Potential biomarkers for acute and chronic rejection after lung transplantation

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Ju mer man tänker, ju mer inser man att det inte finns något enkelt svar.
– Nalle Puh

Abstract

Chronic rejection in the form of bronchiolitis obliterans syndrome (BOS) is the main hindrance for long-term survival after lung transplantation. Repeated acute rejections (AR) constitute a major risk factor for developing BOS. The aim of this thesis was to expand the knowledge of the biological processes underlying AR and BOS and to identify potential immunological biomarkers for these conditions. The following specific research questions were posed:
Are alterations in IL-18 concentration associated with AR (paper I)?
Are there local pulmonary changes in the protease/anti-protease balance in BOS (paper II)?
Is the neutrophil mobilizing cytokine IL-26 involved in AR and BOS development (paper III)?
Does composition of particles in exhaled air (PEx) differ between BOS/non-BOS (paper IV)?
Methods: In a biobank of collected bronchoalveolar lavage (BAL) samples from lung transplant recipients (LTRs), we identified patients, with or without AR and BOS respectively, who were carefully matched. The matching procedure included preoperative diagnosis, age, gender, type of and time after transplantation to avoid the influence of confounding clinical factors. Inflammatory cells and soluble mediators involved in the inflammatory process were analyzed in BAL samples (paper I-III). In paper IV, PEx composition in LTRs and healthy controls was investigated with a novel method that enables non-invasive sampling from the distal airways.
Results: There were no changes in IL-18 concentration or correlation between IL-18 and lymphocyte percentages in BAL samples from patients with AR (paper I). Increased net gelatinase activity and a clear correlation between activity and concentration of the gelatinase MMP-9 (but not MMP-2) as well as a correlation between activity and neutrophil percentages were found in BAL samples from BOS patients (paper II). It was also found that the concentration of IL-26 in BAL samples from patients with BOS (but not AR) was increased and intracellular IL-26 was detected in alveolar macrophages and lymphocytes (paper III). Finally, surfactant protein A (SP-A) was lower in PEx from BOS patients compared to stable LTRs and LTRs, in particular BOS patients, exhaled a higher amount of PEx than healthy controls (paper IV).
Conclusions: These findings forward evidence that local unopposed gelatinase activity, likely to be accounted for by the gelatinase MMP-9 from neutrophils, and the neutrophil mobilizing cytokine IL-26 from macrophages and lymphocytes, are involved in BOS development. The results also show that PEx composition differs between stable LTRs and patients that develop BOS. The clinical utility of PEx as a non-invasive diagnostic tool in the follow up after lung transplantation and the possibility of targeting MMP-9 and IL-26 for early detection, monitoring and possibly even treatment of BOS warrant further study.

Key words: lung transplantation, graft rejection, bronchiolitis obliterans syndrome, IL-18, MMP-9, IL-26, surfactant protein

Populärvetenskaplig sammanfattning på svenska

Lungtransplantation kan vara en livsförlängande åtgärd för patienter med lungsjukdom då alla andra behandligsmöjligheter har uttömts. Resultaten har successivt förbättrats men endast 54% av patienterna lever 5 år efter transplantationen. Långtidsöverlevnaden är lägre än för de flesta andra organtransplantationer som njure, lever och hjärta, men jämförbar med överlevnaden efter tarmtransplantation.

Både lungan och tarmen står i direkt kontakt med omgivningen och har ett aktivt immunsystem för att skydda kroppen från infektioner och skadliga ämnen. Många likheter finns mellan hur immunförsvaret attackerar sjukdomsframkallande ämnen och icke kroppsegna organ. Den ökade ”försvarsberedskapen” i lungan och tarmen bidrar med största sannolikhet till den sämre prognosen vid transplantation av dessa organ. För att förmå kroppen att acceptera den transplanterade lungan behandlas patienterna med mediciner som dämpar immunförsvaret. Att förhindra avstötning av det nya organet och samtidigt bibehålla ett fungerande infektionsförsvar är dock en svår balansakt.

Kronisk avstötning är den dominerande orsaken till begränsad överlevnad. Det är en inflammation i de små, perifera luftvägarna som leder till ärrbildning och tilltäppning av de minsta luftrören vilket i sin tur leder till en successiv förlust av lungfunktion.

I dagsläget finns ingen botande behandling mot kronisk avstötning. Det finns inte heller några kliniskt användbara markörer som kan förutsäga och följa utvecklingen och därmed också ge möjlighet individuellt anpassa den immunhämmande medicineringen. Optimering av behandlingen i ett tidigt skede av avstötningsreaktionen ökar möjligheten att förhindra, eller i alla fall, förlångsamma processen.

Målet med avhandlingsarbetet var att öka kunskapen om de biologiska processer som leder till avstötning samt att identifiera immunologiska markörer med potential att användas för att upptäcka och följa densamma. Frånsett biomarkörer skulle också utvecklingen av nya metoder som på ett känsligare sätt mäter lungfunktionen och som medger upprepad provtagning från de små luftvägarna utan obehag/risk för patienten, bidra till ökad kunskap och därmed ett bättre omhändertagande och förlängd överlevnad.

Delarbete I-III. I uppföljningen efter lungtransplantation ingår bronkoskopier (ett böjligt fiberinstrument med optik förs ned i luftrören). Vid bronkoskopin sköljs koksalt ner och sugs sedan tillbaka. Man får då med sig celler och proteiner från de perifera luftvägarna. Efter genomförda kliniska analyser sparades överblivet material. Detta innebar att vi hade tillgång till över 900 nedfrysta ”sköljprover” från mer än 140 lungtransplanterade patienter.

Det stora antalet prover gav möjlighet att para ihop patienter, en med avstötning och en utan, men i övrigt så lika varandra som möjligt vad gäller ålder, kön, diagnos, operationstyp och tid efter transplantation. Denna matchning gjordes för, att så långt möjligt, undvika att andra faktorer än de vi ville mäta skulle påverka resultaten.

Vid kronisk avstötning ser man en ökning av neutrofiler och makrofager som är två typer av vita blodkroppar. Cytokiner är små proteiner som agerar budbärare mellan immunförsvarets celler. En speciell undergrupp av cytokiner är interleukinerna. I våra studier analyserade vi bl.a interleukin (IL)-26, som nyligen, för första gången, påvisats i lunga och som ökar invandringen av neutrofiler i vävnaden vid inflammation. Vi fann betydligt högre IL-26 nivåer hos patienterna med kronisk avstötning talande för att IL-26 är delaktig i denna inflammatoriska process. Vi fann också bevis för att IL-26 kan produceras av makrofager hos lungtransplanterade patienter.

Det är viktigt att det finns en balans i immunförsvaret mellan vävnadsnedbrytande ämnen, proteaser och anti-proteaser som skyddar vävnaden. Proteaser kan frisättas av både neutrofiler och makrofager. Vi fann en ökad aktivitet hos två proteaser, matrix metalloproteinas (MMP)-9 och neutrofilt elastas (NE). Den ökade aktiviteten hos NE motverkades av anti-proteaser men det gjorde inte aktiviten hos MMP-9 vilket talar för att en ökning av MMP-9 utan motsvarande ökning av anti-proteaser bidrar till utvecklingen av kronisk avstötning.

I **delarbete IV** använde vi en ny metod, utvecklad här i Göteborg. Genom att samla in partiklar i utandningsluft (PEx) får man både ett mått på lungfunktionen och provmaterial från de perifera luftvägarna. En jämförelse gjordes mellan patienter med kronisk avstötning, lungtransplanterade patienter utan avstötning och med en grupp friska personer. Vi fann att PEx insamling är lätt att genomföra även för patienter med dålig lungfunktion och att partikelsammansättningen skiljde sig åt mellan lungtransplanterade och friska kontroller. Vi fann också att surfactant protein-A (SP-A), ett protein som både deltar i immunförsvaret och bidrar till minskad ytspänning i lungblåsorna, var mycket lägre hos patienter med kronisk avstötning.

Sammanfattningsvis visar denna avhandling att IL-26 och MMP-9 i sköljvätska och SP-A i PEx är möjliga markörer för att upptäcka kronisk avstötning. Den visar också att metoden att mäta PEx är enkel att genomföra för denna patientgrupp. För att klargöra om mätning av dessa proteiner kan vara till nytta i den kliniska vardagen och om PEx är ett användbart instrument i uppföljningen efter lungtransplantation krävs dock mer omfattande studier. Fynden är dock ett steg på vägen mot att förstå de komplexa skeenden som leder till avstötning och därmed mot det övergripande målet att förbättra behandlingen och förlänga överlevnaden efter lungtransplantation.

List of papers

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Ericson P, Lindén A, Riise GC.

BAL levels of interleukin-18 do not change before or during acute rejection in lung transplant recipients

Respir Med 2004; 98 (2):159-63.

II. Riise GC, Ericson P, Bozinovski S, Yoshihara S, Anderson GP, Lindén A.

Increased net gelatinase but not serine protease activity in bronchiolitis obliterans syndrome

J Heart Lung Transplant 2010; 29 (7):800-7.

III. Ericson P, Tengvall S, Stockfelt M, Levänen B, Lindén A, Riise GC.

Involvement of IL-26 in bronchiolitis obliterans syndrome but not in acute rejection among lung transplant recipients

Submitted

IV. Ericson P, Mirgorodskaya E, Hammar O, Viklund E, Almstrand AC, Larsson P, Riise GC, Olin A-C.

Low levels of exhaled surfactant protein A associated with BOS after lung transplantation

Transplantation Direct 2016;2: e103.

Contents

Abbreviations

A1AT	α-1-antitrypsin deficiency
ARAD	azithromycin-responsive allograft dysfunction
BAL	bronchoalveolar lavage
BOS	bronchiolitis obliterans syndrome
CD	cluster of differentiation
CLAD	chronic lung allograft dysfunction
CF	cystic fibrosis
CMV	cytomegalovirus
COPD	chronic obstructive pulmonary disease
CyA	cyklosporin A
ELF	epithelial lining fluid
ELISA	enzyme-linked immunosorbent assay
EVLP	ex vivo lung perfusion
HLA	human leukocyte antigen
IFN	interferon
IL	interleukin
IPF	idiopathic pulmonary fibrosis
ISHLT	International Society of Heart and Lung Transplantation
LTRs	lung transplant recipients
MMP	matrix metalloproteinase
MHC	major histocompatibility complex
NK	natural killer (cell)
OB	obliterative bronchiolitis
PAH	pulmonary arterial hypertension
PCR	polymerase chain reaction
PEx	particles in exhaled air, the sample
PExA	particles in exhaled air, the method
PMN	polymorphonuclear neutrophil
RAS	restrictive allograft syndrome
SLPI	secretory leukoprotease inhibitor
SP-A	surfactant protein A
TBB	transbronchial biopsy
TNF	tumor necrosis factor

Introduction

History

In 1963, Dr. James Hardy, Missisippi, performed the first human lung transplantation.[1] The patient survived for 18 days and was later identified by the Miami news as the convicted murderer John Richard Russle.
For several years thereafter, attempts to achieve long-term survival failed, mainly because of the absence of effective immunosuppressive treatment.
Cyklosporine was discovered in Norway in 1969 and lead to the development of an immunosuppression protocol enabling dr. Bruce Reitz, Stanford, to perform the first successful heart and lung transplantation in 1981.[2] The first single-lung and double-lung transplantations that achieved long-term survival of the recipients were reported by dr. Joel Cooper, Toronto, in 1983 and 1986 respectively.[3,4]
In Sweden the era of lung transplantation began in 1990 with transplantations performed both in Gothenburg and Lund.

