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SMOKING AND HUMAN AIRWAY INFLAMMATORY CELLS

**STUDIES WITH FOCUS ON T CELLS IN THE
DEVELOPMENT OF COPD**

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SMOKING AND HUMAN AIRWAY INFLAMMATORY CELLS

STUDIES WITH FOCUS ON T CELLS IN THE DEVELOPMENT OF COPD

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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ABSTRACT

Chronic obstructive pulmonary disease (COPD), the fourth leading cause of death worldwide, is characterized by persistent airflow limitation and a chronic inflammation of the lungs. One of the major risk factors is long-term exposure to cigarette smoking. The key inflammatory cells in the pathogenesis of COPD are macrophages, neutrophils and CD8⁺ T lymphocytes.

The heterogeneity of COPD and the need for identifying patient subgroups is becoming increasingly recognized. Besides, the differences in the immunopathology of current- and ex-smokers with COPD are uncertain. Characterizing the inflammatory mechanisms driving the disease in different subtypes of patients, including differences between men and women, will likely aid diagnostic procedures and tailoring of treatment strategies and caretaking.

To investigate the airway and systemic inflammatory profile and the role for T cells in smoke-induced inflammation and COPD, bronchoalveolar lavage (BAL) fluid, blood and chest high resolution computed tomography (HRCT) scans were collected from a gender- and age-matched cohort of 40 never-smokers, 40 smokers with normal lung function and 38 COPD patients (27 current smokers and 11 ex-smokers). BAL characteristics, including the distribution of inflammatory cells, were assessed. T cells subsets in BAL and blood were phenotyped using flow cytometry, and the levels of cytokines, chemokines and growth factors in BAL fluid were assessed with a multi-plex bead-based assay. HRCT images of the lungs were analyzed for the investigation of morphological patterns and correlated with the cellular inflammatory patterns of the lungs. In addition to univariate analysis, multivariate data analysis with OPLS-modeling was used for discovering within- and between group variations, and potential biomarkers.

The frequencies of several T cell subsets, including CD8⁺ T cells and NKT-like cells, both with cytotoxic capacities, and CD103⁺CD8⁺ T cells, were increased in BAL from smokers, regardless of airway obstruction. In COPD ex-smokers, the levels were similar to those of never-smokers. A more detailed phenotypic characterization of T cells revealed subsets specifically altered in smokers with normal lung function or COPD patients, including FOXP3⁺ regulatory T cells. A type 1 T cell-driven inflammation was indicated for female, but not male, COPD patients. Lung density, as measured by HRCT, was higher in smokers compared to both never-smokers and COPD patients and correlated with the cellular inflammation in the lungs.

Taken together, these findings suggest that current smoking status has a larger impact on the distribution of lymphocytes in BAL than does airway obstruction. A detailed characterization of T cell subsets is important for finding disease-specific alterations. Gender-specific differences among smokers and COPD patients can be detected on a cellular level.

LIST OF SCIENTIFIC PAPERS

- I. **Forslund H**, Mikko M, Karimi R, Grunewald J, Wheelock ÅM, Wahlström J, Sköld CM. Distribution of T-cell subsets in BAL fluid of patients with mild to moderate COPD depends on current smoking status and not airway obstruction. *CHEST* 2014;145(4): 711-22.

- II. Mikko M*, **Forslund H***, Cui L, Grunewald J, Wheelock ÅM Wahlström J, Sköld CM. Increased intraepithelial (CD103+) CD8+ T cells in the airways of smokers with and without chronic obstructive pulmonary disease. *Immunobiology* 2013;218(2):225-31.

*These authors contributed equally.

- III. Karimi R, Tornling G, **Forslund H**, Mikko M, Wheelock Å, Nyrén S, Sköld CM. Lung density on high resolution computer tomography (HRCT) reflects degree of inflammation in smokers. *Respiratory Research* 2014;15:23.

- IV. **Forslund H**, Yang M, Mikko M, Karimi R, Nyrén S, Engvall B, Grunewald J, Merikallio H, Kaarteenaho R, Wahlström J, Wheelock ÅM, Sköld CM. Gender differences in T cells inflammatory profiles of the peripheral airways in COPD correlate with clinical phenotypes. *Manuscript*.

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LIST OF ABBREVIATIONS

APC	Antigen-presenting cell
BAL	Bronchoalveolar lavage
CD	Cluster of differentiation
FACS	Fluorescence activated cell sorter
FEV ₁	Forced expiratory volume in one second
FOXP3	Forkhead box P3
FVC	Forced vital capacity
GOLD	Global initiative for chronic obstructive lung disease
HDS	Higher density spectrum
HLA	Human leukocyte antigen
HRCT	High resolution computed tomography
HU	Hounsfield units
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
LLOQ	Lower limit of quantification
MHC	Major histocompatibility complex
NK cell	Natural killer cell
OPLS	Orthogonal projections to latent structures
PCA	Principal component analysis
Q ²	Seven fold cross-validation predictive value
R ²	Cumulative correlation coefficient
SUS	Shared and unique structures
Tc	Cytotoxic T cell
TGF	Transforming growth factor
Th	T helper cell
TNF	Tumor necrosis factor
Treg	T regulatory cell

1 THE IMMUNE SYSTEM

The immune system defends the body against invading pathogens such as bacteria and viruses, as well as tumor cells, toxic substances and pollutants.

An inflammatory response against infection or irritation is detrimental to the invader but can also cause more harm than good to the host if the response causes excessive damage or become persistent. It is vital that the complex series of pathways of the immune system response are tightly controlled. Disruption of the inflammatory response can cause inhibition of immune cell activation with an increased susceptibility to infection. However, immune system dysfunction can also lead to excessive activation as seen in various inflammatory diseases.

The immune system is typically divided into two distinct but interdependent categories: the innate and the adaptive immune system (Figure 1). The innate immunity, also known as the non-specific immunity, is the first line of defense and is composed of cells and mechanisms that can respond rapidly to a potential threat. The adaptive immunity is a more complex process where antigens (in some cases presented by cells of the innate immunity) are specifically recognized by cells of the adaptive immunity. This mounts a response to antigen including long-lasting memory protecting the host to subsequent encounter [1].

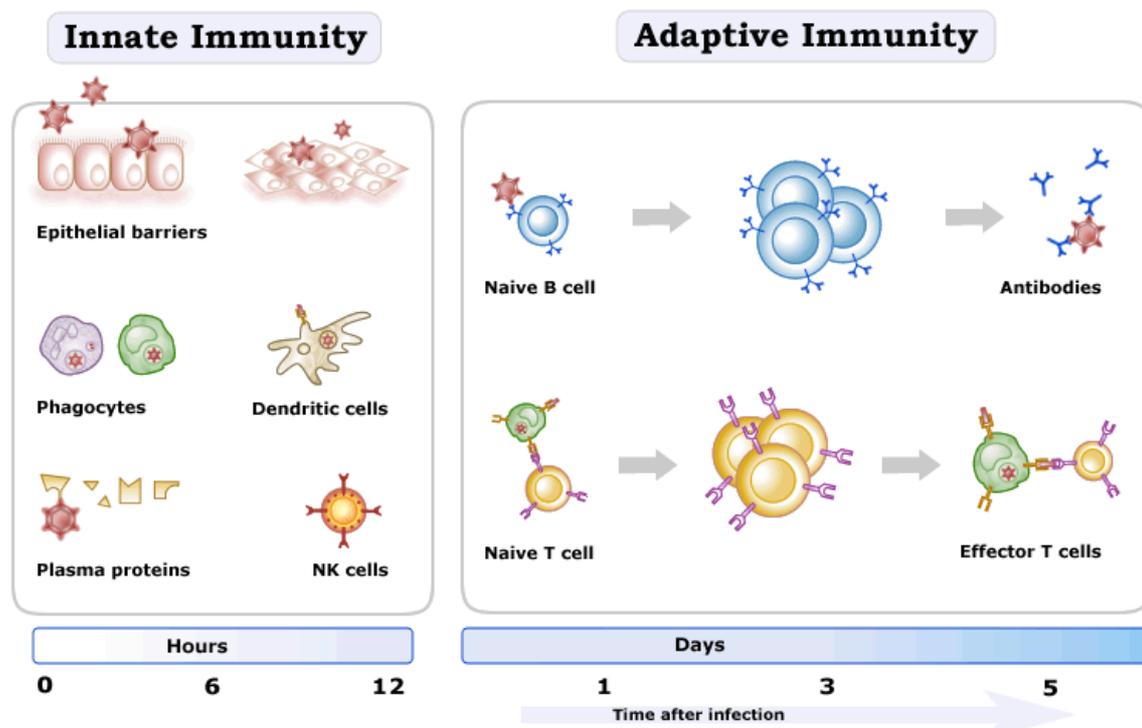


Figure 1. Cells of the innate and adaptive immune system.

