in supra-therapeutic doses (130). Moreover, both salbutamol and budesonide have a weak inhibitory effect on neutrophil migration over a bilayer of epithelial and endothelial cells (131). The discrepancy between these results and our data might partly be explained by a difference in methods. It is likely that the expression of adhesion molecules is of importance in the bilayer model. Formoterol has been shown to decrease the adherence of neutrophils in animal models (132). It is thus conceivable

that the filter assay used in the current study constitutes a too simplistic approach to fully evaluate any potential drug effects.

In addition to the actual chemotaxis studies, expression of adhesion molecules was also assessed (presented above under Preliminary data). Cell surface expressed adhesion molecules (CD11b, CD62L and CD162) were measured on neutrophils from different compartments (blood, sputum and BAL). Our results show that circulating neutrophils from patients with COPD have an increased expression of CD11b as compared to healthy non-smoking subjects. In smokers, irrespective of airway obstruction, sputum neutrophils have an increased CD11b expression compared to circulating neutrophils. Increased expression of CD11b is considered an activation marker in neutrophils and it is related to an increase in several neutrophil functions such as the respiratory burst (64, 133). The increased expression of CD11b on circulating neutrophils in COPD is in agreement with previous studies (64, 104). Also, increased CD11b expression on sputum neutrophils has been described in smokers with COPD (63).

The increased CD11b expression on circulating neutrophils from patients with COPD is thus indicative of activation. This activation appears to remain even as the neutrophil enters the lung, as CD11b expression was increased in sputum neutrophils compared to circulating neutrophils in both smoker groups. The activation of neutrophils, at least in part, may be caused by the smoke exposure as there is evidence that β_2-integrins are up-regulated by *in vitro* exposure of isolated neutrophils as well as by *in vivo* exposure in animal models (134, 135). Our data also show a higher CD11b expression on sputum neutrophils from smokers without COPD compared to the COPD group. It is possible that this lower expression on COPD sputum neutrophils is a sign of exhaustion caused by the general activation of the immune system in COPD. In line with this, CD11b is down-regulated during apoptosis and while the apoptosis rate of circulating neutrophils has been shown to be unchanged in COPD, an increased apoptosis rate has indeed been reported in sputum neutrophils from COPD subjects (72, 136).

While expression of CD11b is increased on the cell surface upon neutrophil activation, L-selectin (CD62L) is shed (64). Our data show a lower CD62L expression on sputum neutrophils than on circulating neutrophils, a finding which further confirms the activation of neutrophils which appears to be brought about by transition into the lungs. The presence of neutrophils expressing the adhesion molecules CD11b, CD62L and PSGL-1 (CD162) was also confirmed in bronchial biopsies by immunohistochemical staining. Neutrophils expressing CD11b have previously been shown to be increased in the submucosa of subjects with COPD compared to control smokers (137).

Our results show no difference between the groups regarding CD162 expression on neutrophils. This finding is contradictory to a previous study where CD162 expression was increased in subjects with COPD (stage I-V). The reason for this discrepancy is unknown, but could possibly be due to differences in study populations. The ligand of CD162, P-selectin, is expressed on activated endothelium as well as on platelets (14). A few studies have measured serum P-selectin in COPD and the results are varied, while one study found no changes, others found increased levels in COPD (138-140). It is difficult to speculate on the cause for this discrepancy, but as the studies which found increased levels of serum P-selectin had larger patient samples (139, 141) it is possible that the absence of differences in the current study is explained by the somewhat small sample size.

E-selectin is expressed on activated endothelial cells and soluble E-selectin has therefore sometimes been interpreted as a sign of endothelial activation. Increased E-selectin expression has been linked to COPD previously both in its soluble form in serum and as percentage of E-selectin positive vessels in bronchial biopsies (142, 143). Nonetheless, our data show no difference in serum and in BAL fluid and sputum supernatant levels were below the detection limit.

Serum ICAM-1 and ICAM-3 were increased in smokers with COPD compared to healthy non-smoking subjects. Previous studies of ICAM-1 levels in serum show conflicting results, with reports of both increased and decreased levels in subjects with COPD (104, 142). However, previous findings of increased ICAM-1 levels were found in COPD patients with a disease severity similar to that in present study population. Serum ICAM-1 has also been used as a marker of systemic inflammation, thus the increased levels of ICAM-1 observed here further supports the idea of an on-going systemic inflammation in the COPD group.