The development of immunosuppression therapy

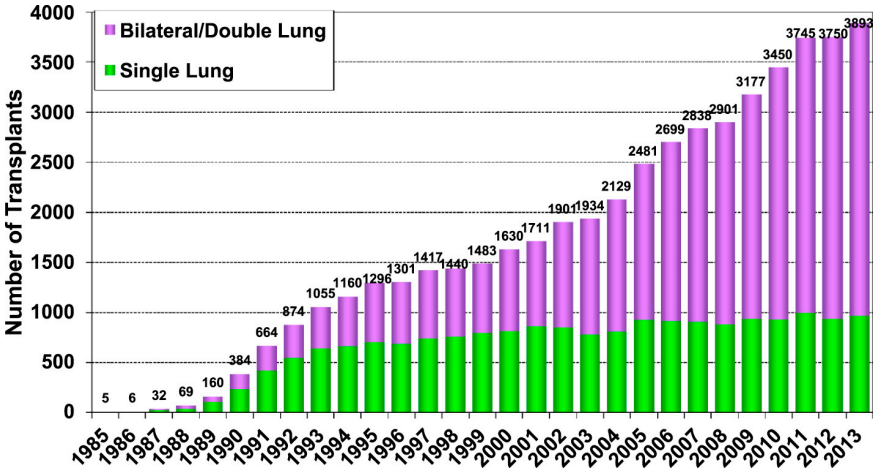
Organ transplantation generally and lung transplantation specifically was revolutionised by the introduction of cyclosporin A (CyA) with its relative potency and T-cell selectivity.[2,5] To enable effective immunosuppression without excessive myelosuppression whilst minimizing specific toxicities, a ‘‘standard’’ triple regime for immunosuppression (initially CyA+steroids+azathioprine) became routine treatment for lung transplant recipients (LTRs).[6] This standard regimen has developed over the years by reduction of the total steroid dose, increasingly changing calcineurin inhibitor to tacrolimus, and often using mycophenolate mofetil instead of azathioprine. These measures taken to, as far possible, maximize the efficacy with which the alloimmune response is suppressed whilst keeping drug related toxicities at a minimum.

Lung transplantation today

The International Society of Heart and Lung Transplantation (ISHLT) has since 1985 collected data from centers where lung transplantations are performed. Up to date, approximately 55,000 patients have been transplanted and the number reported per year is steadily increasing.

In the beginning of the transplant era, the standard procedure was to perform single lung transplantation for chronic obstructive pulmonary disease (COPD), α-1-antitrypsin deficiency (A1AT) and idiopathic pulmonary fibrosis (IPF), while double lung transplantation was reserved for cystic fibrosis (CF) and pulmonary hypertension. Over time there has been a shift towards double lung transplantations for all indications (Figure 1).[7]

Figure 1. Number of reported adult lung transplants by year and procedure type



The Registry of the International Society for Heart and Lung Transplantation: Thirty-second Official Adult Lung and Heart-Lung Transplantation Report -2015
The Journal of Heart and Lung Transplantation, Volume 34, Issue 10, 2015, 1264–1277
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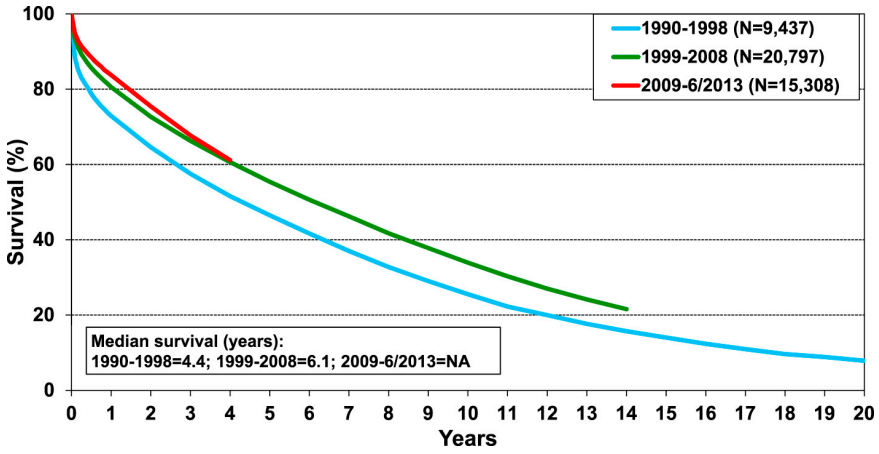
Patient survival has improved over the years, predominantly due to increased short-term (3-month) survival. Better recipient selection, organ preservation, surgical technique, immunosuppressive treatment, infection control and foremost better intensive care management have all contributed to this development.[8]
Despite this, the overall long-term outcome is still poor, with a median survival of 5,7 years and a survival rate of 54 % at five years after the transplantation (Figure 2).

Figure 2. Adult lung transplant recipient Kaplan-Meier survival, stratified by era



The Registry of the International Society for Heart and Lung Transplantation: Thirty-second Official Adult Lung and Heart- Lung Transplantation Report -2015
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Chronic rejection in the form of bronchiolitis obliterans syndrome (BOS) is the main long-term survival limiting complication. Factors that improve survival include; double lung transplantation, pre-transplant diagnosis of CF, recipient age < 55 years, positive donor-recipient height difference and higher transplant centre volume (>30 transplantations/year).[7]

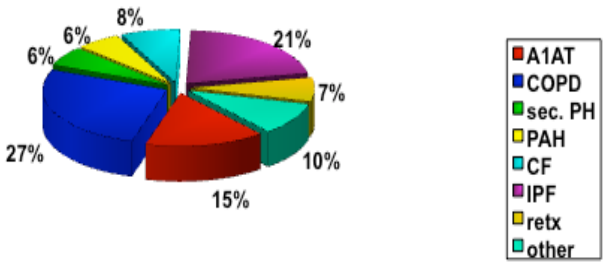
Lung transplantation at Sahlgrenska University Hospital

Since the first lung transplantation in Gothenburg in 1990 more than 670 transplantations have been performed. Recently, on average 40 patients/year have been transplanted at Sahlgrenska.
The data from our centre reflect the international trend with an increasing number of transplantations and an even more pronounced shift towards double lung transplantation. Our results stand up well in comparison with the international data with a survival rate of 70% five years after surgery.
The number of patients referred to Sahlgrenska for lung transplantation investigation has also increased over time. After investigation, around 25% of the referred patients are accepted and listed for transplantation.
The mortality of the patients waiting for transplantation has averaged 6% over the last five years (compared to 21% 10 years earlier), a low percentage by international standards.

The lung transplant recipient

Lung transplantation is the ultimate option for selected patients with end-stage lung disease and is considered when all other treatment possibilities are exhausted. Various pulmonary disorders develop terminal respiratory failure. However, only a few conditions generate the majority of the candidates for transplantation (Figure 3).

Figure 3. Diagnoses of lung transplant recipients at Sahlgrenska University Hospital 1990-2015



A1AT, α-1-antitrypsin deficiency; CF, cystic fibrosis; COPD, chronic obstructive pulmonary disease; IPF, idiopathic pulmonary fibrosis; PAH, pulmonary artery hypertension; retx, re-transplantation; sec. PH, secondary pulmonary hypertension (mainly Eisenmenger)

Chronic obstructive pulmonary disease (COPD) is a chronic inflammatory lung disease, generally related to tobacco smoking, causing obstructed airflow from the lungs. It manifests as *chronic bronchitis*: inflammation of the lining of the bronchial tubes, and *emphysema*: a gradual detoriation of the alveolar structure of the lung, resulting in impaired lung function.

α-1-antitrypsin deficiency (A1AT) is a genetic disorder leading to decreased activity of the serine protease inhibitor α-1-antitrypsin. Severe A1AT deficiency causes panacinar emphysema or COPD in adult life in many people with this condition, especially if exposed to cigarette smoke.

Idiopathic pulmonary fibrosis (IPF) is a disease of unknown ethiology that gradually impairs both the volume and the gas exchange capacity of the lungs. The interstitium is thickened with scarring, making it difficult for oxygen to reach the capillaries. The scarring also makes the lungs "stiff" and hard to inflate.

Cystic fibrosis (CF) is an autosomal recessive genetic disorder affecting mucus producing cells. The ion transport over the epithelium is abnormal causing thick secretions. Mucus plugging in the airways, chronic infection and inflammation, leads to bronchiectasis and destruction of the lung tissue.

Pulmonary artery hypertension (PAH) is a disease where the small vessels of the lung loose their elastic properties and become narrowed. This creates a higher vascular resistance in the lungs and eventually causes right-sided heart failure. The disease can be idiopathic or associated with congenital heart disorders (see below), viral infections, drug treatments or chronic pulmonary embolism.

Eisenmenger is defined as the process in which a long-standing left-to-right heart shunt causes pulmonary hypertension. Fetal screening with echocardiography and surgical intervention early in life have reduced the incidence of heart defects progressing to Eisenmenger's and thereby the number of patients needing heart-lung transplantation.

The data from ISHLT show that *re-transplantations*, predominantely because of chronic graft failure, have stabilized around 4-5% since 2005.

Recipients with COPD, IPF and CF have contributed the most to the growth in the number of transplantations. However, since 1999, IPF is the pre-transplant diagnosis that increases the most and there has been a slight decrease in the percentage of patients with COPD.[7]

The lung transplant donor

In 1987, an updated legislation for determining death was passed and the concept of brain death was introduced in Sweden (SFS 1987:269). Patients could be declared dead although the circulation was operating and, as a consequence, organ function remained intact. This dramatically changed the prerequisites for organ transplantation and made way for the introduction of lung transplantation as a treatment option for end-stage lung disease in our country.
ISHLT has defined donor criteria, which, if fulfilled, are considered optimal for ensuring good recipient outcome (Table 1).[9]

Table 1. Ideal donor criteria according to ISHLT

Age < 55 years
ABO compatibility
Tobacco history < 20 pack-years
Clear chest radiograph
PA O_2 > 40 kPa
No chest trauma
No aspiration
No previous cardiopulmonary surgery
Absence of purulent secretions

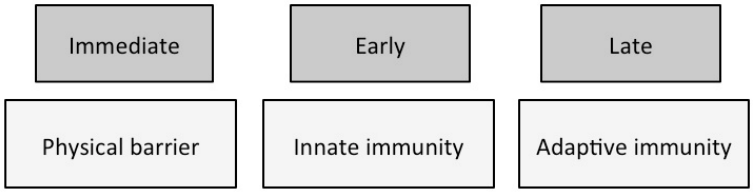
The ideal lung donor is young, previously healthy, non-smoking and without impairment of lung function in the period preceding donation. In clinical practice the donor circumstances are often far from ideal and the majority of donor lungs that are used do not fullfill all ISHLT criteria.[10]

Ex Vivo Lung Perfusion (EVLP) is a method developed to distinguish usable from unusable donor lungs. It is also a potential tool for treatment of donor lungs with reversible pathologies, so that they can be accepted for transplantation. It has been demonstrated that EVLP is safe, enabling marginal lung allografts to be recovered and subsequently used for transplantation, and that the method can contribute to the expansion of the lung donor pool thereby decreasing mortality on the transplantation waiting lists.[11] At Sahlgrenska, EVLP was introduced into clinical practice in 2011.

Lung immunology

A well-functioning immune response has the ability to distinguish between foreign (non-self) and host (self). The host must provide protection against foreign attacks and, at the same time, tolerate all cells within itself (Figure 4).