1.1 THE INNATE IMMUNE SYSTEM

The cells and mediators of the innate immune system are the first line of defense against invasion and can act immediately or within hours after recognizing a potential threat. The major functions of the innate immune system include non-specific recognition of a pathogen, induction of the adaptive immune system and regulation of the adaptive immune system.

Epithelial barriers, humoral factors such as antimicrobial peptides and complement proteins that can recognize pathogens, and cells of the innate defense, accomplish this. Innate immune cells are present in all tissues and include: macrophages, dendritic cells, monocytes, neutrophils, natural killer (NK) cells and mast cells [1, 2].

Neutrophils and monocytes (after differentiation into macrophages at the site of inflammation) are specialized in capturing and ingesting pathogens and cells in search for potential harms, a mechanism called phagocytosis. As pathogens, or parts thereof, are recognized by the receptors on phagocytes, the cells become activated to produce a range of pro-inflammatory cytokines and chemokines that activate and direct other parts of the immune system. Through their role as antigen presenting cells (APCs), macrophages introduce antigens to the T cells of the adaptive immune system. Furthermore, they ingest apoptotic cells before they lyse thereby preventing the release of potentially harmful substances into surrounding tissue. Dendritic cells are also phagocytes and are present in tissues in direct contact with the external environment such as skin and the airways. They are important APCs linking the innate and adaptive immune systems, since they are responsible for the initial activation of T cells. Phagocytes express pattern recognition receptors (PRRs) that recognize conserved structures shared by many pathogens named pathogen-associated molecular patterns (PAMPs). PRRs can also respond to endogenous molecules that are released after cell damage known as damage-associated molecular patterns (DAMPs). Most infections are cleared by the innate immune system, but persistent infections require a more complex defense. Together with the dendritic cells, NK cells can initiate and direct the adaptive immune system, forming a link between innate and adaptive immunity. The NK cells share characteristics of both innate and adaptive immune cells. They are classified as lymphocytes based on their origin as lymphoid progenitors, expression of lymphoid markers and morphology, but are considered components of the innate immune system due to the absence of antigen-specific cell receptors on their surface [1, 3].

1.2 THE ADAPTIVE IMMUNE SYSTEM

In contrast to the innate immune response, the T- and B lymphocytes of the adaptive immune system have a much higher specificity for antigens and can provide an efficient defense able to remember and store information for a quick and increased protection at a subsequent encounter with the same antigen.

Lymphocytes express antigen-specific receptors that recognize specific protein structures and amino acid sequences from the processed antigen. Antigen-specific naive lymphocytes continuously pass through the lymphoid tissues and organs. If they meet and interact with a target cell displaying that specific antigen, they become primed and respond by

differentiation and cell division, generating an army of immune effector cells of the same specificity, designed to eliminate the antigen. On the first encounter with an antigen, it can take up to a week before the response becomes protective. After pathogen clearance, most antigen-specific B- and T cells undergo apoptosis. However, some cells remain and differentiate into memory cells that can mount a more rapid and effective immune response when re-encountering the same antigen [4].

B cells originate and mature in the bone marrow and mediate humoral immune responses. They express antigen-specific B cell surface receptors and after encountering their antigen, the cells start to proliferate and differentiate into plasma cells that secrete antibodies. Antibodies can neutralize toxins and opsonize pathogens in the extra-cellular compartments.

T cells on the other hand provide cell-mediated immune responses. They also originate in the bone marrow but then migrate as immature thymocytes to the thymus, where they mature.

CD4⁺ T helper (Th) cells are activated by extracellular antigens presented by major histocompatibility complex (MHC) class II molecules on APCs (B cells and DCs) to help orchestrate specific immune responses through the secretion of cytokines [5]. For instance, they help boost the effector functions of macrophages so they more efficiently can kill ingested pathogens.

CD8⁺ T (Tc) cytotoxic cells recognize intracellular antigens such as viral antigens presented by MHC class I on APCs [5]. The CD8⁺ T cells, as well as NK cells, kill infected cells directly, either through membranolysis or by the Fas ligand/Fas system. The pathway of membranolysis is mediated by the release of perforin that forms pores in the cell surface of the target cell, and the subsequent entry of granzymes that activate cell-death pathways resulting in rapid death of the target cell. The alternative cell death pathway involves the engagement of FAS (death) receptors on the target cell and the FAS ligand on the killer cell, resulting in caspase-dependent apoptosis [6].

1.2.1 Regulatory T cells

T regulatory cells (Tregs) prevent autoimmunity and also control inflammation during protective immune defenses to prevent tissue damage. They exert their immunoregulatory actions through different mechanisms; by producing the immunosuppressive cytokines transforming growth factor (TGF)- β and interleukin (IL)-10, modulating APC maturation and function, depleting effector T cells and by disrupting metabolic pathways [7, 8].

The two most studied subsets of regulatory T cells are the natural Tregs and the induced Tregs. Both subsets derive from the thymus and circulate in the periphery as differentiated CD4⁺ T cells expressing the transcription factor Forkhead box P3 (FOXP3). Natural Tregs differentiate in the thymus and migrate to the periphery as CD4⁺CD25⁺FOXP3⁺ T cells where they act to maintain self-tolerance. Induced Tregs differentiate in the secondary lymphoid organs and tissues following antigen-presentation by DCs, in the presence of cytokines such as TGF- β and IL-10 [9, 10]. Later, other subsets of regulatory T cells have been identified, including CD8⁺ regulatory T cells [11].

The study of Tregs is hampered by the lack of specific and reliable phenotypic markers. Accumulating evidence suggests that FOXP3 and CD25, as well as other currently used Treg markers (CTLA-4 and absence of CD127 for example) are not strictly specific for Tregs but are also T cell activation markers [8].

1.2.2 Chemotactic T cell recruitment and the Th1/Th2 balance

Chemokines are small cytokines that induce recruitment of leukocytes through specific binding to chemokine receptors. Chemokines and their receptors are key players in the immune defense through their chemotactic and immunoregulatory actions. Recruitment of leukocytes to sites of infection and/or inflammation is essential for the host defense but might also contribute to disease by maintaining and amplifying chronic inflammation.

The nomenclature of chemokines and chemokine receptors is based on the number and spacing of the conserved cysteine residues at the amino terminus of the protein. Among four different families of chemokines, members of the family of CXC chemokines have an intervening non-conserved amino acid between the first two cysteines, whereas members of the CC chemokine family have two adjacent cysteines [12].

T cells can be divided into functionally distinct subsets based on the cytokines they produce, the most traditional being Th1/Tc1 and Th2/Tc2, but later additional subsets were discovered including Th17, Th22 and Th9 [13]. Moreover, the T cell subsets express a distinct set of chemokine receptors specific for only a few chemokines [14, 15].

Th1/Tc1 cells produce IFN- γ , TNF and IL-2. They regulate cellular immunity and antigen-specific presentation, as well as stimulate macrophages, recruit inflammatory cells to the site of inflammation and act on B cells to stimulate antibody class switching. Th1/Tc1 cells are recruited to the site of inflammation via their chemokine receptors CXCR3 and CCR5, which bind to the chemokines CXCL9-11 and CCL3-4 respectively.

Th2/Tc2 cells produce IL-4, IL-5 and IL-13 and regulate humoral immunity and allergy. Th2/Tc2 cells are recruited through the binding of CXCL12 to the chemokine receptor CXCR4 [14-16].

2 THE RESPIRATORY SYSTEM

The respiratory system includes air passages, the lungs and pulmonary vessels. The main function is to supply the body with oxygen from the inspired air and remove carbon dioxide from the circulation. When breathing, air first enters through the nose or mouth, passes through the nasal cavities and continues down the trachea. As the trachea divides into two primary bronchi, each entering one of the two lungs, the air reaches the airway branching of increasingly smaller bronchi. The smallest airways, bronchioles, end up in the tiny air sacs called alveoli. In the alveolar walls, a network of capillaries allow for efficient exchange of oxygen and carbon dioxide between the air and the blood stream (Figure 2) [17].