Transendothelial migration of neutrophils is dependent on PECAM-1 and it has been shown that blocking of PECAM-1 inhibits transendothelial migration of neutrophils (144). Interestingly, we found a trend towards lower serum PECAM-1 in smokers, irrespective of airflow obstruction, with a significantly lower level in smokers without COPD as compared to both controls and the COPD group. It could be speculated that the lower levels in the smoker group are caused by sPECAM-1 binding to endothelial PECAM-1 as part of a protective mechanism, a mechanism that has failed in the COPD group.

It is clear from the literature that soluble adhesion molecules and their function still are a largely unexplored field in COPD. There is no doubt of the potential in adhesion molecules as a target for anti-inflammatory drugs but it is also apparent that more research is required to elucidate their role and function in COPD.

In paper IV the release of CXCR3 ligands by alveolar macrophages was studied. Alveolar macrophages released CXCL9 and CXCL10 upon stimulation with IFN-γ but there was no difference between the three groups, although a trend towards lower CXCL9 and CXCL10 levels in BAL fluid was found in the two smoker groups. CXCL11 levels were below the detection limit in almost all samples. The supernatants from the stimulated alveolar macrophages caused migration of CXCR3 expressing lymphocytes and the migration was reduced by the addition of antibodies against the respective CXCR3 ligands. Thus, it is clear that alveolar macrophages release chemokines capable of eliciting migration by CXCR3 expressing lymphocytes.

An increased presence of CXCR3 expressing $CD8^+$ T-cells has previously been shown in bronchial biopsies (81). As $CD8^+$ T-cells express IFN-γ and the CXCR3 ligands all are induced by IFN-γ, it has been suggested that a loop of IFN-γ producing T-cells and CXCR3 ligands might be self-sustaining constituting an important mechanism for the increased number $CD8^+$ T-cells observed in the COPD airway. Based on the current results, alveolar macrophages do not, however, appear to be responsible for any potential increase in CXCR3 ligands. We found no difference between the groups regarding chemokine levels in BAL fluid, suggesting that if the increased presence of CXCR3 expressing $CD8^+$ T-cells is driven by enhanced chemokine levels, this enhancement is not reflected in the BAL fluid. It is, however, still fully plausible that there is an augmentation of CXCR3 ligands but that this is restricted to the tissue.

The addition of antibodies directed against the respective chemokines showed that all three CXCR3 ligands were involved in eliciting lymphocyte migration. The migration induced by the supernatants was rather weak compared to the maximum response produced by the optimal dose of recombinant chemokine. However, when the levels of chemokine measured in the supernatant were related to similar levels of recombinant chemokine the results were similar. CXCL11 has previously been reported to display the strongest affinity for CXCR3 implying that it might also be the more important chemokine (145). Our results show that the biggest reduction of migration was achieved by the addition of the CXCL11 antibody. This is somewhat confusing as the CXCL11 levels were below the detection limit, but it is also possible that it, to some extent, is explained by the high affinity for CXCR3 by CXCL11.

In contrast to our findings CXCL9 and CXCL11 have previously been found to be increased in BAL fluid from smokers (146). The conflicting results could possibly be explained by a difference it techniques, where the previous study used a smaller total volume (150 mL) for the BAL and a different technique for chemokine measurement (cytometric bead array). Interestingly, a recent study of alveolar macrophages showed a down-regulation of genes typical for M1 macrophages (e.g. CXCL9 and CXCL11) in smokers with normal lung function. These genes were further down-regulated in smokers who had developed COPD (147). These findings are in line with our data where there was a trend towards lower levels, especially of CXCL9, in alveolar macrophage supernatants from smokers with and without COPD. In addition, Shaykhiev *et al* found that CXCL11 was not expressed by alveolar macrophages from all subjects; this was particularly true for smokers (147). This could partly explain why we were unable to detect CXCL11 in most samples.

Taken together the different studies included in this thesis provide evidence that neutrophil function is altered in COPD and to some extent also in smokers who have not developed COPD. The observed increase in neutrophil migration towards two of the important neutrophil chemoattractants, CXCL8 and LTB_4, contributes to the explanation of the increased neutrophil numbers in the COPD lung. As chemotaxis was also increased in smokers without COPD, it is most likely that this effect is brought about by the exposure to tobacco smoke. Several studies have also shown an activation of neutrophils by tobacco smoke or one of its components (e.g LPS). Increased activation of the neutrophils as a cause of the enhanced migration is supported by an increased expression of the neutrophil activation marker CD11b on circulating neutrophils from COPD patients.