Figure 4. Host defense



Innate and adaptive immunity

The *innate arm of immunity* is the oldest component of our host defence from an evolutionary point-of-view. This component quickly acts on a foreign attack on the body, whatever the cause may be. The unspecific response is essential for the detection of viruses, bacteria and other noxious agents. The innate immune system initiates an inflammatory cascade and activates the adaptive immune response leading to the elimination of the foreign threat. Macrophages and neutrophils are critical effector cells in innate immunity.[12]

The *adaptive arm of immunity* (= acquired immunity) is more "sophisticated" and a relatively young component of host defence from an evolutionary point-of-view. It exists only in vertebrates. After an initial exposure to a pathogen (= antigen) the host processes the information and creates an army of programmed effector cells and antigen-specific antibodies. If the host encounters the specific antigen again these effector cells, mainly different types of lymphocytes, are ready to attack. This means that the adaptive immune system represents an immunological memory. Thus, this memory recalls how to encounter specific antigens and can act rapidly and effectively on a repeated exposure.

Immune cells

Macrophages

Macrophages are the most abundant leukocytes (= white blood cells) in the alveolar space, accounting for 90-95% of the alveolar leukocytes in a healthy lung. They originate from the bone marrow as monocytes. After a short period in the bloodstream the monocytes migrate to the tissue, where they become dendritic cells or macrophages, i.e. the alveolar macrophages in the alveolar space.[13]

The macrophages live for several months and patrol the tissue in search of harmful particles and change according to the stimuli of the surrondings. They are phagocytes, antigen-presenting cells and cytokine producers and have been described as orchestrating both acute and chronic inflammation as well as later repair processes. By not presenting all possible antigens to the adaptive immune cells the macrophages contribute to the homeostasis of cells and mediators, thereby avoiding inflammation in a healthy lung.[14]

Like other cells, the macrophage expresses surface proteins that may be used for detection. These are named cluster of differentiation (CD). For alveolar macrophages, CD68 or CD14 are often used for identification.

Neutrophils

The most abundant leukocytes in the body are the polymorfonuclear neutrophil granulocytes, which, together with the macrophages, form the first line of host defence.

The neutrophils are produced in the bone marrow and the average lifespan of inactivated human neutrophils in the circulation has been reported to be between 5 and 90 hours.[15] When called upon by an injured tissue, they marginate (position themselves adjacent to the blood vessel endothelium), adhere to the endothelium, squeeze in between the endothelial cells and migrate to the site of foreign exposure, where they survive for 1–2 days.[16]

The neutrophils contain granulaes, each with different kinds of mediators that can digest tissue and kill microbes, as well as intracellular feedback mediators that downregulate the proinflammatory signals to keep the homeostasis.[17-19] When in the tissue, the neutrophils release their mediators in relation to the type of noxious stimuli, mainly by degranulation of their granulae, by phagocytosis of the foreign antigens or by throwing out neutrophil extracellular traps to capture microbes.[18]

Lymphocytes

The lymphocytes are mononuclear leukocytes, also originating from the bone marrow. There are three main types of lymphocytes; T-cells, B-cells and natural killer (NK) cells. In this thesis we have focused on the T-cells.

The T-cells are phenotypically defined as having CD3 ($CD3^+$). They are divided into two main groups; T-helper (Th) cells, also expressing $CD4^+$ and cytotoxic T-cells (Tc), also expressing $CD8^+$. Cytotoxic T-cells are capable of lysing infected or otherwise damaged cells and T-helper cells play an essential role in coordinating the immune response through their release of cytokines and growth factors that regulate other cells like neutrophils and macrophages.

Depending on the pattern of signals they receive, the naïve $CD4^+$ T-helper cells mature into different subtypes; Th1, Th2, Th17 and regulatory T (Treg) cells. The subtypes have different functions and specific cytokine production.[20]

B-cells are responsible for the humoral immunity by secreting antibodies.

The NK-cells belong to the innate immune system and lack receptors for specific antigens.

Cytokines

Cytokines are small proteins that act as signalling molecules between cells involved in the immune response. The cytokines show a great variety in function and activity and play a crucial part in all inflammatory processes by, for example, controlling cell proliferation and differentiation, extravasation of inflammatory cells from the bloodstream and production of immune cells in the bone marrow. They can also alter or reverse the effects of their fellow cytokines.[21] Lymphokines, interleukins, chemokines, interferons and tumor necrosis factors are all different types of cytokines. Below is a brief description of the cytokines discussed in this thesis.

Interferon-γ (IFNγ)

Interferon-γ is an important activator of macrophages and increases host defenses by upregulating the expression of major histocompatibility complex (MHC), a set of cell surface proteins essential for the adaptive immune system to recognize foreign molecules. It has immunostimulatory and immunomodulatory effects and is a key cytokine in the chronic inflammatory process. Among others, NK-cells, T-cells and also neutrophils can produce IFNγ.[22-24]

Tumor necrosis factor (TNF)

Tumor necrosis factor is an archetype pro-inflammatory cytokine. [21] This cytokine has a variety of systemic effects and, for instance, increases the production of neutrophils in the bone marrow, stimulates the liver to produce acute phase proteins and it is also a potent pyrogen. Locally TNF recruits neutrophils and increases the macrophages production of lysosomal enzymes enhancing intracellular killing. Activated macrophages constitute the main source of TNF but it can also be produced by activated T-helper cells, NK cells and neutrophils.

Interleukin-8 (IL-8)

The C-X-C chemokine IL-8 is an interleukin that stimulates the chemotaxis (i.e. when cells move through its concentration gradient) of neutrophils. It plays a key role in neutrophil recruitment and degranulation in the lung and is associated with BOS development.[25-27]

Interleukin-17 (IL-17)

The archetype Th-17 cytokine is IL-17A; a cytokine that can also be produced by additional T cell subsets, including cytotoxic T cells, and by innate lymphoid cells.[28] Interestingly, IL-17A is strategically positioned at the interphase between innate and adaptive immunity and it induces a range of neutrophil-mobilizing compounds, like the neutrophil-activating cytokine IL-6, the chemokine IL-8 and the growth factor granulocyte macrophage colony-stimulating factor.[29] Moreover, IL-17 might also more generally stimulate neutrophil activity by inducing for example NE and MMP-9 and it has been shown to be involved in both acute and chronic rejection.[30,31]

Interleukin-18 (IL-18)

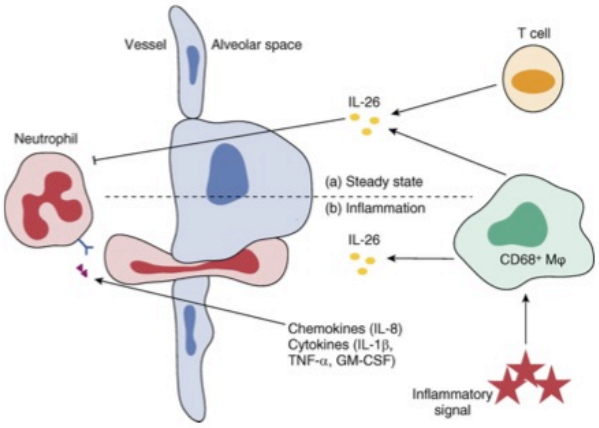
Interleukin-18 is a pro-inflammatory cytokine, belonging to the IL-1 family, mainly produced by activated macrophages.[32] It induces production of IFNγ in several types of immunocompetent cells such as helper T-cells, cytotoxic T-cells and NK cells especially in collaboration with IL-12.[33,34]

Inteleukin-26 (IL-26)

Interleukin-26, (earlier named AK155), is an IL-10 family member produced by Th17 cells, macrophages and other leukocytes.[35-38] IL-26 up-regulates IL-8, TNF and IL-1β production in the target cells indicating that it drives or sustains inflammation and a growing number of studies suggest that IL-26 is involved in a range of chronic inflammatory disorders.[39-41]

Recent findings show that, in the airways, IL-26 is abundantly produced and released by alveolar macrophages and contributes to the local mobilization of neutrophils.[38] It can differentially regulate the immune microenvironment depending on the setting and responder cell type (Figure 5).

Figure 5. IL-26 differentially controls neutrophil recruitment to the lungs



In the steady state, in the absence of IL-8, IL-26 inhibits neutrophil chemokinesis (*a*); however, IL-26 produced by CD68+ macrophages (Mϕ) in the presence of an inflammatory signal and IL-8 induces neutrophil chemotaxis (*b*). GM-CSF = granulocyte–macrophage colony–stimulating factor; TNF = tumor necrosis factor

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Proteolytic homeostasis

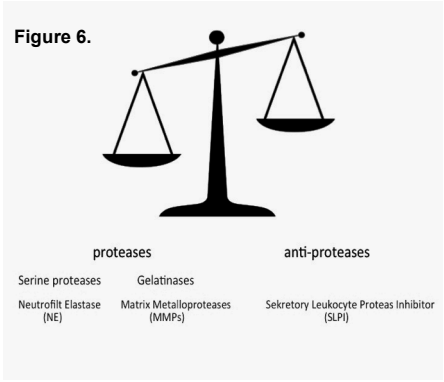


Figure 6.

Proteases, also called proteinases or peptidases, are enzymes that break peptide bonds between amino acids in proteins and peptides. Cleavage can lead to degradation of extracellular matrix and to both activation and inactivation of enzymes. To protect the tissue there are several proteins that inactivate proteases. The protease – anti-protease balance, i.e. the proteolytic homeostasis, plays an important role in both inflammation and healing. There are noumerous proteases and anti-proteases, below is a brief description of those addressed in this thesis (Figure 6).

Serine proteases

Neutrophil elastase (NE), one of the serine proteases, is neutrophil specific and released by the neutrophil after stimulation by e.g. TNF or IL-8. In addition to degrading extracellular matrix, NE has been shown to degrade other proteins such as cytokines and chemokines.[42] Elastin, the substrate of NE, is essential for the structure of small airways, preventing them from collapse. Increased levels of NE are characteristic for several chronic pulmonary diseases, such as COPD, CF, and IPF.[42,43]

Matrix metalloproteinases (MMPs)

The MMPs are a group of proteases that degrade extracellular matrix. In this thesis we analysed the zink-containing gelatinases, MMP-2 and MMP-9, who cleave gelatin. MMP-2 is expressed in several cell types, among them macrophages.[44] MMP-9 is expressed in, for instance, neutrophils and activated macrophages and released as 92kD precursor to be cleaved and activated extracellularly by serine proteases. After activation MMP-9 can cleave IL-8 and TNF into their active forms, thereby promoting inflammatory response.[44]

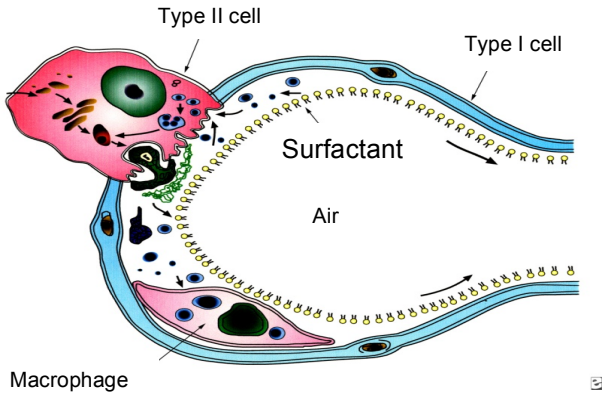
Anti-proteases

One of the main anti-proteases of serine proteases is secretory leukocyte proteinase inhibitor (SLPI). It constitutes one of the major defenses against the destruction of pulmonary tissues by NE. SLPI is produced by epithelial cells and neutrophils.[45]

Lung surfactant

Lung surfactant is a phospholipoprotein complex formed by alveolar type II cells.[46] The proteins and lipids that make up the surfactant have both hydrophilic and hydrophobic regions. By adsorbing to the air-water interface of alveoli, with hydrophilic head groups in the water and the hydrophobic tails facing towards the air, the main lipid component of surfactant, dipalmitoylphosphatidylcholine (DPPC), reduces surface tension. In turn, reduced surface tension leads to increased pulmonary compliance, prevention of atelectasis at the end of expiration and facilitates recruitment of collapsed airways (Figure 7).
Lung surfactant serves as one of the first defence mechanisms the lung can mount against various insults. Surfactant phospholipids are part of the physical mucosal barrier.[47] Surfactant proteins play important rolls in the innate host defence and may serve ass cross-talk proteins between the innate and the adaptive immune systems.[48]

Figure 7. Alveolus



Adapted from Arun K Pramanik, Respiratory Distress Syndrome, Medscape

Surfactant protein-A

Surfactant protein-A is produced in the lung primarily by the alveolar type II cells, and belongs to the collectin super-family serving as an opsonin for bacteria, fungi and viruses. It also participates in the phospholipid homeostasis and in the orchestration of lung immunity by regulating cytokine production from macrophages and neutrophils and by providing direct or indirect modulation of lymphocyte activity and proliferation.[47-49]

Allograft rejection

An allograft is a tissue graft from a donor of the same species as the recipient but not genetically identical. Alloreactivity is defined as the host immune response to non-self donor antigens and, as such, a dominant feature of lung transplantatation where donor organs are not routinely human leukocyte antigen (HLA)-matched (because of organ scarcity and logistic constraints).
The HLA system is a gene complex encoding the major histocompatibility complex (MHC) proteins in humans. It is a set of cell surface proteins essential for the adaptive immune system to recognize foreign, = non-self, molecules which in turn determines histocompatibility.

