From the Department of Public Health and Clinical Medicine, Division of Medicine/Respiratory Medicine
Umeå University, Sweden

T cells in Chronic Obstructive Pulmonary Disease

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Umeå 2010
Is there a lymphocyte hiding between the epithelial cells?
Airway tissue from one of the subjects with COPD.

Cover photo: Lennart Nilsson

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This book is dedicated to my family and
to the memory of my mother and father

“I can do everything through him who
gives me strength” — Phil 4:13
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ABSTRACT

**Background:** Tobacco smoking is the main cause of chronic obstructive pulmonary disease, COPD, but the mechanisms by which cigarette smoke induces COPD are still elusive. T lymphocytes have been implicated in the pathogenesis of the disease, but their role in the airway inflammation in COPD is not fully understood. The aim of this thesis was therefore to address T lymphocyte subsets and their activation in the airways of subjects with COPD, in comparison to smokers with normal lung function (S) and never smokers (NS).

**Methods:** Subjects with moderate to severe COPD were recruited along with controls. They were all non-atopic and clinically stable, without any exacerbation during at least three months prior to inclusion. Only medication with short-acting β2-agonists and/or anti-cholinergic drugs was permitted. All subjects underwent bronchoscopy with endobronchial mucosal biopsy sampling as well as bronchial wash, BW, and bronchoalveolar lavage, BAL, collection. Biopsies were immunohistochemically stained for inflammatory cells and markers. BW and BAL fluids were prepared for differential cell counts. Soluble markers were measured in BW and lymphocyte subsets were determined in BAL using flow cytometry.

**Results:** In biopsies, an increase in epithelial CD3^+ and CD8^+ cells was found in COPD, compared to NS. In BAL fluid, CD8^+ cells were enhanced, whereas CD4^+ cells were reduced in subjects with COPD and S, compared to NS. Furthermore, CD4^+ and CD8^+ cells were more activated both in COPD and S, in terms of increased expression of CD25, CD69 and HLA-DR. NKG2D-expressing CD8^+ T cells in BAL fluid were enhanced in both COPD and S. CD4^+CD25^{bright} cells were upregulated in COPD and S, suggesting the presence of regulatory T cells. Further analyses of T cell subsets with the more specific markers for regulatory T cells, FoxP3 and CD127, indicated a smoking-induced expansion of non-regulatory T cells, which tended to normalize after smoking cessation in COPD. Currently smoking subjects with COPD still expressed high proportions of activated non-regulatory CD4^+ T cells. The data on FoxP3 expression further indicated that the increase in CD25 expression in COPD and S was not only associated with the expansion of regulatory T cells. As CD127 expression is reported to be inversely associated with FoxP3, the data indicate the expansion of a non-regulatory CD25^+ population in smokers and patients with stable COPD. The immunohistochemical staining for the NKG2D ligands MICA and MICB on epithelial cells was unchanged.

**Conclusion:** The results of this thesis suggest a role for CD4^+ and CD8^+ T-cells in clinically stable COPD, indicating that T-cells are of importance in the long-term inflammatory response in COPD. Regardless of current smoking habits, activated CD8^+ T lymphocytes were found to be increased in BAL fluid from subjects with COPD, suggesting that changes in CD8^+ T cells are associated with a persistent immune response and, thus, of importance in COPD pathogenesis. In contrast, the expansion of non-regulatory CD25^+CD4^+ cells in BAL fluid seemed to be preferentially smoke-related. In summary, the data indicate that, among airway T cells, changes in CD8^+ cells seem to be highly associated with COPD pathogenesis, whereas changes in CD4^+ cells appear to be related to cigarette smoke-induced responses. Further, a non regulatory population of helper T cells was identified in BAL fluid of COPD patients, which may contribute to the persistent cytotoxic T cell responses.
Tobaksrökning är den viktigaste orsaken till kroniskt obstruktiv lungsjukdom, KOL. I Sverige ökade rökning efter andra världskriget, vilket återspeglades i en ökning av både sjukdomsförekomst och dödlighet i KOL. Idag röker 14 % av kvinnorna och 11 % av männen, och 2007 avled 2300 personer av sjukdomen i Sverige. KOL var tidigare vanligare hos män än hos kvinnor men idag insjuknar fler kvinnor än män. KOL kommer enligt WHO att vara den tredje vanligaste dödsorsaken i världen år 2030. Mekanismerna bakom hur tobaksrökning orsakar sjukdomen är fortfarande till stora delar okända.

T lymfocyter har i tidigare studier visat sig vara av betydelse för utvecklingen av KOL. Syftet med avhandlingen var därför att studera luftvägsinflammation med fokus på lymfocyter och lymfocytsubtyper hos individer med KOL, i jämförelse med rökare med normal lungfunktion och icke rökare. Målsättningen var också att studera om pågående rökning påverkar luftvägsinflammationen. Individerna med KOL var stabila i sin sjukdom, det vill säga de hade inte någon påvisbar infektion eller försämringstillstånd tre månader före studien. Behandlingen bestod enbart av kortverkande luftvägsvidgande inhalationer vid behov. Via bronkoskopi togs små bitar av slemhinnan (biopsier) från de centrala luftvägarna och genom luftvägssköljningar insamlades bronchial wash (BW) och bronkoalveolärt lavage (BAL). Slemhinnebiopsierna färgades med specifika antikroppar (immunhistokemi) för att identifiera inflammatoriska celler och markörer, och i BAL karakteriserades lymfocyter och lymfocytsubtyper med flödescytometri.

Hos personer med KOL observerades ökat antal cytotoxiska T lymfocyter (CD8+)** i bronkslemhinnan och BAL jämfört med de två kontrollgrupperna. Dessa celler var mer aktiverade hos personer med KOL jämfört med de två andra grupperna.

I BAL fanns ett ökat uttryck av hjälpar T celler (CD4+), och även dessa var mer aktiverade hos personer med KOL jämfört med de övriga grupperna. Ett högre uttryck av CD4+CD25+ celler med misstänkt regulatorisk funktion observerades i KOL-gruppen. För att mer i detalj undersöka fyndet av regulatoriska T celler användes mer specifika markörer för regulatoriska T celler som FoxP3 och CD127. Resultatet tyder på att T cellerna hade mer aktiverande än regulatoriska egenskaper vid KOL, medan de hos en undergrupp av rökarna var av mer regulatorisk karaktär. Hos KOL patienter observerades även en skillnad i uttrycket för CD127 mellan de som röker och de som slutat röka, vilket antyder att rökstopp minskar aktiviteten hos CD4+ cellerna vid KOL.

Uttrycket av aktiveringsmarkörerna NKG2D och CD69 på CD8+ celler var ökat hos KOL och rökare med normal lungfunktion jämfört med icke-rökare. Antalet CD69-receptorer uttryckta på cellytan var också förhöjt, vilket tyder på att enskilda CD8+ celler har en högre aktiveringsgrad i KOL-patienter. Vi fann dock ingen ökning av NKG2Ds ligander MICA och MICB i luftvägsslemhinnan.

Sammanfattningsvis har dessa studier visat att populationerna av CD4+ och CD8+ T celler i luftvägarna är förändrade i den inflammatoriska och immunologiska processen hos individer med kliniskt stabil KOL. Oavsett rökvanor var CD8+ T celler aktiverade i luftvägarna hos patienter med KOL, vilket tyder på att dessa celler sannolikt är viktiga i utvecklingen av KOL. I luftvägarna hos patienter med KOL identifierades även en ökning av icke-regulatoriska CD4+CD25+ T celler, vilka möjligen kan bidra till det bestående cytotoxiska svaret.
## SELECTED ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>A1ATD</td>
<td>α-1 anti trypsin deficiency</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen-presenting cell</td>
</tr>
<tr>
<td>BAL</td>
<td>Bronchoalveolar lavage</td>
</tr>
<tr>
<td>BCR</td>
<td>B-cell receptor</td>
</tr>
<tr>
<td>BW</td>
<td>Bronchial wash</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CD3⁺</td>
<td>Lymphocyte surface antigen for T cells</td>
</tr>
<tr>
<td>CD4⁺</td>
<td>Lymphocyte surface antigen for helper T cells</td>
</tr>
<tr>
<td>CD8⁺</td>
<td>Lymphocyte surface antigen for cytotoxic T cells</td>
</tr>
<tr>
<td>COPD</td>
<td>Chronic obstructive pulmonary disease</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T cell</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cells</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorting</td>
</tr>
<tr>
<td>FEV₁</td>
<td>Forced Expiratory Volume in one Second</td>
</tr>
<tr>
<td>FoxP3</td>
<td>Transcription factor fork head box P3</td>
</tr>
<tr>
<td>FVC</td>
<td>Forced Vital Capacity</td>
</tr>
<tr>
<td>GMA</td>
<td>Glycolmethacrylat</td>
</tr>
<tr>
<td>GOLD</td>
<td>Global Initiative for Chronic Obstructive Lung Disease</td>
</tr>
<tr>
<td>IL-</td>
<td>Interleukin-</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon gamma</td>
</tr>
<tr>
<td>MFI</td>
<td>Median fluorescence intensity</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MIC</td>
<td>MHC class 1 chain-related</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metallo proteinases</td>
</tr>
<tr>
<td>NK</td>
<td>Natural Killer cells</td>
</tr>
<tr>
<td>NKG2D</td>
<td>NK cell group 2D</td>
</tr>
<tr>
<td>PMN</td>
<td>Polymorphonuclear leukocyte</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RTLF</td>
<td>Respiratory tract lining fluid</td>
</tr>
<tr>
<td>TC</td>
<td>Cytotoxic T cells.</td>
</tr>
<tr>
<td>TCR</td>
<td>T-cell receptor</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor-beta</td>
</tr>
<tr>
<td>TH</td>
<td>Helper T cells</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor alpha</td>
</tr>
<tr>
<td>VC</td>
<td>Vital capacity</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
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</table>
This thesis is based on the following papers, which will be referred to in the text by their Roman numerals.

Increased intraepithelial T-cells in stable COPD.
*Respir Med.* 2008 Dec;102(12):1812-8

II. Roos-Engstrand E, Ekstrand-Hammarström B, Pourazar J, Behndig AF, Bucht A, Blomberg A.
Influence of smoking cessation on airway T lymphocyte subsets in COPD.
*COPD.* 2009 Apr;6(2):112-20.

III. Roos-Engstrand E, Pourazar J, Behndig AF, Bucht A, Blomberg A.
Expansion of helper T cells with a non-regulatory function in smoking and COPD
*Submitted*

IV. Roos-Engstrand E, Pourazar J, Behndig AF, Blomberg A, Bucht A.
Cytotoxic T cells expressing the co-stimulatory receptor NKG2D are increased in cigarette smoking and COPD
*Submitted*

*Contributed equally to first authorship*

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INTRODUCTION

CHRONIC OBSTRUCTIVE PULMONARY DISEASE, COPD

Chronic obstructive pulmonary disease (COPD) is characterized by airflow limitation that is not fully reversible, as stated in Global Initiative for Chronic Obstructive Lung Disease (GOLD). The airflow limitation is usually both progressive and associated with an abnormal inflammatory response of the lungs to noxious particles or gases, such as cigarette smoke.

The prevalence of smoking escalated in the middle of the 20th century and the negative effects induced by smoking began to appear. Tobacco smoke is the main cause of COPD but also exposure to biomass fuel used for cooking and heating in low-income countries, has been associated to the development of COPD, as well as other air pollutants.

In Sweden, about 50% of all smokers over the age of 70 years develop COPD and about 90% of all individuals with COPD are, or have been smokers. In Sweden, about 700 000 people suffer from the disease today and around 2 300 people died of COPD in 2007 (Swedish Cause of Death Register). Worldwide, the WHO estimates that 80 million people suffer from moderate to severe COPD. More than 3 million people died of COPD in 2005, which corresponds to approximately 5% of all deaths globally. Most of the knowledge about COPD prevalence, morbidity and mortality is based on studies conducted in high-income countries. However, it is known that almost 90% of the deaths related to COPD occur in low- and middle-income countries.

Earlier, COPD was more common in men but because of increased tobacco use among women in high-income countries and the higher risk of exposure to indoor air pollution (such as biomass fuel used for cooking and heating) in low-income countries, the disease now affects men and women almost equally worldwide. In Sweden, COPD is more frequent among women compared with men and since 2005 more women than men die from COPD (Swedish Cause of Death Register). Thus, COPD has become a woman’s disease (Figure 1).

In 2002, COPD was the fifth leading cause of death worldwide. Approximately 50% of all individuals with COPD are not aware of the disease and its prevalence is thus considerably underestimated. The WHO predicts COPD to be the third leading cause of death in 2030, next after ischemic heart disease and cerebrovascular disease.
Figure 1: Death rate for COPD in Sweden 1987-2007. (Source: Swedish Cause of Death Register).

THE RESPIRATORY SYSTEM

The respiratory system involves two main levels, the upper and lower respiratory systems. The nose and throat (pharynx and larynx) make up the upper system, whereas the lower respiratory tract’s different parts are the windpipe (trachea), bronchi and bronchiole (Figure 2). The main carina is the point where the trachea divides into the right and left lung lobes. The left lung consists of an upper and lower lobe, whereas the right lung also has a separate middle lung lobe. The bronchioles are intralobular airways with a diameter of less than 2mm. Proceeding distally along the respiratory bronchioles, the number of alveolar openings into the bronchiolar wall becomes ever greater until the wall consists of nothing else, and the tube is now termed an alveolar duct. Both the alveolar ducts and alveoli are lined by extremely attenuated squamous epithelial cells. The function of the alveoli is to facilitate the gas exchange, i.e. supply the body with oxygen and remove carbon dioxide from the blood. The air is also warmed up and humidified. There are approximately 24 000 small airways and bronchioles, thousands of which could be narrowed or totally obstructed without a significant loss of lung function. The total area of respiratory airways is about 100 m², comparable to the size of a tennis court. One cubic millimeter lung parenchyma contains around 170 alveoli. In an adult lung, there are 480 million alveoli. The number of alveoli is closely related to the total lung volume whereas alveolar size is not.
Airway tissue

The airway epithelium represents the first physical interface with which inhaled toxins can interact, and is therefore one of the first targets of cigarette smoke. The epithelial cells are covered with cilia. The basement membrane is a thin layer between the epithelial cells and the submucosa. Glands, arteries, smooth muscle and cartilage are all represented in the submucosa and adventitia, which are the layers beneath the basement membrane. Airway epithelial cells can produce mediators that initiate and modulate the host response to inhaled irritants. This inflammatory response can be further amplified by cells infiltrating the epithelium. Intraepithelial T-lymphocytes seem to be of specific interest in this context, as they constitute a distinct T-cell subset with specific activation, regulatory capacity and subsequent capability of adhering to the bronchial epithelium, suggesting a role in an initial defense mechanism and T-cell cytotoxicity.

Mucus, together with the cilia, transports particles up through the throat. Mucus is a mixture of protein, glycoprotein and water and is derived from the secretions of cells from several areas including the alveoli. The respiratory epithelium is covered by a thin layer of fluid, the respiratory tract lining fluid (RTLF), a complex mixture of phospholipids and proteins secreted by a number of different cells.

