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New Aspects of Alveolar Adaptive Immune Responses in COPD

Michiko Mori



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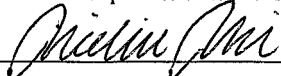
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<p>Abstract</p> <p>Chronic obstructive pulmonary disease (COPD) is characterised by a chronic inflammation in conducting airways and the alveolar parenchyma. The inflammation, which is a consequence of inhalation of noxious gases like tobacco smoke, has traditionally been thought to be driven by innate immune responses. However, more recent studies have revealed an increase in lymphocyte-rich lymphoid aggregates (LAs) and antigen presenting dendritic cells (DCs) in peripheral airways of patients with COPD. These findings suggest that peripheral adaptive immune responses and antigen-driven immune events may contribute to the immunopathology of COPD. However, the involvement of the alveolar compartment has remained unexplored. The aim of this thesis work was to provide new insights into the structural as well as the immunological basis for alveolar antigen uptake and peripheral adaptive immune responses in COPD. To investigate this, detailed immunohistochemical assessments of adaptive immune system components were performed in surgical specimens obtained from patients with different severities of COPD (GOLD stage I-IV), smokers and never-smoking control subjects. Immunohistochemistry and 3D reconstructions of serial sections were used to demonstrate a rich occurrence of alveolar-restricted LAs and reveal that also the vast majority of the bronchiolar- and vascular-associated LAs have alveolar interfaces. Importantly, in COPD patients but not in controls a selective accumulation of langerin⁺ DCs was observed along the alveolar-lymphoid interfaces. Moreover, these alveolar interface DCs had increased luminal protrusions and more physical contact with lymphoid T cells than the corresponding mucosal DCs. A further investigation of multiple DC subsets in different peripheral lung compartments found that in COPD the most marked increase in DC subsets, including BDCA-2⁺, CD1a⁺langerin⁺ and CD11c⁺CD68⁺CD163⁺ DCs, was observed in the alveolar tissue. Notably, the DCs in LA interfaces and alveolar tissue differed from airway DCs by a distinct marker expression profile. The 3D analysis revealed an intricate connection between LAs and lymphatic vessels. Further, a marked increase in the number of alveolar lymphatic vessels was detected in patients with advanced COPD. Importantly, in COPD lymphatic vessels had an activated phenotype as revealed by increased lymphatic expression of CCL21 and D6, both of which are involved in DC transport to lymphoid tissues. In conclusion, the studies in this thesis have discovered that the alveolar parenchyma in COPD lungs contain a novel type of DC-rich alveolar-lymphoid epithelium, elevated levels of multiple DC subsets as well as activated lymphatic vessels. Taken together these findings forward the alveolar parenchyma as an important arena for antigen uptake in COPD. This insight calls for future investigations to find out to what extent the resulting adaptive immune responses contribute to the COPD pathogenesis and how they can be pharmacologically targeted.</p>		
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New Aspects of Alveolar Adaptive Immune Responses in COPD

Doctoral Thesis

by

Michiko Mori



LUNDS
UNIVERSITET

Supervisor: Professor Jonas S. Erjefält

Unit of Airway Inflammation
Department of Experimental Medical Sciences
Faculty of Medicine, Lund University
2013

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Cover image shows a haematoxylin-stained lung tissue section of a patient with very severe chronic obstructive pulmonary disease. Cover design made by Cecilia Andersson.

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*Till mamma, pappa, Mikio, Miyoko
och Magnus*

The task is not to see what others have not seen, but to think what others have not thought about that which everybody sees

Arthur Schopenhauer

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List of Papers

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals. The papers are appended at the end of the thesis.

- I. **Mori M**, Andersson CK, Svedberg KA, Glader P, Bergqvist A, Shikhagaie M, Löfdahl CG and Erjefält JS.
Appearance of remodelled and dendritic cell-rich alveolar-lymphoid interfaces provides a structural basis for increased alveolar antigen uptake in chronic obstructive pulmonary disease. *Thorax*, 2013, 68:521-531. Reproduced with the permission of BMJ Publishing Group Ltd and the British Thoracic Society.
- II. **Mori M**, Andersson CK, Graham GJ, Löfdahl CG and Erjefält JS.
Increased number and altered phenotype of lymphatic vessels in peripheral lung compartments of patients with COPD. *Resp Res*, 2013, 14:65. © 2013 Mori *et al.*
- III. **Mori M**, Andersson CK, Shikhagaie M, Bergqvist A, Löfdahl CG and Erjefält JS.
Expansion of multiple dendritic cell populations in the alveolar parenchyma in patients with chronic obstructive pulmonary disease. Submitted

Some data not included in the papers are added in this thesis.

Papers and book chapter not included in this thesis

Erjefält JS and **Mori M**. “Makrofager och dendritiska celler och deras roll vid KOL”. In: KOL, Kroniskt obstruktiv lungsjukdom. Chapter 4.4. Ed. Kjell Larsson. Lund: Studentlitteratur, 2013.

Swärd K, Sadegh MK, **Mori M**, Erjefält JS and Rippe C. Elevated pulmonary arterial pressure and altered expression of *Ddah1* and *Arg1* in mice lacking cavin-1/PTRF. *Physiol Rep*, 2013, 1(1), e00008.

Al-Garawi A, Fattouh R, Botelho F, Walker TD, Goncharova S, Moore CL, **Mori M**, Erjefält JS, Chu DK, Humbles AA, Kolbeck R, Stämpfli MR, O’Byrne PM, Coyle AJ, Jordana M. Influenza A facilitates sensitization to house dust mite in infant mice leading to an asthma phenotype in adulthood. *Mucosal Immunol*, 2011, 4:682-694.

Andersson CK, **Mori M**, Bjermer L, Löfdahl CG, Erjefält JS. Alterations in lung mast cell populations in patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med*, 2010, 181:206-217.

Mori M, Thelin MA, Ekman M, Larsson-Callerfelt AK, Wess J, Swärd K* and Erjefält JS*. Distribution of muscarinic M5 receptors in man and role in contractility of murine smooth muscle. Manuscript

* These authors share senior authorship

Selected Abbreviations

ALT	alveolar-only associated lymphoid tissue
AP	alkaline phosphatase
BAL	bronchoalveolar lavage
BALT	bronchus-associated lymphoid tissue
BDCA	blood dendritic cell antigen
BRALT	bronchiolar-associated lymphoid tissue
COPD	chronic obstructive pulmonary disease
DAB	3,3'-diaminobenzidine
DC	dendritic cell
FEV ₁	forced expiratory volume in 1 second
FVC	forced vital capacity
GALT	gut-associated lymphoid tissue
GOLD	Global Initiative for Chronic Obstructive Lung Disease
HEV	high endothelial venule
HRP	horseradish peroxidase
LA	lymphoid aggregate
MHC	major histocompatibility complex
MMP	matrix metalloproteinase
SMA	smooth muscle actin
TGF	transforming growth factor
Th	T helper
TLR	Toll-like receptor
TNF	tumor necrosis factor
VALT	vascular-associated lymphoid tissue
VEGF	vascular endothelial growth factor

Introduction

Chronic obstructive pulmonary disease (COPD) represents a global health problem and is predicted to become the third leading cause of death worldwide by 2020. Hence, there is a strong need for more effective treatments of this disease. COPD is characterised by an accelerated decline in lung function and airway inflammation and is most often caused by cigarette smoking. The major pathological features are bronchitis and bronchiolitis (inflammation in the conducting airways) as well as emphysema (destruction of the alveolar walls). While several studies have been performed to increase the understanding of the airway inflammation in the larger airways (i.e. the bronchi), less is known about the inflammation in the small airways (bronchioles). Yet, this region represents the main site for pathological changes in COPD and its exploration is currently an emerging research field. Traditionally, studies on inflammation in COPD have focused on cells involved in innate immune mechanisms. Interestingly, recent evidence also suggests a crucial role for adaptive immune responses and there is a growing interest towards the role of lung lymphoid aggregates and dendritic cells in the pathogenesis of COPD. However, studies of these cells in the peripheral lung have mainly focused on the bronchioles and fundamental knowledge of the adaptive immunity in other peripheral lung compartments is lacking, especially in the alveolar tissue. The overall aim of this thesis work is to provide new insights into the structural as well as the immunological basis for alveolar antigen uptake and peripheral adaptive immune responses in patients with COPD. In order to investigate this, detailed immunohistochemical assessments of lymphoid aggregates, lymphatic vessels, and dendritic cells were performed on peripheral lung tissue samples. Importantly, at the start of this work most of the immunological characterisation, such as the exploration of alveolar lymphoid tissues, lymphatic vessels, and dendritic cell populations, had never been explored in humans and much initial work has been devoted to the development and validation of protocols for their explorations in human paraffin-embedded tissues. The actual studies (studies I-III) were performed using carefully selected patient cohorts that allowed comparisons of immunological features between non-smoking and smoking control subjects and patients with different severities of COPD. The use of large surgical samples offers several advantages, including the possibility to compare multiple anatomical regions within the same tissue section and sampling

of all peripheral parts of the lung, many of which are poorly accessible with other techniques.

Background

Introduction of chronic obstructive pulmonary disease

Chronic obstructive pulmonary disease (COPD) is a global health problem. According to the World Health Organization, 65 million people have moderate to severe COPD. The prevalence is continually increasing and by the year 2020 COPD is predicted to be the fifth leading cause of morbidity and the third leading cause of mortality worldwide^{1,2}. Hence, the medical care costs and suffering from COPD are immense and rapidly increasing. Although COPD is primarily a lung disease, it is often associated with comorbidities that contribute significantly to the disease severity and mortality³. To increase the attention to COPD, the Global Initiative for Chronic Obstructive Lung Disease (GOLD) program was formed in 1998⁴. The first GOLD report was published in 2001 and provided strategies for improvements of diagnosis, management and prevention of COPD. Since then, the GOLD document has been updated and revised in 2006 and 2011.

Definition

COPD is defined by the GOLD (2011) as “*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.*”⁵ The characteristic symptoms of COPD include chronic and progressive dyspnea, cough and sputum production. The persistent airflow limitation is measured by spirometry and is defined as a post-bronchodilator ratio between forced expiratory volume in one second (FEV_1) and forced vital capacity (FVC) of less than 0.70. The classification of the severity of COPD is then based upon the severity of the airflow obstruction measured as FEV_1 expressed as a percentage of the predicted normal values⁵. At the start of this thesis work, the classification of disease severity was based on the 2006 GOLD document⁶ in which the severity of COPD was classified into four different stages: GOLD stage I (mild COPD, $FEV_1 \geq 80\%$ predicted), GOLD stage II (moderate COPD, FEV_1 50-79%

of predicted), GOLD stage III (severe COPD, FEV₁ 30-49% of predicted), and GOLD stage IV (very severe COPD, FEV₁ <30% of predicted) (see figure 1). In the 2011 GOLD document⁵ the staging system has been replaced by a grading system (GOLD I-IV) since FEV₁ is an unreliable marker of severity at an individual level. However, the spirometric classification of COPD severity remains the same.

Risk factors

Cigarette smoking is the far most common cause of COPD. The classical study by Fletcher *et al.*⁷ demonstrated that smoking is associated with an acceleration in the normal decline in lung function compared to never smokers (figure 1). Importantly, their data showed that smoking cessation reduced the rapid rate of lung function decline. It was also evident from this study that not all smokers developed COPD. This notion has been confirmed in other studies^{8,9} and has led to the identification of genetic risk factors for COPD. In this regard the deficiency of alpha-1 antitrypsin is a well-known genetic factor for developing COPD¹⁰. Other risk factors include exposure to indoor air pollution (e.g. burning of wood or biomass fuels for cooking), outdoor air pollution, occupational dust and chemicals, and infections^{5,11-13}.

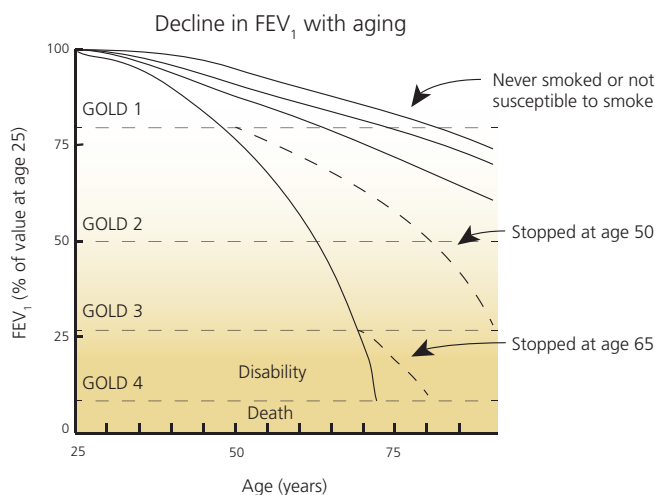


Figure 1

Age related decline in forced expiratory volume in one second (FEV₁) in never smokers and smokers who either do not or do develop COPD⁷. Smokers who develop COPD have a rapid rate in lung function decline, a phenomenon that is reduced by smoking cessation. Dotted horizontal lines indicate the classification of COPD severity according to the Global Initiative for Chronic Obstructive Lung Disease (GOLD), where GOLD I is mild COPD (FEV₁ ≥80% predicted), GOLD II is moderate COPD (FEV₁ 50-79% of predicted), GOLD III is severe COPD (FEV₁ 30-49% of predicted), and GOLD IV is very severe COPD (FEV₁ <30% of predicted)⁵. (Figure by C. Andersson, adapted from reference¹⁴).

COPD pathology

The airflow limitation in COPD is caused by pathological changes that are thought to begin in the bronchioles (peripheral/small airways, <2 mm in diameter) and eventually reach the lung parenchyma¹⁵, but early changes are also observed in the bronchi (central/large airways) and pulmonary vasculature^{14,16,17}. The structural changes in bronchioles include airway wall thickening due to chronic inflammation with specific remodelling processes such as increased smooth muscle mass as well as fibrosis^{16,18} (figure 2A-B). In addition, lymphoid aggregates and luminal exudates (mucus and cell debris) are often present^{18,19}. Together, these changes result in airway narrowing, increased airway resistance and, hence, airflow limitation^{14,18}. In patients with very severe COPD the increased resistance may also be due to a reduced number of terminal bronchioles²⁰. In the lung parenchyma, the permanent enlargement of airspaces is caused by destruction of alveolar tissue (i.e. emphysema) (figure 2C-D). The loss of the alveolar tissue results in reduced gas exchange and reduced lung elasticity (increased lung compliance). The emphysema is thought to result from an imbalance between proteases and anti-proteases, and/or an imbalance between proliferation and apoptosis of structural cells^{12,16,21} but other factors may also contribute. Furthermore, patchy fibrotic lesions are also detected

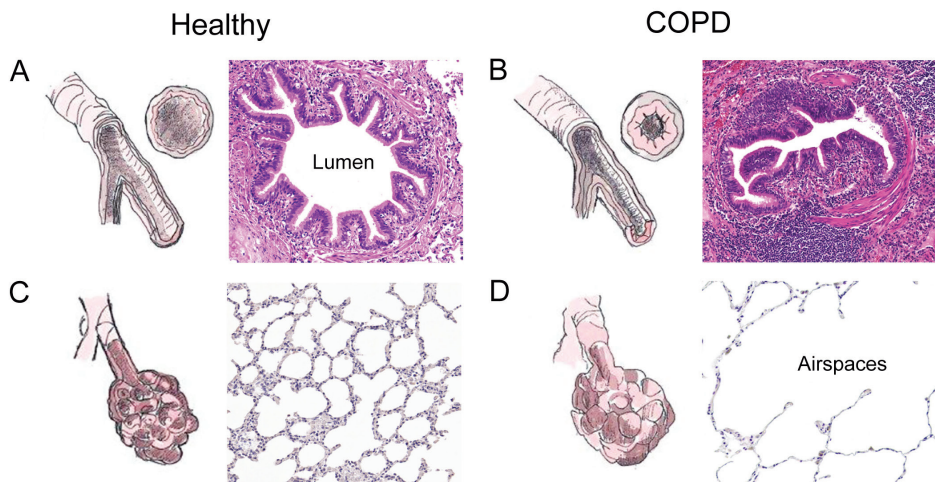


Figure 2

(A) Illustrations show a normal bronchiole and a cross-section of a bronchiole. Photomicrograph shows a cross-section of a bronchiole in normal lung. (B) Illustrations and photomicrograph of an obstructed bronchiole in COPD. The airway wall thickening is due to inflammation and fibrosis. (C) Illustration of normal spherical air sacs (i.e. lung parenchyma) and photomicrograph shows a cross-section of air sacs in normal lung. (D) Illustration of emphysema, which is characterised by enlargement of air sacs. Photomicrograph shows a cross-section of emphysematous parenchyma in COPD. As the alveolar walls are destroyed, the air sacs are turned into larger, irregular structures. This results in impaired capacity for gas exchange. Tissue sections were stained with Mayer's haematoxylin and eosin in A-B, and Mayer's haematoxylin in C-D. (Drawings made by O. Hallgren, photos by M. Mori).

in the lung parenchyma in COPD. The structural changes in the bronchi include increased number of goblet cells and enlarged submucosal glands¹⁴. The changes in pulmonary vessels include enlargement of the intima of arteries and arterioles, as well as muscularisation of arterioles^{17,22}.

Importantly, the presence of the structural changes may vary between patients^{18,23}. For example, in some patients emphysema is more prominent, whereas other patients have a more pronounced inflammation of the airways and less emphysema. In addition, it is likely that the structural changes include micro-anatomical variations and comparisons between lung compartments in the same patient are important to identify any regional heterogeneity within the COPD lung.