Neutrophil numbers as well as CXCL8 levels are increased and correlate to one another in sputum from COPD patients (69). While no difference between the groups was observed in CXCL8 release after stimulation with LPS, the autocrine regulation of CXCL8 release by TNF-α appeared to be altered in subjects with COPD. However, changes in TNF-α release could not be confirmed as the measured levels were rather low. Similarly, serum TNF-α did not differ between the groups (paper II) although there was a relationship between serum TNF-α and migration towards CXCL8 and LTB_4. It is thus conceivable that even very small changes in TNF-α cause changes in neutrophil activation and function.

The lack of difference in CXCL8 release (isolated blood neutrophils) between the groups (paper I) is not contradictive of neutrophils as a predominant source of the elevated CXCL8 levels observed in sputum and BAL-fluid. It may rather indicate that the increase in CXCL8 might be a consequence of increased neutrophil numbers and not by an increased production per cell. It is also possible that neutrophils that have left the circulation have a different pattern of chemokine release compared to circulating neutrophils. Measurement of cell surface expressed adhesion molecules on circulating neutrophils and sputum neutrophils suggests that neutrophils undergo changes, in activation status, as they migrate from the circulation into the lungs. These changes appear to be more pronounced in smokers, irrespective of airway obstruction. It is thus plausible that the further activation of sputum neutrophils is brought about by not only the actual transition but also by the smoke exposure as well as the inflammatory milieu of the lungs.

Our results also show an increase in serum levels of ICAM-1 in subjects with COPD. This indicates that the airway inflammation characterised by increased numbers of inflammatory cells and enhanced expression of CD11b is not restricted to the airways. The systemic impact of the tobacco smoke exposure and the inflammation of the airways are also revealed by the enhanced chemotaxis and CD11b expression on circulating neutrophils. These findings all contribute to our understanding of the mechanisms underlying altered neutrophil function in COPD.

6 CONCLUSIONS

To conclude, the current studies showed alterations of different aspects of neutrophil function in smokers with and without COPD.

The release of different chemokines (CCL3 and CXCL8) was induced by LPS, TNF-α and organic dust, confirming the role of neutrophils as an important source of chemokines capable of influencing other inflammatory cells.

The release of CCL3 and CXCL8 was inhibited by infliximab (anti TNF-α antibody) confirming the role of neutrophil derived TNF-α as a regulator of chemokine release. LPS-induced CXCL8 release was not inhibited by infliximab in smokers with COPD, suggesting that the TNF-α regulation of CXCL8 release is altered in COPD.

Neutrophils from smokers with and without COPD showed an enhanced migratory response to CXCL8 and neutrophils from smokers without COPD also showed an increased migration to LTB_4. Priming enhances several neutrophil functions, including chemotaxis, and the serum levels of the important priming agent TNF-α correlated with migratory response in the smoker groups. In further support of this observation, expression of CD11b, which is often used as an activation marker for neutrophils, was increased on neutrophils from smokers with COPD.

Formoterol increased the LPS-induced release of cytokines (IL-6 and CXCL8) while budesonide decreased the cytokine release (IL-6 and CXCL1) in neutrophils from healthy controls *in vitro.*

Expression of CXCR1 and CXCR2 was enhanced by both formoterol and budesonide. This was, however, not reflected in an increased migratory response.

There was no difference in the levels of CXCR3 binding chemokines released by alveolar macrophages from subjects in the three groups, and there was no difference in the migratory response generated by the supernatants in CXCR3 expressing lymphocytes. This indicates that chemokine release by alveolar macrophages is unlikely to be responsible for the increased presence of CXCR3 expressing lymphocytes observed in bronchial biopsies from subjects with COPD.