Interactions between airway epithelium and other cell types present in the lung parenchyma play an important role in driving pro-inflammatory responses. Epithelial cells themselves can directly express a number of chemokines and cytokines, which promote the infiltration of neutrophils, dendritic cells and T cells in the lung tissue.
SIGNS, SYMPTOMS AND DIAGNOSIS OF COPD

Many of the signs of COPD are caused by the body's attempt to compensate for a damaged respiratory system. Several symptoms develop as a direct result of disease processes, the most common being a chronic cough, sputum production, wheezing and shortness of breath (dyspnea). Often, these symptoms may occur years before lung function declines. A productive cough is caused by inflammation and an excessive production of mucus in the airways. Coughing becomes less effective because of the obstructed airflow and reduced mucociliary clearance due to ciliary destruction. Some subjects with COPD also suffer from osteoporosis due to tobacco smoking and an inactive life. In some cases, this leads to fractures and hospitalization. In patients with more severe COPD, increased breathing effort leads to higher energy consumption and, consequently, a loss of weight. Depression and tiredness is also more common among people with severe COPD, but not everyone has these symptoms. It is very important to diagnose COPD as early as possible, as the progression of the disease may then be prevented. Exacerbations are occasions when the individuals suffer worsening periods of the disease. The major symptoms are increased dyspnea and sputum volume.

The diagnosis of COPD is confirmed with spirometry. The lung function variables used for diagnosis are a) Forced Vital Capacity (FVC), which is the volume of air that can forcibly be blown out after a deep breath and b) Forced Expiratory Volume in one Second (FEV₁) which is the maximum volume of air that can forcibly be blown out during the first second of the FVC maneuver. Both FVC and FEV₁ are measured in liters and the ratio between them is commonly used to diagnose obstructive lung disease. Vital Capacity (VC) is the maximum volume of air a person can exhale at normal speed after a maximum inhalation. Both VC and FEV₁ decrease as a normal consequence of aging but in smokers, lung function decline is often more rapid than in non-smokers. A diagnosis of COPD should be considered in any individual who has dyspnea, chronic cough or sputum production, and/or a history of exposure to risk factors for the disease, especially cigarette smoking.

To distinguish between asthma and COPD, a bronchodilator test is commonly performed. FEV₁ is measured prior to and 15-20 minutes after the administration of a bronchodilator drug for inhalation, for example Salbutamol. The bronchodilator response in COPD is often absent or small, but some patients may have considerable increase in percent of baseline value. However, they always remain obstructive, i.e the airway obstruction is chronic.
Figure 3: Age-related decline in FEV$_1$ with different smoke history.$^{22}$

The Fletcher curve (Figure 3) is commonly used to show the importance of smoking cessation in patients with COPD.$^{23}$ Fletcher et al longitudinally followed a group of men with repeated lung function measurements. The lower curved line shows the rapid decline in lung function in the cigarette smokers. The upper curved line represents the normal decline in lung function by aging, whereas the middle line represents the decline in lung function in smokers who are less susceptible to cigarette smoke. The Fletcher curve indicates that not all smokers are sensitive to tobacco smoke and thus do not develop COPD. In contrast, sensitive smokers will rapidly decline in lung function. Smoking cessation is very important in this group of smokers who will otherwise probably develop COPD and suffer an increased risk of premature death. According to GOLD standards, the different degrees of disease severity in COPD are called mild, moderate, severe and very severe (Table 1).$^1$ The highest measurement of VC or FVC is used to calculate the FEV$_1$/VC or FEV$_1$/FVC ratios. The basal criterion for all stages of COPD is based on a FEV$_1$/VC or FEV$_1$/FVC ratio of < 0.7. In Sweden, FEV$_1$/VC of < 65% is used in patients older than 65 years as FEV% normally declines with age.$^{24}$
INTRODUCTION

<table>
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<th>FEV₁% of predicted</th>
<th>Stage</th>
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<tr>
<td>&gt; 80%</td>
<td>I</td>
</tr>
<tr>
<td>50% &lt; FEV₁ &lt; 80%</td>
<td>II</td>
</tr>
<tr>
<td>30% &lt; FEV₁ &lt; 50%</td>
<td>III</td>
</tr>
<tr>
<td>&lt;30%</td>
<td>IV</td>
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Table 1: Classification of COPD severity by spirometry test according to GOLD. The basal criterion for all stages of COPD is based on a FEV₁/VC or FEV₁/FVC ratio of < 0.7.

TREATMENT OF COPD

It is critically important that patients with COPD quit smoking. Upon smoking cessation, the rate of decline of lung function is reduced (Figure 3) and smoking cessation is the first, best and cheapest way to stop the progression of the disease. Smoking cessation programs and nicotine supplementation are two ways to help these patients quit smoking. The pharmacological treatments which may facilitate the lives for COPD patients are mainly long acting bronchodilators and inhaled corticosteroids. Bronchodilator therapy, such as long-acting anticholinergics and/or beta₂-agonists, plays an important role in the treatment of COPD. The symptoms often decrease, even if the effect on FEV₁ is minimal with drug therapy. Airflow limitation is generally poorly reversible in COPD. Inhaled corticosteroids have anti-inflammatory properties and the potential to play an important role in the treatment of COPD and are used in combination with long-acting beta₂-agonists, especially for patients with severe COPD and a history of frequent exacerbations, i.e. worsening periods of the disease. While many medications are available to treat COPD, no drug has demonstrated effectiveness in halting the progression of the disease. Rather, the goal of drug therapy so far has been to maintain control of symptoms and prevent COPD exacerbations. COPD is not a reversible condition but treatment can slow its progression. Smoking cessation is most important and has been shown to explicitly affect the decline in FEV₁.

HETEROGENEITY OF COPD

Previously, COPD was considered as being only a lung disease but today it is accepted as a systemic (affects organs outside the lungs) and multicomponent disease. Not only exposure to smoke, gases and particles causes the development of COPD; individual defense mechanisms and heredity also play a role. Chronic bronchitis, emphysema, small airway disease, inflammation, mucociliary dysfunction and a systemic component are multifactorial components of importance in COPD (Figure 4).
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**Figure 4: The heterogeneity of COPD. One of these factors or several together can induce the disease.**

Chronic bronchitis

Many smokers with normal lung function and subjects with COPD suffer from chronic bronchitis, a disease defined as a state with cough and chronic sputum production for three months during two consecutive years. Within the epithelial cell layer, there are goblet cells producing mucus. In COPD, increased numbers of goblet cells are found in small bronchi and bronchioles, where normally there are very few. Upon exposure to cigarette smoke, airway epithelial cells undergo a range of structural alterations, resulting in loss of barrier function, a decrease in mucociliary clearance and squamous metaplasia.

Chronic bronchitis in COPD may cause deterioration of the disease as it predisposes the risk for bacterial infections and exacerbations. Symptoms of chronic bronchitis are associated with an inflammatory process involving the glands and epithelium of the central airways. Chronic bronchitis is associated with hyperplasia of both epithelial goblet cells and submucosal glands in the airways. The enlargement of mucus-secreting glands and goblet cell hyperplasia can occur in the absence of airflow limitation. Inflammatory cells release serine proteases that are potent secretagogues for mucus. Oxidants derived from cigarette smoke and released from inflammatory leukocytes may also be involved in the overproduction of mucin by the induction of the MUC5AC gene.

Small airways disease

Small airways disease, or bronchiolitis, includes the pathological changes within the small airways, bronchioles, with a diameter of less than 2 mm, a major site of airway obstruction in COPD. These changes are the result of chronic inflammation and fibrosis in the lungs.
Studies have also shown structural abnormalities in the small airways of smokers, both with and without COPD. The obstruction of the small airways in COPD is associated with a thickening of the airway wall by means of a remodeling process related to tissue repair and a dysfunction in the mucociliary clearance of the innate host defense system, which results in the accumulation of inflammatory exudates in the airway lumen. There is also a relationship between the severity of COPD and the extent of occlusion of the airway lumen by inflammatory mucus exudates.

**Emphysema**

Emphysema, another important component of COPD, is a destructive disease defined pathologically as “the presence of permanent enlargement of the airspaces distal to the terminal bronchioles, accompanied by destruction of their walls without obvious fibrosis”. Emphysema is a long-term progressive disease, which reduces the ability of gas exchange and causes shortness of breath, as a consequence of destroyed alveoli (Figure 5). COPD is often associated with emphysema and the state is irreversible. Emphysematous lung destruction reduces maximal expiratory airflow by decreasing the elastic recoil force that drives air out of the lungs. Destruction of alveolar walls occurs in COPD as a result of protease-mediated degradation of connective tissue elements, particularly elastin, and apoptosis of type I pneumocytes and endothelial cells. There is an association between the degree of emphysema and smoking, defined as the number of pack-years. About 40% of heavy smokers develop substantial lung destruction from emphysema, but emphysema can also be found in individuals with normal lung function.

![Figure 5: Normal vs emphysematous alveoli](image-url)
Heredity and gender differences

As stated previously, smoking is the main risk factor for the development of COPD but genetic factors are also of importance, since only a subset of smokers develops the disease. The individual defense mechanisms are different and are often genetically dependent. In 1963, α1-antitrypsin deficiency (A1ATD) was identified as a genetic factor for COPD. The role of α1-antitrypsin is to protect the lung tissue from destructive enzymes, such as proteases and especially neutrophil elastase, produced by neutrophils. Lack of α1-antitrypsin is one cause of the tissue destruction that leads to emphysema and, further, the development of COPD. It is a rare condition and responsible for only 1 to 2% of the total burden of COPD.41,42

Gender differences have been suggested both in disease prevalence and in response to environmental exposures. Furthermore, it has been shown that the acquisition of 'addiction' to smoking is partly genetically mediated.43 Women may also be more susceptible to the development of severe COPD.44
THE IMMUNE SYSTEM

The defense mechanisms against virus, bacteria, particles and gases are built up at three levels. Firstly, the mechanical or chemical barriers which the pathogens have to penetrate in the airways are the epithelial cells covered with cilia and a mucus layer. The pathogens will get stuck in the mucus and, via the mucociliary clearance system, be transported towards the throat, where they are swallowed or spat out. If the pathogens get through this defense mechanism, the next level of airway defense immediately steps forward: the innate immune or unspecific defense system. Cells such as macrophages and neutrophils are programmed to get rid of the invader by phagocytosis. They are recruited from the blood to the defense site by different mediators and cytokines, a process called chemotaxis. When these cells and mediators are activated, the inflammatory process is started. Then it is time for the third defense level to be involved: the adaptive immune defense or specific immune response. T and B lymphocytes are involved and antibody production is restricted to this defense mechanism. The adaptive immune response generates effector cells specific for a particular microorganism and produces memory cells which can prevent re-infection with the same microorganism. Both innate and adaptive inflammatory and immune responses are involved in the lung inflammation in smokers and patients with COPD.

The innate immune system

Leukocytes, or white blood cells, are cells within the immune system and their duty is to defend the body against both infectious disease and foreign materials. They develop in the bone marrow and differentiate from myeloid stem cells to various leukocyte types. The innate leukocytes include natural killer cells, mast cells, eosinophils, basophils and the phagocytic cells including macrophages/monocytes, neutrophils and dendritic cells. These cells function within the immune system by identifying and eliminating pathogens or other agents that may cause disease (Figure 6).
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Figure 6: Differential cell count smear, including lymphocytes, macrophages and neutrophils. Here, differences in cell size and granularity are illustrated.

Macrophages/Monocytes

Monocytes circulate in the blood and differentiate continuously into macrophages upon migration into the tissue. Macrophages are central in most inflammatory responses with various functions and appearances in different tissues, such as the lung. Within the lung, these cells are called alveolar macrophages and they play a critical role in both innate and acquired immunity. Their main function is to destroy harmful agents invading the body by phagocytosis. These may be microbes such as viruses, bacteria or fungi, but also tumor cells or particles and gases. Activation of macrophages proceeds through contact to the pathogens, by cytokines secreted by activated T helper cells or by other mediators in the inflammatory response. One of the most potent activators of macrophages is interferon gamma (IFN-γ), secreted by activated T helper cells. Activated macrophages secrete various cytotoxic proteins such as matrix metallo proteinases (MMP) as well as cytokines and chemokines, including IL-1, TNF-α, IL-8 and IL-10. Macrophages can also express class II MHC molecules allowing them to function as antigen-presenting cells (APC). Within the body, they survive up to several months.45-47

Neutrophils

Neutrophils are 10-17 µm in diameter, containing granulae full of different types of substances which enable the differentiation of the granulocytes in cell staining. The granulae contains compounds important in the defense mechanism against microbes.46 Neutrophils are the most abundant and most important cell of the innate immune response, i.e. the front line defensive cells. They are a source of reactive oxygen metabolites, inflammatory cytokines, lipid mediators, antibacterial peptides and tissue damaging enzymes.48 When activated, they quickly release enzymes, such as α-defensins and serinproteases, from the granulae and induce phagocytosis. They are actors in the defense in acute inflammation to eliminate microbes. Neutrophils constitute 50-75 % of all leukocytes and they circulate for 7-10 h in the blood, before migration into the tissue, where they have a life span of only a few days.
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Neutrophils are often called polymorphonuclear leukocytes (PMN) because of their characteristic segmented, multilobed nucleus and granulated cytoplasm, which facilitates the differentiation from other granulocytes. In clinical practice, the increase of blood neutrophils (leukocytosis) is often used as a sign of bacterial infection. 45,46,49

Dendritic cells

Dendritic cells (DC) are specialized to take up an antigen, process it and display it for recognition by T lymphocytes. In the lung’s responses to tissue damage or infection, they initiate and orchestrate innate and adaptive immunity. They are recruited from the circulation and migrate toward epithelial surfaces, where they capture antigens and recognize danger signals. After antigen uptake, DCs migrate to regional lymph nodes for presentation of sequestered antigens. During their migration, DCs internalize and process the antigen and upregulate the expression of co-stimulatory molecules on their cell surface, a process referred to as maturation. In the lymph nodes, DCs present the processed antigen to naive T lymphocytes, resulting in the initiation, suppression or termination of adaptive immune responses. 50 DCs are the key cellular link between the innate and adaptive immunity playing a pivotal role as sensors of infection or injury for the initiation of adaptive immune responses. 51,52 Immature dendritic cells alert the adaptive immune system to the presence of incoming pathogens or to tissue injury. These cells mature when the displayed toll-like receptors (TLRs) bind to a ligand. Mature dendritic cells express high levels of class II MHC proteins and the co-stimulatory molecule CD80 and CD86, which direct them to local lymph nodes where they present antigens to T cells. 46,53

Natural killer cells

Natural killer cells (NK cells) develop in the bone marrow and will then circulate in the blood. They are larger than both T and B cells, have distinct cytoplasmic granules, and are functionally identified by their ability to kill certain cell lines, such as lymphoid tumors, without the need of prior immunization or activation. The mechanisms are the same as used by cytotoxic T cells. NK cells are activated in response to interferon and macrophage derived cytokines. NK cells can activate helper T cells type 1 (T_H1) cells by secreting IFN-γ and helper T cells type 2 (T_H2) cells by secreting IL-4. They constitute 5-10% of the lymphocytes in human peripheral blood and are generally detected by the expression of CD56 and CD16 membrane receptors and the lack of CD3. 46,47

Figure 7: Helper T cell, CD4+ and cytotoxic T cell, CD8+ and their stimulating ligands. 47
INTRODUCTION

The adaptive immune system

Lymphocytes are white blood cells, belonging to the adaptive immune system and activated when the innate immune response fails. T, B and NKT cells originate from bone marrow-derived progenitors. Progenitors that migrate to the thymus and receive signals through the Notch receptor commit the T-cell lineage. Lymphocyte specificity and diversity are gained during the process of T-cell receptor (TCR) or B-cell receptor (BCR) generation, key events in the adaptive immune system.