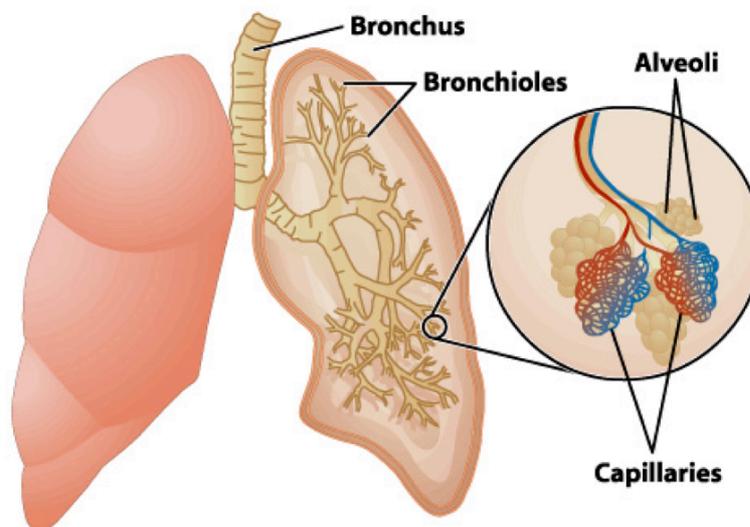


Figure 2. The bronchial tree of the lung and the alveoli covered by a network of capillaries.

The airway epithelium represents the first line of defense of the lung against airborne harmful agents, which include pathogens, toxins and pollutants. In addition to the epithelium providing a physical barrier, ciliated cells and mucus producing goblet cells lining the epithelium enable mucociliary clearance of the foreign particles out of the airways through the cough reflex. Upon recognition of threats, the airway epithelium activates innate and adaptive immune mechanisms. This includes production of antimicrobial substances as well as pro-inflammatory cytokines and chemokines that recruit and activate more inflammatory cells into the airway lumen [18, 19].

3 CHRONIC OBSTRUCTIVE PULMONARY DISEASE

The global initiative for chronic obstructive lung diseases (GOLD) defines COPD as follows: *"Chronic obstructive pulmonary disease (COPD), a common preventable and treatable disease, is characterized by persistent airflow limitation that is usually progressive and associated with an enhanced chronic inflammatory response in the airways and the lung to noxious particles or gases. Exacerbations and comorbidities contribute to the overall severity in individual patients"* [20].

The most common symptoms are shortness of breath and chronic cough. In addition, patients with COPD may experience extrapulmonary effects such as cardiovascular and psychological conditions [21]. An underlying inflammatory process, most often caused by exposures to noxious particles and gases, is believed to drive the disease progress.

3.1 EPIDEMIOLOGY AND RISK FACTORS

COPD is one of the leading causes of morbidity and mortality worldwide representing an extensive economic and social burden [22, 23]. The disease is currently the 4th leading cause of death affecting over 200 million people [24] and estimates show, that by 2030 it will be the third leading cause of death [22]. However, COPD-related prevalence and mortality is most likely underestimated as most information available comes from high-income countries and the vast majority of COPD deaths are believed to occur in low- and middle-income countries. COPD has historically been considered a men's disease, but the prevalence as well as the hospitalization and mortality rates, of women have now surpassed men [25]. Women seem to be more susceptible to the toxic effects of cigarette smoking [26].

In the industrialized world, the major cause of COPD is tobacco smoking. However, not all smokers develop COPD, indicating that the disease development also is related to genetic predispositions. Other risk factors for developing the disease are increasingly being recognized. These include inhalation of other toxic particles and gases through outdoor air pollution, occupational dusts and chemicals, and indoor air pollution from the burning of biomass fuels used for cooking and heating in developing countries [2, 27]. Additional causes of COPD include passive exposure to cigarette smoke, deficiency of alpha1-antitrypsin [28] and impaired lung growth in early life [29]. In the current thesis, the focus is on COPD caused by cigarette smoking.

3.2 PATHOPHYSIOLOGY

The most common symptoms of COPD are dyspnea, chronic cough and sputum production. Primarily two distinct features are responsible for the pathogenesis of the disease and they often coexist; the obstruction of the peripheral airways and the emphysematous destruction of the alveoli [30].

3.2.1 Bronchiolitis

The major site of obstruction in COPD is the smaller airways of less than 2 mm in internal diameter. In the response to repetitive tissue damage from long-term smoking, inflammatory

repair and remodeling mechanisms result in bronchial wall thickening. The toxic smoke also increases mucus production with concurrent luminal plugs. The increased airway resistance in bronchiolitis is thus a combined effect of the remodeling and mucus secretion.

3.2.2 Emphysema

In emphysema, destruction of alveolar walls forms permanent enlargements of the alveoli, resulting in a reduced gas exchange and air trapping, with a decrease in expiratory flow rates (Figure 3). Importantly, emphysematous destruction of the lung elastic recoil results in impaired exhalation, which may result in a buildup of carbon dioxide [31].

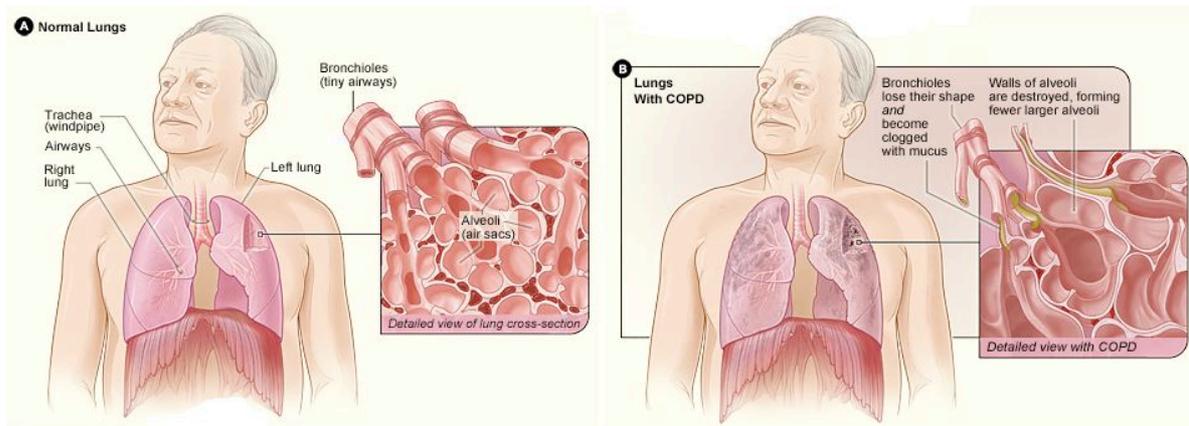
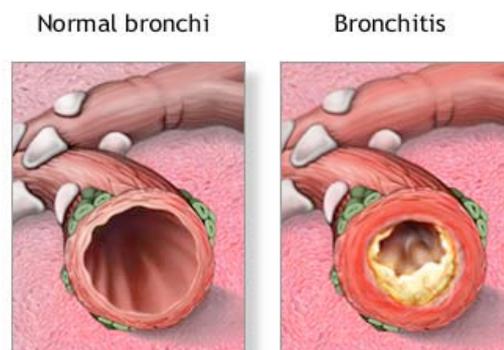


Figure 3. Alveoli in normal lungs (left) and in lungs with emphysema (right). Credit: NIH.

3.2.3 Chronic bronchitis

Chronic bronchitis is a common feature in smokers and COPD patients. It is a clinical diagnosis characterized by inflammation of the bronchial tubes giving rise to excessive mucus production and chronic cough (Figure 4). There are a number of clinical consequences for COPD patients with chronic bronchitis, including accelerated reduction in lung function, higher risk for acute worsenings of the disease and increased risk of mortality [32-34].



ADAM.

Figure 4. Normal bronchial tube and bronchial tube with bronchitis, characterized by an inflamed wall and increased amount of mucus.

3.2.4 Comorbidities

Comorbid conditions such as cardiovascular disease, cancer, osteoporosis and depression are common in COPD. The relationships between these are uncertain and are believed to involve an immune-mediated link between the lungs and circulation. Evidence for this include studies showing significantly elevated levels of several systemic inflammatory markers, such as circulating leukocytes, C-reactive protein and TNF- α , in COPD. The comorbidities are often detrimental, as they can interfere with the COPD management, affect the patient's health status and accelerate the worsening of the disease [35, 36].

3.3 CLINICAL FEATURES

3.3.1 Diagnosis

Diagnosis of COPD is based on the presence of airflow obstruction, which is assessed with spirometry. The essential values of spirometry for COPD diagnosis are the forced expiratory volume in 1 second (FEV₁) and the forced vital capacity (FVC). FEV₁ is the volume of air expired in the first second during maximal expiratory effort and is reduced in obstructive lung disease because of increased airway resistance. FVC is the total volume of air expired after a full inspiration. In patients with obstructive lung disease, FEV₁/FVC is decreased compared to healthy and a post-bronchodilator ratio of FEV₁/FVC below 0.7 confirms the presence of chronic airflow limitation. Most often, the value of FEV₁ used for an individual is FEV₁% predicted, which is derived after correcting for the average FEV₁% in the population for any person of similar age, sex and body composition.