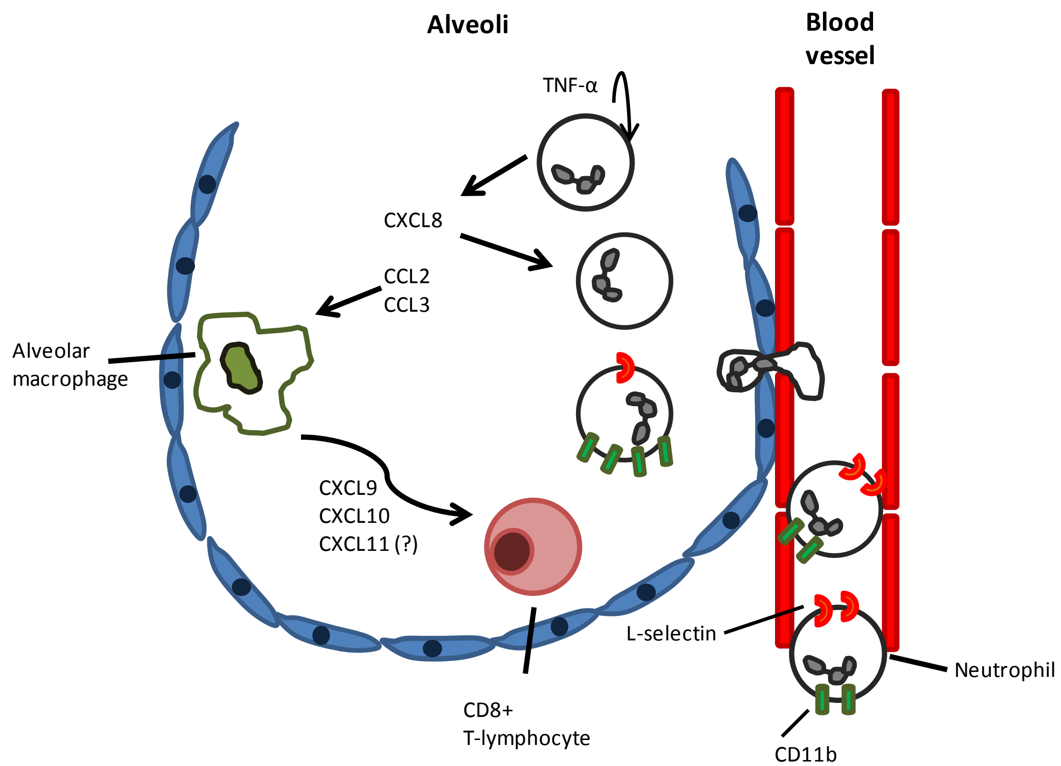


Figure 18. Possible mechanisms by which neutrophil function may be altered in COPD. Chemotaxis towards CXCL8 is increased in smokers, irrespective airway obstruction. Adhesion molecule CD11b is up-regulated on circulating neutrophils from COPD patients; this may further facilitate migration into the lung and indicates that the neutrophils are activated. Moreover, CD11b is increased on sputum neutrophils compared to circulating neutrophils in smokers with and without COPD. Isolated circulating neutrophils release CCL2 and CCL3 which are chemoattractants for macrophages. They also release CXCL8 which is a major chemoattractant for neutrophils. The level of released chemokine is not altered in COPD but the neutrophil derived TNF-α regulation of CXCL8 is altered in COPD.

7 POPULÄRVETENSKAPLIG SAMMANFATTNING

Kroniskt obstruktiv lungsjukdom (KOL) beräknas vara den fjärde vanligaste dödsorsaken i världen idag. Bara i Sverige beräknas mellan 400 000 och 700 000 leva med KOL. Enligt beräkningar från WHO kommer sjukdomen att öka ytterligare. Den vanligaste orsaken till KOL i västvärlden är rökning men andra långvariga exponeringar så som viss yrkesexponering och matlagning över öppen eld, kan också orsaka KOL. Idag kan sjukdomen inte botas men förloppet kan hämmas och symtomen kan lindras med rätt behandling (rökstopp, läkemedel, sjukgymnastik mm).

KOL karaktäriseras av en försämrad lungfunktion. Hos friska personer minskar lungfunktionen med stigande ålder men hos personer med KOL sker detta snabbare än hos friska personer. Lungfunktionsnedsättningen orsakas av en ständigt pågående inflammation i lungan, som påverkar både de stora och de små luftrören och försvårar lufttillförseln till lungorna. Inflammationen orsakar bland annat en ökad slemproduktion i luftvägarna. Slemmet gör det svårare för luften att passera, orsakar hosta och bildar grogrund för upprepade infektioner. Inflammationen kan också bryta ned de små lungblåsorna, därmed bildas hålrum (emfysem), vilket ytterligare försvårar syreupptaget.

En rad olika immunceller finns i ökat antal i lungan vid KOL, bland annat neutrofila granulocyter, alveolära makrofager och cytotoxiska T-celler. Neutrofilerna utgör en central del av immunförsvaret och de reagerar mycket snabbt på signaler som tyder på att kroppen utsätts för fara från bakterier och virus. De har en rad egenskaper som gör dem perfekta för uppgiften, bland annat kan de släppa ifrån sig ämnen som skadar och dödar bakterien. De kan också äta upp bakterier och delar av döda celler. Neutrofilerna kan också kalla på hjälp från andra celler genom att släppa ifrån sig signalämnen, så kallade cytokiner. Nackdelen med neutrofilens egenskaper är dock att de, om de inte kontrolleras noga, även riskerar att skada den egna organismen. Makrofagens egenskaper liknar neutrofilens. De lever dock längre och anses ofta ha större förmåga att kommunicera med andra celler i immunförsvaret. Den tredje celltypen som ses i ökat antal i lungan vid KOL är den cytotoxiska T-cellen som är specialiserad på att identifiera och förstöra celler som infekterats men de har också en god förmåga att påverka andra celler genom att bilda och frisätta olika cytokiner.