Development of T cell

Early T cell precursors do not express the CD4 and CD8 (double-negative) co-receptor and begin recombination of 4 gene segments, α, β, γ and δ, which create the TCR-gene. Recombination begins at the δ, γ, and β loci, and if the expression of the γ/δ TCR is successful, commitment to the γ/δ T cell lineage results. These cells remain double-negative and leave the thymus and reside in lymphoid tissue and airway epithelium. Otherwise, the β loci substitute with α loci to form the pre-TCR. TCR includes CD3 on its cell membrane. As they progress through their development, they become CD4⁺CD8⁻ (double-positive) thymocytes, and finally mature to CD4⁺CD8⁻ or CD4⁻CD8⁺ (single positive) thymocytes, which are then released from the thymus to the peripheral tissue, such as the lung. The interaction with the TCR complex and MHC molecule is restricted by the specificity of the TCR and T cell co-receptor. Helper T cells (CD4) restrict interactions with class II MHC and cytotoxic T cells (CD8) to class I MHC. Only 2% of double-positive cells survive and the majority undergoes apoptosis. TCR-α/β cells comprise 95% of the T-cell population and orchestrate the immune response by the secretion of activating and regulating cytokines, while TCR-γ/δ lymphocytes play a key role in mucosal homeostasis and in response to tissue damage at the epithelial surface. The antigenic molecules that activate γ/δ T cells are still largely unknown.

Cytotoxic T cells (CD8)

Cytotoxic T cells (T_C) represent 30-40% of the α/β T cell population and express the molecule CD8 on their cell surface. The primary function of T_C cells is to kill infected cells by recognition and destruction of cells expressing a stimulating ligand (Figure 7). T_C produces cytokines such as IFN-γ that affect surrounding cells and other leukocytes. T_C is capable of inducing death of infected cells, tumor cells or cells that are otherwise damaged or dysfunctional. T_C can differentiate into two effector phenotypes, T_C1 and T_C2, secreting different cytokine patterns. Both subsets are cytotoxic via the perforin and Fas/FasL pathways and both kill resting and activating B cells. T_C1 cells express IFN-γ, whereas T_C2 cells secrete IL-4 and IL-5.

Helper T cells (CD4)

Helper T cells (T_H) constitute 60-70% of the total TCR-α/β population and are involved in activating and directing other immune cells, and are thus particularly important in the immune system. T_H cells are the coordinating cells of the immune response, recognizing foreign antigen, and activating other parts of the cell-mediated immune response to eliminate the pathogen. CD4 is a co-receptor that assists the TCR in the cell activation, following an
interaction with an antigen presenting cell (Figure 7). They also play a major part in activation of B cells. T_H cells are divided into two main groups, T_H1 and T_H2 in accordance with their main functions. T_H1 subsets, secretes IL-2 and IFN-\gamma. IL-2 stimulate T cell division and cytotoxicity of CD8^+ T cells, by decreasing activation thresholds. IFN-\gamma activates macrophages to kill intracellular pathogens. T_H1 subset is mainly responsible for classic cell-mediated functions, such as delayed hypersensitivity and the activation of cytotoxic T lymphocytes. The other subset, T_H2, secretes IL-4, IL-5 and IL-10, which favor antibody production. This subset functions more effectively as a help for B-cell activation with antibody responses, particularly with allergic reactions (Figure 9).

**Regulatory T cells**

Regulatory T cells (formerly known as suppressor T cells) are a specialized subpopulation of T cells that suppress activation of the immune system and thereby maintain immune system homeostasis and tolerance to self-antigens. Regulatory T cells are a subset of CD4^+ cells that inhibit activated lymphocytes through the release of suppressor molecules, such as IL-10 or TGF-\beta. They are also capable of inducing inhibition by direct cell-to-cell contact mechanisms, through cytotoxic T lymphocyte-associated antigen 4 (CTLA4) interaction with the antigen presenting cell. There are different types of regulatory T cells, but the cells expressing CD25 (IL-2Rα) seem to be central. CD4^+CD25^+ regulatory T cells represent between 1 and 3% of total CD4^+ T cells. There are two different populations of regulatory T cells, natural and adaptive.

CD4^+CD25^+ natural regulatory T cells are generated in the thymus and reside in the blood and peripheral lymphoid tissues. These cells were first defined in 1995 by Sakaguchi and colleagues. Natural regulatory T cells constitutively express the IL-2 receptor (CD25) in accordance with their high dependency on IL-2 for survival. Other regulatory T cell populations may develop extra-thymically from naive CD4^+ T cells under the influence of TGF-\beta, referred to as induced regulatory T cells. Adaptive CD4^+CD25^+ regulatory T cells are generated in the periphery from naive T-cells after encountering antigens presented by APC. Both the adaptive and natural regulatory T cells depend on a gene called transcription factor fork head box P3, FoxP3. CD4^+ cells expressing high levels of CD25 (CD25^{bright}) are suggested to be regulatory T cells (Figure 8) and they are also closely associated with FoxP3 expression.

![Figure 8: CD4^+CD25^{bright} cells are suggested to be regulatory T cells](image)
Figure 9: Overview of the adaptive immune response. An antigen is ingested and processed by antigen presenting cells (APCs). It presents fragments from it to T cells on either their MHC Class I or class II molecules. Helper T cells recognize antigens presented on MHCII, with the help of their expression of CD4 co-receptor (CD4\(^+\)). The activation of a resting helper T cell causes it to release cytokines that stimulate the activity of macrophages, cytotoxic T cells and B cells, the latter producing antibodies. Cytotoxic T cells recognize antigens presented on MHCI, with the help of the co-receptor CD8 (CD8\(^+\)).

Until some years ago, CD25\(^{bright}\) was the only marker for regulatory T cells. Recently more unique markers have been discovered such as FoxP3, an intra-nuclear marker for regulatory T cells.\(^{69}\) Even a cell surface marker has been discovered, CD127, which has also been proposed to have benefits as a unique marker for regulatory T cells.\(^{70,71}\) In particular, CD127\(^{dim}\) expression has been suggested as a specific regulatory T cell marker, whilst CD127\(^{bright}\) expressing cells are non-regulatory T cells.\(^{71}\) Other, less well-established markers for regulatory T cells are CTLA-4 \(^{72}\) and HLA-DR.\(^{73}\) It has been shown that a mutation in the FoxP3 gene causes autoimmune disorders such as psoriasis, arthritis and systemic lupus erythematosus (SLE), allergy and chronic inflammatory diseases.\(^{64,66,74,75}\)

The expression of FoxP3 is mostly restricted to CD4\(^+\) T cells, but some CD8\(^+\) T cells do express FoxP3. The exact function of FoxP3 is largely unknown. It has been suggested that FoxP3 may act as a repressor of transcription with the function of regulating the amplitude of the response of CD4\(^+\) T cells to activation.\(^{76}\) It has also been proposed that all human CD4\(^+\) and CD8\(^+\) T cells may up-regulate FoxP3 and acquire suppressive properties.\(^{77}\) Regulatory T cells may also play a potential role in graft tolerance and inhibit graft rejections. Regulatory T cells can inhibit the activation of helper T cells as well as antigen presenting cells (APC) (Figure10).
**INTRODUCTION**

![Image of immune cells](image)

Figure 10: Down-regulated immune response by regulatory T cells.

### γδ-T cells

γδ T cell is preferentially associated with epithelial tissue and differs strikingly in function from αβ T-cells. In contrast to αβ T-cells, γδ T cells do not recognize antigen as peptides presented by MHC molecules; instead they recognize their target antigens directly, and thus could recognize and respond rapidly to molecules expressed by many different cells. They recognize a large number of diverse antigens without clonal expansion, and it is believed that tissue-associated subsets of γδ T-cells respond to tissue-specific “stress-antigens” primarily derived from the epithelium. The role of these cells is complex, and they seem to have the ability to influence other immune cells and, hence, the outcome of a variety of inflammatory responses. γδ T-cells are abundant in the gastrointestinal mucosa and skin, representing about 30% of the T cell population having an important role in the epithelial repair. Conditions that lead to responses of γδ T-cells are not fully understood but their suggested function is in the first line of defense or as a bridge between innate and adaptive immune responses.

### NKT cells

NKT cells have been classified as T cells with innate immune function, sharing features of both innate and adaptive immune cells. NKT cells are a subset of T cells, and have characteristics of both natural killer cells (NK) and conventional T cells. Like T cells, they have αβ-T cell receptors (TCR), of which most recognize lipid antigens presented on the MHC class I-like molecule CD1d. Like NK cells, they have variable levels of CD16, CD56 and other receptors typical of NK cells, and they can kill other cells. They express the T-cell membrane proteins CD3 and can also express CD4 or CD8. When activated, NKT cells produce large amounts of cytokines, such as IFN-γ, IL-10 and IL-4, much more rapidly than conventional T cells do. The rapid production of cytokines is a manifestation of innate immunity by activating T cells, NK cells, macrophages and B cells. They can regulate immune responses in diverse situations, such as autoimmune diseases, tumor rejection and different types of infections.
### B cells

The main function of B cells is to produce antibodies. The B cell has a membrane coupled antibody called the B cell receptor (BCR). When activated, the B lymphocytes differentiate into plasma cells and produce antibodies with the same specificity as the receptor.\(^{46}\)

### Activation of T cells

The generation of both humoral and cell-mediated immune responses depends on the activation of \(T_H\) cells. The signal of activation starts when T cells recognize their specific peptide, i.e. MHC ligand on APCs. This interaction generates a signal that, together with a necessary co-stimulatory signal provided by CD28, leads to activation and proliferation of the \(T_H\) cell. After co-stimulation, T cell proliferation and differentiation are dependent on IL-2 production by activated T cells themselves.\(^{60}\) A naive T cell recognizes antigen on the surface of an APC and receives the required two signals to become activated. IL-2 driven clonal expansion is followed by the differentiation of the T cell to an armed effector cell status. Activation of T cells changes the expression of the cell surface molecules.\(^{56}\)

\(T_H1\) cells promote macrophage activation by IFN-\(\gamma\) production and contact-dependent stimulation by using a variety of cell surface co-stimulatory ligands, thus playing a major role in intra-cellular pathogen clearance and delayed-type hypersensitivity. \(T_H1\) differentiation is directed by IFNs generated by the innate response to infection and they also secrete IL-2. \(T_H2\) cells produce IL-4, IL-5 and IL-10, stimulating B cell antibody response.\(^{83}\)

Following responses to the helper T cell derived cytokines (IL-2, IFN-\(\gamma\)), \(T_C\) recognize specific antigenic peptide bound to antigen-MHC class I molecule complex, proliferate and differentiate into an effector cell. The affinity between CD8 and the MHC molecule keeps the \(T_C\) and the target cell bound closely together during antigen-specific activation. Naive CD8 T cells can be activated in different ways to become armed cytotoxic effector cells. Dendritic cells are a common activator of CD8\(^+\) cells. These cells can directly stimulate \(T_C\) to synthesize IL-2, driving an autocrine proliferation.

When activated, cytotoxic T cells produce cytotoxic proteins including perforin and granzymes and secrete them at the point of contact with the target cell, resulting in specific killing without bystander cell damage. Perforin is a membrane-disrupting protein that facilitates the ability of granzymes to induce apoptosis of the target cell. In addition to cytolysis, CD8\(^+\) effector cells produce IFN-\(\gamma\) and TNF.\(^{46,56,60}\)
Migration of T cells

Lymphocytes undergo constant recirculation between the blood, lymphoid organs and tertiary extra-lymphoid tissues, such as lung tissue. This recirculation enables the lymphocytes to actually encounter the antigen when they are present in the body. Although the tertiary extra-lymphoid tissues normally contain few lymphoid cells, migration of lymphoid cells into these sites occurs during the inflammatory response. Migration of leukocytes into inflamed tissue or into lymphoid organs requires interaction between cell-adhesion molecules (CAMs) such as selectins or integrins on the vascular endothelium and their receptors on the circulating cells. Homing receptors on lymphocytes interact with tissue specific adhesion molecules such as, vascular adhesins or high-endothelial venules in lymphoid organs on the endothelium in tertiary organs.46

T cell markers

**CXCR3** is a CXC chemokine receptor, expressed on activated T lymphocytes and NK cells and some epithelial cells. CXCR3 is preferentially expressed on T_H1 cells and interferon inducible receptor, CXCL-10, is one activating ligand. **CXCR3**-ligand interaction attracts T_H1 cells and promotes T_H1 cell maturation.84 **CXCR3** is a marker for T_H1 cells and it is suggested that T_H1 lymphocytes in the lungs of subjects with smoking-related disease, drive the progression of emphysema through **CXCR3** ligands.85

**CD25** (IL2-α receptor), is a type I transmembrane protein presented on activated T cells. CD25 is crucial in activation of T cells. Once T-cells encounter an APC with appropriate MHC molecule and peptide, up-regulation of CD25 on the cell surface is encountered as an early activating marker. Between 12 and 24 hours after antigenic challenge CD25 is up-regulated on cells’ membrane and facilitates production of an IL-2 high affinity receptor that initiates cell proliferation and differentiation.86 **CD25** is a constitutively expressed marker and high or bright expression of CD25 on CD4^+^ cells may indicate regulatory T cells.67

**CD69** is a type II transmembrane glycoprotein with a C-type lectin binding domain in the extracellular portion of the molecule. CD69 is one of the earliest up-regulated T cell activation markers but can also be present on other leukocytes. Once expressed, CD69 acts as a co-stimulatory molecule for T cell activation and proliferation. CD69 is a constitutively expressed early activation marker and acts as a co-stimulatory molecule for T cell activation and proliferation, within one or two hours after TCR engagement.87 The precise role for CD69 in immunity has not been elucidated owing to the absence of a known ligand and adequate *in vivo* models to study its physiological function.88 **CD69** is highly expressed on BAL-lymphocytes compared with peripheral blood cells and can be induced on many different cells including T cells, macrophages, eosinophils and neutrophils.89
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**CD127** (IL-7α receptor) is a cell surface receptor expressed on immature B cells, activated T cells, macrophages and vascular endothelial cells. IL-7 plays an important role in the normal development and maintenance of the human immune system. Its effects are mediated via the receptor, IL-7R (CD127). The activation of IL-7 signalling pathway results in survival, proliferation, differentiation and maturation of hematopoietic cells, including B and T lymphocytes. CD127 distinguishes CD25\(^+\) regulatory T cells from CD25\(^+\) activated T cells. CD127 show an inverse correlation with FoxP3, which might reflect the direct transcriptional repression of CD127 by FoxP3.

**FoxP3** (transcription factor fork head box P3) is a member of the fork head or winged helix family of transcription factors. FoxP3 is a unique marker for regulatory T cells. A mutation in the FoxP3 gene can cause IPEX (Immune dysfunction, polyendocrinopathy, enteropathy, and X-linked inheritance) syndrome as well as other autoimmune diseases such as diabetes and thyreoditis. Human FoxP3 is located on the X chromosome encoding scurfen, which binds to the IL-2 promotor and the granulocyte-macrophage colony-stimulating factor (GMCSF) enhancer, near nuclear factor of activated T-cell (NFAT) sites. FoxP3 represses these genes, thus reducing IL-2 production by CD4\(^+\) T cells.