The role of exacerbations and respiratory infections

Exacerbations

Exacerbations of COPD are defined as an acute worsening of symptoms, such as increased cough, dyspnoea and sputum production, and often require changes in treatments²⁴. In the case of severe exacerbation hospitalisation may be necessary⁵. Exacerbations are associated with accelerated decline in lung function²⁵ and, consequently, a major cause of morbidity and mortality of COPD²⁶. The number of annual exacerbations generally increases with increasing severity of COPD²⁷. According to the 2011 GOLD document, the history of exacerbations should be included in the assessment of the severity of COPD⁵. Exacerbations are often induced by bacterial or viral infections, and are associated with increased inflammation of the upper and lower respiratory tract, as well as systemic inflammation²⁴. The bacteria commonly associated with COPD exacerbations include *Haemophilus influenzae*, *Streptococcus pneumoniae*, and *Moraxella catarrhalis*. In addition, *Pseudomonas aeruginosa* appears to be prevalent in patients with severe COPD²⁸. Among the viruses, rhinoviruses and influenza virus are most frequently associated with exacerbations²⁸. In addition, bacteria and viruses may coexist in some exacerbations.

The mechanisms of exacerbation are not clear. Some studies have suggested a role for increased bacterial load in the lower airways²⁹, whereas others have suggested a role for new bacterial strains in COPD exacerbations³⁰. The latter case is supported by the detection of strain-specific immune responses during infections with new strains of *Haemophilus influenzae* and *Moraxella catarrhalis*^{31,32}. In any case, it is clear that the respiratory infections in COPD patients will result in a whole range of immunological reactions. While some of these are necessary to combat the infection, others may be harmful to the host tissue and thus contribute to the pathogenesis. In the COPD field much of the exploration of these responses has been focused on the innate immune processes, but more attention is now directed towards the

adaptive immune responses. However, many basic aspects remain to be investigated, for example how and where foreign antigens are recognized by the immune system and where they activate adaptive immune responses.

The lung microbiome

Recent studies have detected a low-grade presence of microorganisms in the lower respiratory tract of smokers and even healthy lungs, which previously was considered to be sterile³³⁻³⁵. The term lung microbiome is used to describe this resident collection of bacteria that is present in the lungs. It is not known how these microorganisms enter the lung, but microaspiration of the oral flora has been suggested as a potential source³⁶. More recently, studies have shown that patients with COPD have a changed composition of the lung microbiome compared with control subjects^{33-35,37}. However, it remains unknown how this microbiome is changed during exacerbations and whether or not the changed lung microbiome in COPD has an impact on COPD pathogenesis and progression. However, it can be speculated that the low-grade presence of bacteria together with infection-driven exacerbations may increase the antigen load in peripheral lungs, thereby enhancing antigen uptake and induction of immune responses.

Treatments

Smoking cessation is the only effective treatment to reduce the decline in lung function. Although some medications may have a minor effect on lung function parameters, the pharmacological treatment goal for patients with COPD is mainly to relieve symptoms, reduce the number of exacerbations and improve health status. The primary pharmacologic treatments for COPD include bronchodilators (long acting β_2 agonists and anticholinergics) and inhaled glucocorticosteroids. In addition, influenza vaccine is usually recommended in patients with COPD⁵.

Immunopathogenesis of COPD

In COPD, the airflow limitation is caused by chronic inflammatory immune responses in the peripheral lung (figure 3). Importantly, the inflammatory responses increase with the severity of COPD¹⁸. The inflammation is characterised by both innate and adaptive immune cells, including accumulation of neutrophils, macrophages, and T cells (foremost CD8⁺)^{38,39}, as well as B cells in more advanced COPD¹⁸. However, the inflammation in COPD is complex and other cell types, such as natural killer (NK) cells⁴⁰, may also be involved in the immunopathogenesis of COPD. The accumulation of immune cells involves numerous inflammatory

mediators that are released from both immune cells and structural cells of the lung⁴¹. For example, macrophages exposed to smoke secrete chemoattractants involved in the recruitment of immune cells into the lung. In addition, cigarette smoke disrupts the physical epithelial barrier and activates epithelial cells to release several inflammatory mediators, including tumor necrosis factor (TNF)- α , IL-1 β , IL-6, and IL-8⁴². Epithelial cells in bronchioles also produce transforming growth factor (TGF)- β ⁴³, which may induce fibroblast activation and airway remodelling^{44,45}.

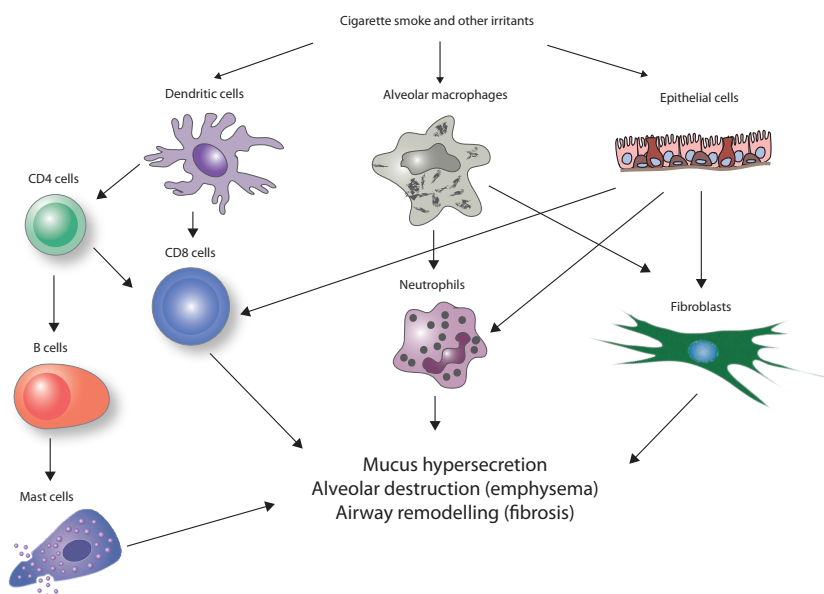


Figure 3
Summary of the innate and adaptive immune cells linking exposure to cigarette smoke and other risk factors to the pathological changes characteristic for COPD. (Figure by C. Andersson).

Innate immune cells

Neutrophils are increased in numbers in both sputum⁴⁶ and bronchoalveolar lavage (BAL) fluid^{47,48} of patients with COPD. In addition, studies have demonstrated that the increase in sputum neutrophils correlates with both the rate of lung function decline⁴⁹ and severity of COPD⁵⁰. Neutrophils have a high capacity for pathogen phagocytosis and are rapidly recruited in response to microbial infections. However, several of the neutrophil-derived products used to destroy invading pathogens may also contribute to some of the pathological changes associated with COPD. For example, activated neutrophils can secrete several proteases, including neutrophil elastase, cathepsin G, proteinase 3, as well as matrix metalloproteinase (MMP)-8

and MMP-9, which stimulate mucus hypersecretion and may also contribute to the destruction of the alveolar walls⁵¹.

Macrophages are thought to play a central role in the pathogenesis of COPD. Upon activation by cigarette smoke, macrophages release several mediators with the potential to influence many of the inflammatory processes in COPD lungs³⁹. For example, macrophages release inflammatory mediators, reactive oxygen species, extracellular matrix proteins, leukotrienes, prostaglandins and MMPs. Patients with COPD have increased numbers of macrophages in bronchial submucosa, bronchiolar epithelium, lung parenchyma, sputum and BAL fluid^{39,52}. The number of macrophages in bronchioles seems to correlate with COPD severity⁵³. In addition, some studies indicate that the number of macrophages in the lung parenchyma correlates with the severity of emphysema⁵⁴. In this regard, it is known that macrophages from COPD lungs produce more MMPs, including MMP-9 and MMP-12, and this may contribute to the alveolar wall destruction³⁹. Apart from their role in inflammation, macrophages have a central role in the host defence against pathogens. Alveolar macrophages are strategically positioned in the air spaces and have a high capacity to clear pathogens as well as other inhaled particles through phagocytosis. In addition, macrophages are important in the phagocytosis and removal of apoptotic cells (so called efferocytosis). However, studies have reported that alveolar macrophages in COPD have a defective phagocytic function, which could lead up to increased bacterial load and necrotic cell debris⁵⁵. An increase in bacterial load in the lower respiratory tract may evoke undesirable immune responses.

Mast cells could also be involved in the immunopathogenesis of COPD. For example, mast cell-derived mediators can initiate inflammatory responses and promote remodelling processes, both of which are implicated in the pathogenesis of COPD⁵⁶. In a recent study it was demonstrated that the progression of COPD into severe disease is associated with several alterations in lung mast cell populations in the peripheral lung, such as changes in density, morphology as well as distribution between major peripheral lung compartments⁵⁷.

Adaptive immune cells

The infiltration of T cells is a characteristic feature of the chronic inflammation in COPD lungs. The number of T cells appears to increase as COPD progresses, suggesting that adaptive immunity is a prominent feature in severe disease^{58,59}. Amongst the lymphocytes, cytotoxic CD8⁺ T cells predominate in COPD lungs and are present in all airway levels and lung parenchyma⁶⁰. The main function of CD8⁺ T cells is to target virally infected cells for destruction by cytolysis or apoptosis. Since patients with COPD often suffer from chronic infections of the lower respiratory

tract it is possible that these pathogens initiate CD8-driven inflammatory responses in the lung. However, it has been suggested that tissue damage in COPD may result from their release of perforins and granzymes. In line with this, studies have demonstrated increased expression of perforin in CD8⁺ T cells in sputum from patients with COPD⁶¹. In addition, the number of CD8⁺ T cells is associated with the number of apoptotic cells in the lung parenchyma⁶².

The numbers of CD4⁺ T cells are increased in the bronchiolar mucosa in severe COPD¹⁸. The role of CD4⁺ T cells in COPD is largely unknown, but could contribute to the inflammatory responses by releasing proinflammatory cytokines. Other possible roles include priming CD8⁺ T cell responses, maintaining memory as well as survival of CD8⁺ T cells³⁹. Among the different CD4⁺ T cell subsets, T helper (Th)1 and Th17 cells appear to accumulate in COPD lungs⁴⁰. Th1 cells in COPD appear to release more interferon γ which may further drive the recruitment of immune cells into the lungs, and Th17-derived cytokines may be involved in the recruitment of neutrophils⁴⁰. Interestingly, immunosuppressive regulatory T cells are decreased in numbers in COPD. It is possible that the imbalance between T cell subsets contribute to the chronic inflammatory responses in COPD.

The numbers of B cells are increased in bronchi⁶³ and bronchioles¹⁸ of patients with COPD. Importantly, more recently the formation of lymphoid aggregates containing B cells and T cells have been described in bronchioles¹⁸ and lung parenchyma⁶⁴ of patients with severe COPD. Indeed, the vast majority of CD20⁺ B cells in COPD lungs reside within lymphoid aggregates. The appearance of peripheral lymphoid aggregates provides important evidence that adaptive immune responses can be initiated locally in the peripheral lung and that antigen presenting dendritic cells may play an important role in linking innate immunity to adaptive immunity in COPD. In this thesis work we hypothesised that better understanding of where dendritic cells are positioned in the peripheral lung in general, and in lymphoid aggregates in particular, would clarify where peripheral antigen uptake takes place and how adaptive immune responses are initiated in peripheral lungs of patients with COPD.

Dendritic cells

Linking innate and adaptive immunity

The activation of adaptive immune responses begins with antigen uptake by dendritic cells (DCs) and generally involves their trafficking to draining lymph nodes⁶⁵ (figure 4). Although other cells, such as macrophages⁶⁶ and B cells⁶⁷, have the ability to take

up antigens, DCs are regarded as the predominant antigen presenting cell type⁶⁸. In a resting state, the airway mucosa is populated by resident DCs. During infection and tissue damage, DCs and monocytes are recruited from the circulation into the lung⁶⁹⁻⁷¹. Mouse studies on airway epithelial DCs^{72,73} as well as recent studies on human bronchial DCs⁷⁴ have shown that DCs extend their membrane protrusions into the airway lumen. It is likely that mucosal epithelial DCs in human express tight junction proteins, similar to mouse DCs, to preserve the integrity of the epithelium^{75,76}. Since most studies on lung DCs have been performed on airway epithelial DCs, it is currently believed that immature DCs, residing in or beneath the airway epithelium, constitutively sample luminal antigens. Immature DCs have a high capacity for antigen uptake (i.e. macropinocytosis, phagocytosis and receptor mediated endocytosis), and low capacity for T cell stimulation^{77,78}. For example, the immature DCs express a variety of endocytic receptors for antigen uptake, such as Fc-receptors and lectin receptors^{65,79}. In addition, DCs sense the environment for pathogens and endogenous danger signals through their expression of pattern-

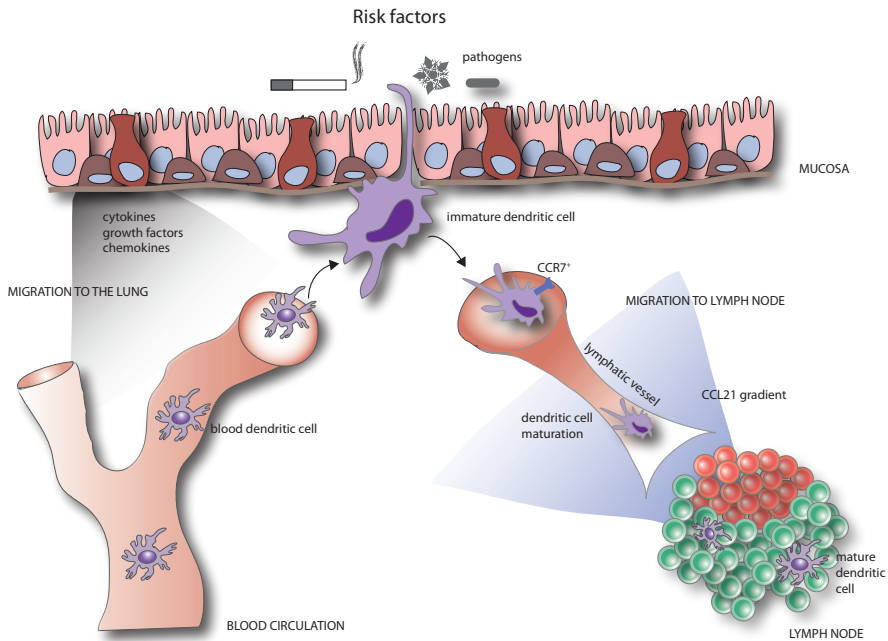


Figure 4

Cytokines, chemokines and growth factors released from the site of inflammation recruit dendritic cells (DCs) and monocytes from the circulation. Newly recruited DCs differentiate into immature DCs, which sample antigens and respond to danger signals in the airway mucosa. Upon antigen uptake, DCs undergo a maturation process in which the antigen is processed and presented on the cell surface. DCs upregulate the chemokine receptor CCR7, which binds to its ligand CCL21 on lymphatic vessels. Antigen-loaded DCs are then transported through lymphatic vessels to lymph nodes, where they interact with T cells to initiate an adaptive immune response. (Figure by C. Andersson, adapted from reference⁶⁵).

recognition receptors, such as Toll-like receptors (TLRs) and C-type lectins^{65,79-81}. Furthermore, factors released within the tissue, such as epithelial-derived thymic stromal lymphopoietin (TSLP), can directly activate DCs⁸².

Antigen uptake results in a process referred to as DC maturation, which includes presentation of antigen-peptide on major histocompatibility (MHC) molecules, upregulation of co-stimulatory molecules, and expression of lymphoid homing receptors. Extracellular antigens are degraded into peptides and loaded onto the MHC-II molecules. The peptide-MHC-II complexes are then transported to the cell surface and presented to CD4⁺ T cells. Intracellular antigens, such as self-antigens and intracellular pathogens, are loaded onto MHC-I molecules and the peptide-MHC-I complexes are presented on the cell surface to activate CD8⁺ T cells. Through so called cross presentation DCs can also present extracellular antigens on MHC-I molecules and activate a CD8 response. The maturation process also involves the upregulation of co-stimulatory molecules for T cells (CD80, CD86 and CD40) and the chemokine receptor CCR7, which binds to its ligand CCL21 expressed on lymphatic vessels⁸³⁻⁸⁵. Thus, antigen-loaded DCs migrate from the peripheral tissue to draining lymph nodes. Currently, studies on DC trafficking in humans are missing. Even basic aspects, such as information regarding the expression of lymphatic CCL21 in COPD have been left unexplored, until recently. In the lymph node, DCs present the peptide-MHC complexes to naïve CD4⁺ and CD8⁺ T cells to induce an immune response. The T cells bearing a T cell receptor that recognises the antigen-derived peptide are then activated by co-stimulatory molecules provided by the DC and undergo clonal expansion. Furthermore, depending on the type(s) of cytokines released by the DC, CD4⁺ T cells differentiate into Th1, Th2, regulatory T cells or Th17 effector cells (figure 5). Thus, DCs not only activate T cell responses, but also induce tolerance to self-antigens and prevent autoimmune responses. The activated T cells then traffic back to the lung where they neutralise the antigens. Other activated T cells remain in the lymph node to stimulate the production of antibody-producing plasma cells^{77,86,87}. In COPD, it is possible that DCs promote a shift towards cytotoxic CD8 T cell, Th1 and Th17 responses⁴⁰.