COPD is classically divided into four stages of severity based on spirometric cut-off values for FEV₁, according to GOLD [20] (Table 1). Stage I (mild) is characterized by mild airflow limitation, and possibly symptoms like cough or mucus production. However, the patient may not suffer from any symptoms and may therefore be unaware of the disease. At stage II (moderate), airflow limitations are worsening and symptoms include shortness of breath. Stage III (severe) and IV (very severe) involve severe airflow limitation and substantially impaired quality of life [37]. In recent years, the impact of symptoms, exacerbation frequencies and co-morbidities on disease severity have been acknowledged, and included in the assessment of COPD [20].

Table 1. Degrees of disease severity in COPD based on spirometry values according to GOLD [20].

Basal criterion for all stages: FEV₁/FVC < 0.7		
Stage		FEV₁ % of predicted
I	Mild	≥80
II	Moderate	50 to <80
III	Severe	30 to <50
IV	Very severe	<30

Based on postbronchodilator values. FEV₁: forced expiratory volume in 1 second; FVC: forced vital capacity.

3.3.2 Treatment

To date, smoking cessation is the most important measure for increased survival in COPD. Besides smoking cessation, and the use of supplemental oxygen in selected patients, no efficacious treatments currently exist to treat or prevent the progression of COPD. Current pharmacological treatments can only relieve the symptoms and complications but do not eliminate the underlying inflammatory process. This underlines the need for a greater understanding of the cellular and molecular mechanisms of COPD and the identification of biomarkers in the search for novel therapies. As subcategories of COPD patients more susceptible to certain treatments exist, for example those with some reversibility after β_2 -agonists [38], identifying patient phenotypes may be needed for customized treatment.

3.4 IMMUNE PATHOLOGY IN COPD

Long-term, cumulative exposure to the noxious particles and gases in cigarette smoke triggers an inflammatory process, and in some predisposed individuals this leads to the development of COPD. Compared to the response to cigarette smoke in individuals without lung disease, COPD patients have developed an abnormal chronic inflammatory process in the lungs. The pathology and clinical course of COPD is the result of complex interactions between the cells of the innate and adaptive immune system (Figure 5) [2].

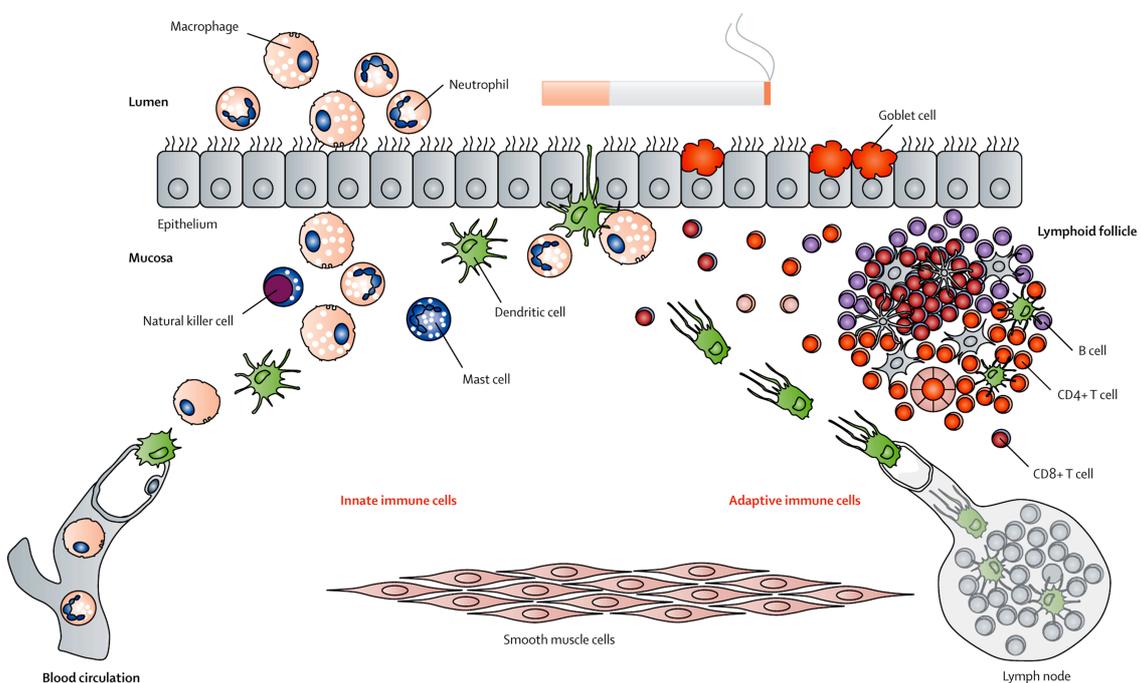


Figure 5. The proposed immune process underlying the pathogenesis of COPD. Reproduced from [2] with permission from The Lancet.

In response to inhalation of toxicants, epithelial cells and macrophages become activated. Macrophages are attracted into the alveolar lumen, engulfing smoke particles and secreting matrix metalloproteinases involved in tissue destruction. Moreover, epithelial cells and macrophages release several chemotactic factors that attract monocytes, neutrophils and

T lymphocytes from the periphery. Dendritic cells activate and orchestrate adaptive immune responses of T cells and B cells. CD8-positive T cells accumulate in the airways and lung parenchyma of COPD lungs [39-42]. These cells, as well as NK cells and NKT-like cells, are able to destroy lung parenchyma through their cytolytic activities [6, 43]. CD4+ T cells increase in the lung in more severe stages of the disease and in emphysema [44, 45], where they recruit and activate other immune cells, including neutrophils, perpetuating the sustained inflammatory process. As T cells respond following antigen recognition, they differentiate in response to the inflammatory milieu to mount a response with a specific cytokine profile [2, 30, 46, 47].

4 AIMS

The overall aim of this thesis was to gain a better understanding of the characteristics of the inflammatory response in the lung and in the blood, to cigarette smoke exposure and in COPD, with emphasis on T cells.

Specific aims were:

Paper I

To study major lymphocyte subsets and the differentiation status of T cells locally in the lung and systemically.

Paper II

To investigate the frequency and state of activation of intraepithelial (CD103+) T cells and the frequency of regulatory T cells in bronchoalveolar lavage (BAL) fluid and blood.

Paper III

To investigate if the morphological patterns of the lungs in response to cigarette smoking and in COPD, as shown by high resolution computed tomography (HRCT) images, associates with the cellular inflammation in the lung.

Paper IV

To elucidate which chemokines and chemokine receptors that may contribute to the recruitment of T cells to the lungs of smokers and patients with COPD and to characterize the Th1/Tc1 and Th2/Tc2 polarization in BAL fluid and blood and finally to relate the findings to clinical features, age and gender.

5 STUDY SUBJECTS AND METHODOLOGY

5.1 STUDY SUBJECTS

The studies in this thesis were carried out on subjects from the Karolinska COSMIC (COPD and Smoking from an OMIC Perspective (COSMIC)) cohort at the Karolinska University Hospital Solna, Karolinska Institutet, Stockholm, Sweden. The aim of the COSMIC study is to investigate and integrate several aspects of COPD and smoking through imaging, transcriptomics, proteomics, metabolomics, and lymphocyte profiling in the context of clinical phenotypes [48-50]. A total of 40 never smokers, 40 smokers with normal lung function and 38 patients with COPD (GOLD I-II) with equal numbers of women and men were recruited with the intent to collect blood and bronchoalveolar lavage (BAL). Among the COPD patients, 27 were current smokers and 11 were ex-smokers, which had refrained from smoking for at least 2 years prior to the inclusion in the study. All subjects were matched in terms of age (45-65 years) and smokers and COPD patients were matched for smoking history. Detailed characteristics of the study subjects and BAL as well as exclusion criteria are described in paper I [51].

5.2 BRONCHOSCOPY WITH BRONCHOALVEOLAR LAVAGE

Bronchoscopy is routinely used in the diagnostic procedure for several lung diseases, including COPD. A bronchoscope is inserted into the airways, usually through the nose or mouth, enabling the physician to investigate the pathology and inflammatory status of the airways. A small camera on the tip of the bronchoscope is used for viewing the airways. Besides, samples from the lower respiratory tract can be obtained from BAL and biopsies (Figure 6).