The nomenclature of chronic allograft rejection has changed over time. Today the picture is somewhat different from when we first started these studies, allthough still applicable on the data presented.

Acute allograft rejection

The cellular mechanisms leading to acute rejection (AR) are only partially understood. There is evidence that T-helper cells in cooperation with antigen presenting cells, such as the alveolar macrophage, play a crucial role in the host response to donor antigens. The production of IFNγ in several types of immunocompetent cells, such as NK-cells, helper T-cells and cytotoxic T-cells is also believed to be a phenomenon of central importance in AR. IFNγ, in turn, decreases the Th2 response and enhances the Th1 response by mobilizing cytotoxic T-cells. [50-52]

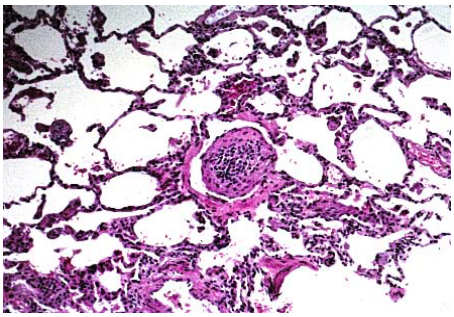
The histopathological classification of acute rejection (AR) in LTRs was first described in 1990 and has only minimally evolved since then.[53,54] Acute vascular cellular rejection on transbronchial biopsies is characterized according to the degree of perivascular lymphocytic infiltrate with minimal (A1), mild (A2), moderate (A3) and severe (A4) infiltration.

The management of life-threatening AR has been a major success. Calcineurin-based maintenance immunosuppression protocols and steroid based augmented immunosuppression strategies have dramatically reduced the rate of histopathological AR and hence graft loss from this condition over the last decades.[55] However, repeated AR still constitutes a major risk factor for developing chronic rejection.[56]

Chronic allograft rejection

Chronic rejection developes in 50% of the LTRs within 5 years post procedure and accounts for about 30% of the mortality between 3 and 5 years after the transplantation.[7]
Originally, chronic rejection was defined as pathological obliterative bronchiolitis (OB) (Figure 8).[54,57,58]

Figure 8. Histological picture of obliterativ bronchiolitis

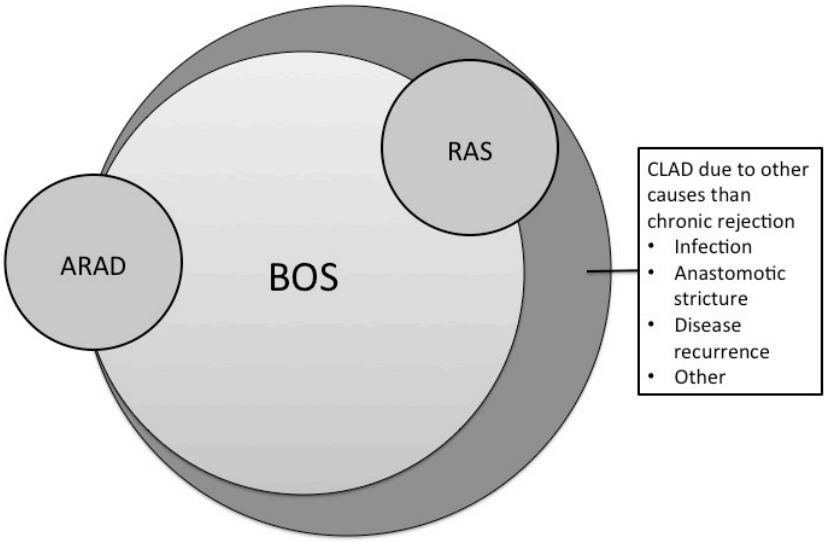


Obliterative bronchiolitis is characterized by inflammation, destruction and fibrosis of small airways leading to proliferation of dense granulation tissue, which organizes to partially or completely fill the lumen of the airway.

Since histological confirmation of OB with bronchoscopy and transbronchial biopsies (TBB) is difficult to obtain, due to the limited biopsy sizes and the patchy appearance of OB, the clinical correlate bronchiolitis obliterans syndrome (BOS) was proposed.
BOS is characterized by an obstructive and persistent pulmonary function decline (decline in FEV_1 of at least 20% of the baseline value, i.e. the average maximum FEV_1 value of two consecutive measurements >30 days apart during the first post-operative year).[59,60]

However, it is apparent that not every chronic decline in FEV_1 is obstructive or irreversible.[61] As a consequence, the term chronic lung allograft dysfunction (CLAD) was introduced to describe any chronic decline in FEV_1 and/or forced vital capacity (FVC), irrespective of its cause (Figure 9).[62]

Figure 9. Chronic lung allograft dysfunction (CLAD) and the different phenotypes of chronic rejection



The descriptive term CLAD includes the different phenotypes of chronic rejection as well as non-chronic rejection causes of graft dysfunction. ARAD should be retrospectively excluded by a 3-month trial with azithromycin, before CLAD can be diagnosed. Specific causes of CLAD should be excluded before CLAD is accepted as a manifestation of chronic rejection and before subphenotyping into BOS and RAS can be performed.
CLAD, chronic lung allograft dysfunction; BOS, bronchiolitis obliterans syndrome; RAS, restrictive allograft syndrome; ARAD, azithromycin-responsive allograft dysfunction

BOS

BOS is the most common form of CLAD, accounting for approximately 70 % of cases and it is the leading cause of mortality beyond the first transplant year.[7,63] Several factors that increase the risk of BOS development have been identified (Table 2).[64]

Table 2. Risk factors for BOS development

Primary graft dysfunction
Acute cellular rejection
Lymphocytic bronchiolitis
Gastro-oesophageal reflux and microaspiration
Infection/colonization (viral, bacterial, fungal)
Humoral rejection
Persistent neutrophil influx and sequestration
Autoimmunity (collagen V sensitisation)
Air pollution
Genetic factors

BOS is associated with local accumulation of the chemokine IL-8 and neutrophils in the airways.[26,27] In addition, epithelial activation is found, associated with increased expression of MMPs and deposition of subepithelial collagens and other matrix proteins in the airway wall.[65,66] Previous studies have suggested that an increase in potentially tissue-destructive gelatinases and serine proteases may signal the development of BOS.[66-70]

There is also evidence that the development of BOS involves interleukin IL-17A released by Th17 cells.[71] However, little is known about the corresponding role of Th17 cytokines other than that of IL-17A. This is particularly true for IL-26, a relatively recently discovered cytokine produced by Th17 cells, macrophages and other leukocytes in the airways.[35-38]

Moreover, a significant reduction of bronchoalveolar lavage (BAL) SP-A content has been found in BOS patients compared with stable LTRs.[72]

In contrast to AR there is no effective treatment for BOS in the majority of cases. Aside from ensuring adequate immunosuppressive treatment it has been shown that a switch from cyklosporin to tacrolimus can potentially decrease the

rate of the decline in lungfunction.[73,74] Stabilization of spirometry results has also been found when switching from azathioprine to mycophenolate mofetil.[75]
A survival advantage can, in some cases, be obtained from treatment with the macrolide antibiotic azithromycin, especially when starting azithromycin during the earlier stages of BOS (BOS Stage 1).[76] Recently the term azithromycin-responsive allograft rejection (ARAD), describing a phenotype of CLAD responding to azithromycin treatment, was introduced.[62] Responders to azithromycin have a higher pretreatment BAL neutrophilia and elevated IL-8 mRNA levels.[77]
In some patients, refractory to all other available treatments, extracorporeal photopheresis can be taken into consideration.[78,79]
However, in the majority of cases, none of the above actions halts the decline in lung function. Selected patients can then be evaluated for re-transplantation as a final treatment option.

Aim

The overall aim of this thesis was to increase the knowledge about the underlying biological processes taking place in acute and chronic rejection and to identify potential biomarkers for early diagnosis and monitoring of these conditions. Ideally the biomarkers are easily detected with non-invasive methods enabling repeated measurements over time, with minimal discomfort and risk for the patient.
The following specific research questions were posed:

Paper I

- Is IL-18, an IFN-γ inducing cytokine that augments the Th1-polarized immune response and is released from activated macrophages in the bronchalveolar space, associated with onset of AR?

Paper II

- Is the net gelatinase or the net serine protease activity, in the bronchoalveolar space, altered in BOS?
- If such alterations exist, are they linked to quantitative changes in the gelatinases MMP-2 and MMP-9 or to the serine protease NE, or to the anti-protease, SLPI?

Paper III

- Is IL-26, a cytokine produced by Th17 cells and macrophages in the bronchoalveolar space, involved in AR and BOS development?
- What are the cellular sources of IL-26 in LTRs?

Paper IV

- Is the non-invasive method for sampling of particles in exhaled air (PEx) feasible in lung transplanted patients?
- Does PEx composition differ between LTRs and healthy controls and between BOS/non-BOS patients?
- Is SP-A in PEx a potential biomarker for BOS?

Patients and Methods

Clinical protocol and participants

In **Papers I-III** the biobank consisted of over 900 frozen (-80°C) BAL samples from more than 140 LTRs collected during 1996-2002.
All transplanted organs were harvested in a similar fashion, surgical procedures and immunosuppression therapy were performed in accordance with the lung transplant program of Sahlgrenska University Hospital. The clinical protocol for postoperative follow-up, at the time for the sample collection, included bronchoscopy with TBB and BAL at regular intervals (1, 2, 3, 4.5, 6, 9, 18 and 24 months after surgery, thereafter yearly) and samples were also taken when clinical signs of worsening of the patient condition appeared, such as radiographic infiltrate, fever, dyspnea, hypoxemia or decline FEV_1.[80,81]
The morphologic evaluation of AR and BOS followed the standard recommendations of the Lung Rejection Study Group of the International Society of Heart and Lung Transplantation.[53] BOS developed in 45% of the patients and was defined as an irreversible decline in FEV_1 of at least 20% of the baseline value, i.e. the average maximum FEV_1 value of two consecutive measurements >30 days apart during the first post-operative year. At the time these samples were collected the term chronic lung allograft rejection (CLAD) and the different phenotypes of CLAD had not yet been established.
Routine BAL and TBB specimen analysis for infectious agents included direct microscopy for cytomegalovirus (CMV) inclusion bodies, Pneumocystis jirovecii, other fungi and mycobacteria. In addition, immunocytochemistry techniques for pneumocystis, CMV and Legionella pneumophila were applied. Cultures for bacteria, including legionella and mycobacteria, fungi and viruses were performed and presence of CMV and respiratory syncytial virus genome was investigated with polymerase chain reaction amplification.