Dendritic cell subsets in the lung

Dendritic cells represent a heterogeneous population of cells derived from the bone marrow. Due to this complexity and the fact that DCs mainly have been explored *in vitro* or in animal models the DC subsets in human lung tissues remain poorly characterised. While several DC subsets have been identified in mice, it has proven to be difficult to identify their human counterparts. The challenges have mainly been related to lack of specific markers and the complex heterogeneity among the tissue DC subsets. Also, the great overlap of marker expression between

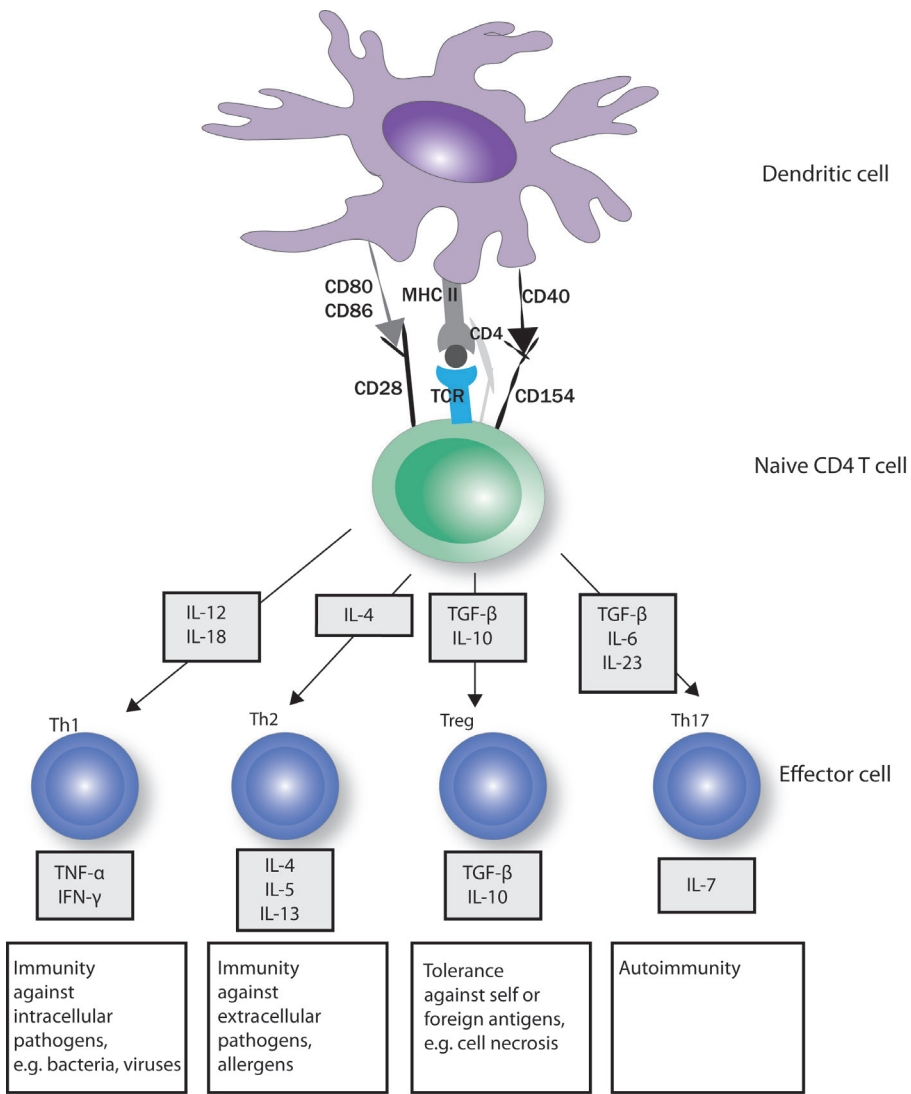


Figure 5

Dendritic cells (DCs) present the peptide-MHC-II complexes to T cells. T cells expressing a specific T cell receptor (TCR) that recognises the peptide are selected. The co-stimulatory molecules expressed on DCs (CD80, CD86, CD40) then interact with the T cell. Depending on the cytokines released by the DC, CD4⁺ T cells differentiate into Th1, Th2, regulatory T cells (Treg) or Th17 cells. (Figure by M. Shikhagaie, adapted from references^{40,86}).

monocytes, macrophages, and DCs⁸⁸ further adds to the technical challenges as the identification of DC subsets usually requires a combination of several markers. The lack of a stringent system to classify DCs into defined subgroups and a shortage of immunohistochemical markers have led to a situation where individual research groups have used different marker combinations and classification to characterise

DC subsets. This in turn makes it difficult to compare results from different studies. However, despite all challenges the DCs can be classified into several subsets. Currently, at least three major subsets of DCs have been identified in the lung; myeloid DC, plasmacytoid DC, and Langerhans type DC (equivalent to Langerhans cells in the skin) (figure 6).

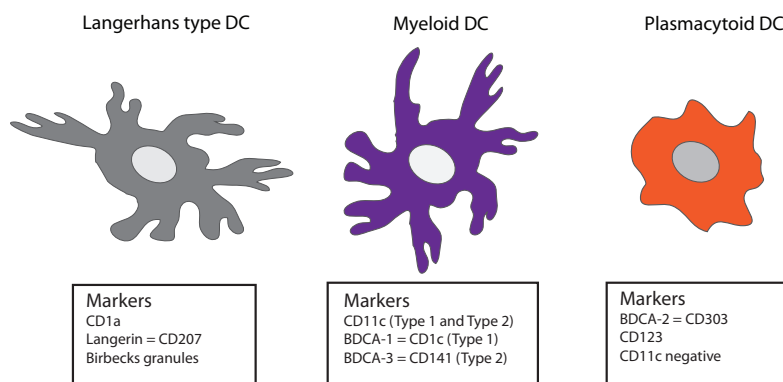


Figure 6
Markers commonly used to identify Langerhans type, myeloid and plasmacytoid dendritic cells in human lung. BDCA, blood dendritic cell antigen.

Myeloid dendritic cells

Myeloid DCs, all expressing CD11c, can be further subgrouped based on their expression of different blood dendritic cell antigen markers (BDCAs). Myeloid DCs type 1 express BDCA-1 (CD1c) and myeloid DCs type 2 express BDCA-3 (CD141)⁸⁹⁻⁹². Myeloid DCs type 2 are generally more abundant than type 1 myeloid DCs⁹². It is important to note that CD11c expression is not restricted to DCs but is also present on monocytes and macrophages^{93,94}. Myeloid DCs isolated from lung resection specimens were found to express mRNA for TLR1-4, TLR6 and TLR8⁹⁵. Thus, this DC subset is thought to be particularly important in the defence against invading microbes. *In vitro* studies on purified human lung myeloid DCs further reveal that both myeloid DCs type 1 and type 2 respond to TLR2 and TLR4 activation by releasing several proinflammatory cytokines, including TNF- α , IL-1 β , IL-6 and IL-8⁹⁵. Furthermore, studies in a mixed leukocyte culture reaction showed that myeloid DC type 1 was a strong inducer of T cell proliferation whereas myeloid DC type 2 showed an intermediate capacity for T cell proliferation⁹⁵.

Plasmacytoid dendritic cells

The plasmacytoid DCs express BDCA-2 (CD303) and CD123, but are negative for CD11c⁸⁹⁻⁹². In contrast to myeloid DCs, plasmacytoid DCs represent a rare

DC subset in the lung⁹². Plasmacytoid DCs express TLR7 and TLR9. Upon activation of these receptors plasmacytoid DCs release proinflammatory cytokines and interferon- α , highlighting their important role in the defence against viral infections^{95,96}. Plasmacytoid DCs are less efficient in antigen processing, compared with myeloid DCs, and hence they are not strong inducers of T cell responses^{95,97}. Instead, several *in vitro* studies suggest that plasmacytoid DCs have important tolerogenic functions by promoting the generation of immunoregulatory T cells⁹⁸⁻¹⁰¹. Taken together, these findings suggest that plasmacytoid DCs are important during viral infections as well as maintaining peripheral tolerance.

Langerhans type dendritic cells

The Langerhans type DCs, originally described in the skin, are recognized electron microscopically by the presence of the organelles called Birbeck granules¹⁰². The surface markers typically expressed on Langerhans type cells include langerin (CD207) and CD1a⁶⁵. More recently Van Pottelberge *et al.*¹⁰³ showed that Langerhans type DCs in the lung are also partially positive for the myeloid DC marker BDCA-1. Hence, it is currently unclear whether or not Langerhans type DCs represent a subgroup of myeloid DCs, although they are often considered as a separate subset⁶⁵. *In vitro* studies have suggested that these DCs may derive from myeloid DC precursors¹⁰⁴, whereas other studies suggest that monocytes, under the influence of certain cytokines, differentiate to Langerhans type DCs¹⁰⁵. Studies in human skin have shown that Langerhans cells are strong inducers of Th1 responses and efficient at cross-presenting antigens to CD8⁺ T cells¹⁰⁶. Another study has revealed that human skin Langerhans cells promoted Th17 responses¹⁰⁷. Therefore, it is likely that Langerhans type DCs have several important roles in COPD.

It is also becoming evident that other types of DCs exist in the lung tissue. In COPD, for example, Van Pottelberge *et al.*¹⁰³ recently identified a DC population that expressed DC-SIGN (CD209) and monocyte markers, but not langerin, CD1a or BDCA-2. The DC-SIGN⁺ DCs did however express the myeloid marker BDCA-3 and may represent a subset of myeloid DCs, but it is also possible that DC-SIGN⁺ DCs should be regarded as a new separate subset of DCs as recent findings in mice demonstrated that monocyte-derived DCs express DC-SIGN¹⁰⁸.

Dendritic cells in COPD

Although there have been great advances in the understanding of the chronic inflammation in COPD, very little is known about the role of lung DCs. However, recent observations of expanded lymphoid aggregates as well as the strong association between adaptive immune cells and COPD severity strongly suggest that DCs are of importance in COPD. To define these roles have turned out to be complicated and

the few studies that have investigated lung DCs in COPD have shown conflicting results regarding the changes in numbers of the different DC subsets, which in turn may reflect differences in sampling and methodological techniques.

Conducting airway dendritic cells

Studies on bronchial biopsies have so far not revealed any differences in number of CD1a⁺ DCs between healthy smokers and smokers with COPD¹⁰⁹. In contrast, evaluation of bronchial specimens using electron microscopy revealed a reduction in the number of BDCA-1⁺ DC in smoking COPD patients compared with ex-smoking COPD patients and never smokers¹¹⁰. In line with the results obtained in bronchi, the number of CD1a⁺ DCs was not changed in bronchioles of smokers or patients with COPD^{103,111-113}. However, several studies have observed an increase in the number of langerin⁺ DCs in patients with COPD compared with controls subjects^{103,113,114}, and the number of langerin⁺ DCs increased with COPD severity¹¹⁴. Moreover, similar to the findings in bronchi, the number of BDCA-1⁺ DCs was reduced in bronchioles of patients with COPD and the numbers appeared to decrease with COPD severity¹⁰³. Furthermore, numbers of BDCA-2⁺ DCs were not changed in numbers in patients with COPD¹¹⁵. Taken together these studies indicate that among the different DC subsets langerin⁺ Langerhans type DCs seem to accumulate in bronchioles of patients with COPD.

Alveolar parenchymal dendritic cells

In contrast to the conducting airways, very little is known about DCs in the alveolar parenchyma in COPD. Conflicting data have been reported with regards to the number of alveolar CD1a⁺ DCs. Soler *et al.*¹¹¹ reported an increase in alveolar CD1a⁺ DCs in smokers compared with never smokers, whereas Tsoumakidou *et al.*¹¹² found no differences in numbers of alveolar CD1a⁺ DCs between never smokers, smokers and patients with COPD. Moreover, numbers of BDCA-1⁺ DCs were not changed between smokers and never smokers¹¹¹. Furthermore, studies on BAL fluid, which samples the alveolar lumen (as well as the conducting airways), have shown an increase in langerin⁺ and CD1a⁺ DCs in smokers compared with never smokers^{116,117}. Although it is evident from these studies that DCs are indeed present in the alveolar tissue, basic information on the different DC subsets in COPD is still missing, including their heterogeneity in marker expression. Importantly, recent findings in mice indicate that alveolar DCs are more efficient in antigen uptake than the airway DCs¹¹⁸. Thus, from this, and the fact that much of the inflammation in COPD is located in the alveolar region, it can be speculated that the alveolar parenchyma is an important site for antigen uptake and induction of adaptive immune responses in this disease.

Recruitment, differentiation and maturation of dendritic cells in COPD

The conflicting results reported regarding DC numbers may also be due to differences in smoking status of study subjects⁷¹. For example, cigarette smoking *per se* may modulate DC numbers in the lung. In a study by Lommatzsch *et al.*¹¹⁹, the number of myeloid DCs, but not plasmacytoid DCs, was increased in human BAL fluid and decreased in blood following a 4 hour period of acute smoke exposure. In addition, the expression of lung homing chemokine receptor CCR5 was downregulated on myeloid DCs in BAL fluid, suggesting that the DCs were mature. Other studies have suggested that acute smoke exposure increases the survival of human monocyte-derived DCs *in vitro*¹¹³. Alterations in chemokine expression within COPD lungs may also modulate the recruitment of DCs. For example, the levels of the DC-attracting chemokine CCL20 have been demonstrated to be increased in lungs of patients with COPD. Further, isolated lung DCs from COPD patients express the CCL20 receptor CCR6, suggesting that CCL20/CCR6 interactions may be involved in the recruitment of DCs into COPD lungs¹¹⁴. In further support of this, smoke exposure of mice deficient in CCR6 resulted in diminished accumulation of lung DCs¹²⁰. Furthermore, a recent study showed that the pulmonary levels of the Langerhans inducing differentiation factor activin-A correlated with the numbers of Langerhans type DCs in bronchioles of patients with COPD¹⁰³. Thus, several local factors within the tissue may favour the differentiation of DC precursors towards a specific DC subset in COPD. This phenomenon may also provide a basis of local and site-specific DC populations within the lung. It can for example be speculated that the differences in the molecular milieu between e.g. the bronchial mucosa and the alveolar tissue would give rise to distinct and site-specific DC compositions in each compartment.

To add the complexity further, several conflicting results have also been reported with regards to the maturation status of DCs in COPD. While studies on tissue sections and sputum samples have revealed decreased maturity of DCs in patients with COPD^{112,121}, studies on resected lung tissue homogenates have shown that the maturity of DCs increased with COPD severity⁹². Furthermore, studies on BAL fluid DCs of smokers revealed an increase in the expression of maturation markers, but a reduced expression of the lymphoid homing receptor CCR7¹¹⁶. Moreover, cigarette smoke may also modulate the function of DCs. For example, in one study cigarette smoke extract reduced interferon- α production by plasmacytoid DCs, which could impair the protection against viral infections¹¹⁵. In the same study, plasmacytoid DCs isolated from patients with COPD produced higher levels of TNF- α and IL-8 upon maturation compared to those from control subjects. In addition, lung DCs isolated from mice exposed to smoke produced high levels of MMP-12¹²². These data indicate that DCs together with macrophages could contribute to alveolar wall destruction in COPD.

Taken together, emerging data indeed suggest that DCs are of importance in the inflammation in COPD. Of note, the antigens driving the immune responses remain to be identified. Nevertheless, it is clear that DCs accumulate in bronchioles of patients with COPD, but still surprisingly little is known about DC subsets in the alveolar parenchyma. Considering the important roles of airway epithelial DCs in the defence against pathogens, it is likely that alveolar tissue DCs have similar roles in the defence against pathogens reaching the most distal parts of the lung. If the immune responses initiated by lung DCs are beneficial or harmful remains to be determined.

Lymphoid aggregates in COPD

Since Hogg and colleagues¹⁸ first described that the progression of COPD from mild to severe disease was associated with a marked increase in the number of bronchioles containing lymphocyte-rich lymphoid aggregates (LAs), several studies have been conducted to increase the knowledge of these structures. These studies have confirmed the presence of LAs in the bronchiolar wall, but have also noted the occurrence of LAs in the alveolar parenchyma^{64,123-125}. It has even been suggested that in patients with severe COPD alveolar LAs are more prevalent than bronchiolar LAs⁶⁴. This notion is based on visualisation of two-dimensional sections and thus it could be argued that some of the alveolar LAs may have physical contact with bronchioles in a three-dimensional context. Since lymphoid tissues are in general rare in lungs of never smokers¹²⁶⁻¹²⁹, LAs found in COPD have been regarded as a form of inducible lymphoid structures that are typically present in chronically inflamed tissues^{130,131}. These new lymphoid structures are termed ectopic lymphoid tissue or tertiary lymphoid organs and are thus distinguished from the secondary lymphoid organs which are usually formed during embryogenesis^{132,133}.