Figure 6. Bronchoscopy with bronchoalveolar lavage.

Bronchoscopy and BAL were performed according to a standard protocol at our clinic [52]. Briefly, a flexible fiberoptic bronchoscope was passed nasally and wedged into a middle lobe bronchus where five aliquots of 50 mL sterile saline solution were instilled and recollected, bringing back BAL fluid.

Cells were separated from the recovered BAL by centrifugation. Leukocyte differential cell counts were determined on cytopsin slides prepared with native pellet and stained with May-Grünwald Giemsa. The cell-free BAL was centrifuged to eliminate cell debris, and the supernatant was stored at -80°C until further use. Bronchial brush biopsies were collected during the bronchoscopy.

5.3 HIGH RESOLUTION COMPUTED TOMOGRAPHY

High resolution computed tomography (HRCT) imaging of the lungs is a non-invasive instrument for detailed visualization and quantification of the organ structure. It is used in the diagnosis of several lung diseases and has proven useful in the diagnosis of pulmonary emphysema [53]. CT attenuation values are expressed as Hounsfield units (HU), which have been arbitrarily chosen to 0 for water density and -1000 for air density.

In paper III, CT examinations of the study subjects were performed. The main focus was the percentage of the lung parenchyma with attenuation between -750 and -900 HU (the high density spectrum, %HDS) to investigate how CT may reflect the enhanced inflammation in smokers.

5.4 MULTICOLOR FLOW CYTOMETRY

In paper I, II and IV, 8-color flow cytometric immunophenotyping of lymphocytes from BAL and whole blood was performed. Flow cytometry measures several characteristics of individual cells, in large numbers, as they pass through a laser beam under laminar flow conditions. Depending on how the laser beam is deflected, the size and granularity of the cell is assessed. The use of fluorescent-labeled monoclonal antibodies to surface or intracellular molecules gives detailed information on the type of cells in the sample.

The majority of cells in BAL fluid are macrophages. Their phagocytosis of dust particles causes a strong autofluorescence that interferes with the fluorescent staining of the lymphocytes, used in flow cytometry. This is particularly a problem in BAL fluid from smokers where macrophages also phagocyte smoke particles. Alveolar macrophages have been shown to increase in number and cause a stronger autofluorescence in smokers [54]. Therefore, the first step after cell isolation from BAL fluid was to deplete the macrophages using carbonyl iron [55].

Cells from blood and BAL fluid were stained with fluorescently labeled monoclonal antibodies in five different antibody panels, and samples were run on a FACSCanto II. Data were analyzed in FACSDiva 6.1.2. Representative flow cytometry dot plots from paper I are depicted in Figure 7.

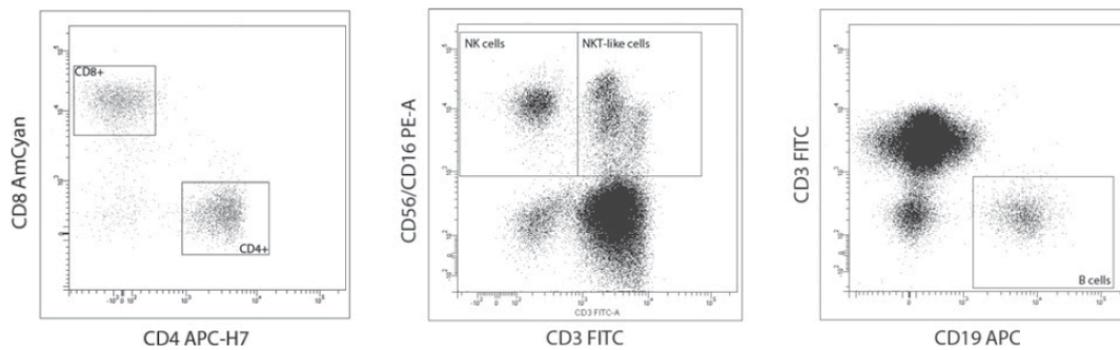


Figure 7. In paper I, a multicolor test for analysis of lymphocytes was used and the distribution of CD8 and CD4-positive T cells, natural killer (NK) cells and NKT-like cells, and B cells was assessed. NK cells and NKT-like cells were identified with a combination of monoclonal antibodies against both CD16 and CD56.

5.5 MULTIPLEX MAGNETIC BEAD-BASED ASSAY

In paper IV, BAL supernatants were concentrated using centrifugal filters and analyzed for chemokines, cytokines and growth factors (listed in paper IV) using multiplex magnetic bead-based assay based on Luminex® xMAP® technology. Multiplex data were analyzed using Bio-Plex Manager software, version 6.0 (Bio-Rad). Samples were run in duplicates. Data out of range or below lower limit of quantification (LLOQ), or sample with high technical variance ($CV > 50\%$) were excluded from all further analyses. Mean-centering and scaling to univariate variance was used to correct for batch effects. Strong intragroup outliers, as identified by Dixon's q-test ($p < 0.05$), were excluded.

5.6 UNIVARIATE DATA ANALYSIS

Data are presented as median (range) in paper I, II and IV, unless otherwise stated. The Mann-Whitney U-test was used when two groups were compared. When more than two groups were analyzed, Kruskal-Wallis test followed by Dunn's multiple comparison test for non-parametric data were performed. A p-value < 0.05 was considered statistically significant. Correlation analyses were assessed with Spearman's rank correlation test and in paper I, the resulting p-values were adjusted for multiple testing using the false discovery rate (FDR) according to Benjamini and Hochberg [56]. An FDR < 0.05 was considered statistically significant. Statistical comparisons and graphs were performed in GraphPad Prism software, version 5.02. In paper III, data are presented as mean and SD. Stata12 software was used for statistical analysis (Stata Corp, College Station, TX, USA).

5.7 MULTIVARIATE DATA ANALYSIS

The classical methods of univariate statistics can often be misleading as in reality; multiple factors can interact and influence the outcome. Today, as an increasing number of studies collect data consisting of variables, multivariate data analysis can be used to extract information from multiple variables by not only taking into account several predictors simultaneously but also co-variance, thereby modeling the property of interest with more accuracy. Multivariate data analysis reduces the dimensionality of the data to aid visualizing and interpreting the results (Figure 8) [57]. The multivariate analyses were performed with SIMCA P+ software version 13 or 14 (Umetrics AB, Umeå, Sweden) applying both unsupervised principal component analysis (PCA) and supervised orthogonal projections to latent structures (OPLS) analysis [58].

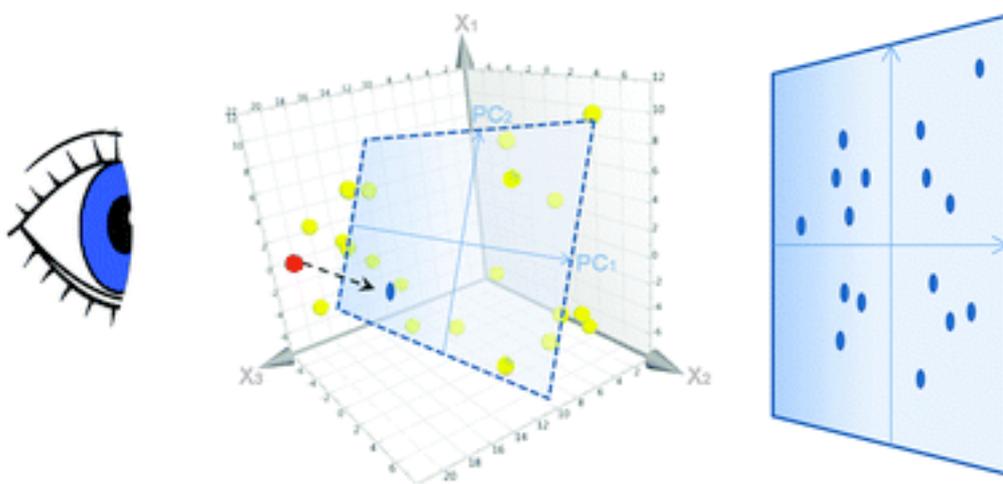


Figure 8. Illustration on how multivariate data analysis reduces the dimensionality of the data to help visualize and interpret the results. Reproduced from Wheelock and Wheelock [57]. Published by The Royal Society of Chemistry.

5.7.1 Principal component analysis

Principal component analysis (PCA) is an unsupervised method suitable for analysis of correlated modeling for a stronger visualization of data. The aim is to find a conversion of an original set of correlated variables to a reduced set of representative latent variables, called principal components [59]. PCA modeling provides an overview of trends in a dataset where correlations among observations and variables can be easily seen, as well as a method for quality control and outlier identification.