De senaste åren har forskningen gjort stora framsteg och man kan nu delvis beskriva den inflammatoriska processen vid KOL. Mycket forskning återstår dock innan bilden blir tydlig. Syftet med avhandlingen är därför att försöka klargöra hur neutrofilens funktion är förändrad vid inflammationen vid KOL och att studera effekterna av två läkemedel (budesonid och formoterol) som ofta används vid behandling av KOL. Avhandlingen belyser också en av de mekanismer genom vilken makrofagen kallar på förstärkning av cytotoxiska T-celler.

I de aktuella delstudierna har prover tagits från friska icke-rökare, rökare utan KOL och rökare med KOL. Proverna har tagits på olika sätt dels i form av blodprover, dels

genom bronkoskopi med lungsköljning (bronkoalveolärt lavage) och i form av upphostningsprov (inducerat sputum).

I det första delarbetet undersöktes hur neutrofiler frisätter en typ av cytokiner, så kallade kemokiner (CCL2, CCL3, CXCL8), som har en attraherande effekt på andra celler. Resultaten visade ingen skillnad i nivåer av frisättning mellan grupperna. Dock kunde frisättningen av CXCL8 inte hämmas med en TNF-antikropp i KOL-gruppen. Detta tyder på att neutrofilens egen reglering av frisättningen av CXCL8 är förändrad vid KOL.

I det andra delarbetet studerade vi neutrofilernas förmåga att vandra mot olika attraherande ämnen, dels CXCL8 och LTB_4 som produceras av neutrofiler och dels fMLP som är ett bakteriefragment. Det visade sig att neutrofiler från rökare med och utan KOL hade en ökad förmåga att vandra mot CXCL8 jämfört med neutrofiler från friska. En orsak till detta skulle kunna vara att neutrofiler hos rökare aktiveras av de reaktioner i kroppen som röken ger upphov till, och därmed lättare svarar på attraherande signaler. Detta skulle delvis förklara varför man hos rökare både med och utan KOL ser en ökning av neutrofiler i luftvägarna.

I det tredje delarbetet studerades effekterna av de två läkemedlen budesonid och formoterol på blod-neutrofiler isolerade från friska personer. Budesonid (steroid) och formoterol (β_2-agonist) används ofta i kombination vid behandling av KOL. Resultaten visade att formoterol stimulerade frisättningen av CXCL1, CXCL8 och IL-6 medan budesonid hämmade frisättningen. Både budesonid och formoterol ökade uttrycket av CXCR2 receptorn. Trots att bindning av kemokiner till CXCR2 ger upphov till migration sågs ingen ökad cellvandring hos neutrofilerna som behandlats med budesonid eller formoterol.

I det fjärde arbetet undersökte vi om alveolära makrofager frisätter en grupp av kemokiner som attraherar cytotoxiska T-celler via CXCR3 receptorn. Vidare undersöktes om skillnader i frisättning mellan grupperna delvis skulle kunna förklara ökningen av cytotoxiska T-celler i lungan vid KOL. Resultaten visar att de alveolära makrofagerna frisätter låga nivåer av CXCR3-bindande kemokiner och att dessa påverkar T-celler till migration. Dock kunde inte några skillnader mellan grupperna ses och slutsatsen blir därför att det inte är de alveolära makrofagerna som, genom denna mekanism, bär huvudansvaret för de ökade antalet T-celler som ses i lungan vid KOL.

Sammantaget visar delarbetena i avhandlingen att neutrofilernas funktion i vissa avseenden är förändrad hos rökare redan innan de utvecklat KOL. Därutöver ses ytterligare förändringar hos neutrofiler från personer med KOL. Gemensamt för de förändringar som påvisats är att de är relaterade till en ökad aktivitetsgrad hos neutrofilerna. En ökad förståelse för mekanismerna bakom inflammationen vid KOL är nödvändig för att man ska kunna hitta nya sätt att stoppa eller bromsa sjukdomen med hjälp av läkemedel.

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