Functional organisation of lymphoid aggregates

Structurally LAs are rather similar to secondary lymphoid organs in that they contain a B cell area surrounded by a T cell area of CD4⁺ and CD8⁺ T cells^{18,131} (figure 7A-C). Studies have also revealed that LAs in COPD contained proliferating cells, which are indicative of germinal centres^{125,134}. Plasma cells have also been observed close to the LAs^{64,125}. In addition, high endothelial venules (HEVs) expressing peripheral lymph node addressin (PNAd), but not mucosal addressin cell adhesion molecule (MAd-CAM) have been observed in LAs¹²⁵. This observation is in line with other studies on lung LAs *in vivo*¹³⁵, and it has been suggested that naïve lymphocytes, instead of memory cells, are recruited through HEVs¹³⁶. Van der Strate *et al.*⁶⁴ further showed

that the majority of the B cells were memory B cells and, similar to secondary lymphoid organs, the LAs contained a network of follicular DCs involved in antigen presentation to B cells¹³⁷. In this study, it was suggested that the B cells had likely been activated by antigens as clonal B cell populations were observed in isolated LAs from patients with COPD. The sequence of immune events taking place in these lymphoid structures was suggested by Hogg and Timens¹³⁸ (as illustrated in figure 7D). It was proposed that LAs are sites where naïve T cells and B cells are activated by antigens. The interaction between these activated cells stimulates B cell proliferation and production of antibodies against the antigen. B cells that express high affinity B cell receptors to that antigen presented on follicular DCs are selected to undergo clonal expansion. These B cells then develop into memory B cells and antibody producing plasma cells. B cells that are unable to bind to the antigen presented on follicular DCs or B cells expressing low affinity B cell receptors to the antigen undergo apoptosis. Taken together, there is strong evidence that LAs are local inductive sites for rapid adaptive immune responses in the lung, and thus the initiation of these immune responses is not spatially limited to the regional lymph nodes.

Although there are several similarities between peripheral lung LAs and secondary lymphoid organs, there are also some important differences. Apart from that LAs do not have fibrous capsule similar to lymph nodes, the most important difference is how they receive antigens. For example, mucosa-associated lymphoid tissues, such as gut-associated lymphoid tissue (GALT) and bronchus-associated lymphoid tissue (BALT), are located just beneath the epithelial layer and are covered by a specialised luminal-lymphoid interface epithelium or lymphoepithelium towards the lumen. This epithelium in general contains clusters of intraepithelial lymphocytes. In GALTs, microfold cells, so-called M cells, are also present in the lymphoepithelium. The M cells are a type of epithelial cells that transport luminal antigens across the epithelium to the lymphoid tissue¹³⁹. However, in COPD, several studies have observed LAs in the outer layer of the bronchiolar wall^{64,123,125}. Since most of these LAs are likely not in physical contact with the bronchiolar surface epithelium, they may acquire airway luminal antigens through other unidentified mechanisms. For example, it has been hypothesised that DCs residing in or beneath the airway epithelium capture luminal antigens and then migrate to LAs, possibly through lymphatic vessels^{131,136,140}. This notion is supported by *in vivo* studies demonstrating that airway luminal antigens were indeed transported to BALT via DCs¹⁴¹. However, it has remained unknown how antigens are transported to LAs in bronchioles and the alveolar parenchyma.

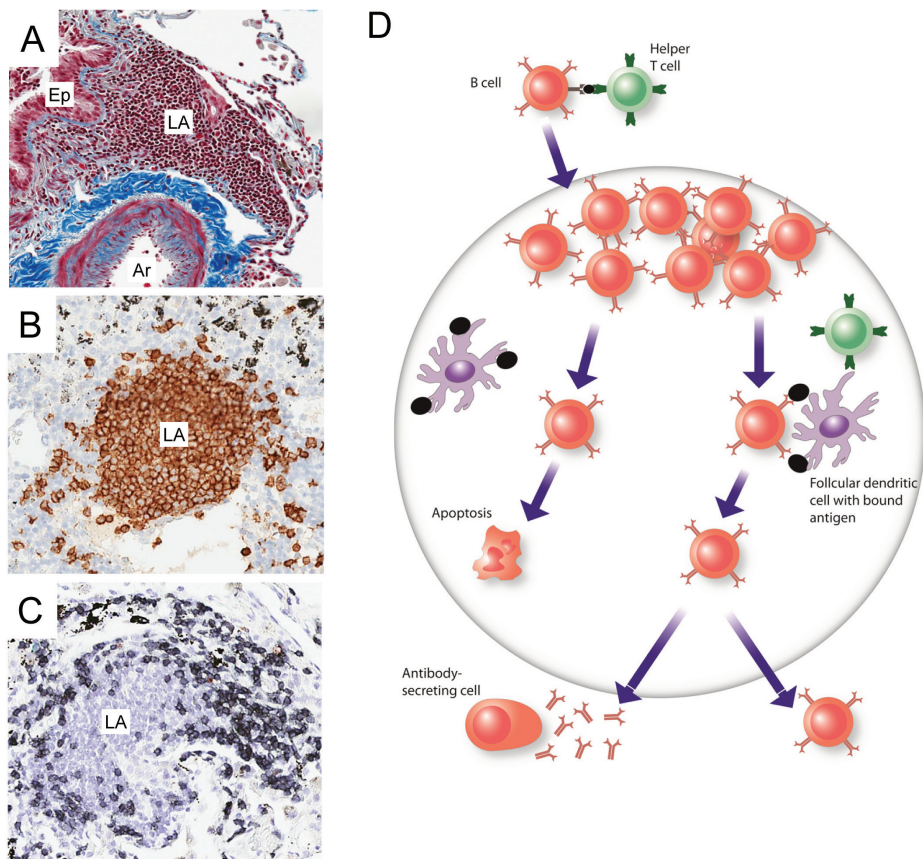


Figure 7
 (A) A Masson's trichrome-stained tissue section illustrating a lymphoid aggregate (LA) in the wall of a bronchiole from a patient with very severe COPD. Ar, bronchiole-associated artery. Ep, bronchiolar epithelium. (B) Lymphoid aggregates mainly consist of CD20⁺ B cells (brown stain) that form the so-called B cell area. (C) CD3⁺ T cells (black stain, including CD4⁺ and CD8⁺ T cells) are present in the T cell area. In B-C, cell nuclei are counterstained with Mayer's haematoxylin (blue stain). (D) The sequence of immune events taking place in LAs. Interactions between antigen-activated T cells and B cells, and follicular dendritic cells result in the generation of memory B cells and antibody producing plasma cells. (Photos by M. Mori. Figure 6D by C. Andersson, adapted from reference¹³⁸).

There is also evidence that other immune cells are present in the LAs and that the cellular composition of LAs is changed in COPD. For example, a histological study revealed that the LA density of CD57⁺ cells was increased in patients with COPD compared with non-smokers and healthy smokers¹²⁵. Moreover, another study observed an increase in FoxP3⁺CD4⁺ T cells in LAs of patients with moderate COPD compared with control subjects, and suggested that these cells may prevent autoimmune responses in LAs¹²³. Plasmacytoid DCs were also identified in bronchiolar LAs¹¹⁵, which is in agreement with previous observations that plasmacytoid DCs are mainly found in lymphoid tissues⁹⁶. Importantly, the number

of plasmacytoid DCs in LAs increased in patients with mild to moderate COPD but not in patients with severe stages of COPD. Considering the tolerogenic and antiviral functions of plasmacytoid DCs, it was suggested that the reduced number in LAs of patients with severe COPD could result in loss of tolerance and impaired defence against viral infections¹¹⁵.

Formation of lymphoid aggregates

Ectopic lymphoid tissues are commonly observed in several inflammatory and infectious diseases as well as autoimmune diseases^{130,136}. In the lung, LAs have been observed in patients with asthma¹⁴², tuberculosis¹⁴³, cystic fibrosis¹⁴⁴, idiopathic pulmonary fibrosis¹⁴⁵, and idiopathic pulmonary arterial hypertension¹⁴⁰. The formation of LAs, a process termed lymphoid neogenesis, begins after chronic exposure to antigen and is followed by an accumulation of T cells, B cells and DCs¹³⁰ (figure 8). The accumulation of cell infiltrates involves interactions between immune cells and resident stromal cells, as well as cytokines and chemokines. Briefly, the cytokine lymphotoxin $\alpha_1\beta_2$ expressed on activated lymphocytes binds to the lymphotoxin β receptor on stromal cells. The activation of lymphotoxin signalling in stromal cells stimulates the release of homeostatic chemokines that recruit naïve lymphocytes as well as DCs. The chemokine ligands CXCL12 and CXCL13 attract B cells expressing their receptors CXCR4 and CXCR5, respectively. In addition, the chemokines CCL19 and CCL21 attract CCR7⁺ naïve T cells and mature DCs to the T cell area^{130,131}. Eventually, these cellular accumulations evolve into organised aggregates with distinct B cell areas and T cell areas, follicular DCs and HEVs¹³⁰.

The exact mechanisms involved in the development of LAs in COPD remain unknown. However, several factors have been identified that may contribute to the LA formation and/or maintenance. For example, studies have shown that CXCL13 is expressed in B cell areas in LAs of patients with COPD^{146,147}. Furthermore, treatment with anti-CXCL13 antibodies prevented smoke-induced LA formation *in vivo*¹⁴⁶. A recent study further demonstrated that IL-17 induced the expression of CXCL13 and was critical for the initial formation of LAs *in vivo*¹⁴⁸. Moreover, increased expression of the cytokine B cell activating factor, which mediates lymphocyte survival and proliferation, has been observed in LAs in COPD compared with control subjects¹²⁴. IP-10/CXCL10 and Mig/CXCL9, both of which are present in lung LAs in COPD, may also contribute to the LA formation by recruiting CXCR3⁺ lymphocytes¹⁴⁹.

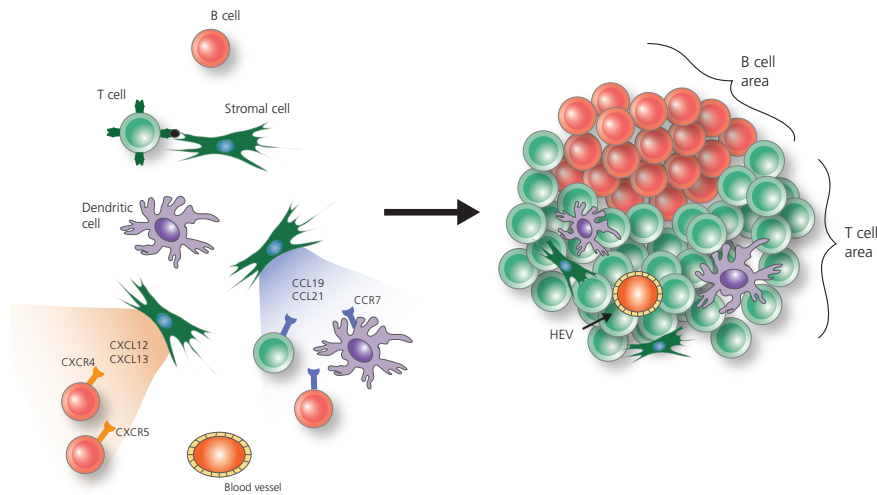


Figure 8

In chronically inflamed tissues complex interactions between immune cells and resident stromal cells stimulate the release of lymphoid chemokines, including CXCL12, CXCL13, CCL19 and CCL21. These chemokines are involved in the recruitment of B cells, T cells and dendritic cells. The accumulation of immune cells eventually evolves into a highly organized lymphoid aggregate with distinct B cell and T cell areas. (Figure by C. Andersson, adapted from reference¹³¹).

Adaptive immune responses in COPD: good or bad?

The antigen(s) triggering the LAs in COPD have not been identified and consequently the role of LAs in COPD is controversial¹³¹. One evident source of antigen may derive from pathogens infecting the lower respiratory tract, especially in patients with severe COPD^{18,131}. In support of this, lung LAs developed in mice infected with *Haemophilus influenzae*¹⁵⁰. In this case the immune responses initiated in LAs may be protective and the formation of LAs may be induced to counteract infections. Moreover, reduced numbers of bronchioles containing LAs were observed in lung resection specimens of COPD patients receiving inhaled and/or oral steroids compared with COPD patients not treated with steroids¹⁵¹. It was later hypothesised that the increased risk of pneumonias in steroid-treated COPD patients may partially be associated with this reduction of lung LA numbers¹³¹. Antigens may also originate from cigarette smoke components¹³¹. For example, chronic cigarette smoke exposure induced lung LA formation in pathogen-free mice⁶⁴. Furthermore, smoke-induced tissue injury may release newly created self-epitopes or altered self-epitopes that may initiate harmful autoimmune responses in LAs⁶⁴. Of note, several autoimmune diseases are associated with the formation of LAs¹³⁶. In addition, elevated levels of antibodies to self-antigens have been found

in COPD, indicating an autoimmune component in COPD⁴⁰. Taken together, it remains to be established whether the overall effects of LAs in COPD are beneficial or harmful in the pathogenesis of COPD.

Lymphatic vessels

Lymphatic vessels are important for extracellular fluid balance and leukocyte trafficking from peripheral tissue to draining lymph node. Whereas lymphatic vessels have been extensively studied *in vivo*, studies on human lymphatic vessels have been hampered by the lack of immunohistochemical markers that discriminate between lymphatic endothelial cells and endothelial cells in blood vessels. However, in the recent past several lymphatic-specific markers have been identified. Although there are some remaining questions whether or not these markers are specific for lung lymphatic vessels, lymphatic vessels have been described in the pleura and along conducting airways as well as major blood vessels¹⁵². The presence of lymphatic vessels in the alveolar parenchyma, however, remains subject to debate since some studies have detected alveolar lymphatic vessels^{152,153} whereas others have not^{154,155}.

Characteristic features and markers for lymphatic endothelium

Lymphatic vessels are structurally very different from blood vessels. In contrast to blood vessels, peripheral lymphatic vessels are blind-ended vessels with a thin vessel wall consisting of a single layer of non-fenestrated lymphatic endothelial cells. The peripheral lymphatic endothelium is also characterised by frequent gaps between the endothelial cells and have no or only a discontinuous basement membrane. The endothelium of the lymphatic vessels is not covered by pericytes or smooth muscle cells, instead the endothelial cells are directly attached to the extracellular matrix via elastic fibres. This allows vessel dilatation as well as preventing the vessels from collapsing. Peripheral lymphatic vessels drain into collecting lymphatic vessels, which are characterised by a smooth muscle layer, continuous basement membrane and valves that prevent back flow of lymph fluid. The lymph fluid is then transported through the collecting lymphatics into the venous circulation¹⁵⁶⁻¹⁵⁸. Commonly used immunohistochemical markers for lymphatic endothelial cells include Prox1, LYVE-1 and podoplanin. Prox1 (Prospero-related homeobox transcription factor) is a transcription factor expressed in lymphatic endothelial cell nuclei only¹⁵⁹. LYVE-1 (lymphatic vessel endothelial hyaluronan receptor 1) is a transmembrane receptor for glycosaminoglycan hyaluronan and is the most commonly used markers of lymphatic endothelial cells, especially peripheral lymphatic vessels^{159,160}. However, LYVE-1 is also expressed on blood vessels, such as lung capillaries and pulmonary

arteries in mice, as well as murine macrophages¹⁵⁹. In addition, proinflammatory cytokines may downregulate the expression of LYVE-1 *in vivo*¹⁶¹. Podoplanin, a mucin-type transmembrane glycoprotein, is also a commonly used marker for lymphatic vessels^{159,162}. The expression of podoplanin is, however, not restricted to lymphatic vessels. Other cells also expressing podoplanin include type I epithelial cells of the lung, epithelial basal cells, myoepithelial cells, mesothelial cells, stromal reticular cells and follicular DCs^{159,163}.

Lymphatic vessels in lung diseases

During inflammation and wound healing, new lymphatic vessels are developed in a process termed lymphangiogenesis, which usually follows angiogenesis¹⁶⁴. This usually involves sprouting of existing lymphatic vessels¹⁵⁸. Whereas inflammation-induced lymphangiogenesis in the lung has been extensively studied in *in vivo* models¹⁶⁴⁻¹⁶⁶, few studies have investigated lymphatic vessel changes in human lung diseases, especially in chronic inflammatory conditions. It is however clear that lymphatic vessels are indeed altered in several lung diseases. For example, recently it was observed that lymphangiogenesis occurs in fibrotic parenchyma of patients with idiopathic pulmonary fibrosis¹⁵⁵. Other studies have revealed a close association between lymphatic vessels and pulmonary sarcoid granulomas¹⁶⁷. Notably, reduced number of lymphatic vessels was reported in conducting airways of subjects who died of severe asthma¹⁶⁸. Moreover, in a recent semi quantitative study that investigated lymphatic vessels in peripheral lungs of patients with moderate COPD it was noted that there was a general increase in the extent of lymphatic vessels¹⁶⁹. However, it still remains unknown whether this increase was different between the lung compartments.

Several mediators may contribute to the formation of new lymphatic vessels in inflammation. Macrophages secrete lymphangiogenic factors such as vascular endothelial growth factor (VEGF)-C and VEGF-D that activate VEGFR-3, which is expressed on lymphatic vessels, and stimulate lymphangiogenesis^{164,170,171}. In a mouse model of airway inflammation, inhibition of lymphangiogenesis through VEGFR-3 resulted in mucosal oedema¹⁶⁴. In the same study, lymphatic vessels, but not blood vessels, persisted after resolution of inflammation. Moreover, proinflammatory cytokines, including TNF- α , induce the expression of VEGF-C¹⁷². A recent study also suggested that TNF- α contributes to lymphangiogenesis in airways *in vivo*¹⁶⁶. Interestingly, under certain circumstances it seems that macrophages may have the ability to transdifferentiate into lymphatic endothelial cells and thus directly contribute to the formation of new lymphatic vessels^{155,173}.