**HLA-DR**’s primary function is to present peptide antigens to the immune system for eliciting or suppressing T\(_H\) cell responses. HLA-DR is the most prevalent class II MHC molecule and was first described as a marker of activated T cells. Patients with chronic autoimmune disease or inflammation display a higher frequency of HLA-DR\(^+\) T cells in the peripheral blood compared with healthy donors. HLA-DR is a late activation marker and is up-regulated 48-60 hours after TCR stimulation.

**NKG2D/MIC** is a receptor-ligand interaction that may provide a mechanistic link between epithelial cell stress and immune cell activation. NK cell group 2D (NKG2D) was identified in 1991 and is a C-type lectin-like, type II transmembrane glycoprotein. The NKG2D receptor is expressed on circulating and tissue lymphocytes. This receptor directly recognizes transformed or infected cells through structurally related ligands that are expressed on the surface of stressed cells. NKG2D receptors are constitutively expressed and almost exclusively on cytotoxic T cells (CTL’s) (CD8\(^+\) T cells, NK cells, NKT cells and γδ\(^+\) T cells) and have an activating function triggered by engagement of MHC class 1 chain-related (MIC) molecule A. They become up-regulated or expressed in stressed conditions, such as tumours, viral and bacterial infections, and in autoimmune conditions. Other ligands to NKG2D are MICB and UL-16 binding protein (ULBP) 1, 2, 3. NKG2D have restricted tissue distribution in intestinal epithelia and are frequently expressed in epithelial tumors. NKG2D serves as a co-stimulatory receptor for TCR-mediated signals. NKG2D and NKG2D ligands also appear to play a role in the pathogenesis of diseases that are associated with type 1 T cell-dependent response against auto antigens, such as celiac disease and rheumatoid arthritis in humans. Normally MIC proteins are constitutively expressed in low levels in epithelial cells in the gut and thymus, endothelial cells and fibroblast.
INFLAMMATORY MEDIATORS

Cytokines
Several cytokines play a role in orchestrating airway inflammation in COPD, through the recruitment, activation and survival of inflammatory cells. Cytokines secreted from T cells regulate the pattern of inflammation, whereas proinflammatory cytokines amplify and perpetuate the inflammatory response. Proinflammatory cytokines such as TNF-α, IL-1β and IL-6 are increased in COPD. Inhaled cigarette smoke and other irritants activate epithelial cells and macrophages to release several chemotactic factors that attract inflammatory cells to the lungs.

TNF-α is secreted by many cells, including epithelial cells, macrophages, T lymphocytes and airway smooth muscle cells. TNF-α has cytotoxic effects, induces cytokine secretion and is responsible for the extensive weight loss associated with chronic inflammation. TNF-α is an inflammatory cytokine and is chemotactic to monocytes and neutrophils.

IL-2 plays a central role in the clonal proliferation of T cells. IL-2 is secreted by T_H1 cells. This cytokine exists in three different forms with various affinities. IL-2 is made up of three subunits, α, β and γ. The α-chain is expressed by activated T cells and is also named CD25. IL-2 promotes the proliferation, differentiation and survival of T cells and NK cells.

IL-4 is produced by activated T_H2 cells, mast cells and NK cells. The target cells are mainly B cells to enable antibody production, but also T cells, macrophages and mast cells. IL-4 has a wide range of functions but its main activity and biological function are to co-stimulate activation of B cells and further to stimulate proliferation and differentiation into different antibody classes. Other functions are to induce proliferation of T cells, and to up-regulate MHC class II expression on B cells and macrophages.

IL-7 plays an essential role in the development of T cells, and promotes survival and proliferation of T cell precursors. Binding of IL-7 to its related receptor, the IL-7 receptor (IL-7R)/CD127, activates multiple pathways that regulate lymphocyte survival, glucose uptake, proliferation and differentiation.

IL-10 is a potent anti-inflammatory cytokine that is released from monocytes and alveolar macrophages in response to inflammatory stimuli. The main function is as a dampener of immune responses, but IL-10 possesses stimulatory activation as well. The induction of regulatory T cells by IL-10 also points towards a crucial role in the establishment of peripheral tolerance.

IFN-γ is secreted by T cells, primarily T_H1 cells and NK cells. The target cells are mainly macrophages and B cells with the purpose of activating or differentiating these cells. IFN-γ induces class switch and inhibits proliferation of T_H2 cells.
Chemokines

Chemokines play an important role in the recruitment of inflammatory cells from the circulation to the airways in COPD. Chemokines are chemotactic cytokines and signal through G-protein coupled serpentine receptors.\textsuperscript{99} Chemokines, their receptors and cell adhesion molecules regulate migration of immune cells into inflamed tissue. Chemokine receptors are categorized broadly into the CXC and CC families and their expression may change due to specific inflammatory signals within the tissue microenvironment.\textsuperscript{100} Pulmonary lymphocytes express high levels of CXCR3 and CCR5.\textsuperscript{85,101} CXCR3 is activated by CXCL9, CXCL10 and CXCL11, all of which are induced by IFN-\gamma. CCR5 is activated by CCL3, CCL4 and CCL5, all of which are elevated in the lungs of COPD. CXCR3 is highly expressed by CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells in lymphoid follicles of subjects with COPD and CXCR3 expression in lymphoid follicles increases with worsening of COPD.\textsuperscript{102}

TISSUE DESTRUCTING SUBSTANCES

Neutrophils, eosinophils and macrophages contain various kinds of tissue destructing substances. For some of them, their main task is to destroy pathogens. Inflammatory cells also secrete proteases, protein degrading enzymes, which destroy the basement membrane and tissue matrix, a way to enable the recruitment of inflammatory cells to the site of inflammation. Release of these tissue destructing enzymes is therefore a consequence of the inflammatory process. Oxidant/antioxidants and protease/antiproteases are important balances in the tissue destructing system.

Oxidative stress

Oxidative stress is caused by reactive oxygen species (ROS) which is a collective term that includes a large variety of free oxygen radicals such as superoxide anion (O\textsubscript{2}•\textsuperscript{-}) and hydroxyl radical (OH\textsuperscript{-}), but also derivates of oxygen such as (H\textsubscript{2}O\textsubscript{2}).\textsuperscript{103} Free oxygen radicals are highly reactive and can trigger an inflammation process. Activation of the transcription factor NFkB can be a result of oxidative stress. NFkB recruits neutrophils that produce ROS and a never-ending circle is started. ROS products can also induce increased mucus production and increased bronchoconstriction.\textsuperscript{104} In healthy subjects, there is a balance between oxidants and antioxidants, keeping the extra cellular environment in a reduced state. Cigarette smoke, which contains massive amounts of oxidants, is an important factor for the development of COPD. Oxidative stress occurs when the burden of oxidants is not well counterbalanced by the antioxidant system. Smokers and COPD patients have higher levels of H\textsubscript{2}O\textsubscript{2} in exhaled breath condensate, a direct measurement of airspace oxidative burden, than ex-smokers with COPD or non-smokers. H\textsubscript{2}O\textsubscript{2} levels in exhaled breath condensate are even higher during exacerbations of COPD.\textsuperscript{105}

Proteases and Antiproteases

Protease-antiprotease imbalance is likely to have an important pathogenic role in the development of emphysema in COPD.\textsuperscript{106} Neutrophils, eosinophils and macrophages contain various kinds of tissue destructing proteases, such as neutrophil elastase, serin proteases and matrixmetallo proteases. They are able to enzymatically degrade a variety of lung matrix proteins in the basement membrane and tissue. In order to protect the tissue, there are
antiproteases, which are able to inactivate the proteases.\textsuperscript{40} The serinprotease granzyme B, predominantly expressed by CD8\textsuperscript{+} cells, is capable of cleaving extracellular matrix (ECM) proteins and the accumulation of granzyme B in the extracellular milieu during chronic inflammation in COPD could contribute to ECM degradation and remodelling, and the emphysematous phenotype in the lung. Increased granzyme B expression is associated with increased COPD severity.\textsuperscript{107} Lack of the antiprotease α-1 antitrypsin is one rare cause of COPD development.\textsuperscript{41,42}

**PATHOGENESIS IN COPD**

Lung inflammation is present in all smokers and comprises T lymphocytes, neutrophils and macrophages. The airway epithelium is one of the first targets of cigarette smoke. COPD is characterized by a chronic inflammatory process predominantly in the small airways and lung parenchyma, with increased numbers of macrophages, neutrophils and T lymphocytes.\textsuperscript{108} The mechanisms and interactions by which tobacco smoke induces the inflammation are elusive, but the outcome is known: mucus production, bronchiolitis and emphysema (Figure 11).

![Figure 11: Possible mechanisms, showing how tobacco smoke may induce inflammatory processes in bronchial mucosa, resulting in mucus production, bronchiolitis and emphysema.\textsuperscript{22}

T cells in COPD

T cells have been implicated in the pathogenesis of emphysema since 1995, when Finkelstein \textit{et al} described a prominent T cell infiltration in the lung tissue of smokers that was strongly
related to the extent of emphysema. They reported an increase in CD3+ T cells in the lung parenchyma, and the most prominent inflammatory cell of smokers. They showed an association between the number of T lymphocytes/mm$^3$ of lung tissue and the extent of emphysema.$^{109}$

**T cells in large airways and lung tissue**

The pulmonary epithelium plays a central role in countering many of the toxic effects of cigarette smoke, limiting lung damage and maintaining adequate lung function.$^{104}$ Both CD4+ and CD8+ cells have been implicated in the development of COPD with increased numbers in the lungs reported in COPD compared to both smokers with normal lung function and never smokers. However, increases in CD8+ T cells in COPD are most frequently reported (Table 2). The enhanced proportion of CD8+ T cells is not discriminated to a special compartment within the lung tissue. The increase in these cells is more general in different tissue compartments, such as large airways, peripheral airway, parenchyma, smooth muscle and bronchial arteries.$^{30,31,101,110-119,120}$ CD8+ T cells are often related to emphysema progression. In 1997, O’Shaughnessy showed a negative association between lung function, assessed as FEV$_1$, and lung CD8+ T cells.$^{118}$ When CD8+ T cells become activated, they secrete enzymes such as granzyme and perforin, which are able to destroy other cells. Fas/FasL binding is another way for CD8+ T cells to destroy surrounding cells such as antigen presenting cells and epithelial cells, which can cause lung damage and emphysema. In agreement with O’Shaughnessy, Lams et al showed a difference in CD8+ T cells between smokers with COPD and asymptomatic smokers. Smokers with COPD displayed an increased infiltration of CD8+ T cells in the large airway sub-epithelium. A negative association between lung function and infiltrating CD8+ cells in the lung tissue was also demonstrated.$^{120}$ Saetta et al studied small airways and showed that the only significant difference in inflammatory cell infiltration asymptomatic smokers and smokers with COPD was between an increase in CD8+ T cells in patients with COPD.$^{113}$ In addition, Saetta et al also reported association CD8+ T cells in the peripheral airways and lung function.$^{113}$ In another study by Saetta et al they investigated the lung parenchyma and pulmonary arteries and found an increase in CD8+ cells in COPD that correlated with lung function.$^{30}$ Several studies have shown higher numbers of cytotoxic CD8+ T cells in patients with COPD compared with smokers without COPD.$^{31,30,113,116,121}$ Increased numbers of CD8+ T cells were also found in small pulmonary arteries of smokers, when compared with non-smokers and this inflammation was greater in smokers with COPD.$^{30,113}$ These findings introduced possible new mechanisms for the pathogenesis of COPD. If T cells were involved, COPD might be an autoimmune disease triggered by cigarette smoking.$^{28,39}$ The underlying mechanism triggering the CD8+ T cells and their functional role in COPD are still elusive.

Lymphocyte numbers, in terms of CD3+ and CD8+ cells, decrease with disease severity and might be due to the emphysema progress.$^{101}$ An explanation to this finding might be that the destruction of lung tissue increases the airspaces; less tissue gives fewer inflammatory cells. Studying the activation of CD8+ T cells is of importance as it is a way to address the inflammatory response in COPD in more detail. Up- or down-regulation of specific membrane proteins can give a hint of further steps in the process of T cells response. There is a lack of studies addressing activation of CD8+ T cells in tissue. Ligands and receptors, such as CXCR3, might also play an important role in the inflammatory response.$^{122}$ Pulmonary lymphocytes express high levels of the interferon inducible receptors, CXCR3 and CCR5, and it has been shown that the expression of CXCR3 is enhanced on CD8+ T cells in the small
airway wall of COPD patients, compared with non-smoking controls, whereas no difference was found between COPD and smokers. In a recently published study, Smyth et al investigated CD8 chemokine receptors and found enhanced release of CCL5 in COPD compared with smokers. In contrast to Saetta et al, they did not find any difference between COPD patients and smokers in pulmonary CD8CXCR3.

### Table 2
Statistically significant differences in T-cell numbers in lung tissue from COPD, smokers and non-smokers

<table>
<thead>
<tr>
<th>Study</th>
<th>CD3</th>
<th>CD4</th>
<th>CD8</th>
<th>Groups studied</th>
<th>Association with FEV₁</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Large airways</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>O’Shaughnessy, 1997¹¹³</td>
<td>COPD &gt; NS</td>
<td>COPD &gt; NS</td>
<td>13 COPD, 11 CB, 5 NS</td>
<td>CD8</td>
<td></td>
</tr>
<tr>
<td>Lams, 2000¹¹⁵</td>
<td></td>
<td>COPD &gt; S</td>
<td>11 COPD, 8 S, 11 NS</td>
<td>CD3, CD8</td>
<td></td>
</tr>
<tr>
<td>Di Stefano, 1998¹¹⁴</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rutgers, 2000¹¹²</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Di Stefano, 2001⁹⁸</td>
<td>mCOPD &gt; sCOPD</td>
<td>mCOPD &gt; sCOPD</td>
<td>9 sCOPD, 9 mCOPD, 14S</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Di Stefano, 2002¹¹¹</td>
<td>COPD, S &gt; NS</td>
<td>eS-COPD &gt; S-COPD</td>
<td>42 eS-COPD, 72S-COPD</td>
<td></td>
<td></td>
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<tr>
<td>Lapperre, 2006¹⁰⁹</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Di Stefano, 2009¹¹⁰</td>
<td>mCOPD &gt; NS</td>
<td>mCOPD &gt; NS</td>
<td>14 sCOPD, 14 mCOPD, 11 S, 8 NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Peripheral airways</strong></td>
<td></td>
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<tr>
<td>Saetta, 1998¹⁰⁸</td>
<td></td>
<td>COPD &gt; S</td>
<td>9 COPD, 7 S</td>
<td>CD8</td>
<td></td>
</tr>
<tr>
<td>Turato, 2002³⁰</td>
<td></td>
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<tr>
<td><strong>Parenchyma</strong></td>
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<td></td>
</tr>
<tr>
<td>Saetta, 1999²⁹</td>
<td></td>
<td>COPD &gt; NS</td>
<td>10 COPD, 6 S, 8 NS</td>
<td>CD8</td>
<td></td>
</tr>
<tr>
<td><strong>Smooth muscle</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baraldo, 2004¹⁰⁷</td>
<td></td>
<td>COPD &gt; NS</td>
<td>8 COPD, 10 S, 8 NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Bronchial arteries</strong></td>
<td></td>
<td></td>
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<tr>
<td>Peinando, 1999¹⁰⁶</td>
<td>COPD &gt; NS</td>
<td>COPD &gt; NS</td>
<td>20 COPD, 12 S, 7 NS</td>
<td></td>
<td></td>
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<tr>
<td>Turato, 2002³⁰</td>
<td></td>
<td></td>
<td>9 COPD, 9 S</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saetta, 1999²⁹</td>
<td>COPD &gt; S, NS</td>
<td>COPD &gt; S, NS</td>
<td>10 COPD, 6 S, 8 NS</td>
<td>CD8</td>
<td></td>
</tr>
<tr>
<td><strong>Lymphoid follicles</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plumb, 2009¹⁰⁵</td>
<td>COPD &gt; S, NS</td>
<td></td>
<td></td>
<td>12 COPD, 11 S, 7 NS</td>
<td></td>
</tr>
</tbody>
</table>

Severe COPD (sCOPD), moderate COPD (mCOPD), smokers (S), ex-smoker with COPD (eS-COPD), non-smokers (NS).