5.7.2 Orthogonal projections of latent structures

In orthogonal projections of latent structures (OPLS) analysis, two matrices of large data sets are connected, the X-block of predictor variables and the Y-block of response variables, with the objective to find the most applicable components of X that can predict Y. In contrast to the more commonly used PCA modeling, OPLS analysis is a supervised method designed to

separate structured noise of the X-block variables unrelated (orthogonal) to the predictive variance of interest (e.g. between patients and controls) and thereby increasing the interpretability of the multivariate model, particularly in deriving the observed group separation back to the variables of interest. The method can be used for discovering within- and between group variations, and may help identify potential biomarkers.

In order to effectively evaluate the usefulness of an OPLS model, a number of model statistics are reported. The performance is reported as cumulative correlation coefficients for the model (R^2) and predictive performance based on seven-fold cross validation calculations (Q^2), as well as cross-validated ANOVA (CV-ANOVA) p-values for the OPLS models [57]. These are highly important, as a visually convincing separation does not always indicate a good model or a high predictive power. Over-fitting data may lead to an impressive graph showing a clear-cut separation of the groups, but without predictive or biological relevance. The scores- and loading plots in Figure 3 in paper IV shows an example of a score plot visualizing the separation of two groups of subjects and a loadings plot depicting the predominant response variables driving the separation.

5.7.3 Shared and unique structure plot

The results from individual OPLS models can be compared through shared and unique structures (SUS) analysis, where the scaled loadings ($p[\text{corr}]$) from two models are correlated (Figure 9). The resulting SUS-plot can be useful to identify biomarkers selective for a certain condition, or to find overlapping biomarkers of structures between two models [60].

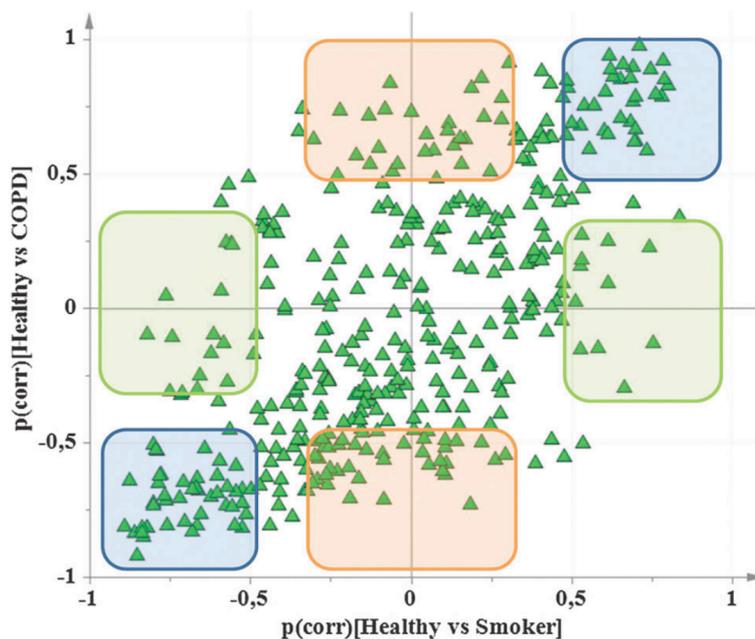


Figure 9..SUS-plot comparing two models for the identification of COPD-specific responses. Response variables (e.g. proteins) unique for either model are found along the outer edges of the axes, specific for smokers in the green boxes and for COPD in the orange boxes. Alterations regardless of COPD diagnosis are located around the positive diagonal indicating that the variables in the blue boxes are not useful as COPD biomarkers. Similarly to univariate correlations, the closer to +1 and -1 the more reliable are the conclusions. Reproduced from Wheelock and Wheelock [49]. Published by The Royal Society of Chemistry.

6 RESULTS AND DISCUSSION

6.1 CYTOTOXIC CELLS IN SMOKING AND COPD

One of the most prominent findings in paper I was the increase in cells with cytotoxic capabilities in BAL fluid from current smokers. Regardless of airway obstruction, current smokers had higher proportions of CD8+ T cells (Figure 10) and NKT-like cells (defined as CD3+ and CD16+ and/or CD56+) in BAL than both never-smokers and ex-smokers with COPD. In addition, most NKT-like cells were CD8+ and there were higher proportions of NKT-like cells among CD8+ T cells, compared with never-smokers and ex-smokers. Increased proportions of NK cells were seen in smokers without COPD only.

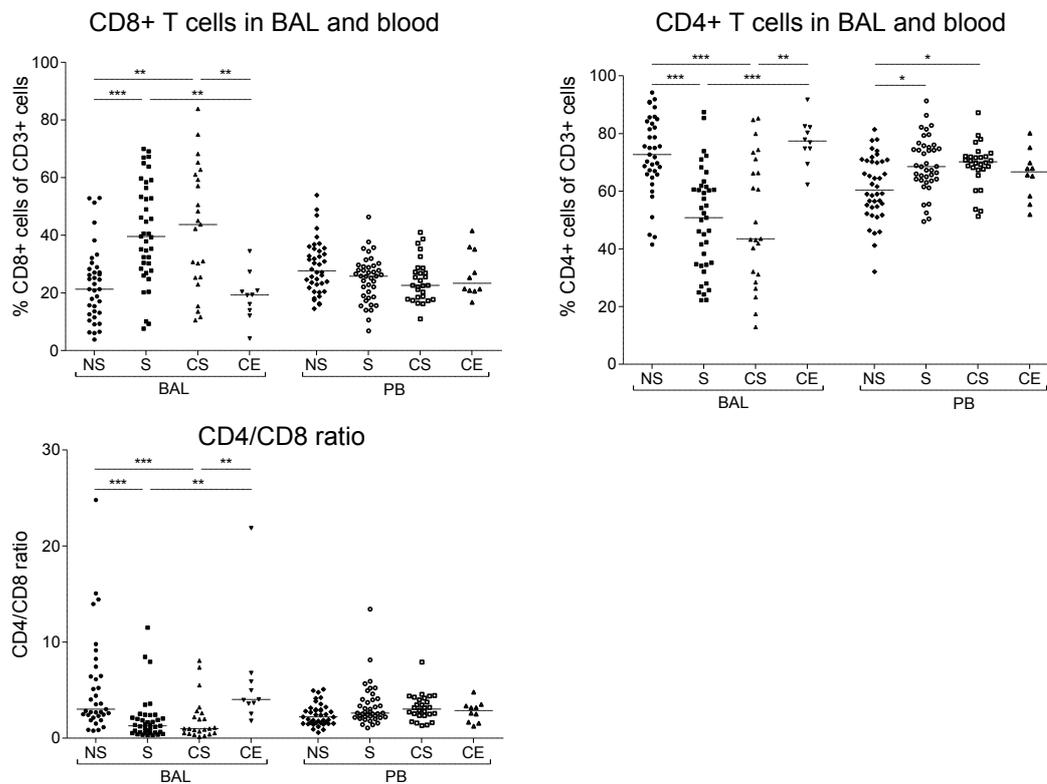


Figure 10. Percentages of CD8+ T cells, and CD4+ T cells and the CD4/CD8 ratio in BAL and peripheral blood (PB) from never-smokers (NS), smokers (S), COPD smokers (CS) and COPD ex-smokers (CE) (paper I).

A number of studies show increased numbers of cytotoxic cells (i.e. CD8+ T cells, NK cells and NKT-like cells) along with higher cytotoxic activity and increased production of perforin and granzyme, in different compartments of the lung, but not in blood, in COPD, [40, 43, 61-63]. These cells are capable of causing the lung tissue destruction seen in COPD patients, both through cytokine secretion and via cytotoxic killing. Our results of a smoke-induced increase in CD8+ T cells, NK cells and NKT-like cells in BAL suggest an increased cytotoxic burden in the peripheral airways of smokers, even before they have developed COPD, that may contribute to the worsening of lung function. Functional studies are needed to investigate their role in smoke-induced inflammation of the lung.

The impact of current cigarette consumption on the proportions of cytotoxic T cells was further demonstrated by a positive correlation between the percentage of CD8-positive T cells in BAL from smokers with COPD and the number of cigarettes smoked per day during the preceding six months. This correlation was even stronger in male smokers with COPD (Figure 11).