Lymphatic vessels and their role in cellular trafficking

Lymphatic vessels not only have a critical role in inflammation by decreasing oedema, but also regulate immune responses by transporting fluid, antigens and selected leukocyte populations from the site of inflammation to draining lymph node^{174,175}. Thus, lymphatic vessels are important in the initiation of adaptive immune responses. The cell types typically present in the lymph fluid include antigen-activated DCs, monocytes and T cells (naïve/memory/effector)¹⁷⁶⁻¹⁷⁹. The migration of these cells into the peripheral lymphatic vessels is mediated through interactions between the chemokine receptor CCR7 and its chemokine ligand CCL21 (also referred to as secondary lymphoid chemokine) (see figure 4). CCL21 is secreted from the lymphatic endothelial cells and acts as a chemotactic ligand for CCR7-expressing DCs, monocytes and T cells and mediates their transport through lymphatic vessels to the lymph node¹⁸⁰⁻¹⁸³. The chemokine receptor D6 is also expressed on lymphatic endothelial cells, but in contrast to other chemokine receptors D6 is a non-signalling receptor and acts as a scavenger for inflammatory chemokines¹⁸⁴⁻¹⁸⁶. Hence, D6 is important for the clearance of inflammatory chemokines on the lymphatic endothelial surfaces and, thus, allows the chemoattractant gradient of CCL21 to act free on CCR7⁺ cells¹⁸⁷. Moreover, studies indicate that the expression of lymphatic CCL21 and D6 may be induced in inflammation^{188,189}. However, whether CCL21 and D6 are expressed on human lung lymphatic vessels and if the expression is changed in COPD remain unknown. Furthermore, in COPD it can also be speculated that lymphatic vessels may transport antigens and DCs to LAs rather than lymph nodes. In support of this, lymphatic vessels have been observed in close proximity to ectopic LAs in the lung^{125,134}.

Aims

The overall aim of this thesis work was to explore the structural and immunological basis for alveolar antigen uptake and adaptive immune responses in the human peripheral lung through detailed immunohistochemical assessments of tissue samples obtained from control subjects and patients with different severities of COPD.

The specific aims of the studies presented in this thesis were:

- To investigate the anatomical localisation of lymphoid aggregates in COPD lungs and perform the first exploration of the interfaces between the alveolar lumen and lymphoid aggregates (paper I)
- To investigate the distribution of lymphatic vessels in peripheral lung compartments of control subjects and patients with COPD, and to seek evidence for an increased immunological activity among lymphatic vessels in COPD lungs (paper II)
- To identify and investigate the distribution as well as marker expression of dendritic cell populations in peripheral lung compartments of patients with COPD (paper III)

Study Subjects and Methods

This section provides a description of the subjects included and an overview of the methods used in this thesis. Further methodological details are presented in the individual papers I-III.

Lung tissue collection

Lung resection specimens from never smokers, smokers without COPD, and patients with GOLD stage I-III COPD were obtained from subjects who underwent lobectomy for lung cancer. A lobectomy is a surgical procedure that removes one lobe of the lung that contains cancerous cells. Only subjects with solitary, well-delineated tumours were included in the study and peripheral lung tissue was collected as far from the tumour as possible to avoid cancerous tissue in any of the samples. Due to difficulty of sampling the peripheral lung, this procedure has repeatedly been used to obtain lung tissue samples^{57,103,115,123,125,190}. Lung tissue from patients with GOLD stage IV COPD was obtained from explant lungs from patients who required lung transplantation (Skåne University Hospital, Lund, Sweden). The patients in the GOLD stage IV cohort did not have lung cancer. All subjects gave written informed consent to participate in the study, which was approved by the local ethics committee in Lund, Sweden.

In total, 31 patients with COPD and 15 control subjects were included. The patients were classified as having COPD when FEV_1/FVC was less than 0.70. Patients with COPD were then grouped into three cohorts based on their disease severity as assessed by FEV_1 % of predicted (according to GOLD criteria⁶); mild COPD (GOLD stage I, $n = 6$), moderate to severe COPD (GOLD stage II-III, $n = 15$), and very severe COPD (GOLD stage IV, $n = 10$). The patients with GOLD stage III COPD ($n = 2$; median value of FEV_1 % of predicted, 44.5%) were rare and, therefore, the tissue samples obtained from these patients were combined with the

samples of the GOLD stage II group. Control subjects with normal lung function were grouped into never smokers (n = 8) and ex-/current smokers (n = 7). The subject characteristics are presented in Table 1.

Table 1. Overview of subject characteristics

	Never smokers	Smokers w/o COPD	GOLD I COPD	GOLD II-III* COPD	GOLD IV [†] COPD
Lung tissue collection	Resection	Resection	Resection	Resection	Transplantation
Total number of subjects	8	7	6	15	10
Total number of tissue blocks analysed	16	14	12	30	30
Gender, men/women	2/6	3/4	4/2	12/3	4/6
Age, years	66 (33-76) [#]	56 (47-68)	68 (56-75)	67 (53-77)	62 (53-66)
Smoking history, pack-years	0	36 (20-80)	39 (25-66)	46 (16-65)	43 (25-60)
Smoking status, ex/current	-	3/4	3/3	10/5	10/0
FEV ₁ , % of predicted	109 (82-141)	95 (82-120)	86 (80-95)	69 (43-78)	23 (15-27)
FEV ₁ /(F)VC, %	82 (66-121)	76 (71-88)	67 (65-70)	58 (41-68)	32 (20-39)
Inhaled β_2 agonists					
Short acting (yes)	-	-	1	2	4
Long acting (yes)	-	-	-	-	4
Inhaled anticholinergics					
Short acting (yes)	-	-	1	2	3
Long acting (yes)	-	-	-	-	5
Inhaled short acting β_2 agonist plus anticholinergics (yes)	-	-	-	-	3
Corticosteroids					
Inhaled (yes)	-	-	-	1	2
Oral (yes)	-	-	-	-	2
Inhaled long acting β_2 agonist plus corticosteroids (yes)	-	-	-	1	7
Mucolytics (yes)	-	-	2	-	5

Values are median (range) or n. * Two patients with GOLD stage III COPD. [†] One patient with unknown medical history. [#] The mean value of the study group is 63 years. COPD, chronic obstructive pulmonary disease; FEV₁, forced expiratory volume in 1 second; (F)VC, (forced) vital capacity; GOLD, Global Initiative for Chronic Obstructive Lung Disease⁶. The severity of COPD is classified into four different stages: GOLD stage I (mild COPD), stage II (moderate COPD), stage III (severe COPD), and stage IV (very severe COPD).

Tissue processing

Tissue samples were immediately immersed in 4% buffered formaldehyde (4% formalin in phosphate buffer, pH 7.2) and fixed for 24 h at room temperature. Samples were then rinsed in 70% ethanol, dehydrated through graded alcohols and xylene, and embedded in paraffin wax.

Histological staining

Mayer's haematoxylin and eosin staining

Tissue sections were stained with Mayer's haematoxylin and eosin. The haematoxylin, a basic dye, stains cell nuclei blue and eosin, an acidic dye, stains cytoplasm pink/red. Stained sections were histologically examined in a blinded manner, and tissue blocks containing all anatomical regions (i.e. bronchioles, large blood vessels and alveolar parenchyma) were selected and used in further analyses. Two to three tissue blocks from separated peripheral lung regions were selected for each subject and multiple serial sections of 4 µm in thickness were generated and then stored at 4°C. Furthermore, Mayer's haematoxylin alone was used as a nuclear counterstain after completion of immunohistochemical staining.

Masson's trichrome staining

In paper I, LAs were assessed on Masson's trichrome-stained sections. Masson's trichrome stains cell nuclei black, connective tissue blue, and muscle fibres, erythrocytes and cytoplasm red. With this stain, LAs were readily recognised and distinguished from other lung structures, such as airways, blood vessels and lung parenchyma.

Immunohistochemistry

Immunohistochemistry is a method based on antibody-antigen binding *in situ* in tissue sections. In this work, several immunohistochemical protocols as outlined below were used to visualise antibody-antigen binding. The choice of staining technique depended on the type of experiment (single or double staining), the type of imaging (bright-field or fluorescence), and the sensitivity of immunohistochemical detection. Briefly, in most experiments, the secondary antibodies used were conjugated to enzymes, horseradish peroxidase (HRP) or alkaline phosphatase (AP), and detected by the addition of chromogenic enzyme-specific substrates, 3,3'-diaminobenzidine (DAB) or Permanent Red, respectively. In double-staining experiments, HRP/DAB (brown stain) and AP/Permanent Red (red stain) were used to visualise two types of antigens on one slide. Multi-staining immunofluorescence was performed using secondary antibodies conjugated to fluorochromes, such as Alexa Fluor 488, 555 or 647. When needed, various amplification methods were applied to enhance the signal from the primary antibody. For example, biotinylated antibodies and subsequent incubation with streptavidin (conjugated to enzymes or fluorochromes)

were applied to increase the detection signal. Also, secondary antibodies conjugated to an enzyme-labelled polymer dextran backbone (polymer/HRP or polymer/AP) were used to increase the sensitivity of the immunohistochemical detection.

The primary antibodies used in this thesis work have been validated extensively, in our laboratory and by others, for use on paraffin sections. The antigen retrieval protocol, antibody concentrations and blocking steps were optimized for each antibody. Optimisation was carried out on positive tissue controls, most often lymph nodes and lung tissue, to ensure that the primary antibody produced a reliable staining which was in accordance with previous studies. On a separate slide, non-specific staining of the secondary antibody was evaluated by omitting the primary antibody. All immunohistochemical stainings were carried out in an automated slide stainer (Autostainer Plus, DakoCytomation, Glostrup, Denmark).

Antigen retrieval

Formalin-paraffin processing of tissue may alter the structure of some tissue proteins, thus making them inaccessible to antibody binding. To unmask antigenic sites, sections were subjected to heat-induced antigen retrieval prior to immunohistochemical staining. The heat-induced antigen retrieval was performed in a pressure cooker (at 120°C for 20 min; Prestige Medical Ltd., Blackburn, England) or water bath (at 97°C for 20 min; Dako PTLINK instrument, Dako, Glostrup, Denmark) using an unmasking retrieval solution suitable for the antibody of interest.

Single immunohistochemical staining

In the standard protocol, single staining immunohistochemistry was performed using Dako EnVision Peroxidase/DAB Detection System kit (K5007). Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide, followed by incubation with primary antibodies for 1 h. Bound antibodies were detected using polymer/HRP-conjugated secondary antibodies, and visualized with DAB (brown) and bright-field microscope. In paper I and III, an additional incubation in EnVision Flex+ Mouse (Linker) (Dako, K8021) was performed to amplify the signal of bound BDCA-2 mouse monoclonal antibodies and the DAB incubation time was increased to 40 min to further increase the sensitivity of the BDCA-2 detection.

Double immunohistochemical staining

In the standard protocol, double staining immunohistochemistry was performed using Dako EnVision Doublestain System kit (K5361). Endogenous peroxidase and

alkaline phosphatase activity was blocked. The first primary antibody was applied for 1 h, and detected with polymer/HRP-conjugated secondary antibodies and DAB (brown stain). After rinsing off excess DAB, sections were incubated with Dako Double Stain Blocking Reagent, which chemically destroys the antigenicity of the first primary antibody to prevent additional binding of secondary antibodies. The second primary antibody was then applied for 1 h, and detected with polymer/AP-conjugated secondary antibodies and Permanent Red (red stain).

In paper III, the double staining protocol (described above) for detection of CD11c⁺ DCs was modified to obscure confounding CD11c-expressing monocytes and/or macrophages (see section below). A mixture of primary antibodies directed against CD68 and CD163 were applied followed by labelling with polymer/HRP-conjugated secondary antibodies and DAB. The high expression levels of CD68 and CD163 in monocytes/macrophages yield extensive formation of dark brown DAB complexes on the cell surfaces that hinder additional binding of CD11c antibodies. After incubation with Double Stain Blocking Reagent, the second primary antibody against CD11c was applied, and detected with polymer/AP-conjugated secondary antibodies and Permanent Red. Thus, monocytes/macrophages appeared dark brown and CD11c bright red.

In paper I and II, an avidin-biotin/streptavidin detection system was applied to increase the detection signals of langerin, CCL21 and D6. In these experiments, the first primary antibody was detected with polymer/HRP-conjugated secondary antibodies using DAB (K5007, Dako). After incubation with DAB, a denaturing solution (DNS001H, Biocare Medical, Concord, California, USA) was applied to prevent additional binding to the first primary antibody. Additional blocking steps with Dako Protein Block Serum Free (X0909) and Dako Avidin/Biotin Blocking solution (X0590) were performed to reduce non-specific binding and block endogenous avidin-biotin activity, respectively. The second primary antibody (anti-langerin, anti-CCL21 or anti-D6) was then applied and detected with appropriate biotinylated secondary antibodies (Vector Laboratories, Burlingame, California, USA), streptavidin-AP (D0396, Dako), and Permanent Red.

In paper I and II, antibodies directed against human podoplanin were detected with polymer/HRP-conjugated secondary antibodies and DAB (K5007, Dako), and AP-conjugated anti- α -smooth muscle actin (α -SMA) antibodies were detected with Permanent Red.

Immunofluorescence staining

In paper I, double staining immunofluorescence was performed using an avidin-biotin/streptavidin detection system. The sections were blocked with Dako Protein Block Serum Free (X0909) and Dako Avidin/Biotin Blocking solution (X0590). The first primary antibody was applied followed by labelling with biotinylated secondary antibodies and Alexa Fluor 555-conjugated streptavidin (Molecular Probes). A denaturing solution (DNS001H) was applied to prevent additional binding to the first primary antibody, followed by incubation with the second primary antibody and visualization with Alexa Fluor 488-conjugated secondary antibody.

In paper III, triple staining immunofluorescence was performed by applying a mixture of primary antibodies of different IgG subclasses (IgG2a, IgG2b and IgG3), followed by a mixture of subclass specific secondary goat-anti mouse antibodies labelled with Alexa Fluor 647, Alexa Fluor 555 and Alexa Fluor 488. DNA-binding Hoechst (H33342) was used as nuclear staining.

Tissue analysis

Digitalisation and generation of virtual slides

A virtual slide is a high-resolution digital image file of an entire microscope glass slide that can be viewed on a computer screen. In this work, the whole lung tissue area on each slide (bright-field) was digitised in a whole-slide scanner (20X/40X objective, ScanScope, Aperio Technologies, Vista, California, USA). In all studies, manual counting and morphometric measurements, including perimeter and area, were performed on the whole-slide images using Aperio ImageScope software.

Investigation of peripheral lung compartments

The main advantage of working with surgical samples is that multiple lung compartments can be examined on the same section and disease-related changes across lung compartments can be assessed. For each subject, the large section area enabled examination of major lung compartments of the peripheral lung (figure 9). On each section, all bronchioles and bronchiole-associated arteries, multiple solitary blood vessels, and large areas of alveolar parenchyma were examined. The definitions of the analysed structures are outlined below:

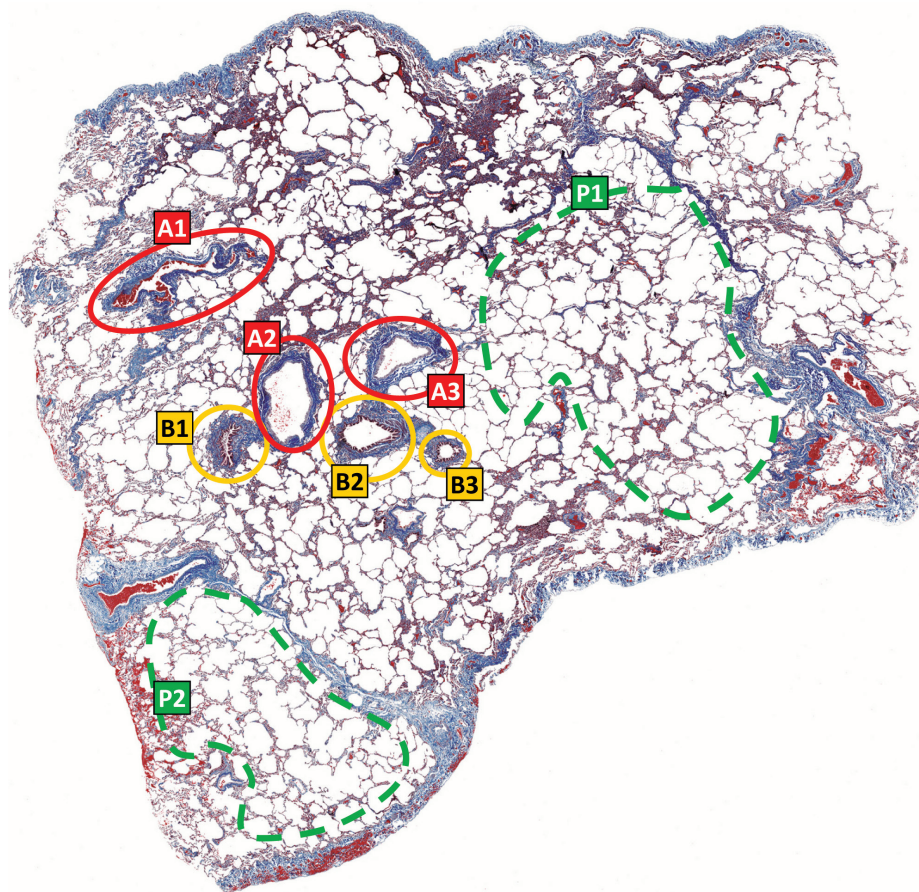


Figure 9
Low magnification overview of a Masson's trichrome-stained tissue section from a patient with very severe COPD. The major lung compartments of the peripheral lung are marked as follows, bronchioles "B1-B3", bronchiole-associated arteries "A1-A3", regions of alveolar parenchyma "P1-P2". (Figure by M. Mori).