Helper T cells, or CD4⁺ T cells, have been shown to be increased in airways and lung parenchyma in COPD patients, but the data are not as convincing as for CD8⁺ T cells. Helper T cells are central players in the immune defense. IL-2 receptor is of importance in the immune response to pathogens, particles or cigarette smoke. IL-2Ra, or CD25, is an auto receptor, meaning that Th cells produce IL-2 which can bind to the receptor on its surface. IL-2Ra expression on CD4⁺ cells implies a regulatory function of the T cells. Recently, these cells have been highlighted as important in autoimmune conditions but their role in COPD needs to be clarified. Transcription factor fork head box P3, FoxP3, is a unique marker for
these cells. Until today, only two studies have addressed this marker in airway tissue in COPD. Isajevs et al found up-regulation of FoxP3 positive cells in large airways in subjects with COPD and asymptomatic smokers compared with non-smokers. Further, a down-regulation of FoxP3 was reported in small airways of subjects with COPD compared with both smokers and non-smokers. In addition, they also found a positive association between FoxP3 and airflow limitation in small airways (p=0.35, p=0.02), whereas the association in large airways was negative (p=-0.32, p=0.01). Lymphoid follicles from subjects with moderate COPD have also been studied with this unique marker. An increase of FoxP3 was found in these subjects but the authors could not exclude the impact of lung cancer as the patients were undergoing investigations for suspected lung cancer. The exact role of regulatory T cells within COPD requires further attention.

Majo et al reported, for the first time, the presence of γδ T cell in the lung parenchyma of smokers, with and without emphysema. The γδ T cell is a lymphocyte previously described in the mucosal surfaces of the gut and in small numbers in the lung, but its function is not yet clear. γδ T cell numbers have also been increased in bronchial glands of smokers compared with non-smokers.

T cells in small airways and BAL

Bronchoalveolar lavage mainly reflects inflammation in the smaller airways and alveoli. Very few studies address inflammatory cell composition in BAL fluid in COPD, as shown in Table 3. Also in BAL, CD8+ T cells seem to be the prominent lymphocyte.

Lately, regulatory T cells have been of interest in the pathogenesis of COPD. The role of these cells in the disease is elusive. There is a lack of studies addressing these cells and conflicting findings are reported. Smyth et al postulate increased levels of BAL regulatory T cells in COPD and smokers, but in fact they have just verified regulatory T cells with FoxP3 in four additional cases. In contrast, Barcelo et al stained for FoxP3 and found that smokers had increased levels of regulatory T cells compared with both COPD patients and never smokers. They also found that regulatory T cells were more frequent in BAL than in peripheral blood in COPD subjects. Smyth et al showed the up-regulation of regulatory T cells in BAL fluid in healthy smokers and COPD patients. Also, a positive association was observed between FoxP3 expression and the number of pack-years, indicating increased regulatory T cells with the burden of smoking. The role of these cells is still elusive and further studies are needed. An additional marker for regulatory T cells has been suggested. CD127 is a cell surface molecule that is postulated as unique for regulatory T cells, especially the CD127dim population. There are no previously published data on this marker in BAL fluid in COPD. In addition, γδ T cells are not well defined in COPD. Only one previously published study addresses γδ T cells in BAL in COPD. Pons et al concluded that γδ T-lymphocytes were significantly increased in BAL in smokers with preserved lung function compared with non-smokers.
Table 3
Statistically significant differences in T-cells expression in BAL fluid from COPD, smokers and non-smokers

<table>
<thead>
<tr>
<th>Study</th>
<th>CD3</th>
<th>CD4</th>
<th>CD8</th>
<th>Groups studied</th>
<th>Association with FEV&lt;sub&gt;1&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pons, 2005</td>
<td></td>
<td></td>
<td></td>
<td>20 COPD, 20 S, 10 NS</td>
<td></td>
</tr>
<tr>
<td>Barcelo, 2006</td>
<td></td>
<td></td>
<td></td>
<td>14 COPD, 16 S, 7 NS</td>
<td></td>
</tr>
<tr>
<td>Smyth, L 2007</td>
<td>COPD &gt; S, NS</td>
<td>COPD &gt; NS</td>
<td></td>
<td>15 COPD, 11 S, 8 NS</td>
<td></td>
</tr>
<tr>
<td>Smyth, L 2008</td>
<td>COPD &gt; S, NS</td>
<td>COPD &gt; NS</td>
<td></td>
<td>26 COPD, 19 S, 8 NS</td>
<td></td>
</tr>
</tbody>
</table>

Smokers (S), non-smokers (NS).

**Neutrophils in COPD**

COPD has often been suggested to be a condition in which neutrophilic inflammation is of major importance. Neutrophils are the predominant cell in the airways. Increased numbers and neutrophil derived enzyme levels have been shown in several COPD studies. Proteinases such as neutrophil elastase (NE), serine proteinases, elastase and proteinase-3 can be released from neutrophils and have the potential to cause emphysema. An association has been shown between emphysema severity and elastase levels in peripheral blood neutrophils. In airway lavage, a similar pattern has been shown. Neutrophils also generate oxidants, which cause tissue damage and cytokine/chemokine release, which can further potentiate inflammation and trigger the immune response. In lung tissue of COPD patients, some studies have shown increased numbers of neutrophils while others have not. This might reflect the short tissue lifespan of infiltrating neutrophils. Neutrophils are located predominantly in the bronchial epithelium and bronchial glands. They are particularly found in the airway lumen, as recovered in sputum or BAL.

**Macrophages in COPD**

There is a 5 to 10-fold increase in the numbers of macrophages in the airways, lung parenchyma and bronchoalveolar lavage fluid (BALF) in patients with COPD (Figure 12). Macrophages play a key role in the defense of the respiratory tract and macrophage numbers in the airways correlate with the severity of COPD. This could be explained by enhanced recruitment of blood monocytes into the lung or impaired clearance of macrophages from the respiratory tract. Alveolar macrophages are activated by irritants, such as cigarette smoke, initiating an inflammatory response through the release of chemotactic factors, such as IL-8, IL-10 and GRO-α, and the recruitment of neutrophils, T lymphocytes and monocytes.

Macrophages have the potential to induce mucus hyper secretion and are suggested to play a role in emphysema progression, via matrix metallo proteases, MMP, production. In particular, MMP-12 has been a focus of special interest. A deficiency of MMP-12, explored in mice, appears to protect against cigarette smoke induced lung destruction. In humans, airway and alveolar macrophage MMP-12 expression in COPD subjects is enhanced compared with healthy controls. In subjects with COPD, several investigators have found increased macrophage numbers in the bronchial submucosa, bronchial glands and small airway epithelium. In lung tissue, an association has been found between alveolar macrophage numbers and the severity of lung destruction in the parenchyma.
INTRODUCTION

Figure 12: Macrophages in BAL fluid from a healthy volunteer (left) and from a smoker (right). The dark staining inside the macrophages in the right picture represents phagocytosed particles from cigarette smoke (Photo: Magnus Sköld).

Current smoking and smoking cessation in COPD

Over 2000 different xenobiotic compounds have been identified in cigarette smoke and it has been estimated that there are $10^{14}$ free radicals in each puff of cigarette smoke. This is a great burden for the respiratory epithelium, which is the first line of defense for inhaled particles and substances. The cellular profile and function of the airway epithelium adapt to protect the lung from the effects of smoking and the inflammatory response. In smokers who develop COPD, these changes contribute to irreversible pathological changes in lung structure and function. The epithelial changes that cigarette smoke induces are associated with the development of chronic bronchitis. Whether this is associated with COPD depends on the degree of epithelial inflammation. It is important to know whether mucosal inflammation is affected by smoking status. Gamble et al support the inclusion of both current and ex-smokers in studies analyzing immunomarkers in COPD.

Autoimmunity in COPD

Autoimmune diseases are chronic inflammatory conditions characterized by the loss of tolerance to self-antigens or development of immunity to foreign epitopes that cross-react to self-antigens. COPD shares many clinical and pathophysiological characteristics of several autoimmune diseases. The inflammation continues after smoking cessation. In addition, the presence of auto antibodies against pulmonary epithelial and endothelial cells has been described in the serum of COPD patients. Van der Straate et al demonstrated the presence of B cell follicles in the parenchyma of patients with emphysema. Indirect evidence of autoimmunity is provided by the presence of circulating antibodies against elastin in COPD and emphysema, along with the observation that CD4+ T cells cultured from lungs of patients with COPD respond to elastin by secreting IFN-$\gamma$ and IL-10. The extent of the response is proportional to the degree of emphysema, and the response can be blocked by MHC class II antibodies, indicating that antigen presentation is involved. A characteristic of autoimmune diseases is their propensity to appear in families and several studies have shown an increased prevalence of airflow obstruction among smokers who are first-degree relatives of patients with COPD.
Role of T cells in COPD?

In summary, T-lymphocytes are implicated in the pathogenesis of COPD, but their role and function remain to be evaluated. CD4+ cells are known as important key cells in immunoregulation, whereas CD8+ cells have cytotoxic properties with unknown function in COPD. The hypothesis is that T cells, T cell subsets and their activation are of importance in the pathogenesis of COPD.
AIMS

The overall aim of this thesis was to investigate airway T cells, T cell subsets and T cell activation in subjects with stable COPD, in comparison with smokers with normal lung function and never-smokers.

The specific aims were:

- To analyze airway inflammation in endobronchial mucosal biopsies, focusing on T lymphocyte subsets.

- To compare airway T cell subsets and their activation in BAL fluid and, furthermore, to investigate whether smoking cessation would affect T-cell subsets in COPD.

- To evaluate airway regulatory T cells using the specific markers FoxP3 and CD127, in BAL fluid, and to examine whether smoking cessation would affect these T cells in COPD.

- To investigate the activation of cytotoxic T cells in stable COPD, as well as the importance of the NKG2D receptor and MIC ligands in smokers and subjects with COPD.
SUBJECTS AND METHODS

SUBJECTS

This thesis is based on four case-control studies; one was performed at the Karolinska Institute, Stockholm (Study I) and the others at the University Hospital, Umeå (Studies II-IV). Subjects were recruited by advertisements, earlier research contacts or by clinical contacts.

The subjects with COPD had moderate to severe COPD according to GOLD criteria (FEV₁ 35-80% of predicted).¹ They all had a smoking history of at least ten pack-years. They were all non-atopic and clinically stable, i.e. without any respiratory tract infection within a six-week period prior to and during the study, along with no history of frequent exacerbations during at least 3 months prior to inclusion. Only medication with short-acting β₂-agonists and/or anti-cholinergic drugs was permitted. Neither long-acting bronchodilators nor inhaled corticosteroids were allowed.

Smokers with normal lung function were age-matched, had normal lung function (>80 FEV₁ % of predicted) and had a smoking history of more than ten pack-years. They were all non-atopic and clinically stable.

Healthy never-smoking volunteers were age-matched, had normal lung function (>80 FEV₁ % of predicted) and were all non-atopic.

Study I

The subjects in this study were recruited at the Karolinska Institute, Stockholm. Fifty-one subjects participated in this study: 22 subjects with moderate to severe COPD (FEV₁ 47-60% of predicted) according to GOLD-criteria, 14 age-matched smokers with normal lung function (S) and 15 non-smokers (NS). Ten of the twenty-two COPD patients and one of the smokers with normal lung function met the criteria of chronic bronchitis, defined as cough and production of mucus on most days for at least three months in the previous two years.²⁷ Patient characteristics for the COPD subjects with chronic bronchitis did not differ compared with those without.

Study II

The participants in this study were recruited at the University Hospital, Umeå. Forty-four subjects participated in this study: 19 subjects with moderate to severe COPD according to GOLD-criteria (FEV₁ 37-60% of predicted), 13 smokers with normal lung function (S) and 12 healthy volunteers with no smoking history (NS). Of the COPD patients, seven were current smokers and 12 were ex-smokers with smoking cessation more than five years prior to inclusion. No differences in smoking habits were found between smokers, COPD ex-smokers and COPD smokers. No subjects suffered from chronic bronchitis.
Study III

Thirty-two subjects were recruited to this study: 9 patients with moderate to severe COPD according to GOLD-criteria (FEV₁ 45-60% of predicted), 14 smokers with normal lung function (S) and 9 healthy volunteers with no smoking history (NS). Of the COPD patients, 5 were current smokers and 4 were ex-smokers, with smoking cessation more than 5 years prior to inclusion. The number of pack-years was higher within the COPD group compared with smokers with normal lung function (p=0.047). No subjects suffered from chronic bronchitis. The subjects in this study were recruited at the University Hospital, Umeå.

Study IV

In this study, the subjects were recruited at the University Hospital, Umeå. Seventy-two subjects participated in this study: 35 patients with moderate to severe COPD according to GOLD-criteria (FEV₁ 35-80% of predicted), 21 healthy volunteers with no smoking history (NS) and 16 smokers with normal lung function (S). Of the COPD patients, 16 were current smokers and 19 were ex-smokers with smoking cessation at least five years prior to inclusion. No subjects suffered from chronic bronchitis. In a sub study, flow cytometry analyses were based on 9 patients with moderate to severe COPD according to GOLD-criteria, 14 smokers with normal lung function (S) and 9 healthy volunteers with no smoking history (NS). Of the COPD patients, 5 were current smokers and 4 were ex-smokers.

METHODS

Pulmonary function test

Dynamic spirometry variables (VC, FVC and FEV₁) were measured pre and 15-20 minutes post bronchodilation with 1mg of terbutalin (Bricanyl® Turbuhaler®; AstraZeneca, Södertälje, Sweden), using a Vitalograph spirometer (Buckingham, UK). At least three satisfactorily performed and well-co-operated measurements of each variable were carried out, according to the recommendations of the American Thoracic Society.¹⁴⁹

Bronchoscopy

Bronchoscopy was performed on an outpatient basis, following an overnight fast. Endobronchial mucosal biopsy sampling and collection of airway lavage fluids were performed. Biopsy specimens were processed into glycol methacrylate resin and inflammatory cells were stained immunohistochemically.

Study I

Pre-medication with morphine-hyoscine was given intramuscularly and lidocaine was applied topically whereupon bronchoscopy (Olympus F Type P30, Tokyo, Japan) was performed with a flexible fibre optic bronchoscope. During bronchoscopy the degree of inflammation was visually assessed using the bronchitis index (BI).¹⁵⁰ This was defined as the sum of scores of
SUBJECTS AND METHODS

the visual appearance of airways according to the presence or absence of abnormal edema, erythema, secretions and friability (0 = normal, 3 = remarkably abnormal). Consequently, the visual appearance of airway inflammation was assessed in a semi quantitative manner on a scale ranging from 0 to 12 with the investigator blinded to the subject’s status (COPD, healthy smokers or normal control). Four to six endobronchial mucosal biopsy specimens were taken from each subject.