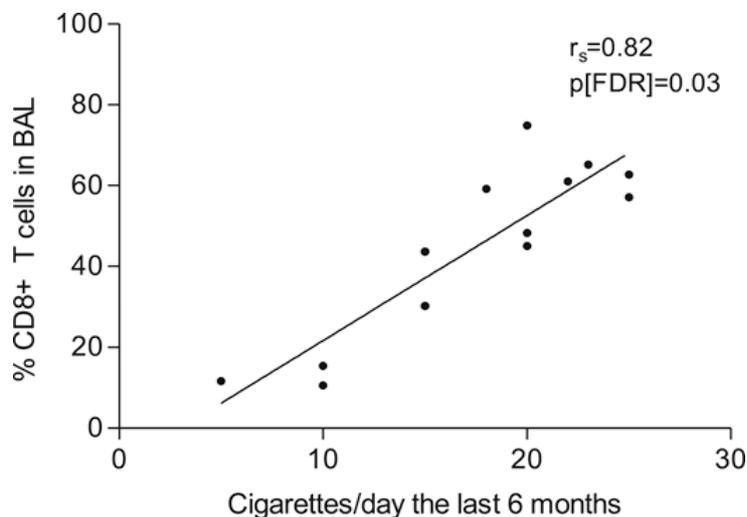


Figure 11. Correlation between the percentage of CD8+ T cells in BAL from male smokers with COPD and the number of cigarettes smoked per day during the preceding six months. p[FDR] = p-value corrected for multiple testing by means of false discovery rate according to Benjamin-Hochberg [56]; r = Spearman rank correlation coefficient (paper I).

In paper **II**, T cells expressing the intraepithelial (CD103+) marker were investigated. The lymphocyte marker CD103 is the αE subunit of the $\alpha E\beta 7$ integrin, which binds specifically to the adhesion molecule E-cadherin expressed in adherent junctions between epithelial cells. T cells expressing CD103 are selectively recruited and retained in mucosal tissues, and are named intraepithelial T cells. Previous studies have found that T cells accumulate in the airway epithelium, but prior to the publishing of paper **II**, their phenotype had not been investigated in more detail. We found higher percentages of CD103+ cells among the CD8+ T cells in BAL from both groups of smokers (Figure 12), suggesting a role for CD8+ T cells in the airway epithelium of smokers. As has been previously shown [64], the proportion of T cells expressing CD103 was higher in BAL than in blood.

More recent findings have suggested that in addition to recruit and maintain T cells to the epithelium, CD103 can have other functions. It has been shown that CD103 can promote migration through epithelial cell layers [65], and has been proposed to be a marker for regulatory CD8+ T cells [66, 67].

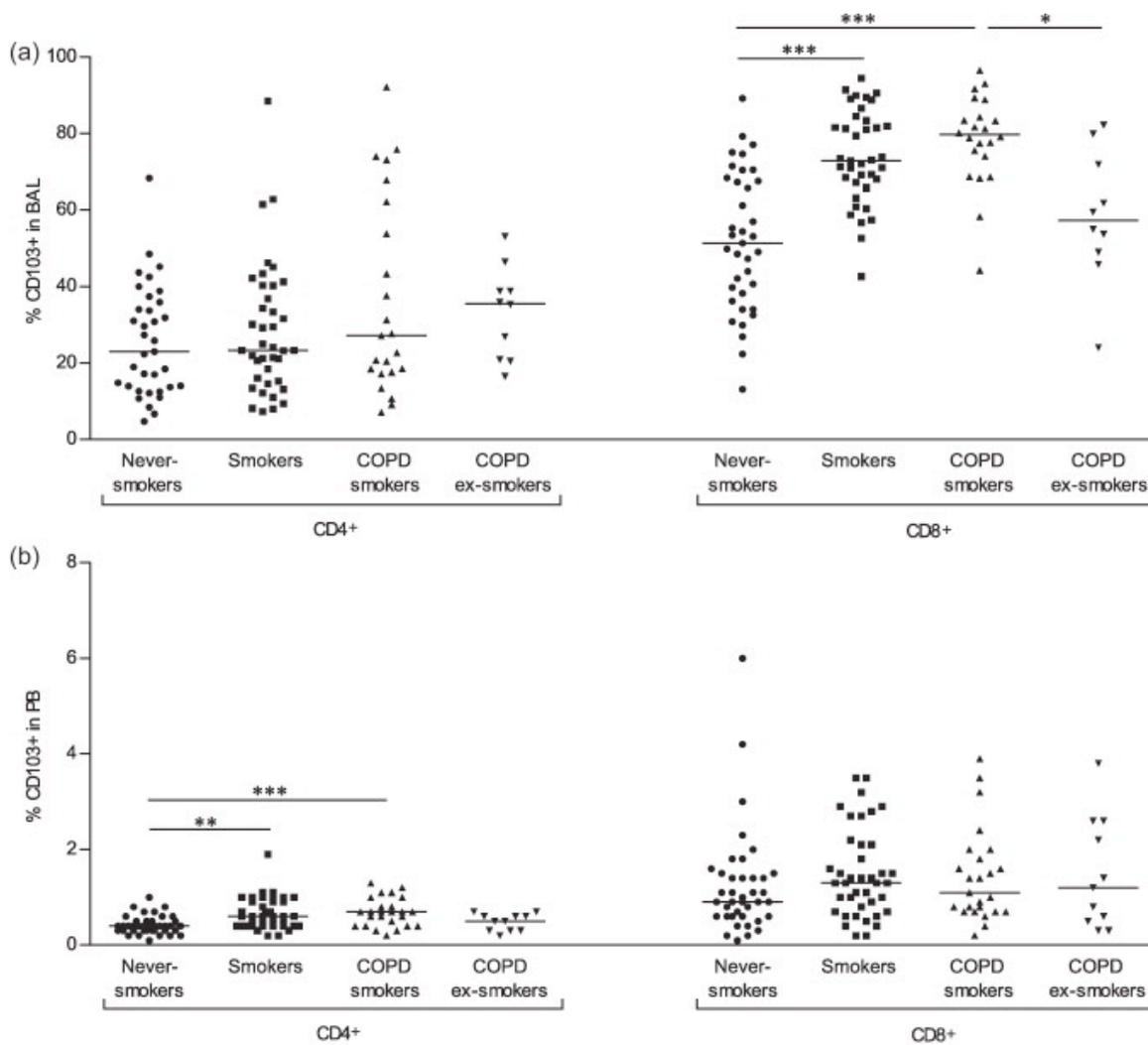


Figure 12. Percentages of CD103-positive cells among CD4+ and CD8+ T cells in BAL and peripheral blood (PB) (paper II).

An additional interesting finding in paper I was that proportions of activated CD69+ naive, central memory and effector CD4+ T cells often were the highest in BAL from smokers with COPD compared to the other three study groups. This shows that the combination of smoking and airway obstruction is related to activated T cell subsets, and that even though the total CD4 T cell number is unchanged, there may be important phenotypic changes.

6.2 REGULATORY T CELLS

Several studies suggest a decreased number and impaired function of regulatory T cells in COPD [68, 69]. We hypothesized that cigarette smoke affects the frequency of regulatory FOXP3+ T cells in the lung and that these changes also reflect development to COPD (paper II). CD4+ T cells from BAL fluid and blood were stained for FOXP3 to identify Tregs. Increased proportions of CD4+FOXP3+ T cells were found in BAL fluid from smokers compared with never-smokers (Figure 13). This upregulation was not seen among smokers with COPD. We reasoned that the increased frequency of Tregs in BAL from smokers with normal lung function has been central for their immune system's ability to counteract the

continuous inflammatory burden, preventing the development of COPD. This finding may give a clue to why some smokers develop COPD and some not.

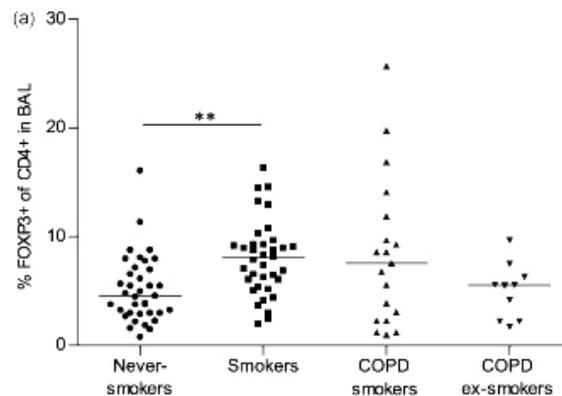


Figure 13. Percentages of FOXP3+ CD4+ T cells in BAL fluid (paper II).