Bronchioles

Bronchioles were defined as non-cartilaginous airways with an internal cross-sectional diameter of <2 mm. In paper II-III, the basement membrane perimeter was assessed by manual cursor tracing and the area of the subepithelial tissue was measured by manually tracing the region extending from the basement membrane to the parenchyma.

Bronchiole-associated arteries

In paper II, the area of the adventitia, the outermost part of the arterial wall, was measured by manually tracing the region extending from the outer border of the tunica media to the alveolar parenchyma.

Solitary blood vessels

Solitary blood vessels were defined as vessels at a distance from bronchioles and bronchiole-associated arteries. In paper I, vessels with a mean cross-sectional internal lumen diameter of $>140\ \mu\text{m}$ were investigated. In paper II, solitary blood vessels were subgrouped based on their internal lumen diameter: $<50\ \mu\text{m}$, $50\text{-}100\ \mu\text{m}$ or $>100\ \mu\text{m}$.

Alveolar parenchyma

The alveolar regions investigated were non-fibrotic and well separated from bronchioles, blood vessels and pleural tissue. Multiple regions of alveolar tissue were manually delineated and the area of the tissue, excluding airspaces, was calculated using Aperio ImageScope.

Quantification of the immunostaining

Automated computerised image analysis permits a rapid and reproducible quantification of immunostainings. Also, this method is useful when it is too time-consuming to manually count cells, such as B cells in LAs. In order to minimise variations in staining intensity, all immunostainings were carried out in an automated slide stainer and stainings were performed in batches containing sections from control subjects and patients with COPD. The image analyses were performed on whole-slide images using Aperio ImageScope software or Visiormorph-DP software (Visiopharm, Hoersholm, Denmark). For each staining, a fixed pixel threshold algorithm was set to calculate the number of pixels corresponding to the immunostaining (i.e. immunopositive) (exemplified in figure 10). The number of immunopositive pixels were expressed either as a percentage of the total number of pixels in the tissue or per lymphatic vessel perimeter. In paper III, the area of the CD11c immunostaining was calculated and normalised to the total area or length of the investigated structure.

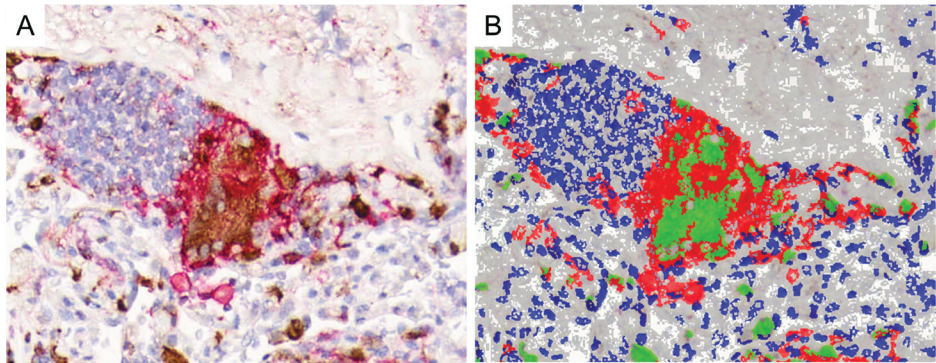


Figure 10

(A) Tissue section double stained for macrophages (CD68⁺ and/or CD163⁺, brown stain) and CD11c⁺ dendritic cells (red stain). Cell nuclei are counterstained with Mayer's haematoxylin (blue stain) (B) Corresponding computerised colour segmentation on the same tissue region using the Visiomorph-DP software and where the structures and immunoreactivity have been replaced with pseudo colours: nuclei in blue, background tissue in grey, CD68⁺ and/or CD163⁺ in green and CD11c⁺ immunoreactivity in red. (Figure by C. Sandén).

New approach for improved cell phenotyping through combined positive and negative selection immunohistochemistry

The present thesis work has applied a methodological innovation, which takes advantage of the physical blocking properties by chromogenic DAB complexes (paper III). This approach, which is described in detail in the online supplement of paper III was used to exclude confounding langerin⁺ and CD68 and/or CD163-expressing cells in order to detect CD1a⁺langerin⁻ and myeloid CD11c⁺CD68⁻CD163⁻ DCs, respectively. While this approach has limitations, the possibility to exclude confounding cells by negative selection offers significant advantages compared to current approaches that normally use single marker detection. Importantly, with this new approach it is not possible to exclude that minor immunoreactivity for the confounding cell marker is also hidden behind the red chromogen used to detect the cell of interest. For example, some of the red CD11c-expressing cells could theoretically have minor immunoreactivity for CD68 and/or CD163 (see figure 10). In any case the level of immunoreactivity would be under the threshold to justify the cells as CD68⁺ and/or CD163⁺. Hence, technically the red cells can thus be regarded as CD11c⁺CD68^{low}CD163^{low} and, in similarity to the nomenclature used in the field of flow cytometry (FACS), this should be regarded as a valid and useful way to phenotype cells.

Serial section-based three-dimensional reconstruction

Three-dimensional reconstructions were generated to reveal the spatial distribution of LAs in the peripheral lung. A series of consecutive 4-micron-thick paraffin sections were stained with Mayer's haematoxylin and eosin, or immunostained for the lymphatic vessel marker podoplanin. Serial images of LAs were captured and manually aligned, and structure-specific segmentation data were generated to create a 3D image in Amira V.5.4.2 software (Visage Imaging Inc., San Diego, California, USA).

Statistical analyses

Non-parametric analyses were applied in all studies. The Kruskal-Wallis test followed by Dunn's multiple comparisons post test was applied for analysis of differences between study groups and the Mann-Whitney rank sum test for analysis between two groups. Correlations were calculated using Spearman's rank method. All statistical analyses were performed using GraphPad Prism V.5.0 (GraphPad Software, San Diego, California, USA).

Summary of Results

Appearance of remodelled and dendritic cell-rich alveolar-lymphoid interfaces provides a structural basis for increased alveolar antigen uptake in COPD

Previous studies exploring LAs in COPD have demonstrated the presence of LAs in relation to bronchioles and the lung parenchyma^{18,64,123,125,149}. Whereas LAs located in the wall of bronchioles may respond to airway luminal antigens, it has remained unknown if LAs acquire antigens from the alveolar lumen. In paper I, we performed a quantitative analysis of LAs in different anatomical locations, and explored the interfaces between the alveolar lumen and LAs in lungs of control subjects and patients with different severities of COPD.

Rich occurrence of LAs in non-airway regions in COPD

Our initial observation was that LAs, defined here as more than 50 contiguous lymphoid cells, displayed a widespread distribution being present in all major peripheral lung compartments. To compare LAs in different lung compartments, we sub-grouped LAs based on their anatomical location: bronchiolar-associated lymphoid tissue (BRALT), vascular-associated lymphoid tissue (VALT) and alveolar-only lymphoid tissue (ALT). Both BRALT and VALT structures had an almost exclusive adventitial distribution. Across all study groups, only an average of 18% of the BRALTs was present just beneath the bronchiolar epithelium. We further showed a rich occurrence of LAs in non-airway regions (figure 11A). The general expansion of LAs, as assessed by total tissue CD20⁺ B cells, correlated with the severity of COPD. Using three-dimensional analysis we were able to confirm the spatial distribution of lung LAs. In COPD, approximately 25% of the LAs were true ALTs, 31% VALTs and the remaining 43% were BRALTs.

Similar cellular composition among anatomically different LAs in COPD

All LAs investigated consisted of a CD20⁺ B cell area surrounded by a CD4⁺/CD8⁺ T cell area. Other leukocytes such as regulatory T cells, plasma cells, neutrophils, monocytes/macrophages were also scattered within the LAs (Table 2). In COPD, the increased presence of Ki-67⁺ cell clusters and CD21⁺ follicular DCs in the B cell areas were indicative of mature LAs. In addition, the immunoreactivity for the B cell chemoattractant CXCL13 was increased in LAs in patients with very severe COPD compared with never-smoking controls (Table 2). There were no differences in the cellular composition or CXCL13 expression among the different types of LAs in COPD.

Table 2. Cellular composition of lymphoid aggregates in all lung compartments

	Never smokers (n=8)	GOLD IV COPD (n=10)	P-value
B cell (CD20 ⁺)	38 (15-77)	66 (28-77)	0.16
T cell (CD4 ⁺)	0.9 (0.3-2)	3 (0.5-9)	0.09
T cell (CD8 ⁺)	33 (19-49)	26 (9-44)	0.51
Regulatory T cell (CD25 ⁺ FoxP3 ⁺)	0.4 (0-1.4)	1.2 (0-2.7)	0.17
Plasma cell (PC ⁺)	4 (1-6)	4 (2-7)	0.95
Neutrophils (MPO ⁺)	1 (1-3)	2 (0.6-4)	1.00
Eosinophils (EG2 ⁺)	0.4 (0.1-3)	0.3 (0.04-1)	0.21
Monocytes/macrophages (CD68 ⁺)	8 (6-14)	5 (2-13)	0.03
CXCL13	2 (1-9)	15 (7-32)	0.01

Values are expressed as % area immunostaining, median (range). Quantification of immunostaining was performed using Aperio ImageScope software.

LAs have interfaces with the alveolar lumen and lymphatic vessels

We also showed that irrespective of anatomical localisation and study group, the vast majority of the LAs had alveolar interfaces (figure 11B). Thus, most bronchiolar-associated LAs had a direct interface with the alveolar lumen and not the airway lumen. In addition, by three-dimensional reconstructions of podoplanin immunostained serial sections, we found an intricate network of lymphatic vessels surrounding the LAs (illustrated in figure 11C).

Remodelling of alveolar-lymphoid interface epithelium in COPD

Further, we observed that the cytokeratin⁺ epithelial layer that covered the LAs at the alveolar-lymphoid interfaces (illustrated in figure 11C) changed from a simple squamous epithelium in control subjects to a more cuboidal and columnar, non-ciliated epithelium in patients with moderate to very severe COPD. As for

the epithelium in conducting airways, both the columnar and cuboidal interface epithelium expressed TLR5.

Selective accumulation of DCs in alveolar-lymphoid interfaces in COPD

Although other DC subsets, including BDCA-2⁺, langerin⁺, CD1a⁺ and CD11c⁺ CD68⁻ CD163⁻ DCs, are present in the alveolar tissue (paper III), there was a selective accumulation of langerin⁺ DCs in alveolar-lymphoid interfaces. Patients with more severe stages of COPD had abundant numbers of interface langerin⁺ DCs compared

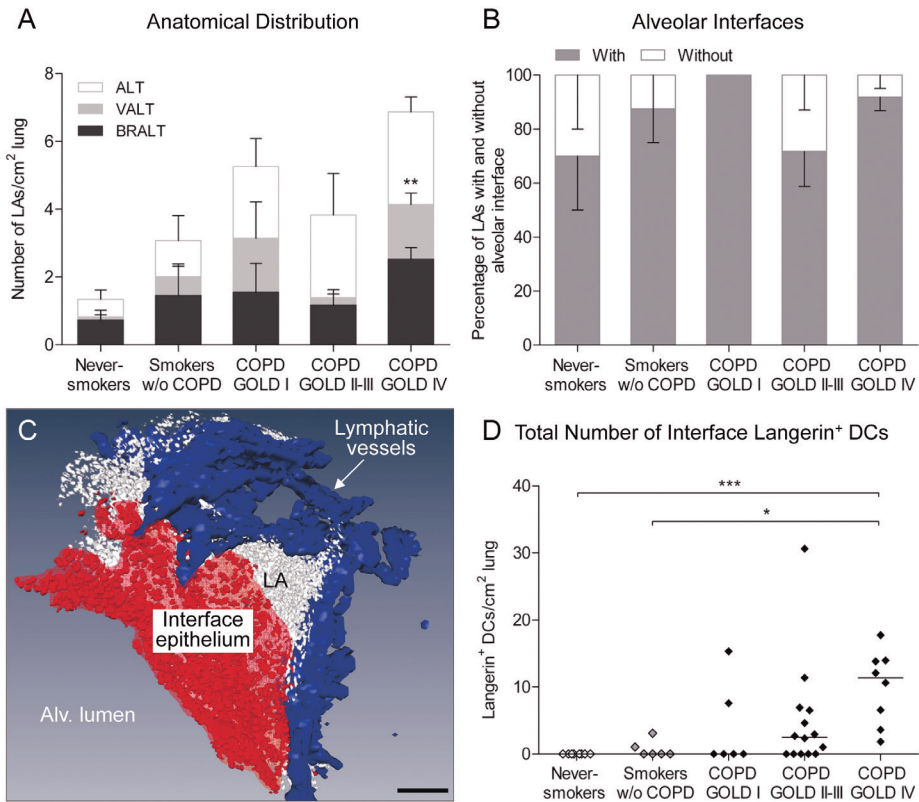


Figure 11

(A) Relative proportion of bronchiolar-associated (BRALT), vascular-associated (VALT), and alveolar-only associated (ALT) lymphoid tissue in the five study groups. Values are given as mean \pm SEM. (B) Percentage of LAs with and without alveolar interfaces, irrespective of anatomical localisation. Values are given as mean \pm SEM. (C) Computerised three-dimensional reconstructions of podoplanin-stained serial sections illustrating the spatial arrangement between the alveolar lumen, lymphoid interface epithelium (red), LA (white), and opposing network of podoplanin⁺ lymphatic vessels (blue). Scale bar: 25 μ m. (D) Total number of langerin⁺ DCs in the interface epithelium, as a combination of LA numbers per cm² lung tissue and mean number of interface langerin⁺ DCs (including squamous, cuboidal and columnar interface epithelium). Horizontal lines indicate medians for each study group.

to control subjects who had no or few such DCs (figure 11D). In COPD, more than 80% of the LA-associated langerin⁺ DCs were present within the interface epithelium or just beneath the interface epithelium and the numbers correlated inversely with lung function. In contrast to airway epithelial-associated langerin⁺ DCs, the interface DCs frequently displayed apical protrusions that reached the lumen. Additionally, several interface DCs had simultaneous contact with lymphoid B cells and T cells.

Increased number and altered phenotype of lymphatic vessels in peripheral lung compartments of patients with COPD

Since we observed a close relationship between LAs and lymphatic vessels, we next expanded the analysis of lung lymphatic vessels to major peripheral lung compartments (paper II). More specifically, we compared the distribution of lymphatic vessels in different lung compartments of patients with COPD and control subjects, and investigated the lymphatic expression of molecules involved in immune cell trafficking. In our experiments, immunostaining for podoplanin was restricted to vessels that lacked immunoreactivity for blood vessel-associated α -SMA, as opposed to LYVE-1 immunoreactivity, which occasionally was detected on pulmonary blood vessels. An antibody directed against Prox1 was further used to confirm the lymphatic identity of podoplanin immunopositive vessels.

Lymphatic vessels exist in all lung compartments including the alveoli

Here we showed that podoplanin⁺ lymphatic vessels were present in all major lung compartments of the peripheral lung, including the wall of bronchioles and blood vessels, and the alveolar parenchyma. Lymphatic vessels were foremost present in the adventitia layer of bronchioles, bronchiole-associated arteries and solitary blood vessels. Intra-alveolar lymphatic vessels were well separated from both bronchioles and α -SMA⁺ blood vessels (figure 12A). There was no difference in location of the lymphatic vessels in patients with COPD versus control subjects.

Expansion of alveolar lymphatic vessels in advanced COPD

Our detailed quantitative analysis, involving over 10 000 individual lymphatic vessels across the five study groups, revealed that the number of lymphatic vessels was significantly increased in the alveolar parenchyma of patients with very

severe COPD compared with never-smoking control subjects (figure 12B). We further showed that the absolute number of lymphatic vessels per bronchiole and bronchiole-associated artery was increased in patients with very severe COPD, but the numbers were not changed after normalisation to tissue area. Furthermore, there was no difference in the mean cross-sectional perimeter of individual lymphatic vessels among the study groups or lung compartments, and only few lymphatic vessels expressed the proliferation marker Ki-67.