Studies II-IV

Pre-medication with atropine was given subcutaneously and topical anaesthesia of the airways was obtained using lidocaine. The subjects were examined in the supine position. Four to six endobronchial mucosal biopsies were taken from proximal cristae. Bronchial wash (BW) was performed by infusing two aliquots of 20 ml of sterile sodium chloride (NaCl), pH 7.3 at 37°C, which was gently sucked back after each infusion. Bronchoalveolar lavage (BAL) was performed in the same way but by infusing three aliquots of 60 ml of sterile sodium chloride and pooled into a tube placed in iced water (Figure 13). The biopsies and recovered fluids were immediately transported to the laboratory for analysis.

Figure 13: Bronchoalveolar lavage fluids (BALF) from a healthy non-smoking volunteer and a current smoker (Photo: Magnus Sköld).

Biopsy processing

Following treatment with protease inhibitors, the biopsies were rapidly cooled and fixed overnight at -20°C. Next day the biopsies were placed in acetone at room temperature for 15 minutes and methylbenzoyl for another 15 minutes. The samples were then immersed in glycolmethacrylate (GMA) monomer with 5% methylbenzoyl at 4°C for six hours, with the GMA solution being changed three times during this period. After this, the biopsies were
embedded into glycol methacrylate (GMA) resin and transferred into flat-bottomed plastic capsules. The biopsies were stored in these airtight containers at -20°C until the staining procedures were initiated.

**Immunohistochemistry**

Immunohistochemistry is a method used to quantify airway tissue inflammation. Cells, endothelial adhesion vascular molecules, cytokines and transcription factors can be immunohistochemically stained. The method is used with different embedding techniques, such as paraffin. Tissue can also be snap frozen and cryo-sectioned. In this thesis, the glycol methacrylate (GMA) resin embedding technique was employed, which makes it possible to cut very thin sections of the tissue (2 μm). Using the GMA technique, the structure of the tissue is very well preserved with an excellent morphology.

The staining and cutting procedures are previously well described by Blomberg *et al.*\(^{151}\) Sections, as thin as 2 μm, were cut and floated on ammonia water (1:500), picked onto 0.01% poly-L-lysine treated glass slides and allowed to dry at room temperature for at least 1 hour. The sections were treated to inhibit endogenous peroxidases by applying a solution of 0.3% hydrogen peroxide in 0.1% sodium azide in distilled water. Non-specific antibody binding was blocked by the use of Dulbecco’s minimal essential medium containing 10% foetal calf serum and 1% bovine serum albumin. Undiluted blocking medium was applied for 30 minutes and then poured off, whereupon mouse monoclonal antibodies were applied and incubated for 16-20h at room temperature. After rinsing with TRIS-buffered saline (TBS), biotinylated rabbit anti-mouse immunoglobulin was added to each section and then incubated for 2h. Subsequently a complex of streptavidin-biotin-horseradish and peroxidase (Dako) was added for another 2h. After rinsing in TBS, aminoethyl carbazole (AEC) in distilled water and hydrogen peroxide were used to induce a red colour, in this manner marking a positive immunoreaction. Diaminobenzidine (DAB) diluted in distilled water and hydrogen peroxide, induced a brown colour indicating a positive immunoreaction. All sections were counterstained with Mayer’s haematoxylin (Figure 14). Primary antibody was omitted on sections serving as negative controls. Two stained sections from each participant were estimated with respect to morphological quality of the bronchial epithelium, and the best of these was used for quantifying positively stained cells. In order to detect mucus secreting cells (goblet cells) in the epithelium, two sections from each subject were stained with Alcian blue.
Quantification of staining in mucosal biopsy specimens

Quantification of stained inflammatory cells was performed separately in the epithelium and in the submucosa using a light microscope with 40x magnification. Areas with mucosal glands, blood vessels, smooth muscle or folded/torn tissue were excluded. The epithelium was defined as the intact area above the basement membrane. The total length of the epithelium and the submucosal area were calculated by use of computer assisted image analysis (Qwin, Leica Q500IW; Leica, Cambridge, UK). Positively stained lymphocytes showed a ring staining pattern for CD3, CD4 and CD8 positive cells (Figure 15). The number of positive stained cells was expressed as cells per millimeter (cells/mm) of epithelium. The DAB stained specimens were analyzed as percent positive staining per square millimeter of epithelium.

*Figure 14: Illustration of the immunohistochemical staining procedure.*
Figure 15: Bronchial tissue including epithelium, basement membrane and submucosa. Positively stained lymphocytes showed a ring staining pattern for, in this case, CD8 positive cells.

Cell preparation
The chilled bronchial wash (BW) and bronchoalveolar lavage (BAL) fluids were filtered through a nylon filter and centrifuged (400 g, 15 minutes, at 4°C). After centrifugation, the cell pellet was separated from the supernatant and resuspended in PBS. The total number of cells was counted and adjusted to a final concentration of $10^6$ cells/ml.

Differential cell counts
Cytocentrifuged specimens were prepared by centrifugation at 450 rpm for 5 minutes. The slides were stained with May-Grünwald Giemsa for cell differential counts. 500 cells per slide were counted using a light microscope at 100 x magnifications and the proportion of non-epithelial cells including macrophages, neutrophils, eosinophils and lymphocytes was established. Mast cells were analyzed on slides stained with acid toludine blue and counterstained with Mayer’s acid haematoxylin, counting a minimum of 12 visual fields at 20x magnification. Based on the total cell concentration and the differential cell counts, the concentration of each cell type was determined as cell/ml.
Flow cytometry analysis

Flow cytometry analysis is a standard method to analyse specific proteins on or within a cell, for example in collected blood or BAL. The purpose is to distinguish between different cell types and to determine specific cell subsets (Figure 16).

Figure 16: Illustration of the principles for flow cytometry.

A laser beam hits each cell and, depending on the cell’s granularity and size, it is possible to differentiate the cells into various populations due to their specific characteristics. Forward Scatter, or FSC, correlates with the cell volume and Side Scatter, SSC, depends on the inner complexity of the cells (Figure 17a). A detector registers the reflection of the light and displays every cell as a dot in the plot. Monoclonal antibodies coupled with fluorochromes, such as fluorescein isothiocyanate (FITC), phycoerytrin (PE), allophycocyanin (APC), phycoerythrin-Cy5 (PE-Cy5) and peridinin chlorophyll protein (PerCP) makes it possible to study specific cell properties. The antibody conjugated to a fluorochrome binds specifically to the cell surface receptors. Depending on the fluorochrome, different detectors will register the emitted light and it is possible to differentiate cell subtypes through different receptor expression and determine the level of expression for the specific receptor (Figure 17b).
SUBJECTS AND METHODS

Figure 17: a, A representative scatter plot of BAL cells. The cells are scattered according to their specific characteristics such as size (FSC) and granularity (SSC).

b, The lymphocytes were further divided into subpopulations with monoclonal antibodies connected to a fluorescein (FITC or PE). The dot plot shows the different cell populations.

By using flow cytometry, lymphocyte subsets were determined in BAL. BAL cells were centrifuged and diluted in PBS to a final concentration of 10^6 cells/ml. For each test, 10 μl of antibody solution was added to 200 μl of cell suspension and allowed to bind for 30 minutes at 4°C in darkness. Red blood cells were lysed with FACS™ Lysing solution (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA), and the remaining cells were washed by adding PBS to the tubes and centrifuged. Cells were then fixed with 500 μl CellFix™ (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA).

After fixation, cells for intracellular staining were permeabilized by using eBioscience Foxp3 Staining Buffer Set and incubated 30 minutes at 4°C in darkness. The cells were washed with permeabilization buffer. 10 μl of antibody solution was added to the cells and allowed to bind for 30 minutes at 4°C in darkness. After washes with permeabilization buffer, the cells were suspended with 300 μl PBS and stored overnight.

In Study II, BAL cells were collected and analysed using a FACScan™ (Becton Dickinson) whereas in Studies III-IV, BAL cells were collected and analyzed using a FACSCalibur™ (Becton Dickinson) flow cytometer. When using FACScan™ up to 10,000 total events were collected in lymphocyte gate per sample, whilst 3,000 total events were collected in CD3+ gate per sample with the FACSCalibur™. The lymphocyte population was gated on their physical characteristics in a region according to their characteristic forward scatter (FCS) and side scatter (SSC) profiles. By using monoclonal antibodies marked with different fluorochromes, it was possible to distinguish between various subtypes of cells. The fluorochromes used in this thesis were FITC, PE, PerCP, APC and finally PE-Cy5. As controls, IgG matched antibody subtypes were conjugated with these fluorochromes. The percentage of different cell types was counted out of gated lymphocytes. The quantification of CD25 bright cells was performed as previously described (Figure 8).67,124 The more specific gate settings were performed with either CD3 gate or CD4 gate. The analyses of CD127 were performed as described in Figure 18.
SUBJECTS AND METHODS

Figure 18: Flow cytometry analysis of CD127. Based on the lymphocyte gate CD4+ cells were identified. Positive cells were calculated in the dot plot.

Soluble markers

The BAL and BW supernatant samples were analyzed for MIC A and MIC B. Commercial kits from Elisa kits, R&D Systems, Abingdon United Kingdom, were used.

Statistical analysis

This thesis is based on non-normally distributed data and therefore the statistical analyses were performed using non-parametric test. The statistical programme SPSS was used for statistical estimations. Flow cytometry data were analysed using CellQuest Software (Becton Dickinson, San Jose, CA, USA).

Statistical comparisons between the three groups were made using the Kruskal-Wallis test and a p-value of less than 0.05 was considered significant. If this test indicated significance, the Mann-Whitney U-test was used for post-hoc analysis for comparison between two groups, with corrections of p-values according to Bonferroni (a p-value less than 0.017 was considered significant). Correlations in Study I were calculated using Spearman’s rank correlation test. The level of significance considered was 0.01. If the result was statistically significant, the probability value (p-value) has been given. In order to distinguish between COPD and smoking-related effects, a subgroup analysis was carried out within the COPD group. The ex-smoking COPD group was compared with both the smoking COPD group and the never smoking group, using the Mann-Whitney U-test. Here, a p-value of less than 0.05 was considered significant. Correlation analyses in Study II were performed with the Pearson correlation test whilst in Study III the Spearman correlation test was used.
RESULTS

Study I

Bronchitis index

The COPD group as well as smokers with normal lung function displayed higher (p<0.001 for both) bronchitis index than the non-smoker group.

Biopsy findings

In the COPD group, intraepithelial CD3\(^+\) lymphocytes numbers, expressed as mm\(^{-1}\) epithelium, were higher compared with smokers with normal lung function and with non-smokers, but the difference was not of statistical significance (p=0.058; Kruskall-Wallis). The number of intraepithelial CD4\(^+\) lymphocytes \(\cdot\) mm\(^{-1}\) epithelium was significantly higher in the COPD group compared with smokers with normal lung function, along with a trend towards significance in non-smokers (p=0.005 and p=0.036 respectively). The number of intraepithelial CD8\(^+\) lymphocytes \(\cdot\) mm\(^{-1}\) epithelium was significantly higher (p=0.017) in the COPD group compared with the non-smoking controls, without a significant difference compared with smokers with normal lung function (Figure 19).

**Figure 19:** Representative immunohistochemical staining of tissue biopsies for CD4\(^+\)(a) and CD8\(^+\)(b) cells in the bronchial epithelium of patients with chronic obstructive pulmonary disease. As expected, the staining is in the form of a ring outlining the cells.
Study II

**Differential cell counts in BAL**

The numbers of total leukocytes in BAL were increased in smokers with normal lung function, compared with never-smokers (p=0.002). Among the leukocytes, macrophage (p<0.001), mast cell (p=0.001) and neutrophil numbers (p=0.012) were increased. Compared with smokers with normal lung function, subjects with COPD had a decreased number of leukocytes in BAL (p=0.001), which was due to a lower number of macrophages (p=0.001). Smoking COPD subjects had increased numbers of BAL macrophages (p=0.003) and total leukocytes (p=0.014) compared with ex-smoking COPD subjects.

**BAL lymphocyte subsets**

Here, significantly increased percentages of CD8⁺ and TCR-γ/δ⁺ among gated lymphocytes as well as increased expression of CD69, HLA-DR and CD25 on CD8⁺ cells were found in subjects with COPD and smokers with normal lung function, compared with never-smokers, along with decreased percentage of CD4⁺ cells. The expression of CD25 on CD4⁺ T cells was significantly enhanced in subjects with COPD and smokers with normal lung function compared with never-smokers, whilst the expression of CD69 and HLA-DR was not significantly altered on CD4⁺ T cells. Among COPD patients and smokers with normal lung function, the percentage of CD4⁺CD25bright cells was enhanced, compared with never-smokers. In contrast to all other markers, the percentage of CD4⁺CD25bright cells was significantly decreased in COPD patients compared with smokers with normal lung function (Figure 20).

In ex-smoking subjects with COPD, decreased expression of TCR-γ/δ⁺, CD8⁺CD25⁺ and CD4⁺CD25bright cells was found in BAL, compared with smoking COPD subjects. In contrast, the percentage of CD8⁺ cells was increased in COPD ex-smokers compared with never-smokers. Activation markers, such as CD69, HLA-DR and CD25 on CD8⁺ cells were also significantly enhanced in ex-smokers with COPD compared with never-smokers.
Study III

Differential cell counts in BAL

Smokers with normal lung function displayed higher numbers of total leukocytes in BAL compared with never-smokers (p<0.001), related to an increased number of macrophages (p<0.001). Even compared with COPD, smokers with normal lung function had higher numbers of macrophages (p<0.014). When the group of COPD patients was divided into current smokers and ex-smokers, increased numbers of BAL macrophages (p<0.05), were found in smoking COPD patients compared with ex-smoking COPD patients.

BAL lymphocyte subsets

The proportion of CD4^+CD25^+ and CD4^+CD25^{bright} cells was enhanced in smokers with normal lung function, compared with never-smokers (p=0.007 and p=0.001 respectively). CD127^+CD25^+ expression among gated CD4^+ cells was also significantly enhanced in COPD patients and smokers with normal lung function compared with never-smokers (p=0.003 and p=0.001 respectively). In contrast, the percentage of FoxP3 on CD25^+ cells among gated
CD4$^+$ was decreased in smokers with normal lung function compared with never-smokers (p=0.009) (Figure 21).

**Figure 21:** Flow cytometry analysis of BAL T-lymphocytes, given for non-smokers (NS), smokers with normal lung function (S) and COPD. Proportion of FoxP3$^+$ and CD127$^+$ among CD4$^+$ T cells expressing CD25. Within the COPD group, ● indicates ex-smokers and Δ smokers. Significance levels are noted as ** p<0.01 and *** p<0.001. COPD smokers have increased proportions CD127/CD25 among CD4$^+$ cells compared with COPD ex-smokers (p=0.027) and never-smokers (p=0.003). Data are given as median and IQR.

Ex-smoking COPD patients expressed a decreased percentage of CD127$^+$ BAL cells compared with smoking COPD (p=0.027). The expression of CD127$^{dim}$ on CD4$^+$CD25$^+$ T cells was increased in smokers, compared with non-smokers (p=0.002) (Figure 22).