As described above, CD103 can be a marker for regulatory T cells [67]. The CD4+FOXP3+ T cells described in paper II did not express CD103. When we analyzed the combination of the activation and differentiation markers CD69, CD27 and HLA-DR on CD103+ T cells (paper II), we found, within the CD8+CD103+ cells, a decrease in a subset of CD27+CD69- T cells with a lower HLA-DR expression in BAL from both groups of smokers. A subset of CD8+ T cells with, among others, these characteristics has previously been described as regulatory T cells that exert their suppressive functions via direct cell to cell-contact [67]. The decrease of this subset in the lungs of smokers might cause an increased susceptibility to persistent damage in the lung.

Tregs play a critical role in regulating inflammatory responses, and a deficiency in function and/or numbers can lead to the development and progress of autoimmune and chronic inflammatory diseases [70, 71]. Autoimmunity is caused by insufficiently controlled B- and T-cell responses to self-antigens. Circulating antibodies to, among others, elastin and Hep-2 epithelial cells have been shown to be increased in levels and more prevalent in COPD patients compared to controls [68, 72]. These and other findings suggest an autoimmune component in COPD pathogenesis, and may explain the enhanced and persistent inflammatory response after smoking cessation [73, 74].

6.3 HIGH RESOLUTION COMPUTED TOMOGRAPHY

HRCT is a well-established method for morphological characterization of pulmonary emphysema [75]. Increased lung density on CT has been suggested to mirror inflammation in the lung parenchyma [76]. In paper III, CT images of the COSMIC study subjects were used for the quantification of inflammatory changes due to chronic smoking and in early stages of COPD. The lung density measurements focused on the attenuation range between

-750 and -900 HU (percentage higher density spectrum (%HDS)) on inspiratory CT-scans. Typical patterns seen on CT scans from a never-smoker, smoker and COPD patient are shown in Figure 14.



Figure 14. Axial inspiratory CT scans of three 54 year old females, representing typical patterns for never-smokers (left), smokers (middle) and COPD patients (right) in this study (paper III).

Lung density was higher in smokers with normal lung function compared to both never-smokers and COPD patients (Figure 15). A possible explanation for the lack of differences between never-smokers and COPD patients may be that in the lungs of COPD patients, areas with decreased lung density (i.e. emphysema) and areas with increased lung density (i.e. accumulation of inflammatory cells) may occur in the same patient but in different regions of the lung. This would result in an average density of the lung comparable to a never-smoker's. CT imaging technique could be a valuable tool for the detection of early changes in the lungs of smokers.

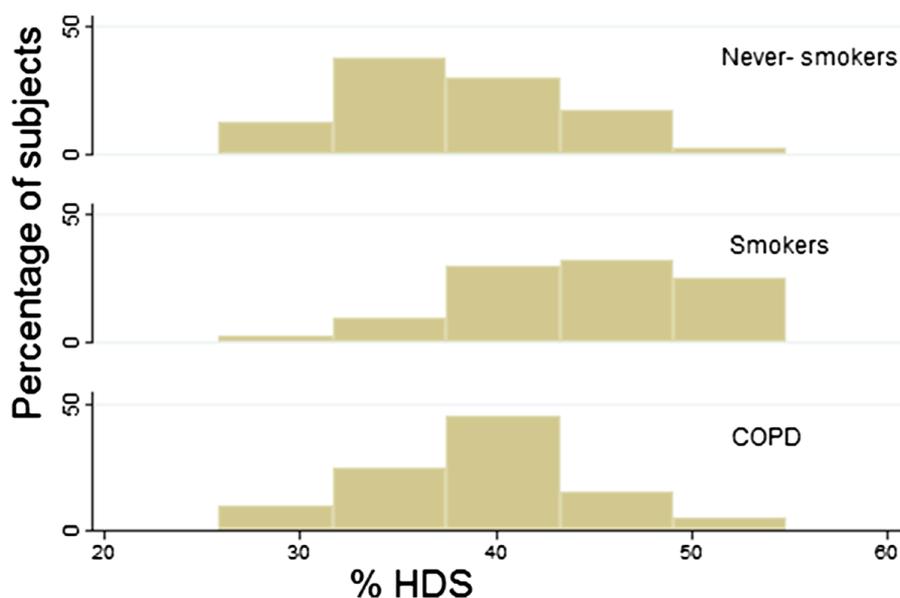


Figure 15. The relationship between lung densities measured as attenuation -750 HU to -900 HU (%HDS) for never-smokers, smokers and COPD patients (smokers and ex-smokers) (paper III).

6.4 T CELL RECRUITMENT TO THE LUNG

In paper **IV**, factors important in the recruitment of T cells to the lung in smokers and COPD patients compared to never-smokers were investigated, and the cytokine milieu in BAL from these individuals was assessed. We especially focused on the Th1/Th2 balance. COPD is generally considered a Th1/Tc1-driven disease [2], but there are studies implicating a role also for Th2/Tc2 cells [77].

In the present study, the Th1-associated chemokine receptor CCR5 was found to be more abundant on CD4+ T cells in BAL fluid from "healthy" smokers compared to never-smokers. This finding suggests a CCR5-mediated recruitment of CD4+ T cells to the lungs of smokers without COPD. We further show decreased levels of the Th2 cell cytokine IL-4 and the Th2-associated chemokine CXCL12 in BAL fluid from both groups of smokers. Taken together, our findings show that a Th1/Tc1-biased inflammation is not solely related to COPD, but also to smoke-induced inflammation, particularly through the CCR5 chemokine receptor. Mapping out a more detailed picture of the T cell recruitment elements of importance in the development and progression and COPD may give rise to new treatment options targeting the chemokines and/or their receptors.

Supervised OPLS-DA modeling comparing T cell subsets and soluble analytes of smokers with normal lung function versus smokers with COPD was performed in order to separate COPD-related factors from the dominating effects of smoking. Analysis of women and men separately revealed gender differences in the immune processes in COPD. Whereas a strong discrimination was found between smoking women with and without COPD ($R^2=0.50$, $Q^2=0.41$, $p=8 \times 10^{-4}$) with eleven variables indicated as driving the separation between the two groups, a less robust model was found among men ($R^2=0.32$, $Q^2=0.25$, $p=0.016$).

The findings of a strong model for women, but not men, when comparing smokers without COPD to smokers with COPD support previous findings on the BAL cell proteome from the COSMIC cohort [49]. Our results indicate gender-specific molecular differences in the inflammatory response to cigarette smoke and in COPD. It can be speculated that compared to women, men with COPD is a more heterogeneous groups with a higher number of patient phenotypes.

7 CONCLUSIONS AND FUTURE PERSPECTIVES

Besides smoking cessation, current treatment methods for COPD patients include bronchodilators and corticosteroids, but these do not act on the underlying inflammation. A number of treatments targeting the inflammatory process have been developed, but these do not cure the disease. Understanding the molecular and cellular patterns behind the inflammatory mechanisms, and their expression in subgroups of patients, is important for the development of biomarkers and new therapeutic interventions.

As shown by others and us, the inflammatory process in smokers with normal lung function and smokers with COPD show both shared and unique characteristics, and many still remain unclear. The impact of current smoking status on CD8⁺ T cells in BAL that we present in paper I, is not reflected in biopsies from the small and large airways, as well as from the lung parenchyma, where increased numbers of CD8⁺ T cells seem related to COPD only.

This thesis shows, that smoking largely determines the distribution of T lymphocyte subsets in the bronchoalveolar compartment and, however to a smaller extent, in the periphery. Several findings in the included papers revealed a similar characteristic; frequencies of T cell subsets were altered in the same fashion in smokers regardless of airway obstruction. In COPD ex-smokers, the frequencies had returned to those of never-smokers. This highlights the importance of studying present smokers and ex-smokers among COPD patients separately when investigating T cell subsets in BAL and blood.

Even after smoking cessation, a continuous inflammatory process in the lungs of COPD patients is seen. A role for the adaptive immunity with the involvement of T lymphocytes has been suggested, particularly reflected in the lung tissue. Based on the findings in the papers included in this thesis, studies on T cells in BAL fluid requires detailed phenotyping to identify those cells reflecting early pathological changes in the lung. Comparisons of lung tissue biopsies and BAL fluid, ideally from the same individual, would give more information. Functional studies on the different T cells subsets, such as assessing their cytolytic capacity, could give a greater understanding of their role in the disease course.

The heterogeneity of COPD is becoming increasingly recognized. Identifying subtypes of patients, characterized by e.g. genetic, immunological or clinical factors, can lead to a more tailored or individualized treatment. With increasing amounts of data collected and large study cohorts of well-characterized individuals, multivariate data analysis provides a means for extracting and integrating the many variables measured, for the identification of biomarkers and subgroups of patients.

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