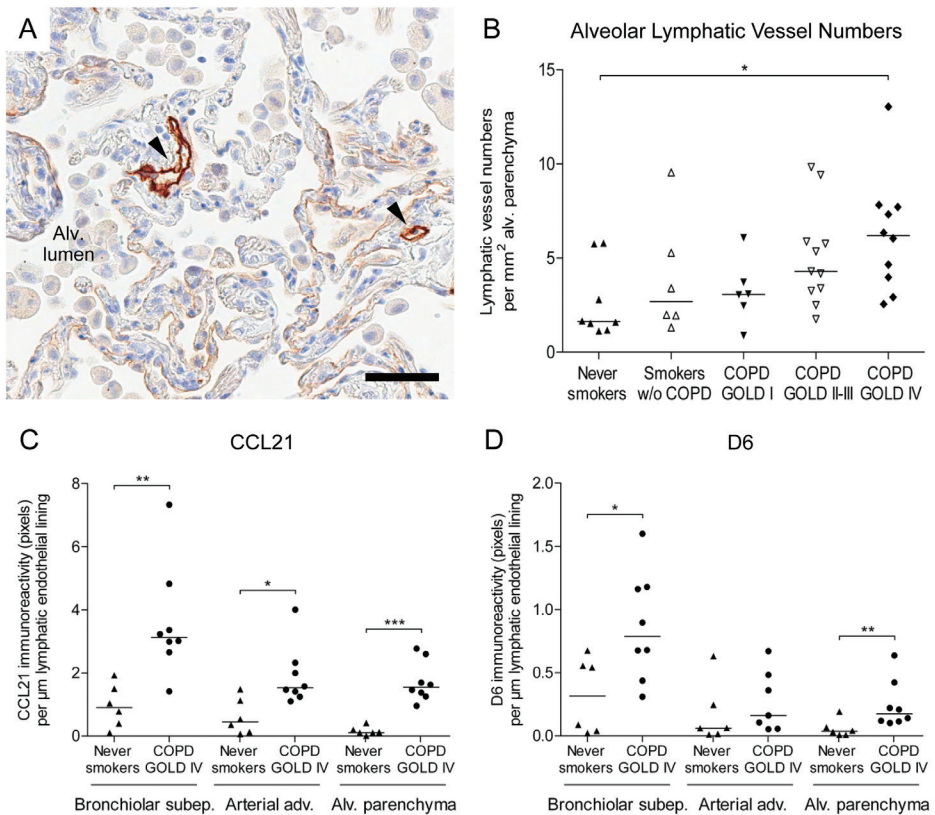


Figure 12
 (A) Podoplanin⁺ lymphatic vessels (brown stain, arrowheads) in the alveolar parenchyma in a section of a patient with very severe COPD. Cell nuclei are counterstained with Mayer's haematoxylin (blue stain). Scale bar: 50 µm. (B) Number of lymphatic vessels in the alveolar parenchyma in the five study groups. Horizontal lines indicate medians for each group. (C) Lymphatic vessel immunoreactivity for CCL21 and (D) lymphatic immunoreactivity for D6. In C-D, data are expressed as the total amount of immunoreactivity per lymphatic vessel endothelial length, in the subepithelial tissue of bronchioles (i.e. the entire wall), adventitia layer of bronchiole-associated arteries, and alveolar parenchyma. Horizontal lines indicate medians for each group.

Increased expression of lymphatic CCL21 and D6 in advanced COPD

We also showed that patients with very severe COPD had increased numbers of CCL21- and D6-immunopositive lymphatic vessels in the alveolar parenchyma compared with never-smoking controls. Computerised image analysis of all individual Prox1⁺ lymphatic vessels revealed further that lymphatic vessels displayed increased levels of CCL21 immunoreactivity in all three lung compartments; bronchioles, bronchiole-associated arteries, and alveolar parenchyma (figure 12C). Similarly, the lymphatic D6 immunoreactivity was increased in the walls of bronchioles and alveolar parenchyma (figure 12D). Further, we observed CCL21-immunopositive lymphatic vessels within LAs.

Immune cell content of lymphatic vessels in COPD

Lymphatic vessels containing luminal cells were observed in all lung compartments, including LAs. We showed that among the intra-lymphatic leukocytes were abundant CD8⁺ T cells and CD11c⁺ DCs, as well as scattered CD20⁺ B cells and CD57⁺ NK/T cells. CD68⁺ monocytes/macrophages were not detected within the lymphatic vessels.

Expansion of multiple dendritic cell populations in the alveolar parenchyma in patients with COPD

In paper I, we demonstrated that the majority of LAs have direct interfaces towards the alveolar lumen and that the alveolar-lymphoid interface epithelium in COPD is selectively infiltrated by langerin⁺ DCs. These findings together with the observation in paper II of increased number of alveolar lymphatic vessels expressing CCL21, which is involved in DC migration, indicate that the alveolar parenchyma is likely to be populated with DCs. To examine this we used known DC markers and combinations of markers to visualise DC subsets in the lung (paper III). We investigated the distribution as well as marker expression of DCs in bronchioles, alveolar parenchyma and LAs of patients with COPD. The DC markers used were langerin, CD1a, BDCA-2 and CD11c. However, CD11c is also expressed on other cell types, including monocytes and macrophages^{93,94}. Therefore, confounding CD11c-expressing monocytes and macrophages were immunohistochemically excluded by staining for CD68 and CD163 prior to staining for CD11c. The area of CD11c immunoreactivity, negative for CD68 and CD163, was quantified using computerised image analysis.

Multiple DC populations are present in bronchioles, alveolar parenchyma and LAs

We identified four populations of lung tissue DCs; langerin⁺ (including langerin⁺CD1a⁺), CD1a⁺langerin⁻, BDCA-2⁺, and CD11c⁺CD68⁻CD163⁻. In bronchioles, DCs were found both in the airway epithelium and in the subepithelial tissue (i.e. lamina propria and adventitia). The alveolar septa were also populated by all DC subsets. We further observed that all alveolar DCs, but BDCA-2⁺ DCs, frequently extended cytoplasmic protrusions into the alveolar lumen (figure 13A-C). These findings were in accordance with our previous observation that alveolar interface DCs frequently extended protrusions into the alveolar lumen. Contrasting the alveolar DCs, the airway epithelial DCs only occasionally reached the airway lumen. Apart from the langerin⁺ interface DCs and CD21⁺ follicular DCs, CD11c⁺CD68⁻CD163⁻ and BDCA-2⁺ DCs were frequently observed in the T cell area in LAs. Only few CD1a⁺langerin⁻ DCs were detected in these structures.

Expansion of several DC populations in the alveolar parenchyma in advanced COPD

We showed that several of the DC populations, including CD1a⁺langerin⁻, BDCA-2⁺, and CD11c⁺CD68⁻CD163⁻, were increased in the alveolar parenchyma of patients with very severe COPD compared with control subjects (figure 13D-F). We also demonstrated that the number of airway epithelial langerin⁺ DCs, partly immunopositive for CD1a, was increased in patients with very severe COPD compared with never-smoking controls. Further we showed that the number of BDCA-2⁺ DCs increased both in the airway epithelium and subepithelium in patients with very severe COPD compared with smokers without COPD and patients with mild to severe COPD. In addition, we found a negative correlation between FEV₁ % of predicted and the number of airway BDCA-2⁺ DCs.

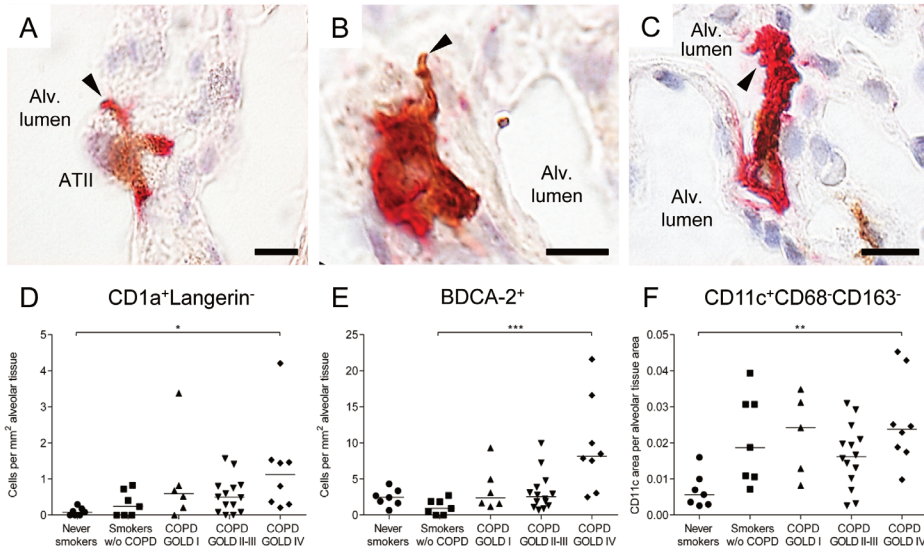


Figure 13

(A) An alveolar langerin⁺ DC (brown stain), partly immunopositive for CD1a (red stain), reaching the alveolar lumen (arrowhead). ATII, alveolar type II cell. (B) An alveolar langerin⁺ DC (brown stain), partly immunopositive for CD1a (red stain), with a podosome that has direct contact with the alveolar lumen (arrowhead). (C) An alveolar CD11c⁺ DC (red stain) which also expresses low levels of CD68 and/or CD163 (brown stain) and extends cytoplasmic protrusions into the alveolar lumen (arrowhead). In A-C, cell nuclei are counterstained with Mayer's haematoxylin (blue stain). Scale bars: (A) 12 μ m; (B) 6 μ m; (C) 9 μ m. (D) Number of CD1a⁺ langerin⁺ DCs per unit area of alveolar parenchyma. (E) Number of BDCA-2⁺ DCs per unit area of alveolar parenchyma. (F) Area of CD11c immunoreactivity expressed per unit area of the alveolar parenchyma. In D-F, horizontal lines indicate medians for each group.

Identification of site-specific langerin⁺ DCs in advanced COPD

The coexpression of CD68 (monocyte/macrophage marker) and CD11c (myeloid DC marker) on langerin⁺ DCs was evaluated in the bronchiolar epithelium, the alveolar parenchyma and the alveolar-lymphoid interface epithelium on triple immunofluorescence-stained sections of patients with GOLD stage IV COPD. The proportion of marker combinations differed within each compartment. For example, the proportion of single positive langerin-expressing DCs (langerin⁺CD68⁻CD11c⁻) was higher in the alveolar parenchyma and the alveolar-lymphoid interfaces. Furthermore, the marker expression profiles also differed between the compartments, as revealed by a higher proportion of triple positive langerin DCs (langerin⁺CD68⁺CD11c⁺) in the bronchiolar epithelium compared with the alveolar parenchyma and the alveolar-lymphoid interfaces.

Discussion

Previous studies on adaptive immune system components, such as LAs and DCs, have mainly focused on bronchioles of patients with COPD and little is known about the alveolar parenchyma. This thesis work provides new insights into the peripheral adaptive immunity in COPD and presents novel aspects of the alveolar parenchyma in the induction of adaptive immune responses: (1) We show that the majority of the LAs have direct interfaces with the alveolar lumen and advanced COPD is associated with a selective accumulation of langerin⁺ interface DCs, many of which extend cytoplasmic protrusion into the alveolar lumen. (2) We demonstrate an intricate connection between LAs and lymphatic vessels, and show an expansion of CCL21- and D6-immunoreactive lymphatic vessels in the alveolar parenchyma in advanced COPD. (3) We also provide evidence that multiple alveolar DC populations are expanded in advanced COPD. Taken together, the studies described in this thesis suggest that the alveolar parenchyma is a site for both antigen uptake and induction of adaptive immune responses in COPD.

Lymphoid aggregates offer a structural basis for enhanced alveolar antigen uptake in COPD

In this thesis work we showed that LAs are present throughout all major lung compartments of the peripheral lung and the general expansion of LAs correlated with the severity of COPD. By subgrouping LAs, based on their anatomical location, it was evident that alveolar-only associated LAs (ALTs) and vascular-associated LAs (VALTs) increased in numbers in advanced COPD, as opposed to bronchiolar-associated LAs (BRALTs), which were not changed between the study groups. In related studies, van der Strate *et al.*⁶⁴ also reported a rich occurrence of non-airway-associated LAs in peripheral lungs of patients with COPD. Thus, our results and those of van der Strate *et al.*⁶⁴ strongly suggest that studies restricted to BRALTs may overlook most of the LAs in COPD lungs. Our study further complements previous observations that most BRALTs are located in the adventitia layer in bronchioles^{64,123,125,190} by showing that in COPD the majority of the LAs had direct interfaces with the alveolar lumen. This finding was observed regardless of the anatomical location suggesting that alveolar-lymphoid interfaces are a

general feature of lung LAs, including BRALTs and VALTs. This unique position of peripheral LAs together with the appearance of a DC-rich alveolar-lymphoid interface epithelium in COPD is intriguing and is suggested to provide a route for alveolar antigen uptake. In that sense the remodelled alveolar-lymphoid interface epithelium, specific for patients with advanced COPD, may be similar to the specialized lymphoepithelium covering the luminal side of most mucosal-associated lymphoid tissues¹³⁹, including those present in the bronchi^{126,136,191}. However, in contrast to the lymphoepithelium in GALTs^{139,192}, we were not able to detect any M cells and there was no obvious lymphocyte infiltration in the alveolar-lymphoid interface epithelium. However, other studies have also failed to identify M cells in peripheral lung LAs⁶⁴. Due to the lack of M cell-specific markers future studies using electron microscopy will be necessary to determine whether or not M cells are present in the interface epithelium. Nevertheless, the selective accumulation of langerin⁺ interface DCs, many of which extended protrusions into the alveolar lumen, suggests an increased capacity for alveolar antigen uptake in patients with COPD.

Lymphoid aggregates as inductive sites for local adaptive immunity

In an attempt to investigate the ability to initiate adaptive immune responses in LAs, we further assessed the cellular composition of these structures. Similar to previous studies^{18,64,123-125,149}, the majority of the cells were B cells, T cells, and DCs. As expected, the LAs in patients with advanced COPD had a dense core of proliferating cells and, compared to control subjects, increased CD21⁺ follicular DCs. This suggests that LAs in COPD are more well developed compared with LAs in controls and may also be indicative of an active antigen presentation in COPD. Further, the finding that the cellular composition was similar irrespective of anatomic localisation is of importance and suggests that LAs have the capacity to initiate immune responses locally in all lung compartments. Taken together, the intimate relationship between the interface DCs and T cell areas as well as the LAs and the extensive network of lymphatic vessels in COPD, is suggested to create a structural basis for a prompt and local induction of adaptive immune responses throughout the peripheral lung. Whether this novel type of structure is beneficial or harmful is currently unknown. As mentioned earlier, the antigen(s) triggering the LAs in COPD have not been identified. It is, however, possible that the chronic low-grade presence of lung pathogens³⁵, together with the defective alveolar macrophage phagocytosis⁵⁵ and frequent infection-induced exacerbations²⁸ in patients with COPD may constitute potential sources of alveolar antigens in the peripheral lung. In light of this, we showed that the alveolar-lymphoid interface epithelium expressed TLR5. The expression pattern of TLR5 was similar to that in bronchiolar epithelium of patients with COPD (unpublished observations by M

Shikhagaie *et al.*). Importantly, TLR5 is known to recognise flagella of *Pseudomonas aeruginosa*^{193,194}, that are commonly detected in the lower respiratory tract of patients with severe stages of COPD²⁸. However, apart from pathogens, self-antigens or cigarette smoke components may also initiate immune responses in LAs^{60,64,195-197}. Together these studies suggest that the antigens captured by the alveolar-lymphoid interface DCs are expected to be multifaceted.

Evidence for increased cell trafficking in the peripheral lung in COPD

A recent study reported that the lymphatic vessels are more numerous in peripheral lungs of patients with moderate COPD¹⁶⁹. The question whether this expansion of lymphatic vessels is present in all anatomic regions of the peripheral lung or not has remained unknown. In this regard, we have shown in this thesis work that among the major peripheral lung compartments the most pronounced increase in lymphatic vessel numbers occurred in the alveolar tissue of patients with advanced COPD. The presence of alveolar lymphatic vessels in control subjects has been a subject of debate owing to conflicting data^{154,155,198} however, similar to our findings, others^{152,153} have detected low numbers of alveolar lymphatic vessels in normal lungs. Nevertheless, the expansion of alveolar lymphatic vessels in advanced COPD was intriguing. This finding, together with the observation of increased absolute lymphatic numbers in the wall of bronchioles and bronchiole-associated arteries, suggests that lymphatic vessels are formed in all major lung compartments in patients with advanced COPD.

It is currently unknown what induces the formation of lymphatic vessels in COPD lungs. However, there are increasing evidences that immune cells as well as structural cells in the lung constitute a rich source of lymphangiogenic factors. For example, studies in a mouse model of airway infection with *Mycoplasma pulmonis* have revealed the expression of VEGF-C on airway epithelial cells, type II alveolar epithelial cells and macrophages¹⁶⁴. In the same study, neutrophils, airway smooth muscle cells as well as macrophages were found to express VEGF-D. In addition, proinflammatory cytokines such as TNF- α can also promote the proliferation of lymphatic vessels^{166,172}. Thus, it is possible that the chronic inflammatory processes in the COPD lungs create an environment that could stimulate the formation of new lymphatic vessels. Another possible explanation for the increase in lymphatic vessel numbers in advanced COPD could be related to the recurrent respiratory infections in this patient group²⁸. For example, *in vivo* studies have shown that infection of the mouse airway epithelium by *Mycoplasma pulmonis* leads to an extensive airway lymphangiogenesis^{164,166,199}. In addition, lung lymphatic endothelial cells in culture can recognize pathogenic components through their expression of multiple TLRs, including TLR2-4, and produce proinflammatory cytokines, such as IL-1 β ²⁰⁰, which in turn may trigger lymphangiogenic responses²⁰¹. Although we could not

detect an on-going lymphangiogenesis, as indicated by the low numbers of Ki-67-expressing lymphatic vessels, it is evident from *in vivo* studies that newly formed lymphatic vessels are long lasting¹⁶⁴ and steroid resistant¹⁹⁹. Thus, the increased numbers of lymphatic vessels in patients with advanced COPD, who received inhaled corticosteroids, may result from previous lymphangiogenesis. However, future studies are needed to investigate the expression of VEGFR-3, the receptor for lymphangiogenic factors VEGF-C and VEGF-D.

Regardless of the initiating stimuli, the increased number of lymphatic vessels is likely to have several implications in COPD. The general increase in lymphatic vessel numbers may reflect the need to decrease oedema, which otherwise could affect the integrity of the tissue, particularly in the alveolar walls. The increased clearance of extracellular fluid could also promote the removal of inflammatory mediators, antigens and pathogens from the site of inflammation. However, if lymphatic vessels formed in inflammation have a decreased capacity to clear fluid due to an altered structural phenotype^{165,202} they could contribute to the inflammation. Future investigations of the structural characteristics of lymphatic vessels in COPD lungs remain to be performed. Furthermore, the increased numbers of lymphatic vessels may improve immune cell trafficking to draining lymph nodes. In this regard we showed that the immunoreactivity for lymphatic CCL21 as well as lymphatic D6 was increased in patients with advanced COPD. Together these results may suggest that the increased expression of D6, which dampens the inflammatory environment by clearing inflammatory chemokines from lymphatic surfaces¹⁸⁷, allows CCL21 to act as a chemotactic ligand for CCR7⁺ lymphocytes, monocytes and DCs that enter the lymphatic vessels^{179,182,203,204} and, hence, increase the capacity for cellular trafficking from the peripheral lung.