**Figure 22:** Flow cytometry analysis of BAL T-lymphocytes, given for non-smokers (NS), smokers with normal lung function (S) and COPD. Proportion of CD127$^{dim}$ among CD4$^+$ T cells expressing CD25. Within the COPD group, ● indicates ex-smokers and Δ smokers. Significance levels are noted as ** p<0.01. Data are given as median and IQR.
Study IV

**Biopsy findings**

The number of epithelial CD3$^+$ lymphocytes was increased in both smokers with normal lung function and in the COPD group, compared with never-smokers (p=0.006 and p=0.005 respectively). Epithelial CD8$^+$ lymphocyte numbers were higher (p=0.001) in the COPD group compared with never-smoking controls.

**Differential cell counts in BAL**

Smokers with normal lung function had higher numbers of total leukocytes in BAL than healthy never-smokers had (p<0.001). Among leukocytes, the number of macrophages was increased (p<0.001). The numbers of neutrophils were increased in smokers with normal lung function compared with never-smokers (p=0.007), whereas mast cells were increased compared with patients with COPD (p=0.003) within the COPD-group. Smokers expressed increased numbers of BAL macrophages (p<0.001) compared with ex-smokers.

**BAL lymphocyte subpopulations**

Subjects with COPD and smokers with normal lung function expressed enhanced percentage of CD8$^-$NKG2D$^+$ cells among gated CD3$^+$ cells, compared with never-smokers (p=0.001 and p=0.002 respectively). The percentage of CD8$^-$CD69$^+$ cells, among gated CD3$^+$ cells, was enhanced in subjects with COPD and smokers with normal lung function, compared with never-smokers (p=0.012 and p=0.001 respectively). In addition, CD69$^+$CD8$^+$ median fluorescence was increased in COPD subjects and smokers with normal lung function, compared with never-smokers (p=0.007 and p=0.001 respectively) (Figure 23).
RESULTS

Figure 23: Flow cytometry analyses of BAL T cells in never-smokers (NS), smokers with normal lung function (S) and COPD. Data are given as percent and median fluorescence intensity, MFI, of gated CD3⁺ cells. Among the COPD group ● indicates ex-smokers, whilst ∆ indicates smokers. A p-value below 0.017 is considered significant. Significance levels are noted as * p<0.017, ** p<0.01, *** p<0.001. Data are given as median and IQR.

Soluble markers

Soluble MICB (sMICB) was undetectable in BW from smokers with normal lung function, whereas in never-smokers sMICB was detectable (83; 0.0-269 pg/ml, p=0.002) (Figure 24).

Figure 24: Analysis of sMICB in bronchial wash (BW) of never-smokers (NS), smokers with normal lung function (S) and COPD by ELISA. A p-value below 0.017 is considered significant. Significance levels are noted as ** p<0.01. Data are given as median and IQR.
DISCUSSION

DISCUSSION OF METHODS AND PROCEDURES

Subjects

Even if COPD is a common disease, the recruitment of subjects fitting the inclusion criteria of the studies has been difficult and time-consuming. The rather strict inclusion criteria were chosen in order to recruit a group of COPD patients with a stable disease, without history of chronic bronchitis (except Study I), frequent exacerbations and other treatment than short-acting bronchodilators on demand. The criteria for inclusion, in the present studies, were chosen to get as pure group of subjects with COPD as possible, with no interfering circumstance. In the beginning, only subjects with COPD and never-smokers were included. However, it became apparent that a “healthy” smoker group was needed to evaluate the smoke-induced effects. Additional subjects have also been included after Study II was published and new endpoints have been addressed. This is the reason why the number of subjects varies in all the different studies.

Bronchoscopies

Bronchoscopy makes it possible to investigate the inflammatory status of the airways. The method is invasive but makes it possible to sample the bronchial mucosa and lower airways without general anaesthesia of the subjects, using only local anaesthesia. This method is a unique way of reaching the human airways and a reference method to investigate airway pathology. Flexible video bronchoscopy enables sampling of both airway lavages and endobronchial mucosal biopsies. The different sample techniques represent different parts of inflammatory sites. BAL and BW provide information of the cellular and soluble constituents in the airway lumen, while bronchial biopsy enables detailed morphological investigations of events in the airway wall and allows identification of the relationship between inflammatory responses and airway structure.

Biopsies

To obtain an endobronchial biopsy specimen of good quality, a skilled broncoscopist as well as sharp-edged forceps are critical. Endobronchial mucosal biopsies are very small, approximately 1-3 mm, at the time of sampling and after dehydration they shrink further in size. It is impossible to predict the quality of the biopsy at the time of sampling. Therefore, four biopsies are collected and they are all primarily cut and stained with toluidin blue to study how the morphology structure is presented in each biopsy specimen. When cutting the biopsies, it is impossible to predict whether the biopsy quality will improve or deteriorate. It is therefore very important to carry out toluidin blue staining of sections throughout the cutting procedure of a biopsy to ensure the morphology is well preserved. The small biopsies collected with bronchoscopy may be a limiting factor for analyses of several antibodies due to the small amount of tissue. In several other studies investigating inflammation in COPD, lung resection specimens are used. During lung surgery, tissue samples from the more distal parts
are collected. By means of surgery, it is also possible to achieve large tissue samples to enable a detailed morphology of the small airways and alveoli. The small airways belong to the distal parts of the lung, where the airway inflammation in COPD is suggested to be most pronounced. In contrast, by employing bronchoscopy, only small endobronchial mucosal biopsies are collected from proximal airway crypts. Bronchoscopy is a very precise method as the location of the biopsy is exactly known. In bronchoscopy studies, it is possible to select individuals who are stable in the disease, who fulfil the study criterion and, above all, are not on medication.

**BW and BAL**

BW and BAL represent different compartments of the respiratory tract. Theoretically, the small volume of BW (20ml) is considered to represent predominantly the bronchi and bronchioli, whereas the large volume BAL (3x60 ml) predominantly reflects the alveolar spaces. This division is not clear-cut and BW partially samples cells from the alveolar part and BAL partially from the bronchial compartment. In COPD, BW and BAL recovery may be due to the presence of airway remodelling and emphysema. The COPD subjects often have increased production of mucus which can complicate the bronchoscopy procedure. In six COPD subjects, the bronchoscopy was interrupted compared with none in the other two groups. The BAL recovery in subjects with COPD was (38; 32-53%), (median; inter quartile range) in smokers with normal lung function (53; 48-65%) and in never-smokers (50; 38-61%). There was a significant difference between recovery in COPD and smokers with normal lung function (p=0.008).

**Reflections on sampling techniques**

The different sampling techniques give a possibility to study airway inflammatory processes in different compartments within the lung. Inflammation is a dynamic process with inflammatory cells circulating in submucosal blood vessels, through the mucosa and airway epithelium into the airspaces. Information of inflammatory status in these different compartments can give us a better understanding and knowledge about inflammation in COPD at different airways levels.

**Immunohistochemistry**

Several other studies have been performed on lung resection tissue from patients with a COPD diagnosis along with suspected lung cancer, which may bias the results. In Study I and Study IV, endobronchial mucosal biopsies were investigated using the GMA embedding technique. GMA preserves the tissue extremely well and keeps the tissue morphology in excellent condition. To analyse the specimens, the morphology needs to be well-preserved with no torn, folded or damaged tissue. The intact epithelium is defined with basement membrane, layers of epithelial cells and on top, the cilia. Representative submucosa is areas without any mucosal glands, blood vessels or smooth muscle. The criterion for positive staining of lymphocytes is a visible nucleus and at least two thirds ring staining around the cytoplasm. The MICA staining within the epithelium was analyzed as percent positive staining per square millimeter of epithelium.
DISCUSSION

Flow cytometry

In Studies II-IV, bronchoalveolar lavage fluids were analyzed with flow cytometry. In BAL, the prominent cells are macrophages, which in some cases may interfere the flow cytometry analysis by autofluorescence. Especially in current smokers, autofluorescence is reported as a consequence of increased phagocytosis of particles derived from the cigarette smoke. No specific problems with autofluorescence were found and we could not find any differences in autofluorescence between the groups. Study II and Studies III-IV were performed using different flow cytometry equipment, a FACSScan and a FACSCalibur respectively. Flow cytometry generates a lot of data which can be analysed in various ways. It is possible to analyse proportions of cells expressing a specific marker and the intensity of the expression on the cell surface on each cell, given as median fluorescence intensity (MFI). In these studies the lymphocytes were defined by size and granulation using the FSC/SSC plot. A general lymphocyte gate was set and used in all subjects. In some cases, the lymphocyte gate had to be adjusted to fit the lymphocyte cell population. Proportions of cells were analysed in the dot plot and the intensity of specific markers was defined from histograms. In Study II the analysis was performed using the lymphocyte gate, whereas in Studies III and IV the lymphocyte subsets were analysed using more specific gates, i.e., T lymphocytes were defined by using CD3+ and total T helper lymphocytes by CD4+. As the latter gates are based on specific immunostaining, other leukocytes of the same size, such as small monocytes, dendritic cells and mast cells were excluded from the subsequent analysis. During the running of Study III, other studies investigating FoxP3 and CD127 have been published. In these studies they have mixed the antibodies in the same test tube, which is preferable because of the possibility to count these markers on the same cell. In Study III the staining for FoxP3 and CD127 was performed in separate test tubes. As CD127 is an extra-cellular marker, the staining procedure is more rapidly performed.

DISCUSSION OF MAIN RESULTS

The overall aim of this thesis was to investigate airway T cells, T cell subsets and T cell activation in subjects with stable COPD, in comparison with smokers with normal lung function and never-smokers.

Subjects

It is important to note that the COPD subjects participating in these studies were stable, with no recent exacerbation or infection three months prior to bronchoscopy. In Studies II-IV, 35 patients with moderate to severe COPD (FEV1 56; 42-65 % of predicted) (median, inter quartile range), were recruited. They were all well characterized and without anti-inflammatory medication. Unfortunately, several other studies have not clearly defined the subject’s status in terms of medication, history of exacerbations, chronic bronchitis or smoking history. These different characteristics are of importance as they may affect the results and make interpretation of the study results more difficult. Even if COPD is increasing in prevalence, it has been hard to find subjects suitable for the present studies. This may explain why it is not unusual for studies to include very few COPD patients, as well as COPD patients with different clinical phenotypes.
Large airways and lung tissue

Endobronchial biopsies reflect inflammatory cells migrated from the blood stream into the tissue and further migrating through the submucosa and epithelium into the air spaces. In both Study I and IV, increased numbers of CD8\(^+\) cells were found in the bronchial epithelium in subjects with COPD. These findings are in line with previously published data, as seen in Table 2. Several studies also verify the finding of increased CD8\(^+\) cells in airway tissue in COPD.\(^{30,31,101,110-120}\) The repeated results in the present studies give more impact to the findings.

In the “early” studies, a negative association was found between lung function and lung CD8\(^+\) cells,\(^{30,113,118,120}\) indicating that CD8\(^+\) cells may play a role in tissue destruction and emphysema progression. In three out of the four studies, subjects without COPD diagnosis were also included in the correlation analyses. In the well-cited study by O’Shaughnessy\(^{118}\), subjects with chronic bronchitis were included, and Lams et al included smokers with normal lung function.\(^{120}\) Saetta et al included both smokers with normal lung function and chronic bronchitis.\(^{30,113}\) So far, only in one study an association between FEV\(_1\) and CD8\(^+\) cells in parenchyma and bronchial arteries has been found, in subjects with well defined COPD.\(^{30}\) Consequently, the negative association between FEV\(_1\) and CD8\(^+\) cells reported in these previous studies may reflect an association between a smoking-induced T cell response and lung function. It would be more appropriate to carry out correlation analysis in a more well-defined COPD group, in order to actually verify an association between CD8\(^+\) cells and the severity of COPD in terms of FEV\(_1\) decline.

The major question is, what role do CD8\(^+\) cells play in the airways of COPD? The primary function of CD8\(^+\) T cells is to recognize and kill cells expressing ligands stimulating cytotoxicity. Antigens can induce CD8\(^+\) cell activation and expansion, and primed and activated CD8\(^+\) cells migrate into inflamed tissues. The antigen stimulation driving lymphocyte activation in COPD may either be from pathogens such as viruses or bacteria, or altered presentation of self antigens caused by cigarette smoke.\(^{153,154}\) Cytotoxic T cells may be recruited or expanded as a result of cigarette smoke-induced cell stress and pro-inflammatory responses triggered in the lung epithelium. One possible mechanism for such activation is the stress-induced expression of the MHC class I-like receptors on epithelial cells. Alternatively, COPD patients often experience an increase in the frequency of airway infections caused by viruses\(^{154}\) and bacteria,\(^{155}\) which is suggested to be one explanation for the increase in CD8\(^+\) T lymphocytes. There are also alternative theories suggesting that CD8\(^+\) cells recognize self antigens, indicating an autoimmune component in COPD.\(^{28}\)

CD4\(^+\) cells, on the other hand, are not as common as CD8\(^+\) cells in lung tissue in COPD. In Study I, increased numbers of CD4\(^+\) cells were found in COPD, compared with smokers with normal lung function. Interestingly, Lapperre et al found increased CD4\(^+\) numbers in large airways of ex-smoking COPD patients compared with smoking COPD subjects.\(^{114}\) CD4\(^+\) cells play a key role in immunological defence as they are the coordinating cells of the immune response, recognizing foreign antigen and activating other parts of the cell-mediated immune response to eliminate the pathogen. They play a major part in activation of B cells\(^{60}\) and also have an important regulatory function to initiate an immune response or not. CD4\(^+\)CD25\(^{bright}\) cells are proposed to be regulatory cells, but in lung tissue there is a lack of studies investigating these cells. In Study IV, FoxP3, a unique marker for regulatory T cells, was employed but no significant differences were found between the groups (Figure 25).
So far, there are just two studies addressing regulatory T cells, in terms of positive FoxP3^+ expression, in tissue from subjects with COPD. In the study by Plumb et al, lymphoid follicles were stained with both CD4 and FoxP3 on serial sections. This study showed increased numbers of CD4^+FoxP3^+ cells in COPD, suggesting the presence of regulatory T cells. The increase in T regulatory cells might be an indicator of increased immunological activity within lymphoid follicles. In contrast, Isajevs et al found the up-regulation of FoxP3^+ T-regulatory cells in large airways but a down-regulation in small airways in current smoking COPD patients. In addition, a negative correlation was found between FoxP3 in small airways and FEV1%. They further reported that smokers with normal lung function displayed the highest FoxP3 numbers in both large and small airways. Both of these studies were performed on lung tissue from moderate COPD patients undergoing lung resection for suspected or confirmed lung cancer. Those circumstances may affect the results and also make interpretation difficult. COPD patients suffering from cancer may also be on medication which might influence the results.

In Study IV, no up-regulation of MICA was found in bronchial biopsies from smokers or COPD subjects, and MICB was undetectable in all groups. Since the MIC proteins can be shedded from the epithelial cells into the alveolar space, we also analysed soluble MIC (sMIC) released into the airways. We observed that sMICB was decreased or undetectable in BW of smokers with normal lung function and nearly all subjects with COPD, when compared with never-smokers. This observation might be explained by the adsorption of MICB by the large number of NKG2D-expressing cells in the airways of these individuals, or alternatively by the interference of cigarette smoking on the detection of sMICB in BW.