In **Paper III**, to characterize the cellular sources of IL-26 protein, fresh BAL cells were collected from LTRs undergoing routine follow-up bronchoscopy in 2014.

In **Paper IV**, LTRs were included consecutively from November 2012 to May 2014 at the time for their scheduled clinical assessment at six months or more after transplantation. Inclusion criteria were stable condition after lung transplan-

tation, freedom from BOS at the prior visit and absence of significant infection at the study visit. Lung function assessment and collection of particles in exhaled air (PEx) were performed prior to bronchoscopy. Each individual was followed for 12 months after the study visit with spirometry, radiography and clinical assessment, and then categorized as BOS or non-BOS. Healthy subjects participating in the Gothenburg part of the European Community Respiratory Health Survey, matched for age and sex were used as controls. Control subjects were selected if they had neither chronic obstructive pulmonary disease (defined as a post-bronchodilator FEV1/FVC ratio < 0.7) nor asthma (excluding those with physician-diagnosed asthma and those reporting asthma symtoms or taking asthma medication) and were non-smokers.

Patient matching (Paper I-III)

In clinical research, a multitude of clinical factors can affect the variables one sets out to analyse, and possibly disturb the results. Thus, to minimize the influence of confounding clinical factors as far as possible we identified pairs of patients, carefully matched for age, gender, preoperative diagnosis, type of and time after surgery, in our database. When comparing AR vs non-AR all samples with BOS were excluded and when comparing BOS vs non-BOS all samples with AR ≥ grade 2 were excluded. Only samples free from infection were included and the matching procedure was repeated in the same way for each study. (Figure 10 and Table 3 - example from paper III).

Figure 10.

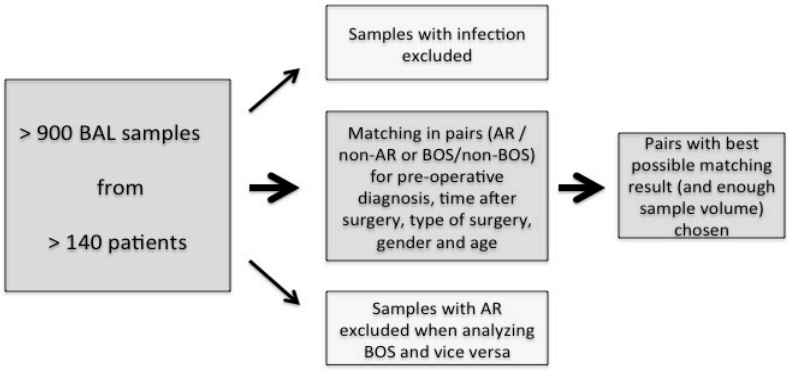


Table 3. Patient characteristics (20 matched pairs*)

Non-BOS (n = 20)				BOS (n = 20)				
Diagnosis	Age	Tx type	Gender	Diagnosis	Age	Tx type	Gender	BOS
A1AT	47	SL	M	A1AT	53	SL	F	3b
A1AT	57	SL	F	A1AT	51	BL	F	2b
CF	31	BL	M	CF	25	BL	M	3b
CF	35	BL	M	CF	21	HL	M	3b
COPD	47	SL	F	COPD	47	SL	F	2a
COPD	47	SL	F	COPD	53	SL	F	2b
COPD	53	SL	F	COPD	47	SL	F	3a
COPD	53	SL	F	COPD	52	SL	F	2a
COPD	55	SL	F	COPD	55	SL	F	2b
COPD	64	SL	M	COPD	56	SL	M	3a
Eisenmenger	32	HL	M	Eisenmenger	16	HL	M	3b
Eisenmenger	30	HL	M	Eisenmenger	35	HL	M	3a
Eisenmenger	40	HL	F	Eisenmenger	51	HL	F	3b
HUVS	39	BL	F	BAC	47	BL	F	3a
IPF	54	SL	F	IPF	51	SL	F	2a
PAH	22	HL	M	PAH	29	BL	M	3b
PAH	43	BL	F	PAH	26	BL	F	2b
PAH	40	BL	F	PAH	33	BL	F	3a
PAH	42	HL	F	PAH	45	HL	F	3b
PAH	47	BL	M	PAH	51	BL	M	3a

BOS, bronchiolitis obliterans syndrome; A1AT, α-1-antitrypsin deficiency; CF, cystic fibrosis; COPD, chronic obstructive pulmonary disease; HUVS, hypocomplementemic urticarial vasculitis syndrom BAC, bronchiolo alveolar carcinoma; IPF, idiopathic pulmonary fibrosis; PAH, pulmonary arterial hypertension; Tx, transplantation; SL, single lung; BL, bilateral lung; HL, heart and bilateral lung; F, female; M, male. *Matched for pre-transplant diagnosis, age, gender, type of surgery and sampling time after surgery.

Ethics

The study protocols that were used in this thesis were approved by the Regional Ethics Review Committee of the University of Gothenburg (Diary No. S-539-03, 1031-13, 390-06, 472-07). Informed consent was obtained from all participants, in accordance with the Helsinki declaration.

Statistics

As normal distribution of data could not be proven with the current sample sizes non-parametric statistical analysis was used throughout all the studies. Mann-Whitney U-test was performed for comparison between groups, Wilcoxon signed rank for comparison of matched data sets and Spearman rank for correlation calculations. We used a SPSS standard statistic program and Graph Pad PRISM 6 ® for the calculations. In Paper IV, each patient was matched to a healthy control using the vmatch SAS macro.[82] Values of $p < 0.05$ were considered to indicate statistical significance.

Bronchoalveolar lavage (paper I-III)

BAL sample collection

Bronchoscopy and BAL sample collection was conducted under anesthesia. In summary, BAL was performed by two infusions with 50 ml of sterile phosphate-buffered saline (PBS) solution (37°C) into a segmental middle lobe or lingula bronchus, with the bronchoscope in a wedged position. The fluid was re-aspirated after each 50-ml infusion, collected in a sterile siliconized container and kept on ice until further processing. After filtering, cellular components were separated by centrifugation and the cell-free supernatant of the BAL sample was removed. The supernatant was frozen at -80°C for later analysis.

BAL- cell differentials

Routine differential counts for BAL-cells were performed in paper I-II. Cytospin slides were prepared from resuspended cell pellets. The relative content of neutrophils, alveolar macrophages, lymphocytes and eosinophils were calculated by counting cells using a standard light microscope and expressed as a percentage of total BAL cells.

Enzyme-linked immunosorbent assay (ELISA)

ELISA was used in all studies for assessment of proteins in BAL (and in PEx, see below). The samples and standards were incubated in a polystyrene microtiter plate with 96 wells pre-coated with antibodies recognizing the protein to be analyzed. The protein was captured by the antibody during incubation, whereafter unbound material present in the sample was removed by washing. A second labeled antibody (detection antibody) was added and bound to a different epitope of the captured protein. After incubation, the excess antibodies were again washed away. The last step was to add a substrate to the wells leading to colour developing proportionally to the amount of labeled antibody bound to the protein in the sample. The reaction was stopped, and the absorbance at a specific wavelength was recorded using a spectrophotometer. A standard curve was obtained by plotting the absorbance versus the corresponding concentrations of the known standards thereby determing the unknown concentrations of the samples. The specific methods for the proteins analysed in this thesis are described in detail in each paper.

Latex bead concentration method (paper II)

Since, in our experience, the commercially available ELISA-kits for assessment of NE concentration did not give reliable results, the samples were sent to Dr. Shigemi Yoshihara, Dokkyo Medical University, Japan for analysis with the latex bead concentration method. In principle, latex particles were coated with antibody fragments against human NE. If NE was present in the test sample, the latex particles agglutinated and and the turbidity in the reaction vessel intensified. The change in turbidity was measured photometrically and the extent of the turbidity is proportional to the NE concentration in the test sample.

Net protease activity (paper II)

The activity of proteases was measured through fluorescence assays. Since MMP-9 is a gelatinase, gelatin was fluorescently tagged and incubated with BAL samples leading to degradation of gelatin according to the amount of active gelatinases in the sample. The fluorescence intensity was then measured. The same principle was used to evaluate the activity of serine proteases using a tagged substrate of serine peptides and measuring the relative absorbance intensity of the degraded peptides after incubation with the BAL fluid.

Zymography (paper II)

Zymography was used to identify and assess the total activity of the gelatinases MMP-2 and MMP-9.
Zymography is based on gel electrophoresis, a method in which molecules are separated by charge and size. By applying an electric field to a matrix loaded with the substrate, in this case a flourescence-conjugated gelatin substrate, the negatively charged molecules are forced to move across the matrix. The migration, visualized by a band, corresponds to a reference level representing the molecular weight. The molecular weight enables identification of the protein. Densitometry, as intensity of the zymography bands, was used to assess total activity.

For the analyses of net protease activity and zymographies the samples were sent to Dr. Gary P. Andersson, University of Melbourne, Australia.

Flow cytometry (paper III)

Flow cytometry was used to identify cellular sources of IL-26. The method allows for the individual measurement of fluorescence from each cell. In principle, the freshly collected BAL cells are marked with antibodies artificially conjugated to fluorochromes. A beam of laser light is directed at a hydrodynamically focused stream of fluid that carries the cells in single file. The cells expressing the marker for which the antibody is specific will be excited by the laser and emit light at varying wavelengths. The fluorescence can then be measured to determine the amount and type of cells present in the sample.

Immunocytochemistry (paper III)

To further determine the cellular sources of IL-26, immunocytochemistry (ICC) was performed using cytospin slides prepared with freshly isolated BAL cells. The method is further described in paper III, but the main principle was to visualize the localization of IL-26 in cells by use of a specific primary monoclonal antibody. The monoclonal antibody was bound by a secondary antibody, containing an enzyme that gave rise to a signal, e.g. color, from an enzyme reaction. This allowed visualization of IL-26 under a light microscope.

Particles in exhaled air (paper IV)

Figure 11. Development of the PExA instrumentation

2003 2006 2016

PExA (Particles in Exhaled Air - the method) is a novel, non-invasive method for monitoring of, and sampling from, the distal airways developed at the Department of Occupational and Environmental Medicine, University of Gothenburg, Sweden (Figure 11).
The sample, i.e. endogenous particles (PEx) are collected using an inertial impactor with subsequent chemical analysis (Figure 12).[83] The particles originate from the epithelial lining fluid (ELF) in the small airways and their protein and lipid profile is similar to that of BAL.[84] It has previously been demonstrated that collection of particles is a valid method for quantifying exhaled SP-A originating from ELF in humans.[85-87]

The procedure is described in detail in paper IV. In summary, prior to PEx sampling subjects inhaled HEPA-filtered air for 2 min to remove particles originating from ambient air. All subjects wore a nose clip throughout the sampling procedure. During sampling, the subjects performed a standardized breathing maneuver allowing for maximum airway closure and reopening.[88] Only the last exhalation was sampled in the instrument. The procedure was repeated until a target volume of 60 L of exhaled air or a sampling time of 30 min was reached. The exhaled particles were sampled on a Teflon membrane and the total number of exhaled particles and total volume of the exhaled breath were recorded. The total mass of the collected samples was calculated based on the number and size of the collected particles. After collection the Teflon membrane was transferred to a polypropylene vial and stored at -80°C until further analysis with ELISA.