A close association between lymphatic vessels and LAs has been observed in other diseases, including inflammatory bowel disease²⁰⁵ and idiopathic pulmonary arterial hypertension¹⁴⁰. In addition, a recent study described lymphatic vessels in relation to LAs in patients with COPD¹²⁵. Our studies provided further evidence for this association by demonstrating an intricate network of lymphatic vessels surrounding LAs, irrespective of lung compartments. Furthermore, our observation that CCL21-expressing lymphatic vessels are present within these structures may also suggest that these vessels provide an entry route of leukocytes into LAs. Taken together, lymphatic vessels may transport leukocytes as well as antigens to LAs, in addition to the draining lymph nodes. Thus, in advanced COPD the expansion of lymphatic vessels in the peripheral lung and the increased expression of lymphatic CCL21 may contribute to the adaptive immune responses initiated in LAs by the continuous transportation of DCs and antigens to these structures.

Alveolar antigen uptake by interstitial alveolar dendritic cells in COPD

An important aspect when studying lung DCs is that the distribution of the different DC subsets is likely dependent on the anatomical localisation⁷¹, and the sampling methods commonly used to study lung DCs, such as lung homogenates, BAL fluid and sputum, do not allow comparison between anatomic lung compartments in the same patient. The few studies that have performed direct histological analysis of lung tissue DCs have primarily focused on the conducting airways of patients with COPD. It is evident from these studies that langerin⁺ DCs accumulate in the mucosa of bronchioles^{103,113,114}, which is in line with our study results, and the numbers correlate with the severity of COPD¹¹⁴. In contrast, very little is known about the distribution of DCs in the alveolar tissue. In this thesis work we showed that, compared to bronchioles, the most marked increase in DC subsets occurred in the alveolar parenchyma. Among these alveolar DC subsets were CD1a⁺langerin⁻, BDCA-2⁺, and CD11c⁺CD68⁻CD163⁻ DCs.

Also, langerin⁺ DCs were detected in the alveolar parenchyma. Our further investigation of the langerin⁺ DCs in patients with advanced COPD revealed that the alveolar and bronchiolar mucosa langerin⁺ DCs could be distinguished by their marker expression. The langerin⁺ DCs in the airway epithelium were identified as a heterogeneous population of DCs; some expressed the monocyte/macrophage marker CD68 and/or the myeloid DC marker CD11c. In contrast, the coexpression of CD68 and CD11c was observed to a much lesser extent on langerin⁺ DCs in the alveolar parenchyma and alveolar-lymphoid interfaces. The reason for this difference in marker expression is currently unknown. Previous studies have reported that blood CD11c⁺CD1a⁺ cells can differentiate into langerin⁺ cells *in vitro*¹⁰⁴ whereas others have reported that peripheral monocytes can differentiate into langerin⁺ cells *in vitro*¹⁰⁵ and *in vivo*²⁰⁶. Furthermore, the coexpression of CD68 and CD11c may be an indicative of an immature subset²⁰⁷. Thus, it remains to be investigated whether the differences in marker expression of CD68 and CD11c may reflect differences in origin, stages of maturation, anatomical location or, perhaps, function. In addition, compared to airway epithelial DCs, the alveolar DCs frequently displayed cytoplasmic protrusions into the alveolar lumen, similar to the alveolar-lymphoid interface DCs. These findings suggest that alveolar DCs through their contact with the alveolar lumen may capture antigens that have reached this compartment. Taken together this thesis work suggests that in addition to bronchioles, the alveolar parenchyma may be regarded as a site for antigen uptake in patients with COPD. In support of this, recent studies involving two-photon microscopy to directly visualise antigen uptake in mouse lungs demonstrated that alveolar DCs rather than bronchial DCs are the dominating antigen presenting cells for capturing luminal antigens in the lung¹¹⁸. Although, this type of comparison in animals does not take into account the predicted increase in bronchial antigen

uptake due to the disturbed epithelial barriers in COPD patients, it is still possible that also in human lungs antigen uptake by alveolar DCs is highly efficient. In addition, it is also possible that in COPD the antigen-loaded alveolar DCs migrate via CCL21-immunoreactive alveolar lymphatic vessels to LAs. However, this hypothesis needs further investigations. For example, in order to enter the CCL21-immunopositive lymphatic vessels the DCs have to express CCR7, the receptor for CCL21^{83,208}. Future investigations of the expression of CCR7 on tissue DCs may elucidate whether or not these DCs enter CCL21⁺ lymphatic vessels in patients with COPD. Furthermore, whereas several factors such as respiratory infections, chemokines, cytokines, and tobacco smoke exposure^{95,103,105,113,114} likely contribute to the recruitment of DC precursor cells, differentiation, as well as activation of DCs in the bronchiolar mucosa in COPD, the factors responsible for the accumulation of alveolar DC subsets remain to be identified.

Concluding remarks

It is evident from the studies described in this thesis work that among the peripheral lung compartments investigated the most prominent findings in LAs, lymphatic vessels, and DC subsets were observed in the alveolar parenchyma of patients with advanced COPD. The reasons for this need further investigations but may suggest a long-term adaptation to chronic stimuli of inflammatory mediators and/or pathogens. In the case of pathogens, the adaptation may ensure a prompt and highly efficient induction of immune responses against pathogens reaching the more vulnerable alveolar compartment. This may be in contrast to the situation in airways, where it can be hypothesised that only tissue-invading pathogens are captured by DCs¹¹⁸. In that sense, the expansion of alveolar lymphatic vessels and alveolar DCs together with the emergence of DC-rich alveolar-lymphoid interfaces would create a structural basis for faster uptake of alveolar antigens, cell trafficking and induction of local adaptive immune responses in LAs. However, it could also be argued that this “immune remodelling” may result in a scenario when “too much” immune responses result in an immunological hyperreactivity that may evoke aggravated immune responses to the frequent respiratory infections. Furthermore, the majority of the patients with advanced COPD were treated with inhaled steroids which may indicate that the therapeutic agents do not readily reach the most distal parts of the lung. In any case, pharmacological intervention of immune events at, for example, alveolar-LA interfaces warrants attention as a potential therapeutic strategy.

It is also evident from this thesis work that there are important regional differences in COPD lungs as findings in bronchioles may not be similar to findings in the alveolar parenchyma. The regional differences were especially evident in the study of DCs, where the marker expression profile was different between DCs in the airway

epithelium and DCs in the alveolar septa. These findings suggested that there are site-specific phenotypes of DCs that are difficult to study with other sampling methods such as lung homogenates, which contain a mixture of cells from different lung compartments. Thus, direct tissue visualisation and comparison between lung compartments seem crucial to increase the understanding of the immunological basis in COPD. The major advantage of performing histological analysis on surgical samples is that it allows comparison between different peripheral lung compartments in the same tissue section. However, since the distal lung is poorly accessible, the lung tissue samples used in this thesis work were collected from subjects undergoing lung resection surgery for lung cancer. In order to avoid cancer tissue in any of the sections, we only included subjects with well delineated solitary tumours and peripheral samples were collected as far from the tumour as possible. Importantly, the most striking findings were found in patients with GOLD stage IV COPD who did not have cancer.

In summary, this thesis work provides novel insights into the peripheral adaptive immunity of COPD and introduces the alveolar parenchyma as an important arena for both antigen uptake and induction of adaptive immune responses. This work is predicted to contribute to the understanding of the initiation of peripheral adaptive immune responses in other lung diseases. Whether the emergence of DC-rich alveolar-lymphoid interfaces together with the changes in lymphatic vessels and DC subsets are beneficial or harmful in COPD remain to be established in future investigations.

Conclusions of the Thesis

- Alveolar-lymphoid interfaces is a prominent feature of all lymphoid aggregates in the peripheral lung
- Lymphoid aggregates in all peripheral lung compartments are surrounded by an intricate network of lymphatic vessels
- Advanced stages of COPD are associated with remodelling of the alveolar-lymphoid interface epithelium where a selective accumulation of langerin-expressing dendritic cells provides a structural basis for efficient and enhanced alveolar antigen uptake
- Alveolar lymphatic vessels are increased in numbers in patients with advanced COPD and have a changed phenotype as demonstrated by increased lymphatic expression of CCL21 and D6
- Multiple alveolar interstitial DC populations, including CD11c⁺CD68⁻CD163⁻, BDCA-2⁺ and CD1a⁺langerin⁻ subsets, are increased in patients with advanced COPD
- The alveolar DC subsets seem to have distinct and site-specific phenotypes that differ from corresponding DC subsets in airway mucosa

Future Perspectives

The studies described in this thesis have provided novel and basic insights into the alveolar adaptive immunity in COPD, with the recognition of alveolar-lymphoid interfaces, lymphatic vessels and DCs. However, several of the issues and discoveries in this thesis remain to be further explored to better understand the role for adaptive immunity in COPD.

Lymphoid aggregates

Since this is the first study to describe the presence of alveolar-lymphoid interfaces and the selective accumulation of interface DCs in disease, one important question for future studies is to establish whether or not this interface is a general feature of all diseases characterised by ectopic lung LAs, including asthma¹⁴², cystic fibrosis¹⁴⁴, idiopathic pulmonary fibrosis¹³⁴ and idiopathic pulmonary arterial hypertension¹⁴⁰. Also, the nature of the alveolar-lymphoid interface needs further investigations in order to increase the knowledge of its function. For example, we showed the expression of TLR5 in the interface epithelium and it is likely that other innate immune receptors also are present. Regarding the function of the interfaces, DCs within the interface epithelium were found to have direct contact with the alveolar lumen which suggests that these cells pick up antigens, but we were not able to perform any functional studies to confirm this assumption. Importantly, it is not known whether the emergence of the alveolar-lymphoid interface is a phenomenon restricted to humans or if it is a common feature of LAs formed *in vivo*. In any case, future studies involving human *ex vivo* imaging of fluorescent-labelled antigens are warranted to investigate the antigen uptake by interface DC. With this technique, it should also be possible to trace the antigen-loaded DCs within the tissue. Future studies should also investigate if the interface DCs could be used as a route for local pulmonary delivery of vaccines against respiratory pathogens that have reached the alveolar compartment. The rationale of using LAs as an entry site for inhaled vaccines has already been proposed for LAs in the bronchial mucosa²⁰⁹. As previously mentioned the functional role for LAs in COPD remains unknown. However, the identification of mediators involved in the formation of LAs may contribute to the understanding of what antigens and signals are involved in the immune responses.

For example, the chemokine CXCL13 is currently being recognised as an important B cell attractant in the development of LAs in patients with COPD^{146,147}. Other mediators are also likely to be involved in the formation of LAs in COPD. In this regard, we are currently investigating a potential role for IL-18 in the development of LAs in mice exposed to chronic cigarette smoke.

Lymphatic vessels

This thesis work has also contributed to the knowledge of lung lymphatic vessels. Future studies are needed to investigate how lymphatic vessels grow and whether the lymphatic vessels surrounding LAs are newly formed or if LAs are actually formed along the lymphatic system in the lung. These studies will increase the understanding of the cellular trafficking in COPD lungs.

Dendritic cells

It is evident from this thesis work that the phenotypic and functional characterisation of lung DC subsets in different anatomical regions need extensive investigations. Future studies investigating the maturation status of alveolar DCs are necessary to understand their role in COPD. Failure of DC migration could result in an accumulation of activated DCs within the tissue which in turn may contribute to the inflammation as well as formation of LAs²¹⁰. In this context, it would be interesting to investigate the DC subsets in draining lymph nodes in COPD.

Populärvetenskaplig sammanfattning (Summary in Swedish)

Kroniskt obstruktiv lungsjukdom (KOL) är en sjukdom som främst drabbar rökare. Sjukdomen utvecklas långsamt och kännetecknas av en ständigt pågående, kronisk inflammation i lungorna som orsakas av tobaksrökens skadliga ämnen. Inflammationen i de små luftvägarna orsakar en bestående förträngning, obstruktion, av luftvägarna. Detta medför att luftvägarna blir mindre elastiska och luftflödet genom de små luftvägarna begränsas. Tobaksröken orsakar även irritation i luftvägsslemhinnan, vilket leder till en ökad slemproduktion och upphostningar. Inflammationen i lungblåsorna, så kallade alveoler, leder till att vävnaden bryts ned och det bildas stora hålrum (emfysem). Eftersom blodet syresätts i lungblåsorna leder denna nedbrytning till en försämrad syreupptagningsförmåga och att personer med KOL upplever andnöd.

Flera av kroppens immunceller samt lungans egna strukturella celler är inblandade i de inflammatoriska processer som driver sjukdomen. De immunceller som har studerats mest i KOL tillhör det så kallade medfödda eller ospecifika immunförsvaret. Det medfödda immunförsvaret utgör kroppens första försvarslinje mot infektioner och vävnadsskador (orsakade av t.ex. tobaksrök). Det som utmärker det medfödda försvaret är att det svarar snabbt och brett mot olika typer av skador. Även immunceller i det adaptiva immunförsvaret är involverade i inflammationen. Med adaptivt menas att immunceller, så kallade lymfocyter, först lär sig att känna igen specifika detaljer hos bakterier och virus. Därefter kan lymfocyterna rikta ett mer specifikt försvar mot dessa inkräktare. Kroppen kan på detta sätt anpassa immunförsvaret mot olika typer av infektioner. För att lymfocyter ska lära sig att känna igen bakterier och virus krävs närvaron av så kallade dendritceller. Dendritceller är grenliknande celler som är strategiskt placerade mot den yttre miljön i lungan och plockar upp oönskade ämnen från inandningsluften. När en dendritcell stöter på något främmande, bär den med sig detta och vandrar via lymfatiska kärl till närliggande lymfkörtel. Väl på

plats presenterar dendritcellen små delar av det främmande ämnet för lymfkörtelns lymfocyter som efter aktivering vandrar till lungorna för att bekämpa infektionen.

Nyare forskning har visat att det bildas lymfkörtel-liknande strukturer i luftvägarna hos KOL-patienter och att det finns ett starkt samband mellan antalet strukturer och sjukdomsutvecklingen av KOL. Forskning har även påvisat en ökad förekomst av dendritceller i lungorna, framförallt i de små luftvägarna. Detta talar för att det adaptiva, specifika, immunförsvaret kan aktiveras lokalt i lungan, dvs. dendritceller behöver inte vandra hela vägen till lymfkörteln utan kan presentera främmande ämnen för lymfocyter lokalt i lungans lymfkörtel-liknande strukturer. Trots ett ökat intresse för dessa strukturer, även kallade lymfoida aggregat, så vet man fortfarande mycket lite om dem. Bland annat vet man inte hur de nås av främmande ämnen. Syftet med denna avhandling har varit att studera strukturen och uppbyggnaden av lymfoida aggregat samt dess relation till lungans dendritceller och lymfatiska kärl. Fokus har varit på de små luftvägarna och den alveolära vävnaden (lungblåsorna). Studierna bygger på avancerad analys av vävnadsmaterial från patienter med olika svårighetsgraden av KOL samt vävnad från rökare utan KOL och icke-rökare.

Resultaten som presenteras i denna avhandling visar att lymfoida aggregat bildas i flera anatomiska regioner i lungorna. Bland annat påvisades en ökad förekomst av de lymfoida strukturer i den alveolära vävnaden hos patienter med svår KOL. Fyndet kunde bekräftas genom tredimensionell rekonstruktion av vävnadssnitten. Vävnadsanalyserna visade även att huvuddelen av de nybildade lymfoida aggregaten, oavsett anatomisk lokalisation, angränsar mot det alveolära luftrummet. Fortsatta analyser av den alveolära-lymfoida angränsningsytan visade en lokal ansamling av dendritceller. Detta fynd var specifikt för patienter med KOL, då angränsningsytan hos friska helt saknade närvaron av dendritceller. I en fortsatt studie på lungans dendritceller fann vi även en ökning av ett flertal dendritcellpopulationer i den alveolära vävnaden hos patienter med svår KOL. Till skillnad från dendritceller i luftvägsslemhinnan har dendritcellerna i den alveolära vävnaden många fler membranutskott som når både luftrummet och aggregatens immunceller. Vidare visade tredimensionella analyser att lymfoida aggregat omges av ett komplext nätverk av lymfatiska kärl. Vi fann också att patienter med KOL har fler lymfatiska kärl i den alveolära vävnaden jämfört med friska. Dessutom visade analysen att de lymfatiska kärnen har en ökad kapacitet för celltransport i lungorna vid KOL.

Sammanfattningsvis visar avhandlingsstudierna att det vid svår KOL bildas nya strukturer som möjliggör upptag av främmande ämnen från det alveolära luftrummet, att ett flertal dendritcellpopulationer ansamlas i den alveolära vävnaden, samt att alveolära lymfatiska kärl har en ökad kapacitet för celltransport. Utifrån detta kan vi konstatera att den alveolära vävnaden har en viktig roll i upptag av främmande ämnen och innehåller strukturer som krävs för att aktivera ett adaptivt immunsvaret i dessa lungregioner.

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