Small airways and BAL

As a consequence of airway immune response, lymphocytes migrate from blood vessels through the submucosa and epithelium, into the air spaces within the lung. This inflammatory cell response is reflected in bronchoalveolar lavage. In Studies II, III and IV, lymphocyte subsets were addressed in BAL. When it comes to differential cell counts, no difference in
lymphocyte populations was shown between the groups, indicating that the detected differences in lymphocyte subsets were found within the lymphocyte population.

γδ T cell numbers have been found to be increased in BAL from smokers with normal lung function, compared with non-smokers and COPD patients. Likewise, we also showed increased expression of these cells in smokers and subjects with COPD compared with never-smokers. When dividing the COPD group into current and ex-smokers, it was found that the increase of γδ T cells was mainly due to smoking habits. This finding indicates that the increase in this lymphocyte subset was related to cigarette smoke rather than to the chronic immune response in stable COPD. It is still unknown whether the expansion of γδ T cells in COPD reflects a common triggering mechanism of cytotoxic T cells, such as the MIC/NKG2D pathway, or is due to any specific responsiveness of this T cell subset.

Few studies have addressed lymphocytes in BAL and those that exist were performed recently. As with airway tissue, CD8+ cells were the most prominent cell in BAL fluid in COPD (Table 3). Regardless of current smoking habits, activated CD8+ T lymphocytes were found to be increased in BAL fluid from subjects with COPD, suggesting changes in CD8+ T cells, associated with a persistent immune response and thus of importance in COPD pathogenesis. In Study II, BAL CD4+ cells were decreased in COPD compared with never-smokers. Other studies investigating T cell subsets in BAL did not find any differences when it comes to airway CD4+ cells. In Studies II and IV it was shown that both CD4+ cells and CD8+ cells were highly activated, in terms of increased CD69, HLA-DR, CD25 and NKG2D expression in COPD compared with the other groups. Even five years after smoking cessation, signs of an ongoing airway inflammation were found in terms of CD8+ cell activation in COPD.

CD8+ cells’ main function is to eliminate infected cells by their secretion of cytotoxic enzymes and Fas/FasL binding. It has been suggested previously that the increase in activated CD8+ cells in COPD is due to increased viral infections or bacterial colonization. Cytotoxic T cells may be recruited or expanded as a result of cigarette-smoke induced cell stress and pro-inflammatory responses triggered in the lung epithelium. There are also alternative theories suggesting that CD8+ cells recognize self antigens, indicating an autoimmune component in COPD.

There are a variety of approaches to identify regulatory T cells. CD25 has been used to identify T regulatory cells, with CD4+CD25bright expression being closely associated with transcription factor FoxP3 expression. FoxP3 is involved in the development of T regulatory cells and is arguably viewed as the most specific marker for regulatory T cells.

When investigating regulatory T cells in BAL, in terms of CD4+CD25bright increased expression was found in both smokers and COPD in Study II. To further evaluate whether these cells had regulatory properties, FoxP3 and CD127 were used as biomarkers (Figure 26). In COPD, analyses of T cell subsets employing these markers indicated a smoking-induced expansion of non-regulatory T cells, which tended to decrease after smoking cessation.
DISCUSSION

**Figure 26:** Flow cytometry analysis on BAL T cells. Proportion of FoxP3 and CD127 positive cells among CD4$^+$ T cells expressing CD25. Among the COPD group ● indicates ex-smokers, whilst ∆ indicates smokers. A p-value below 0.017 is considered significant. Significance levels are noted as ** p<0.01, *** p<0.001. Data are given as median and IQR.

When analyzing CD127$^{\text{dim}}$ as a marker of regulatory T cells, a population of T cells with more regulatory properties was indicated in some smokers, but this was not as obvious in subjects with COPD (Figure 27). It could thus be speculated that T regulatory cells have a protecting function in some smokers, thereby reducing the risk of COPD development. However, in other smokers, smoking induced the expansion of a non-regulatory T cell population, which, on the other hand, may increase the probability of developing COPD.

**Figure 27:** Flow cytometry analysis of CD127 expression on BAL T cells from never-smokers (NS), smokers with normal lung function (S) and COPD. The combined CD127$^+$ and CD127$^{\text{dim}}$ populations are given as percent of gated CD25$^+$CD4$^+$ cells. Among the COPD group ● indicates ex-smokers, whilst ∆ indicates smokers. A p-value below 0.017 is considered significant. Significance levels are noted as ** p<0.01. Data are given as median and IQR.
Currently smoking subjects with COPD expressed high proportions of activated non-regulatory CD4\(^+\) T cells in terms of increased CD127\(^+\)CD25\(^+\) expression. The data on FoxP3 expression further indicated that the increase in CD25 expression was not only associated with the expansion of regulatory T cells. As CD127 expression is reported to be inversely associated with FoxP3, the data indicated the expansion of a non-regulatory CD25\(^+\) population in smokers and patients with stable COPD. The expansion of non-regulatory CD25\(^+\)CD4\(^+\) cells in BAL fluid seemed to be preferentially smoke-related. This figure is an attempt to explain the balance of regulatory and non-regulatory T cells (Figure 28).

**Figure 28: The hypothesis of the balance between regulatory and non-regulatory T cells in smokers with normal lung function and COPD.**

An increased expression of CD127\(^+\) on CD4\(^+\)CD25\(^+\) cells was also found in current smokers with COPD compared with ex-smokers. Despite more than five years since smoking cessation, the expression of CD127 among the CD25 T helper cells tended to be higher in COPD compared with never-smokers, indicating a prolonged immune activation. The FoxP3 expression in BAL was decreased in smokers with normal lung function compared with never-smokers, but there was no significant difference compared with COPD, indicating that a large proportion of CD4\(^+\)CD25\(^+\) cells in smokers do not express FoxP3 and thus are non-regulatory T cells.
In Studies II and IV, activation of T cells in COPD was addressed in terms of CD69, HLA-DR, CD25 and NKG2D expression. When it comes to HLA-DR, different results and levels of expression were found in the two studies. This may be due to the different gating techniques. In Study II, the lymphocyte gate was used to analyze the expression of the activation markers, whilst the analyses in Study IV were performed in the CD3 gate, which is a more specific T-cell gate. This means that the expression of the activation markers out of CD3⁺ cells was more specifically determined in the latter study, whereas all cells in the lymphocyte gate were included in the analysis in Study II. The different results indicate that there are other cells expressing HLA-DR in lymphocyte gate, such as small mast cells, macrophages or dendritic cells. When it comes to the cell surface receptor CD69, both the expression and the median fluorescence intensity (MFI) were increased. This was calculated using the CD3⁺ gate. Therefore, CD69 seems to be a more important activation marker in COPD. However, the precise role for CD69 in adaptive immunity has not been elucidated due to the absence of a known ligand.

CD8⁺ cells and CD4⁺ cells coexist in the different compartments in the airways. On the one hand, regulatory T cells are generally considered to repress immune responses. On the other hand, the presence of activated CD8⁺ T cells suggests that these T cells may directly be contributing to the pathogenesis of COPD. The specific role for CD8⁺ T cells in the lungs of COPD patients is unclear. The primary functions of cytotoxic CD8⁺ T cells are to recognize and destroy cells expressing a stimulating ligand and to produce cytokines such as interferon gamma that can have a direct effect on surrounding cells and other leukocytes.

In differential cell counts there was, not surprisingly, an increase in macrophages in all smokers. Macrophages are important phagocytic cells that clear particles from the tobacco smoke. Neutrophils are proposed to be key cells in COPD, but in these studies there were no differences in COPD compared with the other groups. The lack of a significant increase in airway neutrophil numbers in COPD in the present studies may be due to the fact that the included subjects were stable and without any history of frequent exacerbations. It has also been shown that neutrophils make a rapid transit through the airways and the lung parenchyma.

Smoking habits

Smokers with normal lung function displayed a macrophage type of inflammatory response in the airways. As concluded in Studies II and III, it is very important to interpret the results in relation to smoking habits. Smoking per se is one cause of airway inflammation. When investigating the cellular composition in the development of COPD, it is important to consider this issue. Smoking habits are not always well defined in published studies, which make a full comparison between studies difficult.
Why do only some smokers develop COPD?

All smokers do not display clinically significant airflow obstruction and do not develop COPD. The susceptibility to develop smoke-induced airway inflammation is individual and heredity as well as gender differences are important factors contributing to the development of COPD. It has been shown that the acquisition of ‘addiction’ to smoking is partly genetically mediated. Interestingly, in an epidemiological study, a familial occurrence of emphysema and chronic bronchitis was reported as a more important factor for the development of emphysema and chronic bronchitis than smoking. This finding shows the importance of involving family history in the screening for COPD. Age is also another important factor as the risk of developing COPD increases with age. It is possible that more patients would develop COPD if they did not succumb to other smoking-related conditions such as ischemic heart disease and lung cancer.

Autoimmune component

Several researchers suggest that COPD is an autoimmune disease, in which exposure to tobacco smoke is the triggering agent. Indirect evidence of autoimmunity is provided by Lee et al, showing that emphysema is an autoimmune disease characterized by the presence of antielastin antibody and T-helper type 1 (TH1) responses, which correlate with emphysema severity. A possible autoimmune component to COPD is supported by the marked oligoclonality of CD4+ T cells isolated from resected emphysematous human lung tissue. If T cells, alone or together with other inflammatory cells, are responsible for the lung injury and progression of COPD, it would be as a response to an antigenic stimulus originating in the lung and induced by cigarette smoking. If that were the case, COPD could be considered an autoimmune disease triggered by smoking. Regulatory T cells plays a critical role in the maintenance of peripheral tolerance and the prevention of autoimmunity. More research is needed to clarify the role of autoimmunity in the pathogenesis of COPD.

In summary, the data indicate that, among airway T cells, CD8+ cells seem to be more associated with COPD, whereas CD4+ cells are rather related to current smoking. However, as CD8+ cells co-exist with immunoregulatory CD4+ T cells, pro-inflammatory responses may be balanced by immunosuppressive mechanisms in stable COPD.
SUMMARY OF MAIN RESULTS

➢ The airway epithelium displayed increased numbers of lymphocytes in COPD. The numbers of CD8+ cells were increased compared with non-smokers, whereas CD4+ cells were increased compared with smokers with normal lung function.

➢ In BAL, the expression of CD8+ cells was increased in COPD compared with never smokers. The CD8+ cells were highly activated in terms of increased expression of CD25, CD69 and HLA-DR and these signs of T cells activation were sustained at least five years after smoking cessation, supporting the contention that CD8+ cells may be important in the pathogenesis of COPD. In addition, the expression of CD4+CD25bright cells was increased in smokers and subjects with COPD compared with never-smokers, suggesting the presence of regulatory T cells.

➢ In BAL, the expression of CD127+ out of CD4+CD25+ cells was increased in COPD compared with never-smokers, and currently smoking COPD subjects displayed higher expression of these cells than ex-smokers. There were no differences in FoxP3 expression in COPD compared with either control group. When employing the more specific markers of regulatory T cells, we demonstrated the presence of activated non-regulatory T cells in COPD.

➢ No difference in epithelial MICA or MICB expression was found between the groups. Increased numbers of cytotoxic T cells in COPD were shown in both bronchial epithelium and airway lumen. CD69- and NKG2D-expressing T cells in BAL fluid were enhanced in both subjects with COPD and smokers with normal lung function, indicating that cigarette smoke exposure triggers the expansion of activated cytotoxic T cells, possibly by responding to injured epithelial cells. The cytotoxic T cells remained activated more than five years after smoke cessation of COPD patients, implying they play a role in the chronicity of COPD.
Several studies have addressed T cells in COPD and implied T cells as important players in the development of COPD. However, the role, function and clinical relevance of T cells in COPD still need to be elucidated.

These studies confirm previous findings that CD8⁺ lymphocytes are increased in both tissue and BAL in COPD. CD8⁺ cells have cytotoxic properties. The question is whether their role in COPD is “friend” or “foe”? Friend in the meaning that they destroy infected cells and defend the body against infectious agents, foe if the CD8⁺ cells kill structural cells and induce emphysema, possibly in an autoimmune manner. Thus the effector function of cytotoxic T cells still needs to be elucidated, i.e. by investigating perforins and granzymes in airway tissue and BAL in COPD.

CD4⁺ lymphocytes also need to be further investigated in COPD and, particularly, the CD4⁺CD25bright cell population, reported to have regulatory properties. Other specific markers verifying regulatory cells, such as CTLA-4 and GITR, should be addressed in COPD, as regulatory T cells may play an important role in the immune response, either in tolerance or activation. This thesis gives a new insight into the regulatory/activated status of T cells, and the results indicate an activated status of these cells in COPD. Regulatory T cells may also have a protective function against COPD development in smokers, which could, at least in part, explain why not all smokers develop COPD. As activated T cells were found to be higher in smoking compared with ex-smoking COPD patients, the present data further imply the importance of smoking cessation. However, further studies are needed to elucidate the role of T cell activation/regulation in smokers and patients with COPD.

Investigating inflammation in different compartments of the lung will give increased knowledge and understanding of the inflammatory and immune pattern of COPD. Both CD4⁺ and CD8⁺ cells need to be further elucidated in terms of specific subsets to evaluate stages of development, differentiation, activation as well as effector function in proximal airway mucosa, airway tissue and alveoli. This issue is of importance as COPD affects both proximal airways (chronic bronchitis) as well as more distal parts of the lungs including small airways and alveoli (emphysema). In order to enable comparisons between different COPD studies, it is important to identify and characterize the subjects participating in the studies in detail, in terms of clinical phenotypes such as chronic bronchitis, prevalence of exacerbations including infections, as well as medical treatment. In the present thesis, it was also shown that information on smoking status in COPD is crucial, as smoking per se up-regulates certain airway inflammatory and immune responses.

The clinical implications of the finding of T cell subsets in both smokers and patients with COPD will need further evaluation. The present findings are based on cross-sectional, case-control studies in patients with COPD compared with healthy persons, with the focus on immune and inflammatory endpoints. Given the design of the studies, the data are mainly descriptive and will need to be related to disease development over time. Furthermore, as no association was found between lung function and T cell subsets in COPD in the present thesis, the relevance of the data from a clinical point of view needs to be addressed. This could be achieved in longitudinal studies, in which airway T cell subsets are investigated in smokers and COPD patients and related to clinical parameters followed across time.
CONCLUSIONS

It is concluded that:

- In COPD, increased numbers of cytotoxic CD8$^+$ T cells were detected in both bronchial epithelium and airway lumen. CD8$^+$ cells were activated regardless of current smoking habits, suggesting that CD8$^+$ T cells are associated with a persistent immune response in COPD and therefore of importance in COPD pathogenesis.

- Cytotoxic T cells remained activated at least five years after smoke cessation in COPD patients, implicating a role of these cells in the chronicity of COPD.

- In patients with COPD, smoking increased the proportions of activated CD4$^+$ T cells in the airways, which seem to be reduced after smoking cessation. However, a fraction of smokers without clinical signs of COPD have an increased population of helper T cells with low or absent CD127 expression, suggesting the presence of a regulatory T cell population that potentially can modulate the smoke-induced immune responses.

- CD69- and NKG2D-expressing T cells in BAL fluid were enhanced in both subjects with COPD and smokers with normal lung function, indicating that smoking triggers the expansion of activated cytotoxic T cells, possibly by responding to injured epithelial cells.
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