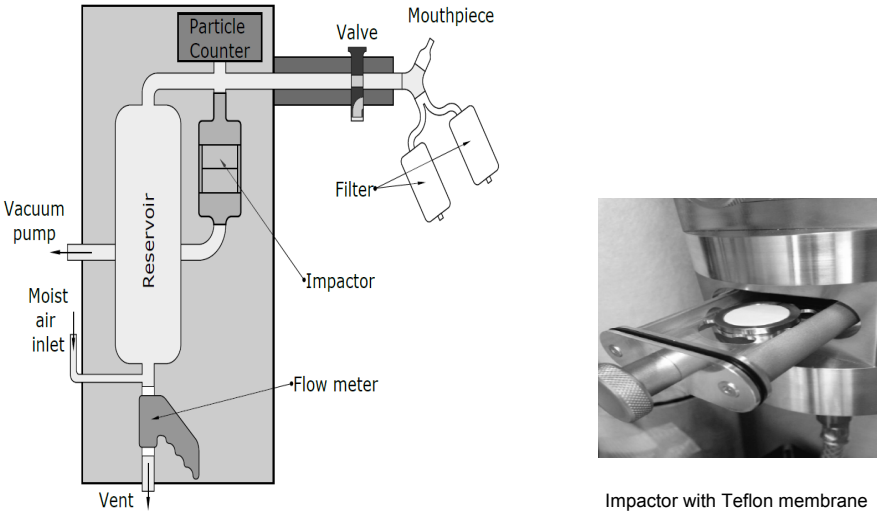


Figure 12. By courtesy of Anna Bredberg
The subject exhales via a mouthpiece and a directional valve into a thermostated box (36°C) containing an exhaled air reservoir, a Grimm 1.108 optical particle counter (Grimm Aerosol Technik GmbH & Co, Ainring, Germany), and an impactor (3-stage PM 10 Impactor, Dekati Ltd., Tampere, Finland). Using a vacuum pump the exhaled air containing particles is drawn through the impactor and the particles are collected by impaction according to their size on the hydrophilic Teflon membrane.

Results

IL-18 (paper I)

In paper I, we identified eight LTRs with AR and matched them in pairs with a control group of LTRs without AR according to the principle described in the methods section. For patient characteristics and matching results see paper I. Cell-free BAL samples collected before, during and after an episode of AR (with corresponding timepoints in the control group) were analysed.

The mean concentration of IL-18 (ELISA) was not increased before or during an episode of AR (Figure 13).

No significant correlation was found for the concentration of IL-18 and the percentages of cell subtypes in BAL. The relationship between IL-18 and BAL lymphocytes is shown in figure 14.

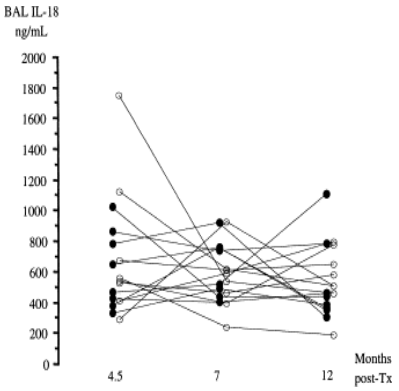


Figure 13. Individual concentration of IL-18 protein in BAL samples at 4.5, 7 and 12 months time after lung transplantation. Filled dots denote subjects with diagnosis of acute rejection ⩾A2 at the 7-month interval. Open circles denote controls without acute rejection.

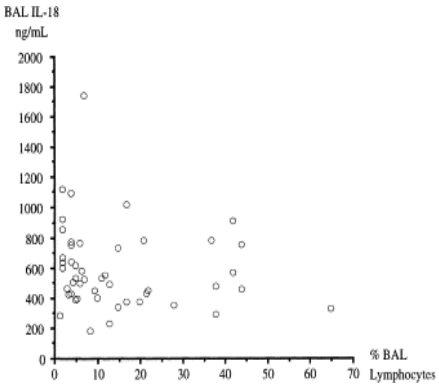


Figure 14. Correlation of individual concentration of IL-18 with percentage of lymphocytes in BAL samples.

Proteolytic homeostasis (paper II)

For this study, we identified cell-free BAL samples from 12 LTRs diagnosed with BOS that could be matched with samples from 12 LTRs without BOS in the same manner as earlier described. For patient characteristics and matching results, see paper II.

We used substrate assay methods of net protease activity, methods that do not remove or inhibit natural anti-proteases. The net gelatinase activity was clearly higher in the BOS group ($p < 0.005$). In contrast, the net serine protease activity did not differ markedly between the groups.

Zymography was performed to identify and assess total activity of gelatinases. The gelatinases were identified as MMP-9 and MMP-2 (Figure 15). Densitometry analysis of the band intensity in zymography gels revealed that both in the BOS and the non-BOS groups, the intensity of the MMP-9 band markedly exceeded that of the MMP-2 band ($p < 0.01$ for both comparisons). For MMP-9, zymographic intensity was clearly stronger in the BOS group (Figure 16A). For MMP-2, however, the intensity did not differ markedly (Figure 16B).

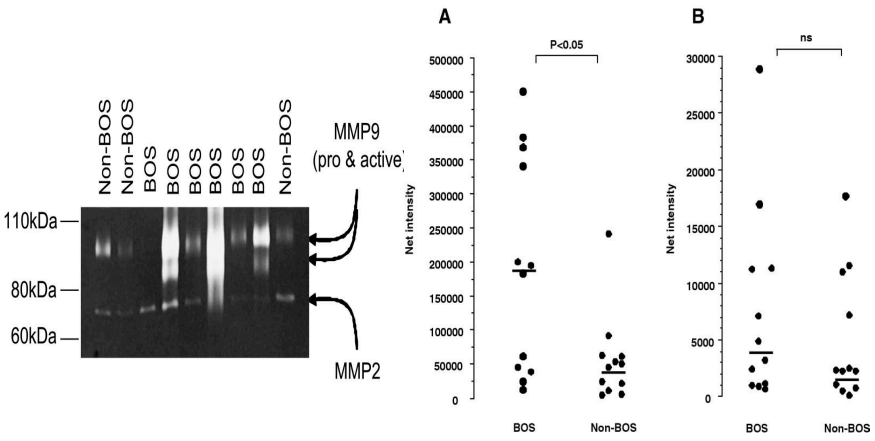


Figure 15 and 16 A+B. Representative zymography gel of bronchoalveolar lavage fluids from patients with or without bronchiolitis obliterans syndrome (BOS) (14) and total gelatinase activity for matrix metalloproteinase (MMP)-9 (15A) and MMP-2 (15B).

The MMP-9 concentrations measured with ELISA and the NE concentrations measured with the latex concentration method were higher in the BOS group than in the non-BOS group ($p < 0.05$ and $p < 0.01$ respectively). However, the MMP-2 and SLPI concentrations (ELISA) did not differ clearly between the groups.

The net gelatinase activity correlated strongly with the concentration of MMP-9 and with the percentage of neutrophils in BAL samples from BOS patients but did not correlate with the corresponding concentration of MMP-2 (Figure 17 A-C).

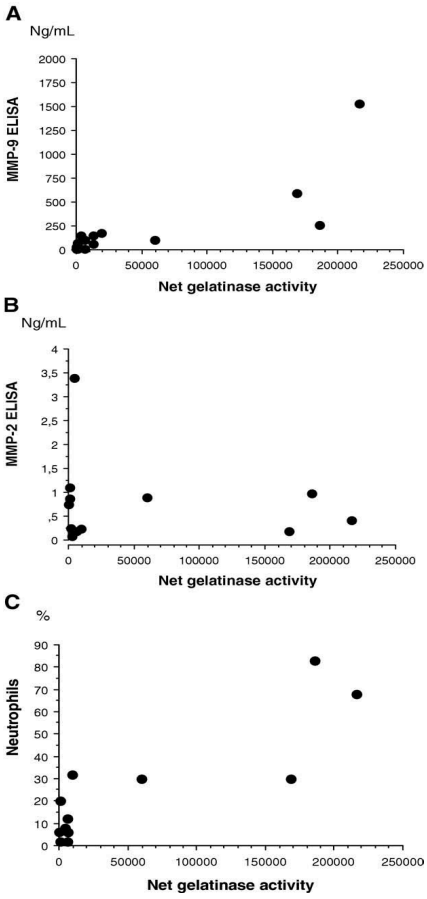


Figure 17. Correlation of (A) net gelatinase activity with the concentration of soluble matrix metalloproteinase (MMP)-9 protein (ρ=0.9, $p<0.01$), (B) net gelatinase activity with the concentration of soluble MMP-2 protein (ρ=0.1, $p>0.05$), and (C) net gelatinase activity with the percentage of neutrophils (ρ=0.8, $p<0.01$) in bronchoalveolar lavage fluid from patients with bronchiolitis obliterans syndrome (BOS) n=12.

Finally, net serine protease activity also correlated clearly with the concentration of NE and neutrophil percentages in BAL from BOS patients.

IL-26 (paper III)

Here, we identified 20 pairs of LTRs, with or without BOS and 12 pairs of LTRs, with or without AR for our cross-sectional investigation. For our longitudinal investigation, we identified 10 pairs (BOS/non-BOS) with BAL samples collected at three occasions. The pairs were again matched as described previously. An example of the result of a matching procedure is shown in table 3 in the methods section.

IL-26 protein concentrations measured by ELISA in cell-free BAL fluid were higher in the BOS group than in the non-BOS group (Figure 18). We found no significant differences when comparing AR to non-AR LTRs.

In the longitudinal investigation, with three sampling occasions, the concentration of IL-26 was low in the pre-BOS period in both groups and increased in the BOS group at BOS diagnosis (Figure 19).

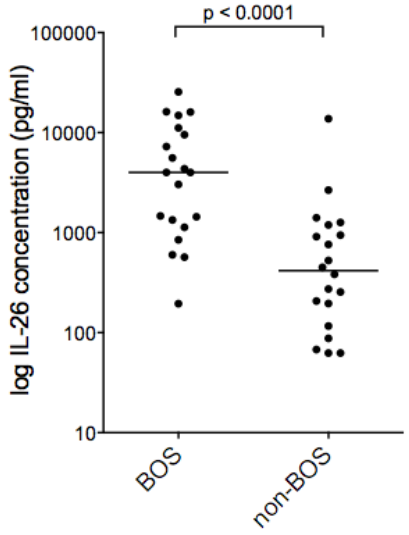


Figure 18. Interleukin (IL)-26 protein concentration (logarithmic scale) in cell-free bronchoalveolar lavage fluid from lung transplant recipients (LTRs) with (*n* = 20) and without (*n* = 20) bronchiolitis obliterans syndrome (BOS) quantified by enzyme-linked immunosorbent assay (ELISA). Bars represent the median.

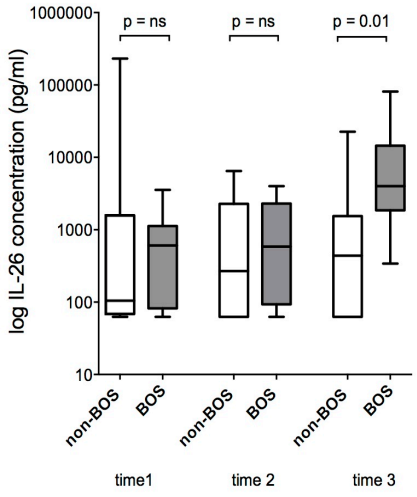


Figure 19. Interleukin (IL)-26 protein concentration (logarithmic scale) in cell-free bronchoalveolar lavage fluid from lung transplant recipients (LTRs) with (*n* = 10) or without (*n* = 10) bronchiolitis obliterans syndrome (BOS) quantified by enzyme linked immunosorbent assay (ELISA). Time one and two before and time three at BOS diagnosis (with corresponding time points in the BOS free group.

To characterize the cellular sources of IL-26 protein we collected fresh BAL cells from five LTRs diagnosed with BOS and seven LTRs without BOS, undergoing routine bronchoscopy.
Flow cytometry staining detected IL-26 protein both in $CD4^+$ and $CD8^+$ cells from BAL in all examined LTRs.
Using immunocytochemistry we found strong immunoreactivity for IL-26 protein in predominantly large mononuclear BAL cells. A specific anti-CD68 antibody was added to prove that these cells were alveolar macrophages. The staining protocol showed, as expected, strong CD68 staining in in large mononuclear BAL cells and furthermore confirmed a co-expression signal for CD68 and IL-26 in BAL alveolar macrophages (Figure 20).

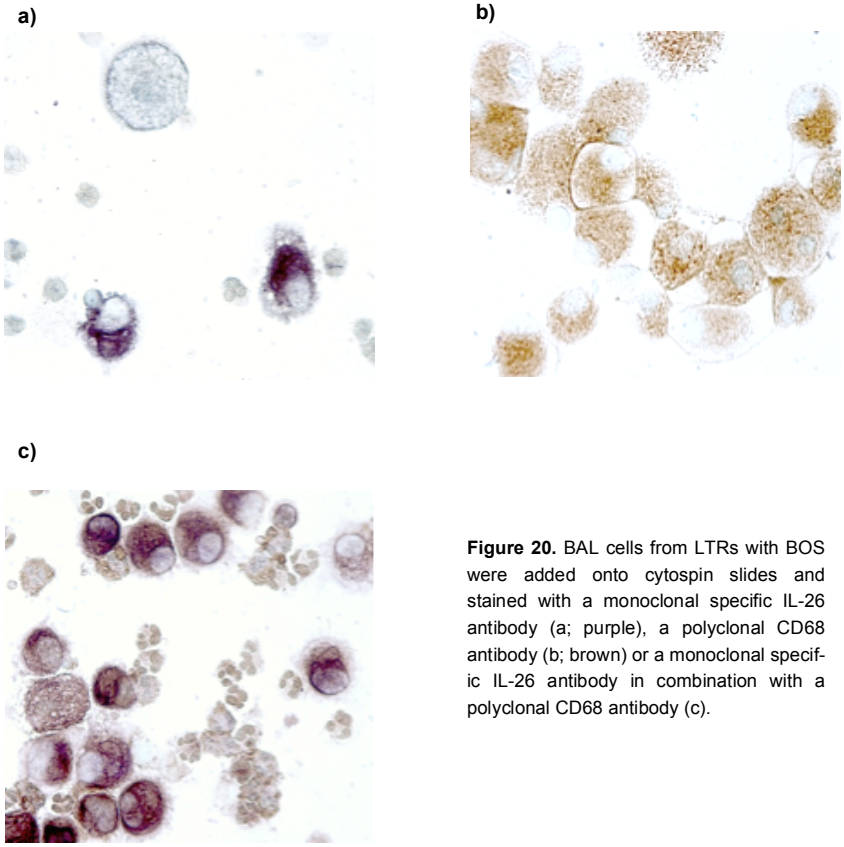


Figure 20. BAL cells from LTRs with BOS were added onto cytospin slides and stained with a monoclonal specific IL-26 antibody (a; purple), a polyclonal CD68 antibody (b; brown) or a monoclonal specific IL-26 antibody in combination with a polyclonal CD68 antibody (c).

PEx (paper IV)

33 LTRs accepted to participate and fulfilled inclusion criteria. 5/33 had recieved a single lung transplant and 28/33 had received two new lungs. 12 months after the study visit they were categorized as stable lung LTRs (*n* = 26), or LTRs who developed BOS (*n* = 7). 2/7 patients where diagnosed with BOS at the study visit and 5/7 within the 12 months after the visit. A comparison between the LTRs and healthy subjects participating in the Gothenburg part of the European Community Respiratory Health Survey, matched for age and sex (*n* = 33), was also conducted. Study subject characteristics are presented in table 4.

Table 4. Study subject characteristics

	non-BOS (*n* = 26)	BOS (*n* = 7)	controls (*n* = 33)
Male/Female (*n*)	15/11	2/5	17/16
Mean age (*years*)	55,4	57,6	56
Type of operation (*SL/BL*)	3/23	2/5	
EVLP (*n*)	6	1	
Pre-op diagnosis (*n*)			
COPD	10	4	
IPF	5	1	
A1AT	4	2	
CF	4		
Other	3		

BOS, bronchiolitis obliterans syndrome; SL, single lung; BL, bilateral lung; EVLP, ex vivo lung perfusion; COPD, chronic obstructive pulmonary disease; IPF, idiopathic pulmonary fibrosis; A1AT, α-1-antitrypsin deficiency; CF, cystic fibrosis.

The amount of PEx collected per liter exhaled air (i.e. mass concentration) was markedly higher among the LTRs (BOS + non-BOS) compared to the healthy controls. There was also a significant difference between the BOS and the non-BOS group (Figure 21 A).

SP-A in PEx was significantly lower in the BOS group compared to the non-BOS group but there was no difference between LTRs (BOS + non-BOS) and healthy controls (Figure 21 B).

Albumin in PEx was significantly higher in the healthy controls than in the LTRs (BOS + non-BOS) ($p < 0.0001$) but there was no difference between the BOS and the non-BOS group (Figure 21 C).

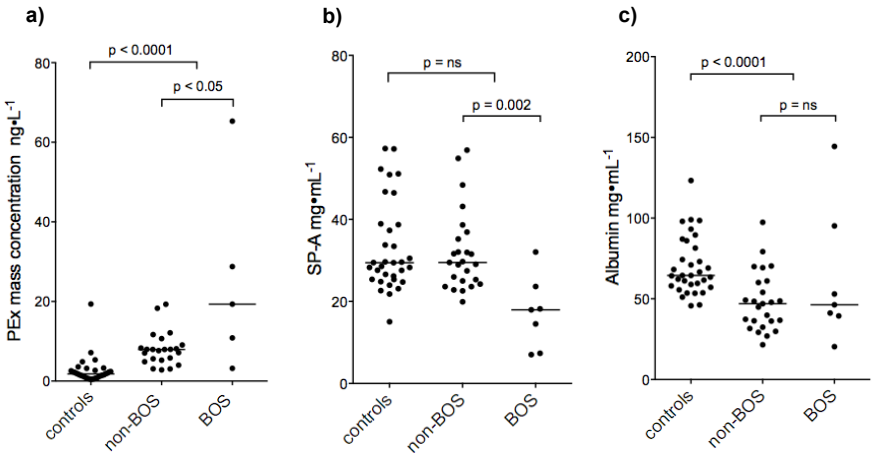


Figure 21. Amount of PEx (ng) collected per liter exhaled air (a)*, SP-A ($mg•mL^{-1}$) (b) and Albumin ($mg•mL^{-1}$) (c) measured by enzyme-linked immunosorbent assay (ELISA) in PEx from healthy controls, stable lung transplant recipients (non-BOS) and patients that developed bronchiolitis obliterans syndrom (BOS). Bars represent the median.
*Due to technical problems while recording particle data, particle mass concentration have missing data for two patients in the BOS group and three patients in the non-BOS group.

The SP-A/Albumin ratio was lower in the BOS group than in the non BOS group ($p = 0.0001$) but there was no significant difference between LTRs (BOS + non-BOS) and healthy controls (see figure in paper IV).

Discussion

Lung transplantation is the final treatment option for patients with end-stage lung disease. Even though the results are continuously improving, survival is still behind other solid organ transplantations. Liver, kidney and heart transplantation have a recipient 5–year survival in excess of 70–80%, whereas lung and small bowel transplantation have a 5–year survival of approximately 50%.
One plausible explanation for this scenario is that both the lung and the intenstine are constantly exposed to the environment and rely on a more active innate immune process for the first line of protection from pathogens and noxious agents. Given the many fundamental similarities with which an immune response attacks both foreign tissue and foreign pathogens, interactions between innate and adaptive immune responses in these organs are likely major detrimental factors contributing to the increased graft dysfunction observed.
The main survival limiting complication after lung transplantation is the development of chronic rejection in the form of BOS. Achieving tolerance of the allograft and at the same time maintaining a potent host response to microbial pathogens is a difficult balancing act. Better diagnostic and therapeutic tools are needed in order to individualise care and increase the efficacy with which the alloimmune response is suppressed whilst avoiding infection and keeping drug related toxicities at a minimum.

The BAL fluid has, aside from its clinical use in assessing infection, been extensively studied to determine its utility in predicting and monitoring of BOS and AR. It is well established that BAL neutrophilia is associated with BOS development.[26,27] Increased IL-8 levels and reduced SLPI in BAL have also been demonstrated independent of airway bacterial colonization.[89] Several studies have shown elevated levels of other cytokines and inflammatory proteins, such as IL-12, MMP-9 and IL-17.[27,31,66,90] However, it is unclear what predictive value these, or the presence of airway neutrophilia, have in the diagnosis of BOS due to the multiple confounding factors. Furthermore, no biomarkers for early detection and/or monitoring that can be utilized in a clinical setting have been identified.

For papers I-III we had access to BAL samples collected during an era when the clinical protocol for postoperative follow-up at Sahlgrenska included frequent routine bronchoscopies (see methods). This resulted in a unique biobank of more than 900 frozen BAL samples from over 140 LTRs. The extent of the material allowed for a careful matching procedure where pairs of patients (with or without AR and BOS respectively), free from infection and matched for age, gender, preoperative diagnosis, type of and time after surgery, could be identified in order to minimize the influence of confounding clinical factors as far as possible.
Since the collection of these samples, the routine follow-up at Sahlgrenska (and worldwide) has changed and the number of protocol bronchoscopies has been significantly reduced. Today, the gathering of such an extensive material would be close to impossible, as would the prospects of getting ethical approval for a study including frequent bronchoscopies on LTRs without a clinical indication. Hence, the development of alternative noninvasive diagnostic methods that enable repeated measurements over time would greatly facilitate our efforts to understand the biological processes occurring in the distal airways during allograft rejection.

The papers included in this thesis have, although with different approaches, all tried to shed some light into the complex world of allograft rejection.
As is often the case in this patient setting, the small sample sizes limits the power of the studies. However, the matching procedure likely reduced the influence of confounding clinical factors as far possible, and in addition, robust non-parametric statistical methods were used. It is therefore reasonable to interpret the findings as clinically relevant.

IL-18 (paper I)

At the time of the initiation of this study IL-18 was a fairly novel cytokine and, to our knowledge, had not been addressed in the setting of lung transplantation before.
Expecting that IL-18, produced by activated macrophages and being a key inflammatory cytokine augmenting the Th1-polarized immune response, would have a potential for being a biomarker for AR, we examined whether alterations in the concentrations of IL-18 in BAL were associated with AR onset.[33,91,92]
BAL samples from a well-defined patient material, where patients with AR $\geq$ A2 were identified and carefully matched in pairs with a control group, were analyzed.

We found no substantial change in IL-18 concentration during an episode of AR, and no correlations between IL-18 and the different inflammatory celltypes in BAL.
The result was somewhat surprising, especially in view of earlier findings in patients with acute graft versus host disease (aGVHD) after allogen bone marrow transplantation, a condition with many similarities to AR. These patients had a four-fold increase in IL-18 levels and high levels of IL-18 strongly correlating with the severity of the disease.[93] In our study we were unable to confirm any such pattern and this may be due to the role of IL-18 being more complex. In fact, whilst IL-18 can enhace natural killer-cell activity and therefore increase the risk of graft rejection, some results indicate it may also protect against AR.[94] In a murine bone marrow transplantation model, antibodies to IL-18 accelerated aGVHD related mortality depending on when in time they were administered. Furthermore, administration of the actual IL-18 protein significantly improved survival suggesting that IL-18 may have a regulatory role rather than simply amplifying the cyokine dysregulation that characterizes aGVHD.[95]

Our retrospective study did not exclude that IL-18 can be involved in the cellular response causing AR in lung transplant recipients. On the other hand, we could not forward any evidence that alterations in the airway concentration of IL-18 are useful for early detection of AR.

An up-to-date reference search revealed that IL-18 is still of some intrest in the context of kidney transplantation. However, probably due to the complexity of its actions, it has not emerged as a clinically useful biomarker for kidney allograft rejection. Concerning other solid organ transplantations there has been virtually no interest and no publications were found on the topic of IL-18 and lung transplantion.

Proteolytic homeostasis (paper II)

Several previous studies indicate that proteases from activated neutrophils in the small airways are involved in the development of BOS. However, only a few address the role of MMPs in BOS.[67-70] These studies analyzed messenger RNA, concentrations, and total activity of soluble protease proteins stripped of their natural inhibitors. This approach, using conditions that dissociate inhibitors, may not optimally reflect the functional protease–anti-protease balance in vivo during BOS.

In our study of matched pairs of LTRs, we used substrate assay methods of net protease activity in BAL, methods that do not remove or inhibit natural anti-proteases. We found a substantial increase in net gelatinase activity in BAL fluid from patients with BOS compared with matched control patients. Moreover, we found a clear correlation between the concentration of the gelatinase MMP-9, but not MMP-2, and the net gelatinase activity. There was also a clear correlation between the net gelatinase activity and the percentage of neutrophils in BAL samples from patients with BOS. In addition, the total activity of MMP-9 by far exceeded that of MMP-2 in BAL from BOS patients. In contrast, the net serine protease activity did not differ markedly between the groups even though we found an increase in the concentration of NE in the BOS group.

Taken together, these findings suggest that local unopposed gelatinase activity is associated with the development of BOS, and it is plausible that the gelatinase activity, at least in part, can be generated by MMP-9 released from neutrophils.
The fact that we did not detect any substantial increase in net serine protease activity in patients with BOS even though we found an increase in the concentration of NE, a potent serine protease, and no increase in the concentrationof SLPI, one of the main anti-proteases of serine proteases, deserves consideration. One feasible explanation is that endogenous anti-proteases, other than primarily SLPI, are able to oppose the increase in the concentration of NE.

It is well known that MMPs play a major role in pulmonary extracellular matrix formation and it has been shown that MMP-9 contributes to the migration of inflammatory cells through the extracellular matrix, the basement membrane, and the endothelial layer in several lung diseases characterized by recruitment of polymorphonuclear neutrophils and parenchymal destruction.[44]
Based on these findings, strategies to attenuate the damaging action of MMPs in the airways have emerged. A specific blockade of MMP-9 has been found to prevent fibroproliferation in murine tracheal allografts and a broad-spectrum MMP inhibitor was effective against airway obliteration, if treatment was introduced early, in another animal model.[96,97] Simvastatin also reduced MMPs in an experimental model of airway remodelling, in line with the clinical (although truly not validated) beneficial finding of statin use after lung transplantation.[98,99]

This study forwarded new evidence that there is an unopposed gelatinase activity in the bronchoalveolar space in BOS likely to be accounted for by MMP-9 from neutrophils. No corresponding evidence was found for unopposed serine proteinase activity, suggesting that endogenous antiproteases may be sufficient to oppose the increase of neutrophil elastase but not that of MMP-9.

IL-26 (paper III)

It is well known that neutrophils are involved in the pathophysiology of BOS and that a high neutrophil count is associated with increased IL-8, a key neutrophil chemotactic factor in the lung.[26,27] There is also evidence that both the development of BOS and AR involve a specific subset of T-helper cells, named Th17 cells.[51,71] However, little was known about the role of Th17 cytokines other than that of IL-17A.
Interleukin-26 is a cytokine produced by Th17 cells, macrophages and other leukocytes in the airways and recently, for the first time, detected in human airways.[35-38] It has been shown that, *in vitro,* IL-26 potentiates neutrophil chemotaxis induced by IL-8, implying that IL-26 amplifies neutrophil mobilization towards inflammation and infection.[38]

To evaluate the role of IL-26 in BOS and AR we again matched patients in pairs according to the principle described earlier. We also collected fresh BAL samples to characterize the cellular sources of IL-26 in this setting.
In a cross-sectional comparison we found higher IL-26 protein concentrations in BAL fluids from BOS (but not AR) patients compared to stable LTRs. To examine how the observed difference developed over time, we analyzed BAL samples collected prior to, and at the time of BOS diagnosis (with corresponding timepoints in the BOS-free group) and found that the concentrations of IL-26 in both groups were low in the pre-BOS period and increased in the BOS group at diagnosis.
Using flow cytometry we detected IL-26 in $CD4^+$ as well as in $CD8^+$ cells in all patients. Immunocytochemistry indicated IL-26 protein predominantly in large mononuclear cells and we verified co-localization of IL-26 and CD68 in the large mononuclear BAL cells, identifying these cells as alveolar macrophages.

Our results are well in line with the findings of Che *et. al*., who first characterized IL-26 in human lung tissue, and a growing number of studies suggesting that IL-26 is involved in a range of chronic inflammatory disorders, including rheumatoid arthritis and Crohn´s disease.[39-41] It has also been shown that IL-26 producing Th-17 cells may constitute up to 30% of infiltrating T-lymphocytes directly isolated in bronchial biopsies from patients with severe asthma. A recently published study found that IL-26 protein concentrations are increased in sputum from pediatric patients with uncontrolled asthma, but without signs of Th2-mediated inflammation, forwarding IL-26 as a potential, novel biomarker of disease severity in this phenotype of asthma.[100,101]

This study is the first to report on IL-26 in rejection after lung transplantation and it demonstrates that IL-26 is markedly increased in BOS but not in AR, implying a critical involvement of IL-26 in the pathology behind BOS.
Since there was a substantial time difference between the sampling points before and at BOS diagnosis one can speculate that we might have demonstrated an increase in IL-26 preceding clinical BOS diagnosis if samples in closer proximity to the time of diagnosis had been available. Such findings would, of course, have strengthened the hypothesis that IL-26 has potential to act as a predictive biomarker for this condition.

PEx (paper IV)

Impaired innate defences are associated with BOS development. SP-A is a component of the innate immune system in the lung and earlier findings in BAL have shown a reduction of SP-A content in BOS patients compared with stable lung LTRs.[102,103,72]
PExA is a non-invasive method for monitoring and sampling of the small airways developed in-house. It has previously been demonstrated that collection of particles is a valid method for quantifying exhaled SP-A originating from ELF in humans.[85-87]
In this study we investigated the potential of SP-A in PEx as an early biomarker for BOS. We also evaluated whether differences in particle composition could be observed when comparing BOS to non-BOS patients and when comparing healthy subjects to LTRs.

The SP-A concentration in PEx was significantly lower in patients developing BOS (two patients diagnosed with BOS at the study visit, five during the follow up period) compared to stable LTRs. The SP-A/albumin ratio was also lower in the BOS group. Bearing in mind the small sample size, this still indicates that SP-A in PEx could be a more sensitive diagnostic tool for BOS detection than spirometry.
LTRs, and in particular LTRs developing BOS, exhaled higher number of particles than matched controls. This might be explained by a higher degree of airway closure and re-opening. One plausible mechanism is that it is related to altered composition of the surfactant giving rise to increased airway surface tension and leading to earlier small airway closure. Another explanation could be that the geometry of the small airways is different in the transplanted lung and/or that loss of alveolar attachments results in increased airway closure or reduced loss of particles during the exhalation.

We also found that LTRs had a lower concentration of albumin in PEx than controls. This could either be due to a true reduction of albumin, or to an increase of another constituent in the surfactant. The question deserves further study and it has previously been shown that the most common phospholipid, dipalmiotylphosphatidyl choline (DPPC), is altered in asthma with an increase in those treated with inhaled glucocorticoids.[104]
The percentage of single lung transplants was higher in the BOS group and another question that remains to be answered is whether the non-transplanted lung contributes to the results? However, this sample size was too small for reliable statistical evaluation.
We found no evidence that grafts treated with EVLP prior to transplantation differed from "standard" grafts with respect to particle mass concentrations or SP-A and albumin concentrations.

In performing this study we found that the collection of particles with the PExA method is feasable in this patient setting. Even patients with a pronounced reduction of lung function were able to perform the breathing manouver and produce enough particles for analysis.

Conclusion

Paper I
Interleukin-18 in the bronchoalveolar space is not markedly altered or related changes in leukocyte subsets during AR.

Paper II
There is an unopposed gelatinase activity in the bronchoalveolar space during BOS that is likely to be accounted for by MMP-9 from neutrophils. There is no unopposed serine proteinase activity, suggesting that endogenous antiproteases are sufficient to oppose the increase of NE but not that of MMP-9.

Paper III
The cytokine IL-26 is markedly enhanced during BOS but not during AR, suggesting that it is involved in the pathology behind BOS. In this clinical setting, IL-26 may be produced by T-helper cells, cytotoxic T-cells and by alveolar macrophages.

Paper IV
Collection of PEx is easy to perform for LTRs, irrespective of lung function. The composition of PEx differs between LTRs and healthy controls and also between BOS/non-BOS patients. Patients developing BOS have significantly lower SP-A in PEx.

Final comments

The interpretation of the inflammatory reaction during allograft rejection after lung transplantation is difficult due to the confounding influence of immunosuppressive treatment and frequent infectious complications, both affecting inflammatory markers.
The lack of specific, predictive and thereby clinically meaningful measures of the alloresponse makes individual titration of maintenance immunosuppression, augmentation of immunosuppression strategies and prediction/management of BOS less than optimal.
Despite extensive research efforts, there is still limited understanding of the immunological mechanisms underlying BOS and AR and there are presently no clinically useful biomarkers available. Apart from identification of biomarkers, the development of new non-invasive diagnostic methods with higher sensitivity for lung function decline than spirometry and less risk/discomfort for the patient than bronchoscopy, could also contribute to new knowledge leading to better care and improved survival rates after lung transplantation.

In the studies included in this thesis MMP-9 and IL-26 in BAL, and SP-A in PEx have emerged as potential biomarkers for BOS. The clinical utility of of targeting these compunds for early detection, monitoring, and possibly even treatment of BOS deserve to be tested in future trials with prospective, longitudinal and interventional approaches.
The PExA method proved to be easy to perform in LTRs, also in patients with severely impaired lung function. However, its role in the follow-up after lung transplantation remains to be established. The method both gives a functional measure of, and samples from, the small airways. This new “window” to the small airways, that is non-invasive and enables repeated measurements over time, could facilitate our understanding of the biological processes occurring during, not only allograft rejection, but all diseases affecting the small airways.

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